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REGULATION OF LEUKOTRIENE BIOSYNTHESIS IN EOSINOPHILIC AND NEUTROPHILIC HL-60 CELLS

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ABSTRACT

Leukotrienes are potent mediators of immediate hypersensitivity reactions and are predominantly produced by myeloid cells. Although the regulatory mechanisms governing leukotriene biosynthesis are largely unknown, cytokines have been shown to upregulate leukotriene production by these cells. GM-CSF primed leukotriene biosynthesis in a dose-dependent manner in eosinophilic HL-60 cells, whereas IL-5 had no effect on leukotriene production in this cell line. GM-CSF did not affect the kinetic parameters of LTC_4 synthase and therefore most likely enhanced the rate of leukotriene biosynthesis by acting upstream of this catalytic event in the biosynthetic pathway. This differential priming ability was not merely due to different receptor populations or differences in the affinity and stability of the ligand-receptor complexes of GM-CSF and IL-5. Receptor binding assays and phosphotyrosine patterns demonstrated that IL-5 is incapable of signal transduction in eosinophilic HL-60 cells, despite the fact that both GM-CSF and IL-5 receptors share a common ß-chain component, necessary for high-affinity ligand binding and signal transduction. The regulation mechanisms that govern leukotriene biosynthesis during myeloid cell differentiation to eosinophils or neutrophils were revealed to be analogous up to the point where the leukotriene biosynthetic pathway diverges. At the stage in the leukotriene biosynthetic pathway where LTA_4 can be converted to either LTB_4 or LTC_4, specific regulators of transcription may become activated as a myelocyte differentiates. LTA_4 hydrolase expression was shown to be up-regulated in neutrophils whereas the activity and protein and messenger RNA expression of LTC_4 synthase were up-regulated in eosinophils. Microsomal GST-II is a novel membrane bound enzyme that possesses LTC_4 synthesizing activity and consequently may also be responsible for regulating the formation of LTC_4. Microsomal GST-II was determined to be the predominant enzyme responsible for LTC_4 production in human endothelial

cells and liver microsomes. While, LTC_4 synthase was confirmed to be the main enzyme capable of catalyzing the conjugation of reduced glutathione to LTA_4 in human lung, platelets, and eosinophilic HL-60 cell membranes. As leukotrienes have been implicated in many pathological disorders, understanding the mechanisms of regulation of their biosynthesis is of great importance.

démontré que la GST-II microsomale est une enzyme essentielle à la production de LTC_4 dans les cellules endothéliales humaines et dans les microsomes hépatiques. Cependant, dans les poumons et les plaquettes chez l'homme ainsi que dans les membranes cellulaires des lignées éosinophiles HL-60, il a été confirmé que la LTC_4 synthase est la principale enzyme qui catalyse la conjugaison du glutathion réduit au LTA_4. Les leucotriènes ayant été impliqués dans de nombreuses affections, la compréhension des mécanismes de régulation de leur biosynthèse est d'une importance capitale.

Dedicated to my parents, brother, and husband for their love and continuous support and encouragement.

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ABBREVIATIONS

ARE	antioxidant response element
BA	*n*-butyric acid
CaLB	calcium and lipid binding
COX	cyclooxygenase
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetraacetic acid
ERK	extracellular signal-regulated kinase
FLAP	5-lipoxygenase activating protein
fMLP	N-formyl-methionyl-leucyl-phenylalanine
GRE	glucocorticoid response element
GST	glutathione *S*-transferase
GSH	reduced glutathione
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
IL	interleukin
JAK	Janus kinase
KPi	potassium phosphate
LDL	low-density lipoproteins
LO	lipoxygenase
LT	leukotriene
LTA_4	5,6-oxido-7,9,11,14-eicosatetraenoic acid
LTB_4	5(S), 12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid
LTC_4	5(S)-hydroxy-6(R)-S-glutathionyl-7,9,trans-11,14-cis eicosatetraenoic acid
LTD_4	5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis eicosatetraenoic acid
LTE_4	5(S)-hydroxy-6(R)-S-cysteinyl-7,9-trans-11,14-cis eicosatetraenoic acid
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MGST	microsomal glutathione *S*-transferase
PAF	platelet activating factor
PBS	phosphate-buffered saline
PG	prostaglandin
PGB_2	prostaglandin B_2
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear cells
rhGM-CSF	recombinant human granulocyte-macrophage colony-stimulating factor
rhIL-5	recombinant human interleukin-5

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRS-A	slow reacting substance of anaphylaxis
STAT	signal transducers and activators of transcription
TGF	transforming growth factor
TNF	tumor necrosis factor
TXA_2	thromboxane A_2
XRE	xenobiotic response element

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1. INTRODUCTION

1.1 Eicosanoids

In animal cells, 20-carbon polyunsaturated essential fatty acids can be converted into a group of compounds termed *eicosanoids*. These eicosanoids are mediators of inflammatory and immunological reactions and include the families of prostanoids, leukotrienes, and lipoxins. The classical substrates for the production of these mediators are dihomo-γ-linolenic acid (20:3w-6), arachidonic acid (20:4w-6), and eicosapentaenoic acid (20:5w-3). These precursor fatty acids contain 3, 4, or 5 double bonds and correspond to the subsequent formation of the 1, 2, or 3 series of prostanoids, respectively (1, 2). Similarly, the 3, 4, or 5 series of leukotrienes can be formed from the above essential fatty acids with the exception of dihomo-γ-linolenic acid (20:3w-6). Leukotrienes require the 5-6 *cis* double bond in the precursor fatty acid and are therefore formed from mead acid (20:3w-9), (20:4w-6), or (20:5w-3) (2). These essential polyunsaturated fatty acids can be obtained directly from the diet (meat, fish, or seaweed) or derived from linoleic acid (18:2w-6) and γ-linolenic acid (18:3w-6) through desaturation and elongation events which usually occur in the liver (2, 3).

Arachidonic acid is the main precursor for eicosanoid biosynthesis as it is usually the most prevalent 20-carbon polyunsaturated fatty acid found in mammalian cells. Thus, the term *eicosanoids* was coined to include all products of arachidonate metabolism. Although the abundance of arachidonate results in the predominant formation of the 2- and 4-series of prostanoids and leukotrienes, respectively, the overall basal levels of intracellular free arachidonate remain very low (1). Arachidonic acid is efficiently and preferentially incorporated into the *sn*-2 position of phospholipids in cell membranes. Thus, the free concentration of arachidonate available for eicosanoid biosynthesis is dependent on the release of arachidonate by phospholipases versus the rate of its re-

esterification by acyl transferases (4).

1.2 Phospholipases

The main phospholipase pathways considered responsible for the liberation of arachidonate from membrane phospholipids are the direct action of a phospholipase A_2 (PLA_2) or the parallel activation of phospholipase C and diglyceride lipase (5). However, phospholipase A_1 and receptor activation of phospholipase D, phosphatidylcholine-specific phospholipase C, and low-density lipoproteins (LDLs) can also generate free arachidonic acid. As well, phospholipases C and D and arachidonic acid itself can activate protein kinase C (PKC) which has been demonstrated to reduce the rate of reacylation of arachidonate thereby enhancing free arachidonic acid levels (6, 7). Phospholipases play important roles in signal transduction and can be activated either by direct interactions of growth factors, hormones, autocoids (cytokines and eicosanoids), or other chemical mediators with cell-surface (cytokine and/or G-protein coupled) receptors or via an increase in intracellular free calcium concentration as a result of physical stimuli (8, 9)

PLA_2 activity is mainly responsible for the release of arachidonic acid in most cell types either directly or indirectly from phosphatidylinositol and/or phosphatidylcholine (10). These enzymes catalyze the hydrolysis of arachidonic acid from the *sn*-2 position of membrane phospholipids as well as the release of lysophospholipid, the precursor to platelet-activating factor (PAF), another inflammatory mediator. PLA_2s are also the rate limiting enzymes for the formation of prostaglandins and leukotrienes. Thus, understanding the regulation of these enzymes is very important due to the various biological effects that these mediators display.

The PLA_2s are a structurally diverse group of enzymes that consist of multiple secretory, cytosolic, and calcium-independent forms. There are presently nine groups of PLA_2s (11). The secretory pancreatic type IB PLA_2

(13-15 kDa), secretory nonpancreatic type IIA PLA_2 (13-15 kDa), and high molecular weight, calcium-dependent, cytosolic type IV PLA_2 (85 kDa) represent the foremost-characterized mammalian PLA_2s. The first and most well understood PLA_2s to be studied were the secretory type I PLA_2s ($sPLA_2$s) isolated from snake venoms and mammalian pancreas. In mammals these enzymes primarily function in digestion, however, pancreatic type I PLA_2 is also expressed in non-digestive tissues (spleen and lung) (12) and cells (vascular smooth muscle and fibroblasts) (13, 14) and displays cell proliferating and chemotactic activities (13-18). The type IIA PLA_2 has been detected in a variety of cell types (19) and is proposed to play a critical role in inflammation as it was initially isolated from platelets and synovial fluid from rheumatoid arthritis patients. In support of its function in inflammation, type II PLA_2 expression is up-regulated by proinflammatory stimuli (TNF, IL-1, IL-6, lipopolysaccharide, or cAMP-elevating agents), and can be down-regulated by glucocorticoids (dexamethasone) and the anti-inflammatory cytokine, TGF-ß (19, 20). These $sPLA_2$s contain seven disulfide bridges to stabilize their tertiary structure and are therefore sensitive to disulfide reducing agents. The type II PLA_2 does not display any fatty acid selectivity.

In contrast to the $sPLA_2$s, the cytosolic 85 kDa PLA_2 ($cPLA_2$) enzyme preferentially hydrolyses arachidonate from the *sn*-2 position of phosphatidylcholine and phosphatidylethanolamine. $cPLA_2$ may also be important in the arachidonic acid cascade as its expression and activity are elevated by inflammatory cytokines and it has been shown to mediate agonist-induced arachidonic acid release. $cPLA_2$ has been purified and cloned from various tissues and cell types, including the human pro-monocytic cell line, U937, and rat macrophage cell line, RAW 264.7 (for review see (21)). This enzyme is highly regulated by calcium concentrations and sequencing analysis demonstrated that $cPLA_2$ contains an amino-terminal calcium and phospholipid binding (CaLB) domain that is responsible for calcium-dependent membrane association in several

proteins (PKC, PLC-γ, GAP, and p53). Calcium-mediated translocation of $cPLA_2$ from the cytosol to its membrane substrate is essential for enzymatic activity, however, this activity can be further enhanced by phosphorylation of $cPLA_2$. Various agonists in different cells have been demonstrated to phosphorylate $cPLA_2$ resulting in an upregulation of its activity (21). Specifically, ERK1 (extracellular signal-regulated kinase) and ERK2 MAP (mitogen-activated protein) kinases phosphorylate $cPLA_2$ at Serine-505 (8). Thus, both increased intracellular calcium levels and serine phosphorylation of $cPLA_2$ are required for full activation of $cPLA_2$. As mentioned above, cytokines are also capable of regulating $cPLA_2$ activity post-translationally and/or transcriptionally. The promoter region of $cPLA_2$ contains several potential *cis*-elements, such as AP-1 and AP-2 (phorbol-ester-response elements), PEA 3, NF-κB, and NF-IL-6, that may account for cytokine regulation of $cPLA_2$ expression (22-26). Serine-228 is the active site of $cPLA_2$ and is also required for catalysis (8). $cPLA_2$ does not contain disulfide bridges, as in the type I and II $sPLA_2$s and is therefore capable of working under reducing conditions.

Mammalian cells also contain calcium-independent forms of PLA_2 ($iPLA_2$) which are abundant in myocardium (8) as well as other organs, including brain, lung, liver and spleen (27). The myocardial $iPLA_2$ is a 40 kDa cytosolic protein that displays enzymatic selectivity for plasmalogen-containing phospholipids, the predominant phospholipid in myocardium. This enzyme may be involved in the pathogenesis of myocardial disease (19, 28, 29).

1.3 Arachidonic acid metabolism

Once arachidonic acid is liberated from membrane phospholipids it can be converted to a number of chemical mediators such as prostanoids, leukotrienes, and lipoxins. The cyclooxygenase pathway leads to the formation of prostanoids and was the first arachidonic acid

metabolic pathway elucidated (30). Cyclooxygenase (COX), also known as prostaglandin G/H synthase or prostaglandin endoperoxide synthase, is the first enzyme involved in the production of prostanoids. This enzyme possesses two catalytic activities: the cyclooxygenase activity (generation of PGG_2 from arachidonic acid) and the peroxidase activity (reduction of PGG_2 to PGH_2). COX has recently been determined to be present in two distinct forms, COX-1 and COX-2. COX-1 is a constitutively expressed protein involved in housekeeping functions encompassing regulation of vascular homeostasis, modulation of renal function, and protection of the gastric mucosa, whereas, COX-2 is an inducible isoform involved in differentiative processes such as inflammation and ovulation (31). The prostanoids include the prostaglandins and the thromboxanes and the most common forms of these mediators produced by arachidonic acid metabolism are: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2. Prostanoids have various biological activities in multiple cell and tissue types (for review see (30)).

The lipoxygenase pathway leads to the formation of another large family of lipid mediators, the leukotrienes, and the less familiar, lipoxins. Lipoxins are formed by the coordinate participation of 5- and 12/15-lipoxygenases via the putative intermediate 15-hydroxy-LTA_4, whereas, the leukotrienes are formed strictly by the activity of 5-lipoxygenase (32). These mediators have been implicated in the pathogenesis of allergy and inflammation.

1.4 Leukotrienes: leukotriene biosynthetic pathway

Leukotrienes are potent lipid mediators of immediate hypersensitivity reactions. They are formed by the conversion of free arachidonic acid by 5-lipoxygenase into the labile allylic epoxide LTA_4. LTA_4 is an intermediate for the formation of both LTB_4 and LTC_4. LTA_4 can be hydrolysed to LTB_4 by LTA_4 hydrolase or LTA_4 can be conjugated to glutathione (GSH) to form

LTC_4. Sequential removal of glutamate and glycine by γ-glutamyltranspeptidase and dipeptidase, subsequently produces LTD_4 and LTE_4, respectively (Figure 1.1).

1.4.1 Formation of LTA_4: 5-lipoxygenase and FLAP

5-lipoxygenase (5-LO) catalyzes the first two steps in the biosynthesis of leukotrienes. The first step involves the incorporation of molecular oxygen at carbon 5 of arachidonate resulting in 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoate (5-HPETE). This intermediate is then dehydrated to the allylic epoxide LTA_4, by the second catalytic activity of 5-LO, LTA_4 synthase activity (34, 35). 5-LO was initially characterized in rabbit leukocytes (36) and its dual catalytic activities were subsequently demonstrated using 5-LO purified from potato tubers (37). 5-LO has since been purified from human sources, porcine leukocytes, rat basophilic leukemia cells, and murine mastocytoma cells (34). Although this enzyme is mainly present in myeloid cells it is also expressed in keratinocytes and lymphocytes (38, 39). 5-LO is a 78 kDa soluble protein that requires calcium and ATP for enzymatic activity (40-44). Additional factors (44-46) have also been reported to be required for full enzyme activity such as hydroperoxy fatty acids, phosphatidylcholine, or various cytosolic and membrane factors in cell-free systems (41-44, 46, 47). Increases in intracellular calcium result in the translocation of 5-LO from the cytosol or nucleus to the nuclear membrane (48, 49). This translocation is reversible in the presence of chelators (50) and is associated with the activation of cellular leukotriene biosynthesis with the subsequent irreversible inactivation of 5-LO (51).

Interestingly, the patterns of 5-LO distribution and movement may differ depending on the cell type (52, 53). In human resting polymorphonuclear (PMN) leukocytes, 5-LO is stimulated to translocate from the cytosol to the nuclear membrane by calcium ionophore A23187. 5-LO has been demonstrated to interact with actin/α-actinin *in vitro* (54)

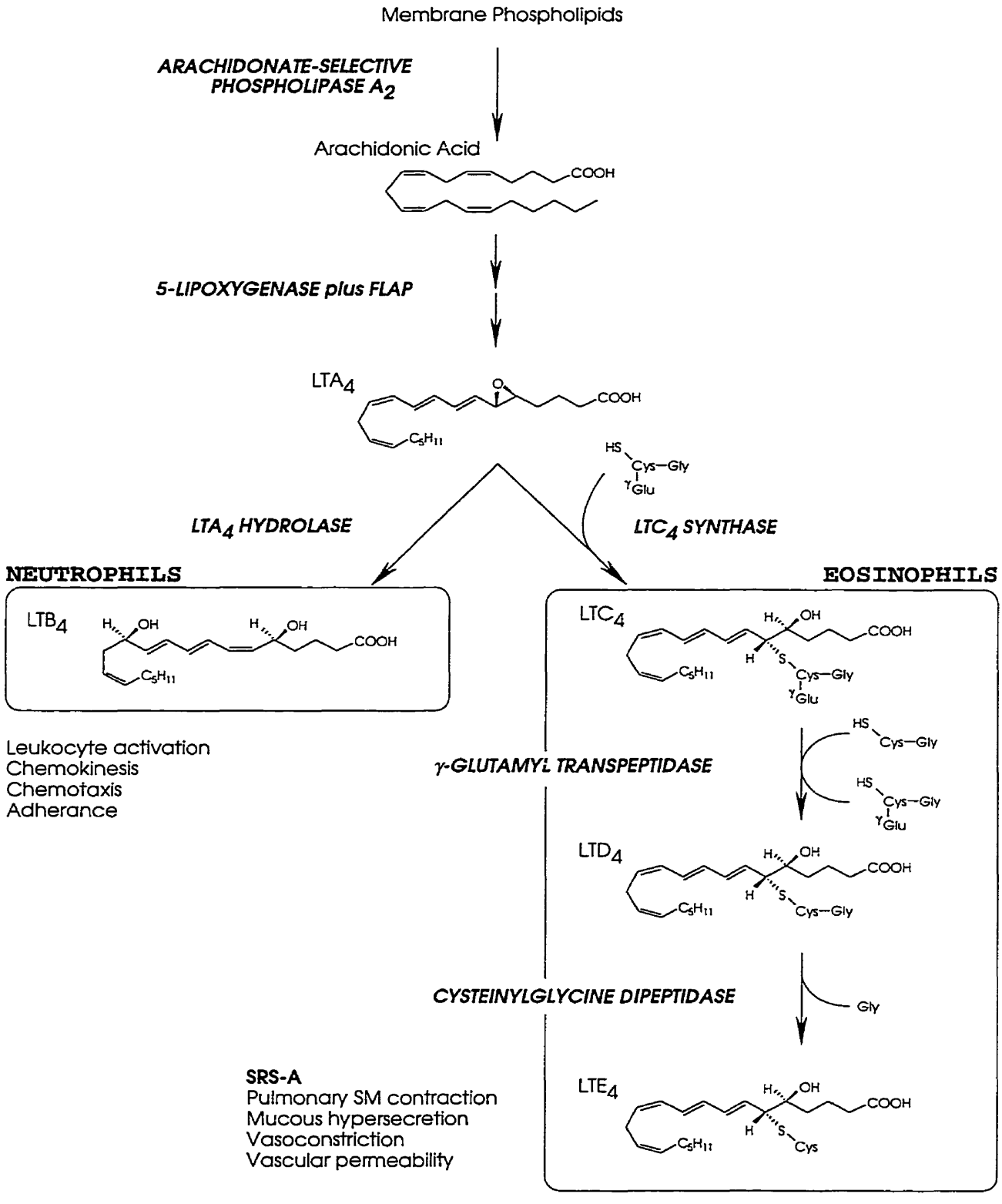

Figure 1.1. Leukotriene biosynthesis (adapted from Nicholson, D.W., 1993, (ref. 33))

and this association with the cytoskeleton may aid in the translocation of 5-LO to specific sites on the nuclear envelope. Recently, Lepley and Fitzpatrick have also shown that 5-LO catalysis and translocation may be modulated by tyrosine kinases (54, 55). In other scenarios, 5-LO resides in the nuclear compartment of resting cells. Alveolar macrophages contain both cytosolic and nuclear forms of 5-LO. In these cells A23187 only stimulates the nuclear form to translocate from the euchromatin region to the nuclear envelope (56). Rat basophilic leukemia cells also contain both cytosolic and nuclear forms of 5-LO, however, A23187 stimulates translocation of both pools to the nuclear envelope (57). *In vivo* evidence has also been reported for nuclear 5-LO localization in alveolar macrophages obtained from patients with idiopathic pulmonary fibrosis (58). In addition to leukotriene formation, 5-LO may have nuclear functions such as transcriptional activation or repression. (53).

In whole cells, 5-LO also requires the presence of 5-lipoxygenase activating protein (FLAP) to form leukotrienes from endogenous substrate. FLAP is an 18 kDa membrane bound protein that resides in the nuclear envelope of PMN leukocytes and macrophages (48, 56). FLAP was originally discovered through its ability to bind with high affinity to MK-886, an indole inhibitor of leukotriene biosynthesis (59). MK-886 inhibits leukotriene biosynthesis in intact cells, but not in cell-free systems, by inhibiting the membrane association of 5-LO (60). Thus, FLAP is thought to act as an arachidonic acid transfer protein that presents 5-LO with arachidonic acid (61, 62). FLAP has been cloned and is expressed in cells of myeloid origin including PMN leukocytes, monocytes, macrophages, eosinophils, and basophils (63). FLAP is also present in B-lymphocytes and transformed B-cell lines (39). The FLAP gene was cloned (64) and consists of 5 small exons and 4 large introns that span 31 kilobases (kb). The promoter region contains a possible TATA box, AP-2, and GRE (glucocorticoid response element) binding sites with no significant homology to the 5-LO gene. These genes are also not necessarily

coordinately regulated.

5-LO has been cloned and expressed in mammalian cells (65-67). The cDNAs of mammalian 5-LO cloned to date are from human (65, 66), rat (68), mouse (69), and hamster (70). The 5-LO gene was also isolated (71) and determined to reside on chromosome 10. The gene is greater than 82 kb consisting of 14 exons and 13 introns. The promoter region contains the putative regulatory elements Sp1(GC-box), myb, AP-2, and NF-κB (71-73).

Recently, 5-LO 'knockout' mice have been developed and characterized. These 5-LO-/- mice do not display a phenotype different from control mice (74). As expected, 5-LO was not essential for fetal development or survival. Surprisingly, there was no difference in leukocyte counts from 5-LO-/- mice compared to controls (74, 75) even though inhibitor studies have linked leukotrienes to myelopoiesis and colony stimulating factor-induced myeloid colony formation (76, 77). Other studies with 5-LO-/- mice confirmed previous findings that were obtained using leukotriene inhibitors. 5-LO products were determined to be required for the development of airway hyperresponsiveness in mice and were partially involved in eosinophil recruitment to antigen challenge (Irvine, C. G., Tu, Y.-P., Sheller, J. R., and Funk, C. D. (1996) manuscript submitted). 5-LO-/- mice also demonstrated a potential beneficial role of leukotrienes in the host defence against parasite and bacterial infections (Secor, W. E., Powell, M. R., Morgan, J., Wynn, T. A., and Funk, C. D. (1996) manuscript submitted) (Urban, J. F., Funk, C. D., and Finkelman, F. D., unpublished data) (78).

1.4.2 Non-enzymatic metabolism of LTA_4

LTA_4 can be metabolised non-enzymatically to 6-*trans*-LTB_4, 6-*trans*-12-*epi*-LTB_4, 5(S),6(R)- and 5(S),6(S)-diHETE (79) or stereospecifically converted to 5(S),6(R)-diHETE by cytosolic epoxide hydrolase (80, 81). LTA_4 is also the common precursor for the synthesis of LTB_4 and LTC_4 (82, 83).

1.4.3 Formation of LTB_4: LTA_4 hydrolase

LTA_4 hydrolase catalyzes the hydrolysis of the unstable epoxide LTA_4 to produce LTB_4, a potent chemoattractant (79, 84). This monomeric cytosolic enzyme was first purified from human peripheral leukocytes and was determined to be distinct from other known epoxide hydrolases (85). LTA_4 hydrolase is widely distributed. It has been isolated from a number of different cells and tissues of various species (86) and its expression has been detected in many different cells including those that do not express 5-LO. This diverse expression of LTA_4 hydrolase supports the physiological significance of transcellular metabolism of LTA_4 to LTB_4 (87). The human LTA_4 hydrolase cDNA has been cloned (88, 89) and expressed in various cell systems (90). LTA_4 hydrolase has a molecular mass of 69 kDa and its amino acid sequence revealed the presence of a zinc-binding motif that is common to certain aminopeptidases (91-93). Accordingly, LTA_4 hydrolase was later classified as a zinc metalloenzyme that displays both epoxide hydrolase and aminopeptidase activities (94-96). In contrast to 5-LO, LTA_4 hydrolase does not require cofactors for its hydrolase activity (85), however, chloride ions are necessary for its aminopeptidase activity (97) and the zinc-binding ligands, histidine-295, histidine-299, and glutamate-318, are essential for both enzyme activities (98). LTA_4 hydrolase activity is controlled by suicide inactivation by covalent binding of the enzyme to its substrate, LTA_4 (99-102). LTA_4 hydrolase may also be regulated by putative *cis*-acting elements found in the promoter region of the LTA_4 hydrolase gene. Mancini and Evans recently characterized the gene structure of LTA_4 hydrolase (103). The gene consists of 19 exons, is greater than 35 kb, and was localized to chromosome 12q22. The 5'-flanking region consists of consensus sequences for AP-2, and two XRE (xenobiotic response element) binding sites. The possibility of more than one transcription initiation site has been proposed and the fact that the zinc-binding residues that make up the zinc-binding domain are divided between exons 10 and 11 may

provide a further mechanism for regulation of this enzyme through RNA splicing events. Recently, an alternatively spliced mRNA form of LTA_4 hydrolase has been identified (104). This shorter isoform has a calculated molecular mass of 59 kDa and a distinct C-terminus. The sequences that encode the zinc-binding motif and the peptidase active site, however, are identical in both LTA_4 hydrolase mRNAs. Therefore, the LTA_4 hydrolase and aminopeptidase activities are most likely intact. The diversified C-terminus of the new isoform, however, might modify and/or add new biochemical characteristics to the LTA_4 hydrolase enzyme.

1.4.4 Formation of LTC_4: LTC_4 synthase/glutathione *S*-transferases

LTC_4 synthase is the rate-limiting enzyme for the biosynthesis of the cysteinyl leukotrienes, LTC_4, LTD_4, LTE_4 (82, 83), mediators of immediate hypersensitivity. This enzyme is an integral membrane protein that catalyzes the conjugation of LTA_4 to reduced GSH to form LTC_4. LTC_4 synthase is specific for the biosynthesis of LTC_4 and has been demonstrated to be distinct from all previously known cytosolic or microsomal glutathione *S*-transferases (GSTs) (105-110). LTC_4 synthase has been partially purified from guinea-pig lung and human platelets (110, 111) and purified to homogeneity from various cell lines and human lung tissue (108, 109, 112, 113). Nicholson *et al.* purified the enzyme as an 18 kDa protein and determined that it functions as a homodimer by gel filtration chromatography (108). The purified enzyme is unstable and requires GSH for stabilisation, as well as, magnesium and phosphatidylcholine as cofactors for activity. These discoveries were essential for the complete purification of LTC_4 synthase (109). In contrast to LTA_4 hydrolase, LTC_4 synthase is expressed in a limited number of cell types. LTC_4 synthase activity is present in eosinophils, basophils, mast cells, monocytes/macrophages, platelets, vascular smooth muscle cells, endothelial cells, hepatocytes, and various leukemic cell lines including HL-

60 cells (108, 109, 112, 114-124). Platelets, vascular smooth muscle cells, and endothelial cells do not express 5-LO, therefore transcellular metabolism is required for LTC_4 formation in these cells. Thus, LTC_4 can be produced by endogenous substrate in 5-LO expressing cells or by exogenous substrate in cells that lack 5-LO. Western-blot analyses have confirmed the presence of LTC_4 synthase in human lung tissue, eosinophils, KG-1 cells, and platelets using a polyclonal antibody raised against purified LTC_4 synthase (113). Further analyses have also revealed that LTC_4 synthase is mainly perinuclear in distribution in alveolar macrophages in human lung sections (113).

Human and mouse LTC_4 synthase were recently cloned (125-127). No nucleotide sequence homologies between LTC_4 synthase and cytosolic or microsomal GSTs were observed. Instead, LTC_4 synthase displayed 31% identity to FLAP at the amino acid level. The predicted transmembrane domains of LTC_4 synthase and FLAP overlapped. One of the regions of high homology between these enzymes corresponded to the putative inhibitor binding region in FLAP. In support of the similar structures of LTC_4 synthase and FLAP, the FLAP inhibitor, MK-886, also inhibited LTC_4 synthase activity. Due to the homology between FLAP and LTC_4 synthase, it is speculated that LTC_4 synthase may present LTA_4 to GSH in a similar manner to that which FLAP may present arachidonic acid to 5-LO. Also, LTC_4 synthase is a very basic protein (pI of 11.05) which may generate a favourable microenvironment for the conjugation of LTA_4 to GSH since these compounds will spontaneously conjugate under alkaline conditions (110, 128).

The cDNA of LTC_4 synthase encoded a protein with two PKC consensus sequences confirming the previous identification of a PKC site in the N-terminus by Edman degradation of the purified protein (108). Similarly, LTC_4 synthase activity has also been shown to be negatively regulated by PKC activity (129, 130). In contrast to the protein expression of LTC_4 synthase, Northern-blot analyses demonstrated that the mouse

RNA transcript is widely distributed (126). The human LTC_4 synthase transcript is present in eosinophils and KG-1 cells, but not detected in either PMN leukocytes or Raji cells (125).

The gene for LTC_4 synthase was recently cloned and characterized (131, 132). Although the gene is only 2.52 kb in length, all of the intron/exon junctions align with those of the 31 kb FLAP gene. Thus, it seems that LTC_4 synthase and FLAP belong to a distinct gene family. Similar to that suggested for LTA_4 hydrolase, several transcriptional start sites at 66, 69, and 96 base pairs upstream of the ATG translation start site were detected (131). In contrast, Bigby *et al.* detected a single transcription initiation site 78 bp 5' of the coding region (132). The promoter region consisted of putative DNA-binding motifs for Sp-1, AP-1, AP-2, ets factors, and CREB/ATF (131, 132). The first intron also contains a STAT-binding motif (132). In contrast to the localization of FLAP and 5-LO genes to chromosomes 13 and 10, respectively, the LTC_4 synthase gene was localized to the q35 region of chromosome 5 (131, 132). Interestingly, this region of chromosome 5 appears to be important in allergic and inflammatory disorders and will be discussed in more detail below.

Recently, a novel LTC_4 synthesizing membrane bound protein, microsomal GST-II, was discovered (133). This enzyme displayed 44% identity to LTC_4 synthase, 33% identity to FLAP, and only 11% identity to microsomal GST-I at the amino acid sequence level (Figure 1.2). These proteins all displayed similar hydrophobicity plots which may indicate that these proteins have analogous structures. Due to the above similarities these proteins may be part of a gene family that encodes membrane bound proteins important for leukotriene biosynthesis or cellular detoxification by GSH conjugation. Microsomal GST-II is a 16.6 kDa protein with a calculated pI of 10.4. This protein displays both LTC_4 synthase activity as well as general GST activity through its ability to conjugate GSH to both LTA_4 and 1-chloro-2,4-dinitrobenzene. Northern-blot analyses demonstrated a wide tissue distribution for the transcript. Thus, based on its

```
                    1                                                   60
microsomal GST-II   .....................MaGNsiLLAAVsiLSacQQsYFALQVgkARlKykVtP
    LTC4 synthase   .....................MkdeVaLLAAVTLLgVllQaYFsLQVisARRafrVsP
             FLAP   ..................mdqetvGNVvLLAiVTLiSVvQngFFAhkVehesRtqngrs
 microsomal GST-I   mvdltqvmddevfmafasyatiilskmmlmstAtafyrltrkvFanpedcvAfgKgenak

                    61                                                  120
microsomal GST-II   PavTGsPEFERVfRAQQNCVEfYPiFiiTLWmAGwyFnQVfAtcLGLVYiyGRhlYFwGY
    LTC4 synthase   PltTGpPEFERVYRAQvNCsEyfPlFLATLWvAGifFhegaAAlcGLVYLFaRlrYFqGY
             FLAP   fqrTGtlaFERVYtAnQNCVdaYPtFLAvLWsAGLlcsQVpAAfaGLmYLFvRqkYFvGY
 microsomal GST-I   kylrtddrvERVrRAhlNdlEniipFLgigllysLsgpdpstAiLhfrlfvGariYhtia

                    121                                                 180
microsomal GST-II   seaAkkRitGfrlSlgILaLLtLLgALGIaNsFLdeyLdlniakkLRrqf.........
    LTC4 synthase   arsAqlRlaplyaSaRaLwLLvaLAALGLlahFLpaaLraallgrLRTllpwa.......
             FLAP   lgertqstpGyifgkRIilfLfLMsvaGIfNyyLifffgsdfenyikTisttisplllip
 microsomal GST-I   yltplpqpnralsffvgygvtlsMAyrlLksklyl.....................
```

Figure 1.2. Comparison of homology at the amino acid level (adapted from Scoggan *et al.*, 1997, (ref. 134))

lack of substrate specificity and its wide transcript distribution this novel membrane bound protein was termed *microsomal GST-II*. Following its classification as a microsomal GST this protein was also found to possess GSH-dependent peroxidase activity, supporting its classification as a microsomal GST. Thus, this new enzyme may have important roles in both leukotriene biosynthesis and in cellular detoxification by GST activity.

GSTs are a family of enzymes that catalyze the conjugation of reduced glutathione to a variety of electrophilic substrates (for review see (135-140)). These enzymes have important roles in cellular protection by acting as detoxifiers of xenobiotic metabolites and endogenous hydroperoxides. By conjugating glutathione to electrophilic compounds these enzymes act as an intracellular detoxifying system. These enzymes also have GSH-dependent peroxidase activity that protects cells from endogenous organic hydroperoxides that are produced during oxidative stress. In addition, GSTs bind to hydrophobic compounds such as heme, bilirubin, polycyclic aromatic hydrocarbons, and dexamethasone, indicating that these enzymes may also serve as intracellular carrier proteins. These enzymes are widely distributed in most forms of life and have a wide tissue distribution. According to the nomenclature proposed by Mannervik, the GSTs have been classified into five groups (141). The soluble GSTs, α, μ, π, θ, and the microsomal GSTs. To date there are four gene families that encode the cytosolic GSTs, α, μ, π, θ. Only one form of the enzyme has been identified in both the π and θ classes. Within the α and μ classes there are 5 and 11 isoforms, respectively. These α and μ isoforms usually function as homo or heterodimers of subunits that have molecular masses in the range of 25-27 kDa. Their expression differs from one tissue to another and they may become activated by inducers of drug metabolism. Rushmore and Pickett have focused on the mechanism by which xenobiotics transcriptionally activate GSTs. Rat GST subunit 1 (Ya) was the most extensively studied gene in which five regulatory elements were found in the promoter region: hepatocyte nuclear factors 1 (HNF1)

and 4 (HFN4), XRE, GRE, and antioxidant response element (ARE) (137).

The μ family has been determined to be the most proficient cytosolic GST family to produce LTC_4 (142). Several studies, however, have indicated that the dominant enzymes responsible for LTC_4 production are particulate.

To date, there are three forms of microsomal GSTs, microsomal GST-I, LTC_4 synthase, and microsomal GST-II. Microsomal GST-I was the first membrane bound GST discovered and is characterized by its N-ethylmaleimide-inducible activity (143). This enzyme displays all of the typical GST features, although, it functions as a homotrimer consisting of 18 kDa subunits (136, 144, 145). LTC_4 synthase is a unique membrane bound enzyme that has been distinguished from all previously known cytosolic and microsomal GSTs (105-110). LTC_4 synthase is an exception to the general criteria of GSTs. Even though LTC_4 synthase catalyzes a GST-like activity, it is restricted to the biosynthesis of LTC_4. This enzyme has a narrow substrate and tissue distribution and does not possess GSH-dependent peroxidase activity. Microsomal GST-II is the newest membrane bound GST discovered to date (133). This enzyme is unique due to its ability to efficiently conjugate GSH to both LTA_4 and 1-chloro-2,4-dinitrobenzene. Thus, microsomal GST-II may play important roles in both cellular detoxification and cysteinyl leukotriene formation.

1.4.5 Metabolism of LTC_4

Once LTC_4 is produced it has been reported to be exported to the extracellular space via active transport (146-148). LTC_4 is then metabolised to LTD_4 by a specific γ-glutamyltranspeptidase enzyme that removes the glutamyl moiety from glutathione (149, 150). γ-glutamyltranspeptidase resides at the external site of the plasma membrane (151) and has recently been cloned (152). LTE_4 is formed by removal of the glycine residue from LTD_4 by a dipeptidase (153-155) and is one of the major urinary metabolites

in man. The cysteinyl leukotrienes are rapidly eliminated through hepatic inactivation or via renal excretion (156).

1.4.6 Transcellular leukotriene synthesis

The process whereby donor cells release LTA_4 to recipient cells, which can further metabolise LTA_4 to either LTB_4 or LTC_4, is known as transcellular metabolism of LTA_4. It has been well established that transcellular metabolism of LTA_4 occurs in many different cell types (117-121, 157, 158). The unstable epoxide LTA_4 is stabilized by albumin and phospholipid bilayers thereby possibly increasing its half life (159, 160). Thus, LTA_4 released from donor cells may have a sufficiently prolonged half life in the extracellular environment that allows recipient cells to further metabolise LTA_4 to either LTB_4 or LTC_4. Once again, the restricted expression of 5-LO to cells of myeloid lineage and the wide cellular distribution of LTA_4 hydrolase is a physiological indicator of the potential for transcellular metabolism.

1.5 Biological actions of leukotrienes

1.5.1 Cysteinyl leukotrienes

The cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, are the components of the *slow-reacting substance of anaphylaxis* (SRS-A) (161). The biological activity of these potent smooth muscle contracting agents was first described in 1940. Kellaway and Trethewie determined that mediators released after antigen challenge of sensitized guinea-pig lungs caused contraction of guinea-pig ileum (162). This contraction was slow and long lasting as compared to histamine and Brocklehurst confirmed the formation of this mediator in induced anaphylaxis (163). Thus, the term *slow-reacting substance of anaphylaxis* was introduced. The chemical

structure of SRS-A, however, was not elucidated for another 40 years. In 1979-80, Samuelsson and coworkers determined that SRS-A consisted of three 20-carbon fatty acids that were conjugated to glutathione with subsequent modifications of the peptide portion (161, 164). These mediators were LTC_4, LTD_4, and LTE_4, and are commonly termed *cysteinyl leukotrienes*. The term, *leukotriene*, was used to describe these compounds as they were originally reported as products of leukocytes that contained a conjugated triene structure (165). Subsequently, the bioactions of chemically synthesized cysteinyl leukotrienes were investigated in animal clinical pharmacologic studies. Cysteinyl leukotrienes were determined to contract vascular, gastrointestinal, and respiratory smooth muscle (166-168). In the vasculature, these leukotrienes constrict arterioles and increase the permeability of venules resulting in plasma exudation (169-171). Cys-LT_2 receptor activation, however, can lead to smooth muscle dilation mediated through nitric oxide release. These mediators also stimulate bronchial smooth muscle contraction and are 100-1000 times more potent than histamine (172-174). Cysteinyl leukotrienes are also capable of stimulating mucus secretion in the respiratory tract (175) as well as impairing the mucociliary clearance (176, 177). These changes result in bronchial wall thickening and airway narrowing. Thus, cysteinyl leukotrienes can mimic the important characteristics of bronchial asthma. The role of these mediators in the pathology of asthma has also been supported by numerous other studies. Increased levels of cysteinyl leukotrienes were detected in sputum (178), BAL fluid (179), plasma (180), nasal secretions (181), and urine (182) from asthmatics. Inhalation of cysteinyl leukotrienes elicits airway obstruction, similar to that observed in asthmatic patients (183-185). Asthmatic patients are hyperreactive to cysteinyl leukotrienes and allergen challenge of asthmatic lung tissue causes release of leukotrienes in amounts that correlate with bronchial contraction (186). Finally, specific leukotriene receptor antagonists and 5-LO inhibitors alleviate the severity of symptoms

in asthma patients (for review see (187-189)).

Cysteinyl leukotrienes and LTB_4 may also play a role in cell proliferation. Leukotrienes may regulate the proliferation of epithelial cells (190), smooth muscle cells (191), and myeloid progenitor cells. LTC_4 production was found to be increased in patients with chronic myelogenous leukemia (192, 193) and LTC_4 stimulated GM-CSF-induced myelopoiesis in normal human bone marrow cells (194). As mentioned earlier, leukotriene inhibitor studies have also implicated leukotrienes in myelopoieses and colony stimulating factor-induced myeloid colony formation (76, 77), although 5-LO$^{-/-}$ mice appeared to have normal leukocyte counts (74, 75).

Cysteinyl leukotrienes mediate their biological actions by interacting with specific guanosine-binding-protein-coupled receptors and in turn elicit an intracellular signalling mechanism that is mediated by phosphoinositide hydrolysis and inositol 1,4,5-triphosphate-dependent calcium release (195). The receptors may couple to multiple G-proteins and cys-LT_1 is known to couple to at least two G-proteins, one pertussis toxin sensitive and one insensitive (167). Two specific cysteinyl leukotriene receptors have been described to date, cys-LT_1 and cys-LT_2. The relative binding affinity that these receptors display for the cysteinyl leukotrienes is: LTD_4=LTC_4≥LTE_4 at cys-LT_1 receptor and LTC_4=LTD_4>>LTE_4 at cys-LT_2 receptor. Several studies have demonstrated the presence of a homogeneous population of cys-LT_1 receptor in isolated human airway preparations (167). In contrast, a homogeneous cys-LT_2 receptor population exists in pulmonary vasculature (196).

1.5.2 LTB_4

The structure of LTB_4 was determined by Borgeat and Samuelsson, in 1979 (79). Soon after, LTB_4 was identified as a potent chemotactic and aggregating agent that was synthesized from neutrophils stimulated with

calcium ionophore, A23187 (197). LTB_4 has since been demonstrated to be a potent mediator of inflammation and affects both lymphocyte and leukocyte functions (84). LTB_4 stimulates leukocyte-chemotaxis, chemokinesis, aggregation, degranulation, and adherence to endothelial cells (198-201). In addition, LTB_4 plays a role in immunoregulation by stimulating the activation, proliferation, and differentiation of human B-lymphocytes (202, 203) and by augmenting human NK cell cytotoxicity (204, 205). LTB_4 can also induce T suppressor cell activity (206) and stimulate lymphocyte production of cytokines, such as IL-1 in human monocytes (207) as well as IL-2 and INF-γ in lymphocyte cultures (208).

LTB_4 acts through a specific guanosine-binding-protein-coupled receptor that has both high and low affinity states. The high-affinity receptor state mediates chemotaxis, adherence of neutrophils to endothelial walls, and transient calcium mobilization. The low-affinity receptor state mediates degranulation, lysosomal enzyme release, and long-term calcium mobilization (209, 210). The high affinity site and the low affinity site appear to correspond to G-protein coupled and uncoupled forms of the receptor (211-213). The B-LT (LTB_4) receptor couples to a pertussis toxin-sensitive G-protein which mediates the functional responses of LTB_4 via phosphoinositol hydrolysis.

1.6 Hematopoietic cytokines

1.6.1 Myeloid cells

Myeloid cells are the major producers of leukotrienes. Specifically, eosinophils predominantly produce cysteinyl leukotrienes, whereas, neutrophils primarily produce LTB_4. The development of these cells is known to be regulated by various cytokines that are produced from activated T cells, macrophages, and stromal cells (214). The hematopoietic cytokines, GM-CSF, IL-3, and IL-5, are among the cytokines responsible for the

proliferation and differentiation of progenitor stem cells into mature myeloid cells. IL-3 and GM-CSF interact with a wide variety of hematopoietic cells, from early to late stages of differentiation. IL-5, however, exclusively stimulates the proliferation and differentiation of eosinophils, basophils, and some murine B-cells (214, 215). Not only are these cytokines committed to regulating the growth and development of pro-myelocytes, they have also been implicated in the activation of terminally differentiated cells. Cytokines are able to modulate the activation of leukotriene biosynthesis in mature myeloid cells. GM-CSF or IL-5 can prime cells (eosinophils and other cells) causing an enhancement in leukotriene biosynthesis upon activation (216, 217). The mechanism by which these cytokines activate leukotriene biosynthesis is ambiguous. These cytokines, however, are capable of inducing protein tyrosine phosphorylation of several signalling molecules and mediate their actions through specific receptors.

1.6.2 Hematopoietic cytokine receptors

The receptors for GM-CSF, IL-3, and IL-5, all belong to the hematopoietic cytokine receptor superfamily. They are components of a subgroup of the type I cytokine receptor superfamily that includes receptors for IL-6, IL-11, IL-12, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin-1, which share the gp130 chain (218, 219); and IL-2, IL-4, IL-7, IL-9, and IL-15, which share the γ_c chain (220, 221). These receptors are composed of one or two common structural motifs in their extracellular domains that consist of 200 amino acids encompassing two repeats of a fibronectin type III domain. The N-terminal domain contains four conserved cysteine residues, and a unique WSXWS sequence is present in the C-terminal domain. Reconstitution of the functional receptors using cloned receptor subunit cDNAs has proven that IL-3, GM-CSF, and IL-5 receptors are heterodimeric

(222). These heterodimeric receptors each consist of a cytokine-specific α-chain (60-80 kDa glycoprotein) and a common ß-chain (120-130 kDa glycoprotein) component (Figure 1.3). The α-chain component is required for the specific binding of the distinct ligands to their respective receptors and is capable of low-affinity ligand binding in the absence of the ß-chain component. The ß-subunit cannot bind any cytokine on its own, but confers high-affinity ligand binding in the presence of the α-subunit and is essential for signal transduction. The ß-subunit consists of two repeats of the common motif in its extracellular domain and two motifs termed, *box-1* and *box-2*, in its large (432 amino acid) intracellular domain. The intracellular domain of the α-subunits is much smaller, approximately 50 amino acids, and comprises a conserved amino acid sequence, RLFP, proximal to the transmembrane domain. Even though the cytoplasmic domains of both the α- and ß-subunits are required for signal transduction (224-227), neither contains any intrinsic enzymatic activity, such as tyrosine kinase activity. This discovery is intriguing since these receptors induce rapid tyrosine phosphorylation of several cellular proteins (228).

1.6.3 Signal transduction pathways

The signal transduction pathways elicited by IL-3, GM-CSF, and IL-5 are just beginning to be elucidated. These signalling mechanisms involve numerous signalling molecules and at least four separate prominent pathways can be outlined for GM-CSF signal transduction (229) (Figure 1.4). These pathways are the Ras-MAP kinase pathway, JAK-STAT pathway, PI 3-kinase pathway, and finally a fourth pathway is surfacing that involves molecules known to be triggered by GM-CSF, including c-fes, c-yes, pim, PKC, and CREB. Any interrelationships between these major signal transduction pathways remain to be elucidated. The pathways that operate in distinct cell types are being investigated and their specificity may involve the activation of more than one pathway.

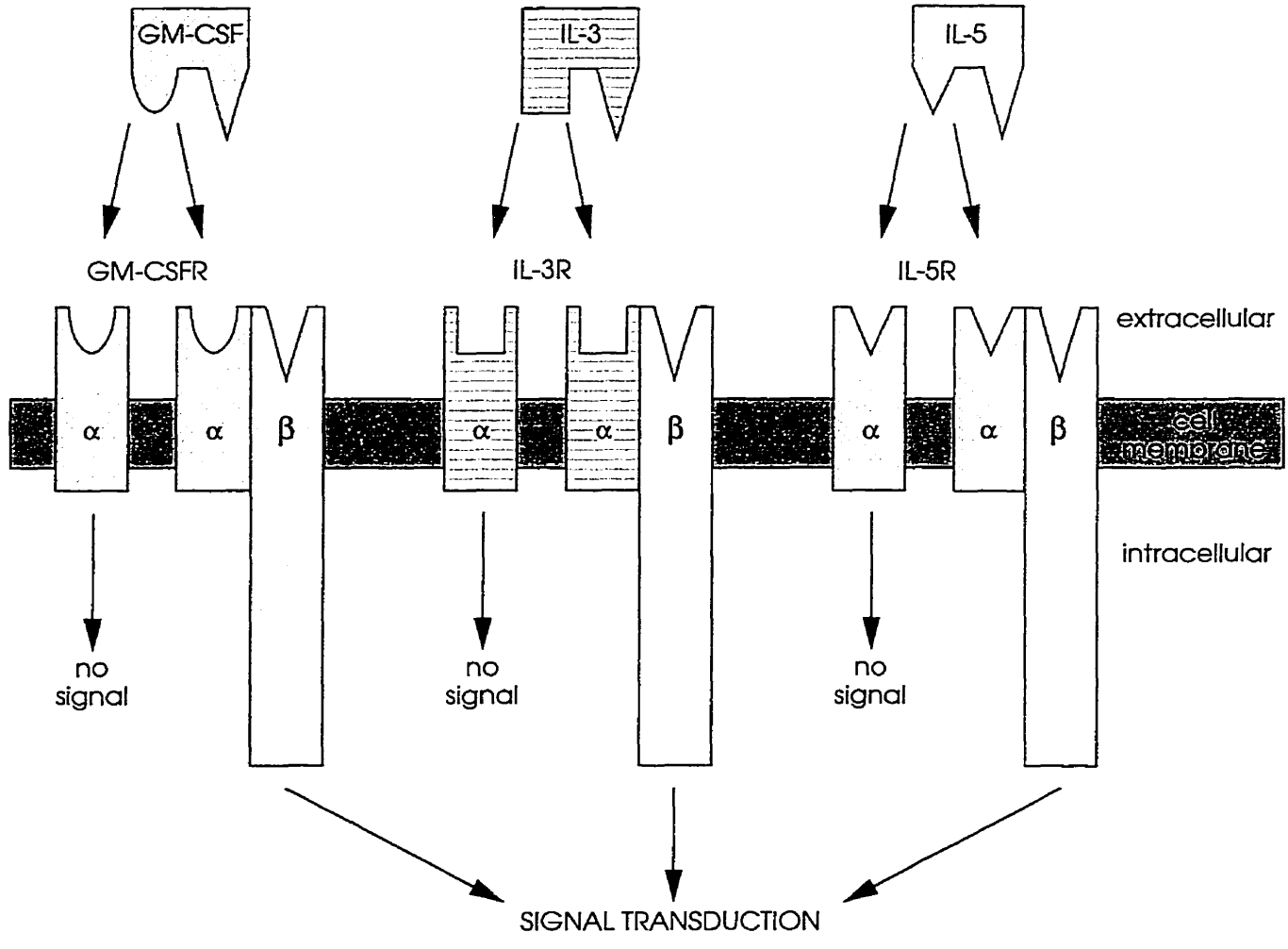

Figure 1.3. Interaction of GM-CSF, IL-3, and IL-5 receptors with their ligands (adapted from Goodall *et al.*, 1993, (ref. 223))

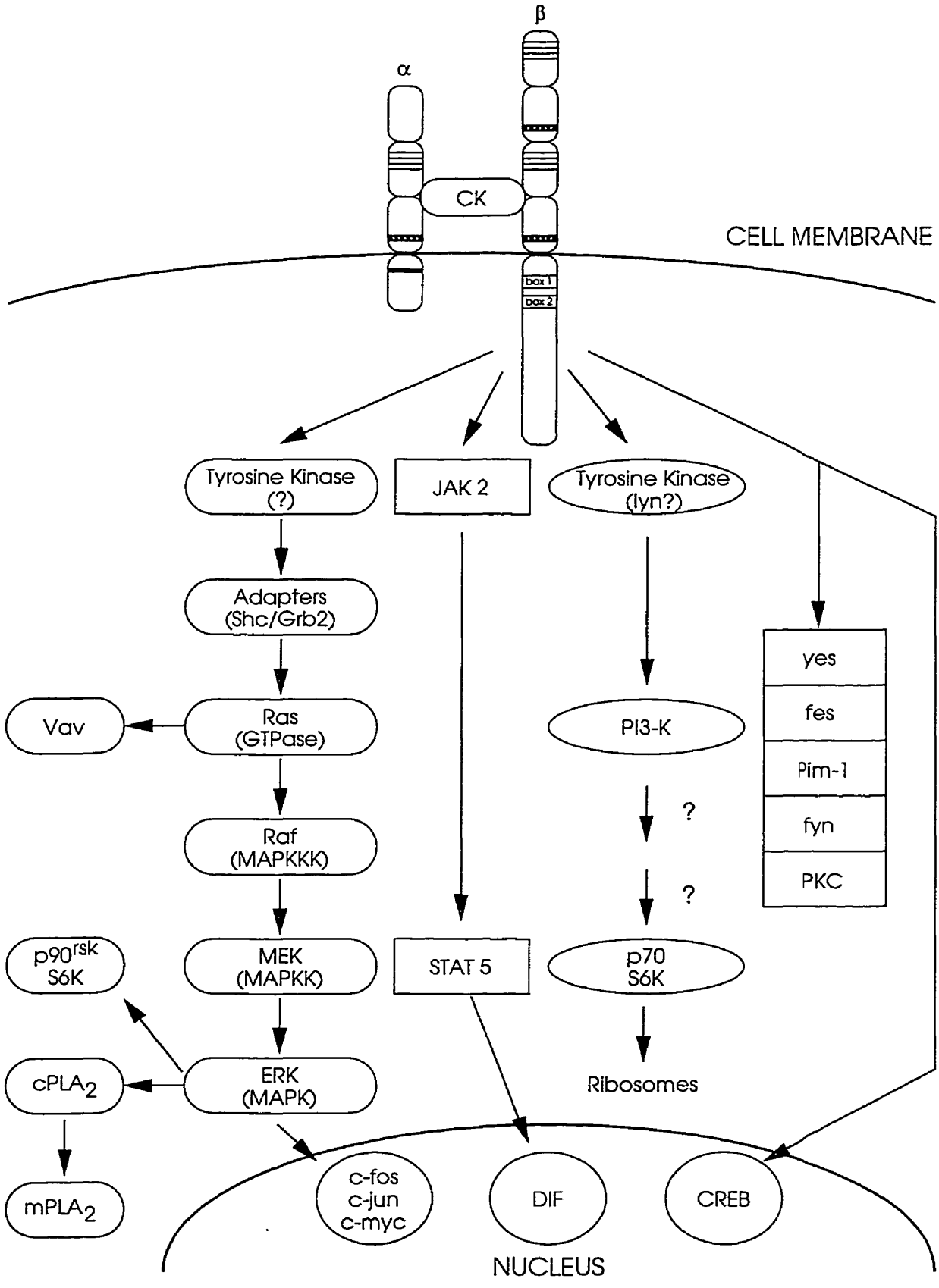

Figure 1.4. Hematopoietic cytokine receptor signal transduction (adapted from Gomez-Cambronero, J. and Veatch, C., 1996, (ref. 229))

The first pathway depicted for IL-3, GM-CSF, and IL-5 signalling was the Ras-MAP kinase pathway. In this pathway intracellular signalling molecules, including the ß-subunit, are sequentially activated. Initially, small adaptor proteins (Shc/Grb2), which are mainly composed of SH2 and SH3 (Src Homology) domains, are activated by an unknown tyrosine kinase. The identity of a tyrosine kinase that interacts with the ß-subunit or is immediately activated by cytokines has been under continuous investigation. Bruton's tyrosine kinase (Btk) has been shown to be involved in IL-5 signalling in B cells, however, it is not known if a similar tyrosine kinase acts during IL-3/GM-CSF signalling in myeloid cells (230). Similarly, Lyn, Fps/Fes and Tec tyrosine kinases have been implicated in IL-3/GM-CSF signalling in certain cell types (231-233). The role of these kinases in the IL-3/GM-CSF signalling pathway, however, remains unknown since some cells that lacked these tyrosine kinases were also able to respond to IL-3/GM-CSF. Although the tyrosine kinase that activates Shc is not known, phosphorylation of Shc by GM-CSF is recognized to require the tyrosine residue at 577 of the ß-subunit (234). These adaptor proteins, Shc/Grb2, form a complex with Sos, a Ras GDP/GTP nucleotide exchange factor, and this complex binds to the tyrosine-phosphorylated ß-subunit resulting in the recruitment of Sos to the cell membrane where Ras activation occurs. Sos has also been shown to be recruited to its substrate via PTP-1D, a protein tyrosine phosphatase, indicating that activation of Ras may be mediated by multiple pathways. Ras is a small molecular weight, membrane-associated, GTP binding protein that is involved in signalling. The GTP-bound active and GDP-bound inactive states are mediated by responses to extracellular signals. The guanine conversion of Ras can be controlled by activation of its intrinsic GTPase activity, by proteins such as GAP or by stimulation with Sos or Vav (235, 236). In hematopoietic cells GM-CSF/IL-3 induce tyrosine phosphorylation of Vav which may couple these receptors to Ras (237). Vav is thought to serve the same function as the Shc/Grb2/Sos complex by activating Ras directly (238, 239), however, recent data has indicated that

Vav is more effective at activating Rac-1 than Ras proteins (240). Thus, the role of Vav in activating Ras is unclear. Also in hematopoietic cells GM-CSF has recently been shown to promote the formation of an additional receptor-associated complex, Shc/Grb2/p140. The function of p140 is currently unknown, however, it is constitutively phosphorylated on tyrosine residues and exists complexed to Grb2 (241).

The elevation of Ras-GTP leads to the phosphorylation and activation of Raf, a serine/threonine kinase, with ensuing activation of MAPK kinase, a protein-tyrosine/threonine kinase. MAPK kinase subsequently regulates MAP kinase, a serine/threonine kinase, leading to activation of phospholipases ($cPLA_2$), other kinases (ribosomal S6 kinase $p90^{rsk}$), and various transcription factors (c-fos, c-jun). In addition, MAPK kinase is involved in the translocation and sequential activation of 5-LO, as stated earlier.

The membrane proximal box-1 region of ß-subunit cooperatively works with the distal part of the ß-subunit to maintain the function of the above pathway. While, the membrane distal region of ß-subunit is required for activation of the Ras-Raf-MAPK pathway (234, 242), the membrane proximal region of ß-subunit is required for activation of the JAK-STAT pathway. This second pathway, is now known to play a critical role in cytokine signalling (243). It is initiated by the tyrosine phosphorylation of the ß-subunit receptor by JAK (Janus kinase or Just Another Kinase). The JAK family consists of JAK1, JAK2, JAK3, and Tyk2. These 120-140 kDa soluble cytosolic proteins contain five regions of homology in the N-termini and possess a kinase-like domain and a tyrosine kinase domain at the C-terminus. JAKs associate with the intracellular region of the ß-subunit and are thought to become activated when cytokines bind to their receptors and initiate receptor dimerization (244). Dimerization causes JAKs to come into close proximity, enabling them to crossphosphorylate and activate one another and subsequently phosphorylate the common ß-subunit. This activity may account for the tyrosine kinase activity that is not intrinsic to the

IL-3/GM-CSF/IL-5 receptor cytoplasmic domains. Different JAKs associate with cytokine receptors and have been implicated in mediating signalling by these receptor molecules. In the IL-3/GM-CSF/IL-5 cytokine signalling system, JAK2 is associated with the common β-subunit (244). GM-CSF induces rapid tyrosine phosphorylation of JAK2 in several myeloid cell lines and in neutrophils (245, 246). Activation of JAK2 by GM-CSF correlates with increased cell proliferation and the activation of STAT5 (Signal Transducers and Activators of Transcription), as well as, the recently identified STAT5-like proteins, STAT5a and STAT5b (246, 247). IL-3/GM-CSF/IL-5 mainly utilize STAT5a and STAT5b, which are highly homologous and have indistinguishable functions (248, 249). JAKs phosphorylate and activate STATs, 80-120 kDa DNA-binding proteins. Phosphorylated STATs dimerize, translocate to the nucleus, bind to distinct DNA sequences, and affect the transcription of specific genes. To date, seven members of the STAT family have been reported. Although, each cytokine activates a relatively distinct set of JAKs and STATs, there does not appear to be a specific association between JAKs and STATs themselves. STATs possess an SH2 domain and can be recruited to the tyrosine-phosphorylated receptors, indicating that the specificity of STATs activated by a given cytokine may be determined by the cytoplasmic domain of the cytokine receptor. The final step in this pathway is the recruitment of another specific gene transcription factor, the differentiation-induced factor (DIF), and the concomitant activation of DNA transcription.

Interestingly, in human eosinophils, JAK2 constitutively associates with the IL-3/GM-CSF/IL-5 β-subunit and is tyrosine phosphorylated after stimulation with IL-5. Although JAK2 activation occurs in this system, the nuclear factor STAT1 is induced by IL-5 in eosinophils (250, 251). It is possible that the JAK-STAT1 pathway plays a role in the activation of IL-5-inducible genes in eosinophils.

Activation of PI 3-kinase is the third pathway described. This pathway is also initiated following phosphorylation of the β-subunit of the GM-CSF

receptor and results in an accumulation of phosphatidylinositol-(PI)-3,4,5-triphosphate and the activation of ribosomal S6 kinase (p70 S6K) via a PI 3-kinase (252, 253). PI 3-kinase contains an SH2 domain which may link this protein to activated cytokine receptors.

The latest pathway to arise describes a variety of other molecules that are activated by GM-CSF, such as c-fes, c-yes, pim, PKC, and CREB.

1.7 Asthma

Asthma is characterized by reversible airway obstruction, bronchial hyperreactivity, and airway inflammation (188). The pathology of this disease is very complex and involves many lipid mediators and cytokines that are produced by a variety of proinflammatory cells, especially mast cells, eosinophils, and T-lymphocytes. Bronchial biopsies from patients even with mild asthma demonstrated inflammatory changes characterized by a cellular infiltration of eosinophils, mast cells, and activated T cells, as well as epithelial damage and desquamation (254, 255), with the expression of cytokines, such as IL-3, IL-4, IL-5, and GM-CSF (256-258). The consequences of these inflammatory responses are increased vascular permeability, stimulation of local and central neural reflexes, epithelial damage, increased mucus production, smooth muscle hypertrophy, and airway wall remodelling (259).

Asthmatic responses to allergen challenge consist of both an early phase and a late phase. The early asthmatic response occurs immediately following antigen challenge and is most likely due to the release of bronchoconstrictive mediators (LTC_4, LTD_4, histamine) from IgE-activated mast cells. The late asthmatic response occurs several hours after antigen challenge and is characterized by an influx of activated proinflammatory cells (TH_2 type T cells, eosinophils, and neutrophils) with a subsequent release of mediators (188). IL-5 and GM-CSF are among the mediators released and are speculated to stimulate hematopoiesis associated with

the inflammatory reaction (228). These cytokines have been implicated as mediators in the pathology of asthma (260) for their ability to cause vasculature and lung eosinophilia, prime eosinophils for enhanced metabolic activity (increased LTC_4 generating capacity and migration capacity), as well as activate eosinophils resulting in the release of cytotoxic granule proteins; all of which are characteristics of asthma. IL-5 and GM-CSF transcripts have also been detected in lung tissue of asthmatics, but not in normal individuals. Similarly, circulating factors (IL-3, IL-5, and GM-CSF) were only present in the circulation of asthmatic patients (261), indicating that these cytokines may be key factors in the development of asthma.

1.7.1 Glucocorticoids

The recognition of the importance of inflammation in asthma has lead to the use of inhaled corticosteroids as the mainstay of asthma therapy (262). Although glucocorticoids are the most effective anti-inflammatory agents currently available for the treatment of asthma, the molecular mechanisms of steroid action are just beginning to be elucidated. The mechanism of action of glucocorticoids is presumably through activated glucocorticoid receptors (GR) (263, 264). Glucocorticoids easily cross cell plasma membranes and can bind to glucocorticoid receptors in the cytoplasm. These complexes then translocate to the nucleus where they bind to specific glucocorticoid response elements (GRE) and induce gene transcription of anti-inflammatory proteins (ie. β_2-adrenergic receptor, lipocortin, neutral endopeptidase, and inhibitors of plasminogen activator). The primary activity of glucocorticoids, however, may be their ability to inhibit gene transcription. Glucocorticoids can suppress gene transcription of pro-inflammatory proteins by binding directly to negative GREs or by interacting with transcription factors (ie. AP-1, NF-κB (increased IκBα transcription) and

CREB). Glucocorticoids can also suppress gene expression by decreasing the stability of specific mRNA transcripts. Collagenase, elastase, plasminogen activator, nitric oxide synthase, COX-II, and most cytokine and chemokine genes are among the glucocorticoid-suppressed genes. Four classes of cytokines have been described to be released in asthma: 1) non-specific endothelial activators (TNF-α and IL-1), 2) specific endothelial activators (IL-4 and IL-13), 3) cytokines that promote eosinophilia (IL-3, GM-CSF, and IL-5), and 4) chemokines (RANTES, MCP-3, eotaxin, MIP-1α, CK-ß10). Thus, glucocorticoids may be efficacious for the treatment of asthma due to their inhibitory effects on several proinflammatory cytokines (for review see (265)).

Although glucocorticoids are relatively safe and effective for the treatment of asthma, these drugs do display some deleterious effects (266). Glucocorticoids may suppress growth in children and possibly cause immunosuppression in the elderly. High doses (>4 mg/day) can cause adrenal suppression and long term use may result in bone loss. Glucocorticoids also display mild side effects such as: development of pharyngeal and laryngeal candidiasis, hoarseness, xerostomia (dry mouth), throat irritation and dysphonia. These adverse effects in combination with poor patient compliance and bad technique have prompted the discovery of alternative agents for the treatment of asthma. The main group of alternative agents are directed against leukotrienes. To date there are four classes of drugs that interfere with leukotriene synthesis or activity. These drugs are either synthesis inhibitors, which block the actions of 5-LO or FLAP, or receptor antagonists, which inhibit the actions of the cysteinyl leukotrienes or LTB_4. The cysteinyl leukotrienes have been particularly implicated as important mediators in the pathophysiology of asthma and have become the leading targets for potential new asthma therapies. Although many mediators are likely to play a role in inducing the characteristic features of asthma, there is now compelling evidence for an important role for cysteinyl leukotrienes in asthma.

1.7.2 Leukotriene inhibitors

Leukotriene biosynthesis inhibitors suppress the formation of both cysteinyl leukotrienes and LTB_4 by either directly blocking the catalytic activity of 5-LO (Zileuton, ABT-761 and Z-D2138) or by binding to and inactivating FLAP (MK-0591 and MK-886). In clinical trials, Zileuton has exhibited sustained improvement in lung function and appears to affect the underlying inflammatory process as demonstrated by its steroid-sparing activity (189, 267). FLAP inhibitors bind to FLAP and prevent the translocation of 5-LO to the membrane resulting in an inhibition of leukotriene biosynthesis (268). MK-886 blocks both FLAP and LTC_4 synthase, however, it is not known if this inhibition of two consecutive steps in the biosynthetic pathway improves its efficacy (189). MK-0591 blocked the early phase of antigen-induced bronchoconstriction by 79% and the late phase by 39% (269). These results were similar to those of several cysteinyl leukotriene receptor antagonists and most likely represent the maximum effect that leukotriene inhibitors can display in asthma therapy.

There are two classes of leukotriene receptor antagonists, cysteinyl leukotriene receptor antagonists and B-LT receptor antagonists. The former block the activity of LTC_4, LTD_4, and LTE_4 at the cys-LT_1 receptor (zafirlukast, pranlukast, verlukast, montelukast) and have been shown to be extremely efficacious by their ability to reduce LTD_4-induced bronchoconstriction and to diminish the early and late phase response after allergen exercise challenge. B-LT receptor antagonists competitively block the activity of LTB_4 (U-75,302). Although the role of LTB_4 in asthma is still inconclusive, guinea-pig and rat models of asthma have demonstrated that a specific B-LT receptor antagonist (U-75,302) reduces both eosinophil influx into the lung (270) and the increased bronchial responsiveness to allergen challenge (271). A recent study in man shows B-LT receptor antagonists have an effect on neutrophil influx with no clinical activity. It is unknown if leukotriene synthesis inhibitors have an advantage over leukotriene

receptor antagonists.

Since leukotrienes have been demonstrated to play an important role in the pathology of asthma, understanding the mechanism of regulation of leukotriene biosynthesis is extremely important and may lead to novel therapeutic targets!

2. RESEARCH OBJECTIVES

Leukotrienes are potent mediators of inflammatory and allergic immediate hypersensitivity reactions and are primarily synthesized by myeloid cells. LTC_4 synthase and the recently discovered protein, microsomal GST-II, are enzymes catalyzing the first committed step for the conversion of LTA_4 to the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, which comprise the slow reacting substance of anaphylaxis. These leukotrienes are responsible for bronchoconstriction, mucus hypersecretion, vasoconstriction, and vascular permeability changes, which are important characteristics of bronchial asthma. Although the biosynthesis and biological effects of these mediators have been well characterized, little is known about the mechanisms controlling their regulation. Understanding the regulation of the biosynthesis of these compounds could lead to the development of new therapeutic agents for the treatment of asthma. Leukotriene biosynthesis has been shown to be governed either by direct phosphorylation, or by gene regulatory mechanisms that alter gene transcription and/or translation. Thus, the objectives of this study were:

1. To study the effects of cytokines on leukotriene biosynthesis in a new eosinophil cell line, HL-60#7/BA, to ascertain the mechanism of cytokine regulation of leukotriene biosynthesis in eosinophils.

2. To investigate the control of leukotriene biosynthetic enzymes during differentiation of myeloid cells to either eosinophils or neutrophils.

3. To determine the physiological location of microsomal GST-II.

4. To investigate the relative importance of microsomal GST-II versus LTC_4 synthase in the production of LTC_4 in various human tissues and cells that have been previously demonstrated to possess LTC_4 synthesizing activity.

3. STATEMENT OF CONTRIBUTIONS

*Manuscripts and Authorship:

Candidates have the option of including, as part of their thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (eg. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

*reproduced from *Guidelines for Thesis Preparation*, Faculty of Graduate Studies and Research, McGill University

This thesis is composed of 3 manuscripts and the contributions of co-authors and myself are described below:

A. Scoggan, K.A., Ford-Hutchinson, A.W. and Nicholson, D.W. (1995) Differential activation of leukotriene biosynthesis by granulocyte-macrophage colony-stimulating factor and interleukin-5 in an eosinophilic substrain of HL-60 cells. *Blood* 86 (9): 3507-3516.

All of the experiments described in this paper were planned and performed by myself. This manuscript was also written by myself. The work was supervised by Drs. D.W. Nicholson and A.W. Ford-Hutchinson.

B. Scoggan, K.A., Nicholson, D.W. and Ford-Hutchinson, A.W. (1996) Regulation of leukotriene-biosynthetic enzymes during differentiation of myelocytic HL-60 cells to eosinophilic or neutrophilic cells. *European Journal of Biochemistry* 239: 572-578.

All of the experiments described in this paper were planned and performed by myself. This manuscript was also written by myself. The work was supervised by Drs. D.W. Nicholson and A.W. Ford-Hutchinson.

C. Scoggan, K.A., Jakobsson, P.-J., and Ford-Hutchinson, A.W. (1997) Production of leukotriene C_4 in different human tissues is attributable to distinct membrane bound biosynthetic enzymes. *Journal of Biological Chemistry* 272: 10182-10187.

All of the experiments described in this paper were planned and performed by myself. This manuscript was also written by myself. The work was supervised by Drs. P.-J. Jakobsson and A.W. Ford-Hutchinson.

4. MANUSCRIPT A

Differential Activation of Leukotriene Biosynthesis by Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-5 in an Eosinophilic Substrain of HL-60 Cells

Blood 86: 3057-3516 (1995)

Leukotrienes are key players in pathological disorders. Thus understanding the mechanism of regulation of their biosynthesis is of great importance. This paper presents evidence that GM-CSF primes leukotriene biosynthesis in a dose-dependent manner in eosinophilic HL-60 cells by enhancing the rate of leukotriene formation at an earlier catalytic event than LTC_4 synthase in the biosynthetic pathway. This paper also presents evidence that priming of leukotriene biosynthesis may be differentially regulated by GM-CSF and IL-5, as IL-5 had no effect on leukotriene biosynthesis in this cell line. This differential priming effect was not due to differences in receptor populations, affinities, or ligand-receptor complex stabilities of GM-CSF and IL-5. Phosphotyrosine patterns suggest that IL-5 is incapable of transducing a signal even though IL-5 and GM-CSF share a common beta chain component of their receptors that is required for signal transduction. This unique cell line may permit the dissection of distinct events responsible for specific intracellular signals transduced separately by GM-CSF or IL-5.

DIFFERENTIAL ACTIVATION OF LEUKOTRIENE BIOSYNTHESIS BY GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND INTERLEUKIN-5 IN AN EOSINOPHILIC SUBSTRAIN OF HL-60 CELLS

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ABBREVIATIONS

LT	leukotriene
LTA_4	5,6-oxido-7,9,11,14-eicosatetraenoic acid
LTB_4	5(S), 12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid
LTC_4	5(S)-hydroxy-6(R)-S-glutathionyl-7,9,trans-11,14-cis eicosatetraenoic acid
LTD_4	5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTE_4	5(S)-hydroxy-6(R)-S-cysteinyl-7,9-trans-11,14-cis eicosatetraenoic acid
PGB_2	prostaglandin B_2
PKC	protein kinase C
rhGM-CSF	recombinant human granulocyte-macrophage colony stimulating factor
rhIL-5	recombinant human interleukin-5
PLA_2	phospholipase A_2
5-LO	5-lipoxygenase
FLAP	five-lipoxygenase activating protein
ECL	enhanced chemiluminescence

ABSTRACT

Cytokines can stimulate eosinophils to produce cysteinyl leukotrienes in the lung which provoke tissue destruction associated with asthma. Priming of an eosinophilic substrain of HL-60 cells (HL-60#7) with recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) prior to ionophore challenge was found to produce an apparent 45% increase in total leukotriene production in a dose-dependent manner (ED_{50}=150 pM) which could be accounted for by a decrease in the time required for maximal formation of leukotrienes. GM-CSF had no effect on the kinetic parameters of LTC_4 synthase and therefore probably acts upstream of this catalytic event. Incubation with interleukin-5 (IL-5), however, had no effect on leukotriene biosynthesis. This differential priming ability was not a consequence of different receptor populations or differences in the affinity or stability of the ligand-receptor complexes of GM-CSF and IL-5. GM-CSF and IL-5 each displayed similar populations of high-affinity binding sites and neither GM-CSF nor IL-5 were able to cross-compete for the other's receptor binding sites. Analysis of phosphotyrosine patterns suggest that IL-5 is incapable of transducing a signal in eosinophilic HL-60#7 cells even though IL-5 and GM-CSF receptors mediate signal transduction via a common ß-chain component which is also necessary for high affinity binding. Overall, this unique system may permit the dissection of distinct events responsible for specific intracellular signals transduced separately by GM-CSF or IL-5.

INTRODUCTION

Leukotrienes (LTs), which are derived through the 5-lipoxygenase pathway of arachidonic acid metabolism, are lipid mediators of inflammation and immediate hypersensitivity (1-5). LTB_4, a mediator of inflammation, has been shown to activate leukocytes, stimulating chemokinesis, chemotaxis, and cell adherence, whereas the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4 (collectively comprise the slow-reacting substance of anaphylaxis) can induce bronchoconstriction, mucous hypersecretion, vasoconstriction, and vascular permeability changes and are thus thought to be important mediators of bronchial asthma (for review see (4-8). Because leukotrienes have important pathological implications, determining the mechanism by which their synthesis is regulated is of considerable interest.

It has been demonstrated that cytokines are not only involved in the differentiation and proliferation of pro-myelocytes, but that cytokines also modulate the activation of leukotriene biosynthesis in mature myeloid cells (9-12). Priming of eosinophils and other cells with GM-CSF or IL-5 has been shown to enhance leukotriene production following stimulation with calcium ionophore through a mechanism that is incompletely understood (13,14). GM-CSF, IL-3 and IL-5 receptors all belong to the hematopoietic growth factor receptor superfamily. These heterodimeric receptors each consists of a cytokine-specific α-chain component and a common ß-chain component (15-21). The α-chain is believed to be responsible for the specific binding of the distinct ligands to each receptor and in the absence of the ß-chain is capable of binding the ligand with low affinity without signal transduction (16,18,20-24). Both the α-chain and the ß-chain, however, are required for high-affinity binding of the ligand to the receptor, resulting in activation of the signalling pathway, probably through the intracellular portion of the ß-chain (17,21,25,26). An understanding of the ligand-receptor mechanism for up-regulation of leukotrienes in eosinophils is

important since eosinophils are a major source of cysteinyl leukotriene production (27,28).

Evidence suggests that IL-5, released by activated T-cells in the lung (29), can activate mature pulmonary eosinophils. This can result in up-regulation of the production of mediators, such as leukotrienes and free radicals, and the release of cytotoxic granule contents including major basic protein. IL-5 can also serve to increase eosinophil migration into the lung by enhancing differentiation of myeloid precursor cells towards eosinophils and by acting as a chemotactic agent for circulating eosinophils (30).

In the present study, the effects of cytokines on a new eosinophilic cell line, HL-60#7, were investigated to ascertain the mechanism of regulation of leukotriene biosynthesis in eosinophils.

MATERIALS AND METHODS

Materials

HL-60 cells (CCL 240) were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media, antibiotics, fetal bovine serum, *n*-butyric acid, phorbol 12-myristate 13-acetate, acivicin, A23187, bovine serum albumin, phenylmethylsulfonyl fluoride, leupeptin, soybean trypsin inhibitor, and pepstatin were purchased from Sigma Chemical Co. (St. Louis, MO). LTA_4-methyl ester was synthesized by the Department of Medicinal Chemistry at the Merck Frosst Centre for Therapeutic Research. Sep-Pak C_{18}-light cartridges and Novapak C_{18} HPLC columns were from Waters Chromatography (Milford, MA). Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), recombinant human interleukin-5 (rhIL-5) and carrier free IL-5 were obtained from R & D Systems (Minneapolis, MN). [^{125}I]GM-CSF and [^{125}I]IL-5 were from New England Nuclear (Mississauga, Ontario) or prepared using IODO-BEADS Iodonation Reagent from Pierce (Rockford, Illinois). HPLC solvents were from BDH (Toronto, Ontario). PGB_2 was purchased from Cayman Chemical Co. (Ann Arbor, MI). The anti-phosphotyrosine monoclonal antibody, 4G10, was from Upstate Biotechnology Incorporated (Lake Placid, NY). Enhanced chemiluminescence (ECL) kit and horseradish peroxidase-linked goat anti-mouse antibody were obtained from Amersham Life Sciences (Buckinghamshire, England). Sodium dodecyl sulfate and Tween-20 were from BIO RAD (Richmond, CA). Triton X-100 and pre-made blocking buffer (Superblock) were purchased from Pierce (Rockford, Illinois). Sodium orthovanadate was from Fisher Scientific (Fair Lawn, NJ) and Tris/HCl was from BRL (Gaithersburg, MD). All other reagents were of analytical grade and were purchased from Sigma.

Growth and Differentiation of Eosinophilic HL-60#7 Cells

The HL-60/MF211#7 cell line (abbreviated HL-60#7 below), a substrain of HL-60 cells with a high propensity for eosinophilic differentiation, has been developed by long term subculturing under alkaline conditions and continuous selection of substrains expressing eosinophilic markers when cultured in the presence of butyric acid (31). This pro-eosinophilic cell line was propagated by subculturing at a seed density of 0.2 x 10^6 cells / mL into fresh sterile RPMI 1640 (supplemented with 0.2% (w/v) sodium bicarbonate and 0.03% (w/v) L-glutamine) containing 50 U / mL penicillin and 50 µg / mL streptomycin, and 10% (v/v) fetal bovine serum (Sigma, Hybri-Max, not heat-inactivated). Cultures were grown at 37°C in a humidified atmosphere containing 6% CO_2 in either 175-cm^2 culture flasks or spinner flasks (25 rpm). To differentiate the HL-60#7 cell line into eosinophilic cells, cultures were seeded at 0.2 x 10^6 cells / mL in fresh medium (described above) and supplemented with 0.4 mM *n*-butyric acid (added from a 150 mM stock in ethanol). The cells were grown without further subculturing for up to 7 days. (Note that differentiation in spinner cultures lagged behind that in T-flasks by 1-2 days, and that differentiated cells could not be propagated). Cells were harvested by centrifugation at 500 x g for 15 min at room temperature. The resulting pellet was washed by resuspending the cells in Dulbecco's phosphate buffered saline (dPBS) and re-sedimenting them at 500 x g for 15 min.

Cytokine Priming and Measurement of Leukotriene Production in Ionophore-Challenged HL-60#7 Cells

Butyric acid differentiated HL-60#7 cells were harvested, washed with Dulbecco's phosphate buffered saline (dPBS), and resuspended at 20 x 10^6 cells / mL in dPBS containing 1 mM EDTA and 1 mg / mL D-glucose. Unless otherwise indicated, cells were incubated with 200 pM cytokine for 30 min at 37°C then $CaCl_2$ (2 mM final concentration) was added to the mixture which was incubated for an additional 8 min with constant orbital

shaking. Calcium ionophore, A23187, was added to a final concentration of 1 μM and the reaction was stopped after 3 min at 37°C by combining the mixture with an equal volume of ice-cold methanol containing 0.2 nmol / mL prostaglandin B_2 (as an internal HPLC standard).

After allowing the terminated reaction mixture to stand for at least 15 min at 4°C, precipitated proteins were removed by centrifugation for 15 min at 150 x g at 4°C. The supernatant was diluted with H_2O to a final volume of 10 mL and respun for 15 min at 150 x g at 4°C. Leukotrienes were purified from the resulting supernatant by applying it to a Sep-Pak C_{18} cartridge (Waters Associates), which had been pre-conditioned with 10 mL H_2O, 10 mL methanol then 10 mL H_2O, at a maximum flow rate of 1 mL / min. After washing the cartridge with 20 mL H_2O, the bound leukotrienes were eluted with 1 mL methanol. The eluent was dried by vacuum centrifugation and resuspended in 200 μL HPLC mobile phase (acetonitrile: methanol: water: acetic acid at 54:14:28:1, pH 5.6). Leukotrienes were then resolved by isocratic reverse-phase HPLC on a Novapak C_{18} column (3.9 x 150 mm, Waters associates) in the mobile phase at a flow rate of 1.2 mL / min. The column effluent was monitored by on-line measurement of the absorbance at 280 nm. Leukotrienes were identified by retention-time matching to synthetic standards, and quantities were adjusted to the recovery of the internal standard, PGB_2.

Measurement of Leukotriene C_4 Synthase Enzymatic Activity

Butyric acid differentiated HL-60#7 cells were harvested, washed with Dulbecco's phosphate buffered saline (dPBS), and resuspended at 10 x 10^6 cells / mL in dPBS containing 1 mM EDTA and 1 mg / mL D-glucose. Cells were incubated with 200 pM cytokine for 30 min at 37°C and subsequently with $CaCl_2$ (2 mM final concentration) for 8 min at 37°C as described above. Acivicin (2.5 mM) and LTA_4 (5 μM unless otherwise indicated) were added to the incubation mixture and after 4 min at 37°C with gentle shaking the reaction was stopped by combining the mixture

with an equal volume of cold methanol containing 0.9 nmol / mL prostaglandin B_2. Leukotrienes were enriched by solid phase extraction and resolved by isocratic HPLC as described above.

Analysis of GM-CSF and IL-5 Binding to HL-60#7 Cells

HL-60#7 cells, differentiated by growth in the presence of butyric acid for 5-6 days, were harvested, washed with dPBS, and resuspended to a final density of 40 x 10^6 cells / mL in dPBS containing 0.1% (w/v) bovine serum albumin (BSA) and 0.2% (w/v) sodium azide. Unless indicated otherwise, equilibrium binding mixtures containing 4 x 10^6 cells and 1.0 nM radioiodonated ligand ($[^{125}I]$rhGM-CSF or $[^{125}I]$rhIL-5) were incubated in the presence or absence of 100-fold molar excess rhGM-CSF or rhIL-5, respectively, for 60 min at 37°C with gentle orbital shaking. Cells were harvested by centrifugation at 6,600 x g for 90 sec and washed twice with 1 mL dPBS containing 0.1% (w/v) BSA. Resuspended pellets and combined supernatants were counted for 1 min in a gamma counter to measure the cell associated and free radioactivity, respectively. Specific binding to differentiated HL-60#7 cells was deduced by subtracting non-specific radioligand binding (samples with rhGM-CSF or rhIL-5 present) from total radioligand binding (no rhGM-CSF or rhIL-5 present).

Western Blot Detection of Phosphorylated Tyrosines

Butyric acid differentiated HL-60#7 cells were harvested and treated with 200 pM rhGM-CSF, rhIL-5, or dPBS (as a control) for 30 min at 37°C as described above. An equivalent aliquot (600 µL) from each incubation mixture was removed, centrifuged for 10 sec at 16,000 x g, and resuspended in 180 µL of cold solubilization buffer (50 mM Tris/HCl (pH 7.7), 1% (v/v) Triton X-100, 10% (v/v) glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na orthovanadate, 1 mM Na molybdate, 40 µg /mL PMSF, 1 µM pepstatin, 0.5 µg / mL leupeptin, 10 µg / mL soybean trypsin inhibitor). The solubilized sample was rapidly centrifuged for 1 min at 16 000 x g at

4°C. The supernatant was transferred to a tube containing 1/4 the volume of 4 x sodium dodecyl sulphate (SDS) sample buffer and immediately incubated for 1 min at 90°C. The samples were then run on a 14% Novex SDS/polyacrylamide gel then electroblotted onto nitrocellulose. The transfer efficiency was visualized by Ponceau S staining. The membrane was soaked for 1 h at 37°C in a non-specific site-blocking agent (Pierce Superblock), and subsequently exposed to 0.1 μg / mL 4G10 anti-phosphotyrosine monoclonal antibody for 1 h at 37°C. The membrane was then washed three times with Tris-buffered saline containing Tween 20 (25 mM Tris/HCl (pH 7.6), 0.15 M NaCl, 0.1% (v/v) Tween 20) for 15 minutes each at 25°C, and treated with a horseradish peroxidase-linked goat anti-mouse antibody (dilution 1:20 000) in Tris-buffered saline containing Tween 20 for 30 minutes at 37°C. After three washes, the blot was developed using enhanced chemiluminescence (ECL kit, Amersham International plc), according to the manufacturer's instructions.

RESULTS

rhGM-CSF priming increases the rate of leukotriene formation in ionophore challenged eosinophilic HL-60#7 cells differentiated with butyric acid

A pro-eosinophilic substrain of the myelocytic leukemia cell line HL-60 (HL-60#7) was differentiated with butyric acid and used as a model for studying leukotriene biosynthesis in eosinophils. These cells resemble eosinophils in many different aspects. Morphologically, butyric acid differentiated HL-60#7 cells have prominent eosin-staining granules that contain eosinophil major basic protein and eosinophil peroxidase. Functionally, eosinophilic HL-60#7 cells are chemotactic and respond to mediators with similar rank orders of potency as eosinophils (RANTES> PAF> LTB_4> C5a> LTD_4> fMLP). These cells also express pertinent cytokine and leukotriene receptors (IL-5, RANTES, GM-CSF, LTB_4 and LTD_4) as well as relevant cysteinyl leukotriene biosynthetic enzymes such as LTC_4 synthase (31).

As has been observed for eosinophils isolated from peripheral human blood, this cell line synthesizes predominantly cysteinyl leukotrienes when stimulated with the calcium ionophore A23187 (Figure 1) (13). Since cytokines appear to mediate activation of leukotriene biosynthesis in mature myeloid cells (9-12), the effects of cytokine pre-treatment on this eosinophilic cell line were investigated. Priming of HL-60#7 cells with rhGM-CSF was found to enhance ionophore-stimulated leukotriene biosynthesis in a dose dependent manner (ED_{50}=150 pM) reaching a maximum at 200 to 400 pM rhGM-CSF (Figure 2a). HL-60#7 cells incubated with PBS (control), rhGM-CSF or rhIL-5 for 30 min and prior to calcium ionophore challenge showed that rhGM-CSF priming increased total leukotriene biosynthesis by about 45% whereas rhIL-5 had no effect on the formation of leukotrienes (Figure 2b). Leukotriene biosynthesis could not be primed with concentrations of IL-5 tested in the range of 3 pM to 1 nM (data not

shown).

Since cytokines are involved in cellular differentiation as well as activation, their effects may differ depending on the stage of cell maturation. To initiate differentiation, HL-60#7 cells were cultured for a varying number of days with butyric acid, which converts pro-eosinophilic cells towards mature eosinophilic cells. The ability of these cells to produce leukotrienes after ionophore challenge was then measured with or without rhGM-CSF or rhIL-5 pre-treatment (Figure 3). After 5 days of culturing cells with butyric acid, the rhGM-CSF priming effect was maximal while rhIL-5 pre-treatment had no effect on leukotriene production regardless of the stage of differentiation.

To determine if rhGM-CSF affected the amount of leukotrienes produced or their rate of biosynthesis, HL-60#7 cells were pre-incubated in the presence or absence of rhGM-CSF and then after varying times subsequent to ionophore challenge total leukotriene formation was measured (Figure 4a). In the absence of rhGM-CSF priming leukotriene biosynthesis occurred at a steady rate reaching a maximum 10 min after ionophore challenge. Pre-treatment with rhGM-CSF increased the rate of leukotriene production without affecting the total amount of leukotrienes formed. In both cases subsequent degradation was observed. The maximum leukotriene production in rhGM-CSF primed cells occurred 3 min after ionophore-challenge. Similar results were obtained measuring only cysteinyl leukotrienes (Figure 4b).

Kinetic parameters of LTC_4 synthase are not modulated by GM-CSF or IL-5 pre-treatment

It is possible that rhGM-CSF may enhance leukotriene formation by up-regulating one of the enzymes in the leukotriene biosynthesis pathway. LTC_4 synthase is the first committed enzyme in the cysteinyl leukotriene half of this biosynthetic pathway. As such, this enzyme may play a pivotal role in the regulation of leukotriene biosynthesis in cytokine-treated eosinophilic

cells. LTC_4 synthase has recently been purified to homogeneity and its N-terminal amino acid sequence subsequently deduced (32). A protein kinase C consensus sequence was identified within the amino-terminus of LTC_4 synthase and consistent with this, LTC_4 synthase has been shown to be negatively regulated following cellular PKC activation (33). In order to determine if cytokine-stimulated phosphorylation events could modulate leukotriene biosynthesis in HL-60#7 cells, the effects of rhGM-CSF or rhIL-5 pre-treatment on the K_m and V_{max} of LTC_4 synthase in HL-60#7 cells was investigated (Figure 5). HL-60#7 cells were pre-treated with rhGM-CSF or rhIL-5 for 30 min and subsequently incubated in the presence of varying concentrations of LTA_4, a co-substrate for LTC_4 synthase, and acivicin, an inhibitor of the γ-glutamyl transpeptidase which converts LTC_4 to LTD_4. After a 4 min incubation at 37°C, the amount of LTC_4 produced was measured by isocratic reverse-phase HPLC. The kinetic parameters, K_m and V_{max}, of LTC_4 synthase were found to be unaltered when HL-60#7 cells were pre-treated with either rhGM-CSF or rhIL-5. Thus, these cytokines do not affect cysteinyl leukotriene production by altering the activity of LTC_4 synthase.

Differential activation of eosinophilic HL-60#7 cells by GM-CSF and IL-5

rhGM-CSF was able to prime leukotriene production in eosinophilic HL-60#7 cells whereas rhIL-5 did not (see above). This finding was unexpected since we have previously demonstrated that eosinophilic HL-60#7 cells contain high-affinity (K_d = 19 pM) IL-5 binding sites (31) as well as mRNA for both the α and ß subunits of the IL-5 receptor (Scoggan *et al.*, unpublished). The characteristics of ligand binding and signal transduction for both GM-CSF and IL-5 were therefore investigated.

i) Similarity of binding sites for GM-CSF and IL-5 on eosinophilic HL-60#7 cells

One explanation for the differences in priming of leukotriene formation by GM-CSF versus IL-5 may be a differential in their respective

receptor populations. Accordingly, receptor binding assays were performed on eosinophilic HL-60#7 cells using (^{125}I)rhGM-CSF or (^{125}I)rhIL-5.

Initially, the time course of appearance of (^{125}I)rhGM-CSF or (^{125}I)rhIL-5 binding sites on HL-60#7 cells at various stages of eosinophilic differentiation was investigated. Cells were cultured for a varying number of days in butyric acid and then subsequently incubated with radioligand in the presence or absence of 100-fold excess of unlabelled competing ligand to ascertain the non-specific and total binding, respectively. Specific binding was deduced by subtracting the non-specific binding from the total bound. (^{125}I)rhIL-5 and (^{125}I)rhGM-CSF specific binding to HL-60#7 cells increased at similar rates with increasing days in culture with butyric acid, reaching a maximum after 5 days of differentiation (Figure 6). This result corresponded to the optimum priming effect previously observed with rhGM-CSF (Figure 3). Since maximum specific binding was obtained after culturing HL-60#7 cells for 5 days with butyric acid, these 5 day differentiated cells were used to produce saturation binding curves.

HL-60#7 cells were incubated with varying concentrations of (^{125}I)rhGM-CSF (Figure 7a) or (^{125}I)rhIL-5 (Figure 7b) in the presence or absence of 100-fold excess of unlabelled competing ligand and the specific binding was again determined by the difference between the total bound and the non-specific bound. Upon Scatchard transformation of the resulting saturation binding isotherms, it was found that GM-CSF and IL-5 each displayed a single population of high-affinity binding sites on eosinophilic HL-60#7 cells with dissociation constants of 86 pM and 25 pM, respectively. GM-CSF and IL-5 receptor binding sites were also shown to be present on the cells in equivalent abundance with about 200 receptor sites per cell. Therefore the differential priming effects of rhGM-CSF versus rhIL-5 on leukotriene biosynthesis cannot be explained by a difference in the population of high-affinity receptors.

ii) GM-CSF and IL-5 receptor populations in eosinophilic HL-60#7 cells cannot be cross-competed

GM-CSF and IL-5 cytokine receptors both consist of a specific α-chain and a common ß-chain component (15-21). On the surface of the cell membrane, these subunits must somehow form a complex, either before, or after ligand binding. One possibility that might account for the differential priming effect of GM-CSF versus IL-5 on leukotriene formation may be due to a difference between the stability of the respective GM-CSF and IL-5 ligand-receptor complexes, although the similarity of K_d values argues against this possibility. A more stable ligand-receptor complex may facilitate the receptor's ability to transduce a signal, leading to increased leukotriene biosynthesis after ionophore challenge, as a result of cytokine priming. In order to determine if the GM-CSF ligand-receptor complex is more stable than that of IL-5, and thereby facilitates the priming of leukotriene biosynthesis, competition receptor binding assays were performed. The ability of rhGM-CSF and rhIL-5 to compete for IL-5 and GM-CSF receptor binding sites, respectively, was measured. Equilibrium binding mixtures containing eosinophilic HL-60#7 cells and (^{125}I)rhGM-CSF (Figure 8a) or (^{125}I)rhIL-5 (Figure 8b) were incubated in the presence of varying concentrations of unlabelled rhIL-5 or rhGM-CSF. Neither rhGM-CSF nor rhIL-5 were capable of competing for the other's receptor binding sites despite the fact that the two receptors share a common ß-chain component. This data, along with the determination of similar dissociation constants for both receptors, indicates that there is no difference between the stability of the ligand-receptor complexes of GM-CSF or IL-5 on HL-60#7 cells.

iii) Tyrosine phosphorylation patterns suggest that GM-CSF and IL-5 transduce signals differently.

Since receptor binding studies were not able to resolve the difference in the ability of rhGM-CSF and rhIL-5 to prime leukotriene biosynthesis, the possibility that these cytokines may have specific signalling pathways affecting leukotriene formation was investigated. GM-CSF and IL-5 are known to rapidly induce tyrosine phosphorylation, indicating the involvement of protein tyrosine kinases which may be important for signal transduction (34-38). Thus, to determine if GM-CSF and IL-5 have different signalling mechanisms, phosphotyrosine patterns produced from incubating eosinophilic HL-60#7 cells with either PBS (control), rhGM-CSF or rhIL-5 were analyzed using a specific phosphotyrosine monoclonal antibody, 4G10 (Figure 9). The pattern of phosphorylated tyrosines exhibited by rhGM-CSF incubation was different from that produced by treatment with rhIL-5. Several proteins were found to be tyrosine phosphorylated in the presence of rhGM-CSF that were not detected from cells treated with rhIL-5. Thus, the tyrosine phosphorylation patterns suggest that GM-CSF and IL-5 signal differently in this cell line.

DISCUSSION

Eosinophils are major producers of cysteinyl leukotrienes in the lung and have been implicated in the tissue destruction associated with the late phase of asthma (8,39,40). The biosynthesis of leukotrienes by these cells has also been shown to be enhanced by the presence of cytokines (13,14). Thus, the control of leukotriene production, through cytokine signal transduction mechanisms, may substantially affect the pathogenicity associated with eosinophil activation. In this study, the effects of cytokines on leukotriene formation in eosinophilic HL-60#7 cells were investigated in order to investigate their mechanism of regulation of leukotriene biosynthesis.

Similar to previous results with eosinophils derived from circulating blood, pre-treatment of eosinophilic HL-60#7 cells with GM-CSF primed the calcium-ionophore stimulated biosynthesis of total leukotrienes in a dose-dependent manner. Interestingly, this enhancement appeared to be due to an increase in the rate of leukotriene formation rather than the overall amount of leukotrienes produced, although the possibility that high concentrations of ionophore may mask later events cannot be excluded. These results correlate well with the ability of this cytokine to enhance leukotriene biosynthesis in other systems such as neutrophils and basophils as was demonstrated by the up regulation of five-lipoxygenase activating protein (FLAP) mRNA by GM-CSF in neutrophils (41). Since the release of such mediators is almost instantaneous upon stimulation, the priming of leukotriene biosynthesis could increase the quantity of mediators immediately available for release in activated cells.

The enhancement in the rate of leukotriene biosynthesis by GM-CSF was initially speculated to be caused by an increase in the activity of LTC_4 synthase. LTC_4 synthase was a probable candidate since it represents the first committed enzymatic step in the leukotriene biosynthetic pathway leading to the production of cysteinyl leukotrienes. Eosinophils

predominantly produce the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, and this bias in leukotriene formation has been characterised to be an increase in the activity of LTC_4 synthase upon terminal differentiation towards an eosinophil (31). Further analysis of LTC_4 synthase lead to the discovery of a protein kinase C consensus sequence present in the N-terminus of this protein (32) and that PKC activation negatively modulated activity (33). GM-CSF may consequently be capable of priming leukotriene biosynthesis by up-regulating LTC_4 synthase via its PKC consensus sequence through phosphorylation/dephosphorylation events. Unexpectedly, however, neither GM-CSF nor IL-5 had any effect on LTC_4 synthase activity. The kinetic parameters, K_m and V_{max}, were unaltered in the presence of GM-CSF or IL-5 indicating that these cytokines do not enhance leukotriene biosynthesis by increasing the activity of LTC_4 synthase. Recently, it was demonstrated that GM-CSF pre-treatment of neutrophils enhanced 5-lipoxygenase and FLAP expression (41,42). It has also been shown that phospholipase A_2 activity in neutrophils can be up-regulated by GM-CSF (43). Furthermore, GM-CSF priming may elevate the pool of arachidonic acid (44,45). Thus, GM-CSF pre-treatment of eosinophilic HL-60#7 cells may enhance leukotriene biosynthesis of ionophore-stimulated cells by up-regulating the activity and expression of proteins that occur earlier on in the biosynthetic pathway.

One of the most interesting observations of this study was that GM-CSF was capable of priming leukotriene biosynthesis in eosinophilic HL-60#7 cells, whereas IL-5 had no effect on leukotriene formation. This result was in contrast to previous experiments that demonstrated both GM-CSF and IL-5 priming of leukotriene production in eosinophils (9,10,13,14). Receptor binding studies confirmed that the differential priming effect observed between GM-CSF and IL-5 was not simply due to differences in receptor populations or affinities for their respective ligands. Both GM-CSF and IL-5 high-affinity receptors were present on these cells in equal populations. The difference in the ability of GM-CSF versus IL-5 to prime leukotriene

biosynthesis was also demonstrated not to be due to a discrepancy in the stability of their respective receptor complexes. Despite the fact that these heterodimeric receptors share a common ß subunit that is necessary for high-affinity conversion and for signal transduction, neither GM-CSF nor IL-5 were capable of competing for the other's receptor binding sites, indicating that the stability of the ligand-receptor complex was not a factor in determining the leukotriene priming ability of GM-CSF over IL-5 .

Since the differential priming effect could not be resolved by receptor binding studies, it was postulated that GM-CSF and IL-5 may have distinct signalling mechanisms in these cells. Analysis of phosphotyrosine patterns produced in the presence of GM-CSF or IL-5 suggest that these cytokines may be signalling differently or that IL-5 is unable to transduce a signal in this cell line. At least three distinct bands were tyrosine phosphorylated by treatment with GM-CSF that were not present in IL-5 treated or control cells. These observations suggest that the specific α-chains of the receptors may play a role in diverse signalling mechanisms. Other studies have shown that although the α-chain is incapable of signalling alone, it is required for the ß-chain to signal (46,47). It has been suggested that the α-chain confers specificity not only to receptor binding but to the signal transduced by the receptor as well (48). Another possible explanation for differences in the ability of GM-CSF versus IL-5 to signal intracellularly is the possibility of alternative splicing events that occur following transcription of the receptors' mRNAs. HL-60#7 cells may contain an alternate form of the IL-5 receptor α-chain that is capable of high-affinity binding but not intracellular signalling, although neither Northern blots nor the cDNA sequences of the α-and ß-receptor subunits cloned from this cell line show any abnormalities (data not shown).

In summary, GM-CSF was able to prime leukotriene biosynthesis in eosinophilic HL-60#7 cells by enhancing their rate of leukotriene production in a dose-dependent manner. GM-CSF had no effect on the activity of LTC_4 synthase but may have up-regulated the activity of 5-lipoxygenase or

phospholipase A_2 enzymes that occur earlier in the leukotriene biosynthetic pathway. Unexpectedly, IL-5 was incapable of a similar priming effect. This unconventional priming effect was not a result of different receptor populations or differences in the affinity or stability of the ligand-receptor complexes of GM-CSF and IL-5. Analysis of phosphotyrosine patterns suggest that IL-5 is incapable of transducing a signal in eosinophilic HL-60#7 cells even though IL-5 and GM-CSF both share a common ß-chain component of their receptors that is believed to be responsible for signal transduction. Overall this unique system may permit the dissection of distinct events that are responsible for the specific intracellular signals transduced separately by GM-CSF or IL-5.

FOOTNOTES

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FIGURE LEGENDS

Figure 1. Like eosinophils, butyric acid-differentiated HL-60#7 cells predominantly produce cysteinyl leukotrienes. Butyric acid-differentiated HL-60#7 cells were harvested, washed with dPBS, and resuspended at 20 x 10^6 cells / mL in dPBS containing 1 mM EDTA and 1 mg / mL D-glucose. After a 30 min pre-incubation with 200 pM GM-CSF, 200 pM IL-5, or vehicle, cells were incubated for an additional 8 min with 2 mM calcium. Subsequently, cells were ionophore-challenged with 1 μM A23187 for 10 min at 37°C to stimulate the production of leukotrienes and reactions were stopped by combining the mixtures with an equal volume of ice-cold methanol containing 0.2 nmol / mL PGB_2. Leukotrienes were purified by solid phase extraction with Sep-Pak C_{18} cartridges and resolved by isocratic reverse-phase HPLC as described in "Materials and Methods". The resolution and retention times of leukotrienes and their metabolites produced by eosinophilic HL-60#7 cells in the absence of cytokine pre-incubation are shown. A representative chromatogram (n>10) is shown.

Figure 2. (A) Pre-incubation with 200-400 pM rhGM-CSF results in maximum leukotriene production by butyric acid differentiated HL-60#7 cells. Eosinophilic HL-60#7 cells were incubated for 30 min at 37°C with varying concentrations of rhGM-CSF. Calcium (2 mM final concentration) was subsequently added to the mixtures which were incubated for an additional 8 min. The cells were then challenged with 1 μM calcium ionophore, A23187, for 10 min at 37°C and reactions were stopped by combining the mixtures with an equal volume of ice-cold methanol containing 0.2 nmol / mL PGB_2. Leukotrienes were purified by solid phase extraction with Sep-Pak C_{18} cartridges and resolved by isocratic reverse-phase HPLC as described in "Materials and Methods". Data is expressed as the mean of triplicate samples +/- S.E. and is a representative experiment of two. **(B) Priming of HL-60#7 cells with rhGM-CSF but not**

with IL-5 enhances leukotriene biosynthesis. Eosinophilic HL-60#7 cells were incubated for 30 min at 37°C with vehicle, 200 pM rhGM-CSF or 200 pM rhIL-5, then calcium (2 mM final concentration) was added to the mixture which was incubated for an additional 8 min. Calcium ionophore, A23187, was added to a final concentration of 1 μM and the reaction was stopped after 3 min at 37°C by combining the mixture with an equal volume of ice-cold methanol containing 0.2 nmol / mL PGB_2. Leukotrienes were purified by solid phase extraction with Sep-Pak C_{18} cartridges and resolved by isocratic reverse-phase HPLC as described in "Materials and Methods". Data is expressed as the mean of 4 separate experiments (n=4) each done in triplicate +/- S.E. and analyzed using Kruskal-Wallis and Mann-Whitney U statistical tests.

Figure 3. The maximal priming effect of rhGM-CSF occurs after differentiating HL-60#7 cells with butyric acid for 5 days. HL-60#7 cells were cultured for 0, 1, 3, 5, or 7 days with 0.4 mM butyric acid. The cells were harvested, washed in dPBS and incubated for 30 min at 37°C with vehicle (○), 200 pM rhGM-CSF (●) or 200 pM rhIL-5 (■). Calcium (2 mM final concentration) was subsequently added to the mixtures which were further incubated for an additional 8 min. The cells were then challenged with 1 μM calcium ionophore, A23187, for 3 min at 37°C and reactions were stopped by combining the mixtures with an equal volume of ice-cold methanol containing 0.2 nmol / mL PGB_2. Leukotrienes were purified by solid phase extraction with Sep-Pak C_{18} cartridges and resolved by isocratic reverse-phase HPLC as described in "Materials and Methods". A representative experiment of two is shown in which each point represents the average of duplicate samples.

Figure 4. GM-CSF enhances the rate of leukotriene formation but not the total amount of leukotrienes produced. Eosinophilic HL-60#7 cells were incubated for 30 min at 37°C in the presence (●) or absence (○) of 200

pM rhGM-CSF. Calcium (2 mM final concentration) was subsequently added to the mixture which was incubated for an additional 8 min. The cells were then challenged for varying times with 1 μM calcium ionophore, A23187, at 37°C. Reactions were stopped after 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 min. by removing an aliquot of the mixture and dispersing it into an equal volume of ice-cold methanol containing 0.2 nmol / mL PGB_2. Leukotrienes were purified by solid phase extraction with Sep-Pak C_{18} cartridges and resolved by isocratic reverse-phase HPLC as described in "Materials and Methods".

Figure 5. The kinetic parameters, K_m and V_{max}, of LTC_4 synthase are not modulated by GM-CSF or IL-5 pre-treatment. Eosinophilic HL-60#7 cells were incubated with vehicle (O), 200 pM rhGM-CSF (●) or 200 pM rhIL-5 (■) for 30 min at 37°C and subsequently with $CaCl_2$ (2 mM final concentration) for 8 min at 37°C. After the addition of acivicin (2.5 mM final concentration) to the incubation mixture, varying concentrations of LTA_4 were added to initiate the reaction. Reactions were terminated after 4 min at 37°C by combining the mixture with an equal volume of ice-cold methanol containing 0.9 nmol / mL prostaglandin B_2. Leukotrienes were enriched by solid phase extraction and resolved by isocratic reverse-phase HPLC as described in "Materials and Methods". K_m and V_{max} values were calculated based on the Lineweaver-Burk (double-reciprocal) plot of the data (inset). A representative experiment of three is shown.

Figure 6. Time course of expression of GM-CSF and IL-5 specific binding sites on butyric acid-differentiated HL-60#7 cells. HL-60#7 cells were differentiated for 0, 1, 3, 5, or 7 days with 0.4 mM butyric acid. Equilibrium binding mixtures containing 4 x 10^6 cells and 1.0 nM (^{125}I)rhGM-CSF (●) or 1.0 nM (^{125}I)rhIL-5 (■) were incubated in the presence or absence of 100-fold molar excess of rhGM-CSF or rhIL-5, respectively, for 60 min at 37°C with gentle shaking. Cells were harvested by centrifugation, washed and

resuspended in 1 mL dPBS containing 0.1% (w/v) BSA. Resuspended pellets were counted for 1 min in a gamma counter to measure the cell associated radioactivity. Specific binding to differentiated HL-60#7 cells was deduced by subtracting non-specific radioligand binding (samples with rhGM-CSF or rhIL-5 present) from total radioligand binding (no rhGM-CSF or rhIL-5 present). Data is expressed as the percent of maximum specific bound. A representative experiment of two is shown.

Figure 7. High-affinity receptor binding sites for both IL-5 and GM-CSF are in equal abundance on HL-60#7 cells differentiated for 6 days with butyric acid. Receptor binding experiments were performed as described in "Materials and Methods". Eosinophilic HL-60#7 cells were incubated with varying concentrations of (^{125}I)rhGM-CSF (specific activity 4.6 x 10^9 cpm / nmol, panel A) or (^{125}I)rhIL-5 (specific activity 5.9 x 10^8 cpm / nmol, panel B) in the presence or absence of 100-fold molar excess of rhGM-CSF or rhIL-5, respectively, for 60 min at 37°C with gentle shaking. Cells were harvested by centrifugation, washed and resuspended in 1 mL dPBS containing 0.1% (w/v) BSA. Resuspended pellets and combined supernatants were counted for 1 min in a gamma counter to measure the cell associated and free radioactivity, respectively. Total receptor binding was determined in the absence of 100-fold molar excess of competing ligand (O) and non-specific receptor binding was ascertained in the presence of 100-fold competing ligand (❑). Specific receptor binding (●), was deduced by subtracting the non-specific bound from the total bound. The dissociation constants and maximum number of binding sites were determined by Scatchard plots using the equation $B/F = -1/K_D * B + B_{max}/K_D$. A representative experiment of four is shown.

Figure 8. Cross-competition binding analysis of GM-CSF and IL-5 receptor sites on butyric acid-differentiated HL-60#7 cells. Equilibrium binding mixtures containing 4 x 10^6 eosinophilic HL-60#7 cells and 0.1 nM

(^{125}I)rhGM-CSF (specific activity 6.0 x 10^9 cpm / nmol, Panel A) or 0.1 nM (^{125}I)rhIL-5 (specific activity 1.6 x 10^{10} cpm / nmol, Panel B) were incubated in the presence of varying concentrations of rhIL-5 (■) or rhGM-CSF (●) for 60 min at 37°C with gentle shaking. Cells were harvested by centrifugation, washed and resuspended in 1 mL dPBS containing 0.1% (w/v) BSA. Resuspended pellets were counted for 1 min in a gamma counter to measure the cell associated radioactivity. Data is expressed as a percent of the maximum bound. A representative experiment of two is shown.

Figure 9. Different phosphotyrosine patterns were obtained following GM-CSF or IL-5 pre-treatment of eosinophilic HL-60#7 cells. Butyric acid differentiated HL-60#7 cells were incubated with 200 pM rhGM-CSF (lane 2), 200 pM rhIL-5 (lane 3), or vehicle (lane 1) for 30 min at 37°C. Samples were resolved on 14% SDS/polyacrylamide gels, electroblotted to nitrocellulose and then tyrosine phosphorylated proteins were visualized by immunoblotting using the anti-phosphotyrosine monoclonal antibody, 4G10, followed by enhanced chemiluminescence detection, as described in "Materials and Methods". A representative experiment of three is shown.

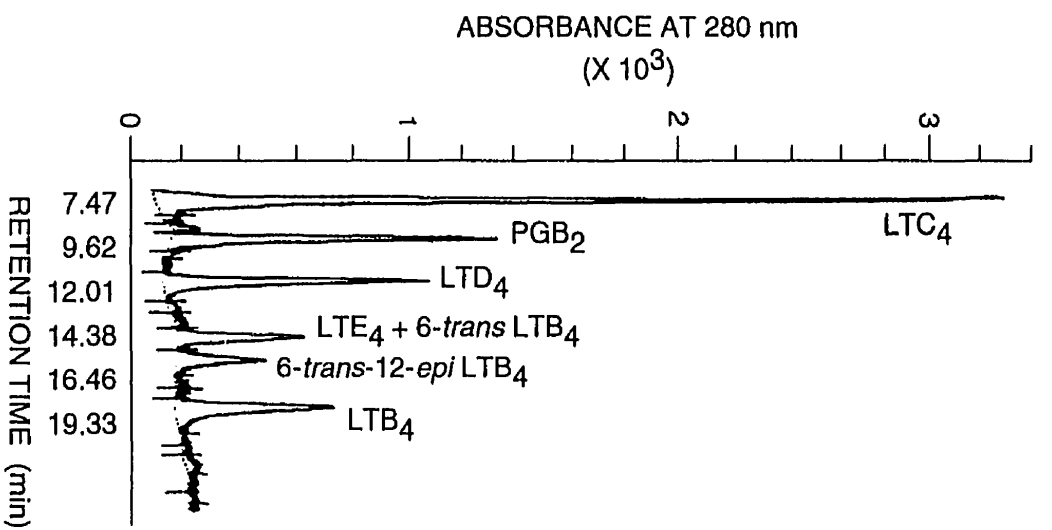

ABSORBANCE AT 280 nm
(X 10^3)
0
1
2
3
RETENTION TIME (min)
7.47
9.62
12.01
14.38
16.46
19.33
LTC_4
PGB_2
LTD_4
LTE_4 + 6-*trans* LTB_4
6-*trans*-12-*epi* LTB_4
LTB_4

Figure 1

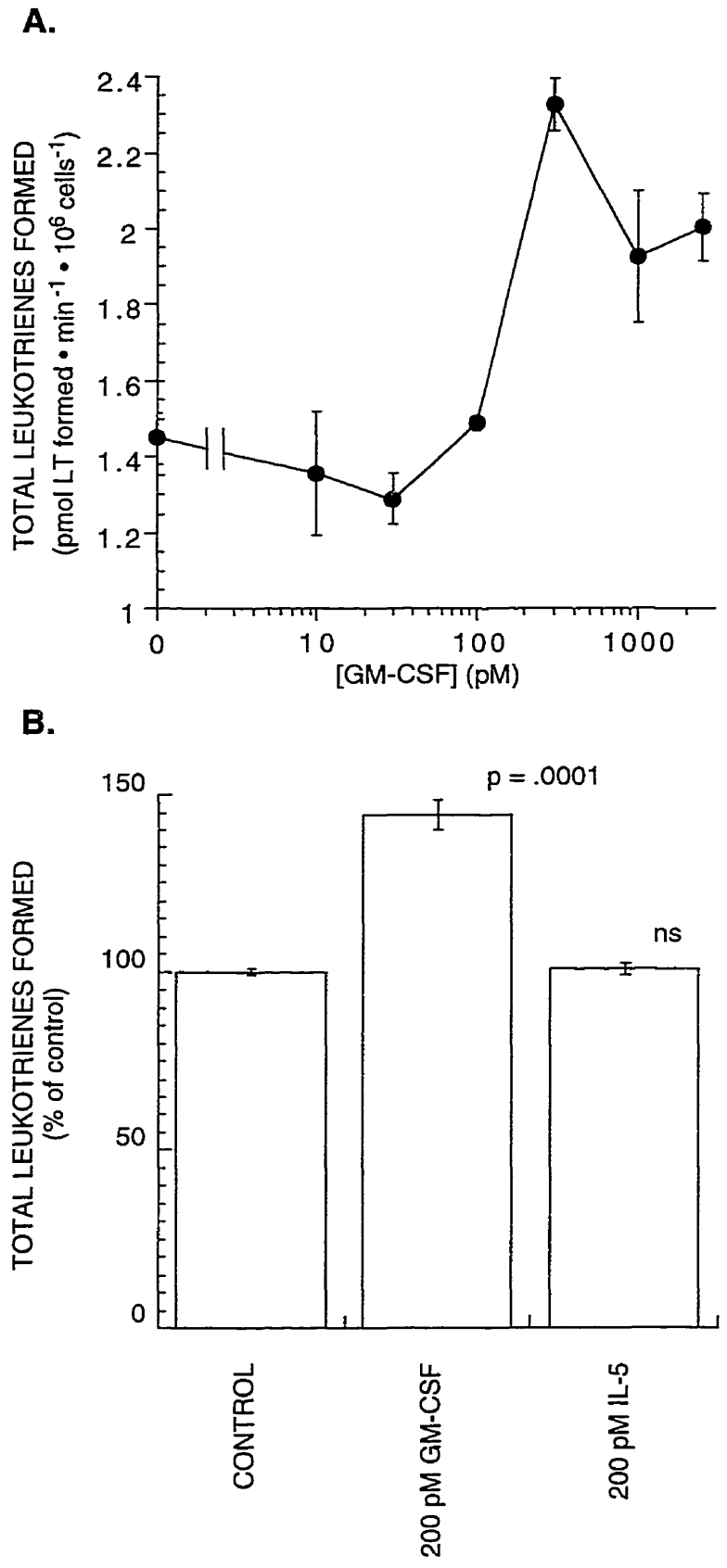

A.
TOTAL LEUKOTRIENES FORMED
(pmol LT formed • min-1 • 10^6 cells-1)
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0
10
100
1000
[GM-CSF] (pM)
B.
TOTAL LEUKOTRIENES FORMED
(% of control)
150
100
50
0
p = .0001
ns
CONTROL
200 pM GM-CSF
200 pM IL-5

Figure 2

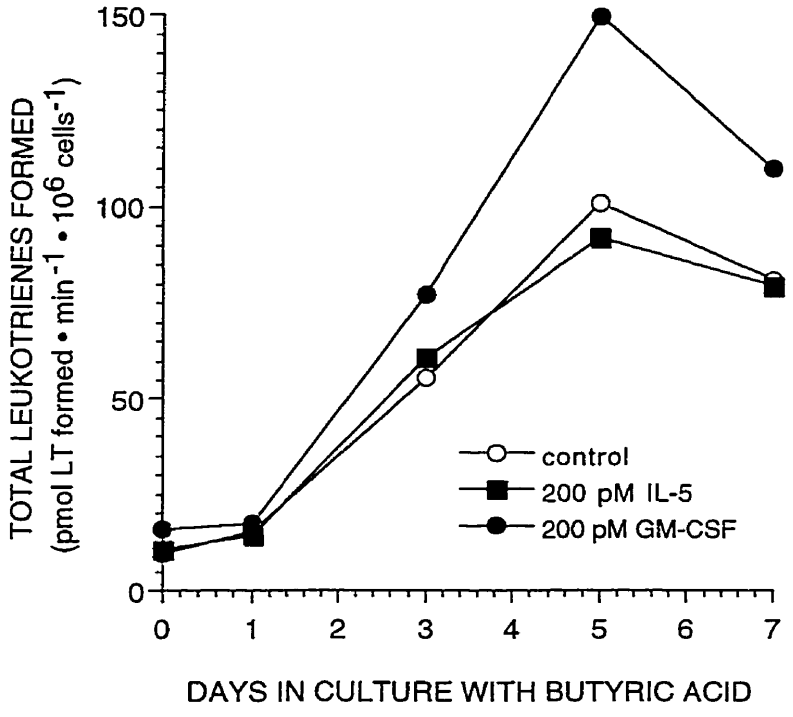
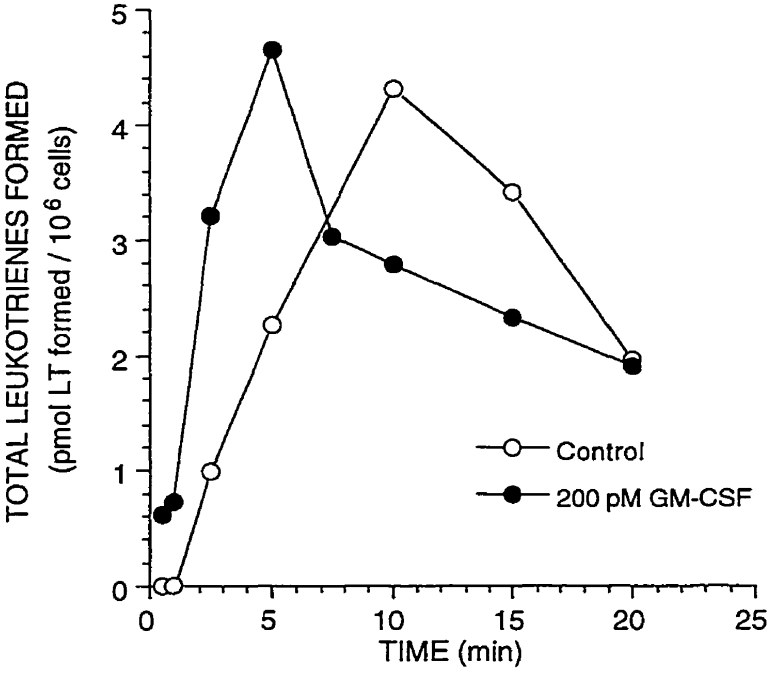

150
100
50
0
TOTAL LEUKOTRIENES FORMED
(pmol LT formed • min^{-1} • 10^6 cells^{-1})
0
1
2
3
4
5
6
7
DAYS IN CULTURE WITH BUTYRIC ACID
control
200 pM IL-5
200 pM GM-CSF

Figure 3

A. Total Leukotrienes



B. Cysteinyl Leukotrienes

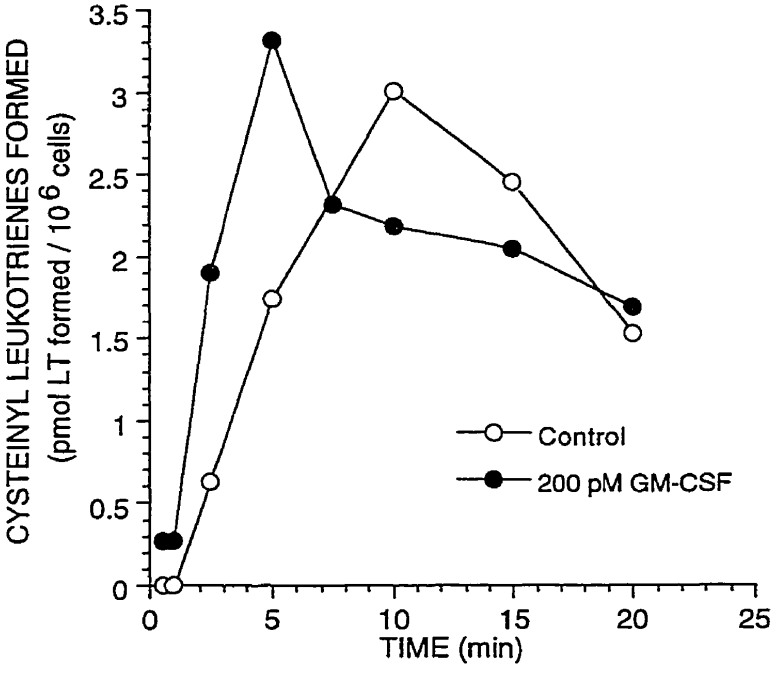


Figure 4

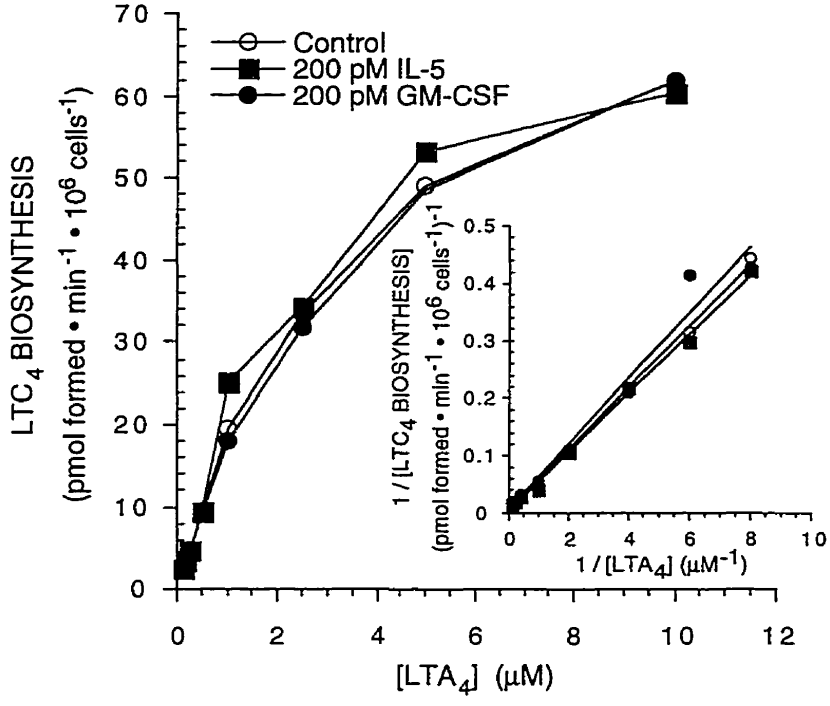

Control
200 pM IL-5
200 pM GM-CSF
LTC4 BIOSYNTHESIS
(pmol formed • min-1 • 10^6 cells-1)
[LTA4] (μM)
1 / [LTC4 BIOSYNTHESIS]
(pmol formed • min-1 • 10^6 cells-1)-1
1 / [LTA4] (μM-1)

Figure 5

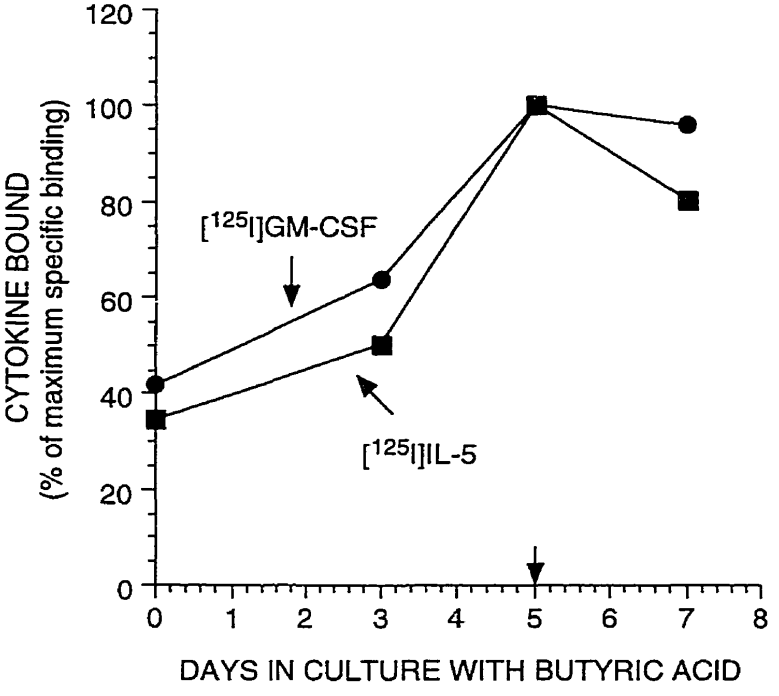
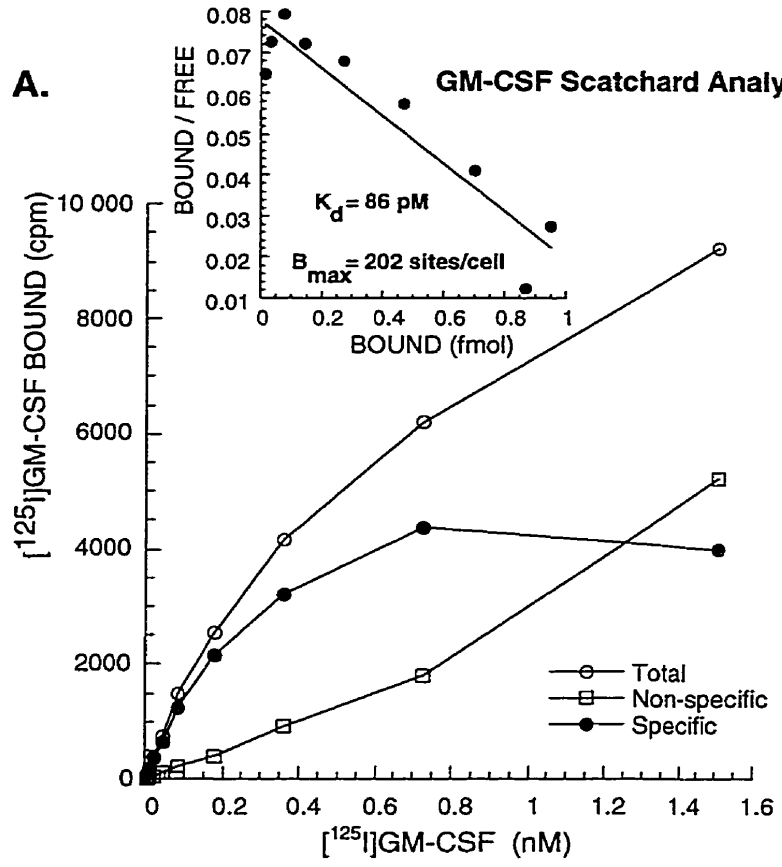

120
100
80
60
40
20
0
CYTOKINE BOUND
(% of maximum specific binding)
[125I]GM-CSF
[125I]IL-5
0
1
2
3
4
5
6
7
8
DAYS IN CULTURE WITH BUTYRIC ACID

Figure 6


A.
GM-CSF Scatchard Analysis
BOUND / FREE
BOUND (fmol)
K_d = 86 pM
B_{max} = 202 sites/cell
[125I]GM-CSF BOUND (cpm)
[125I]GM-CSF (nM)
Total
Non-specific
Specific

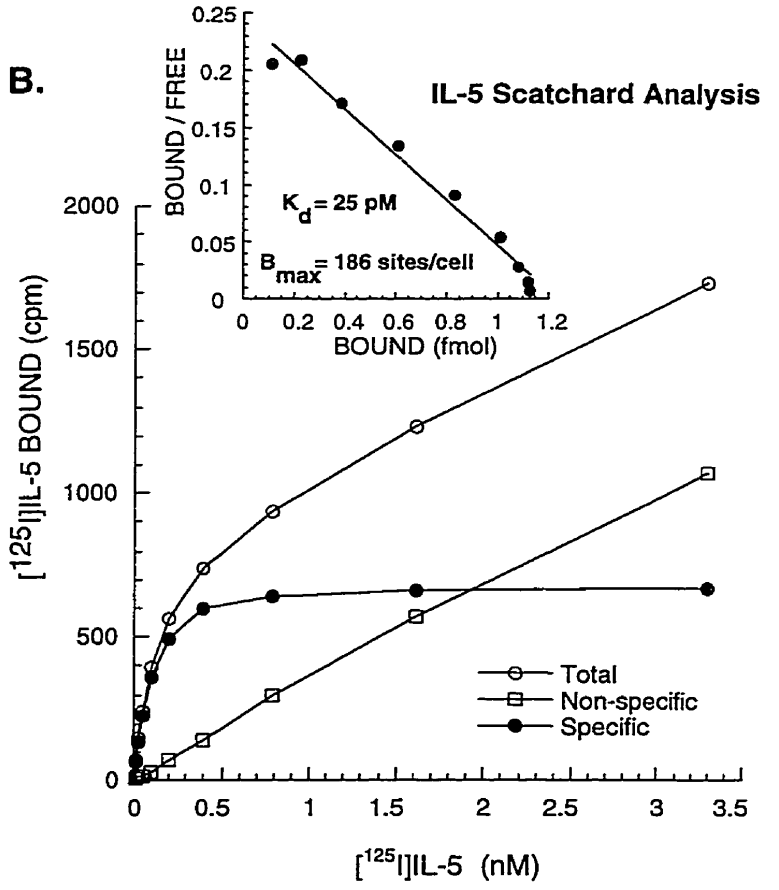
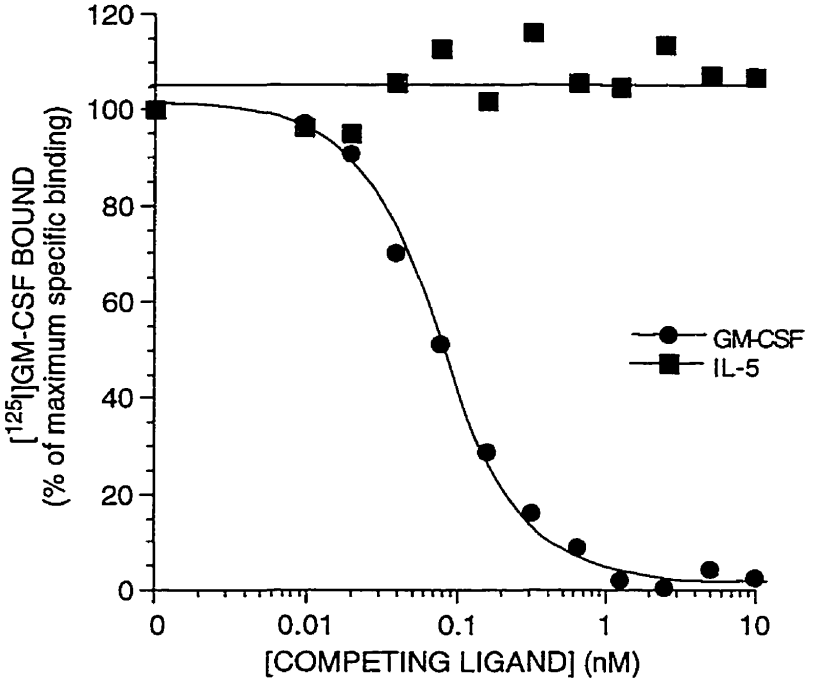

B.
IL-5 Scatchard Analysis
BOUND / FREE
BOUND (fmol)
K_d = 25 pM
B_{max} = 186 sites/cell
[125I]IL-5 BOUND (cpm)
[125I]IL-5 (nM)
Total
Non-specific
Specific

Figure 7

A.


120
100
80
60
40
20
0
[125I]GM-CSF BOUND
(% of maximum specific binding)
0
0.01
0.1
1
10
[COMPETING LIGAND] (nM)
GM-CSF
IL-5

B.

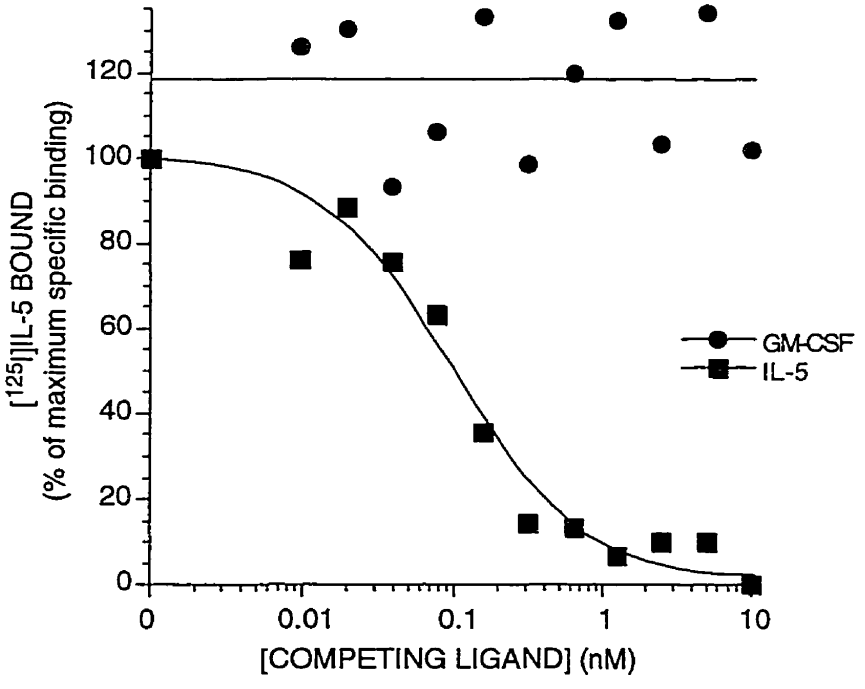

120
100
80
60
40
20
0
[125I]IL-5 BOUND
(% of maximum specific binding)
0
0.01
0.1
1
10
[COMPETING LIGAND] (nM)
GM-CSF
IL-5

Figure 8

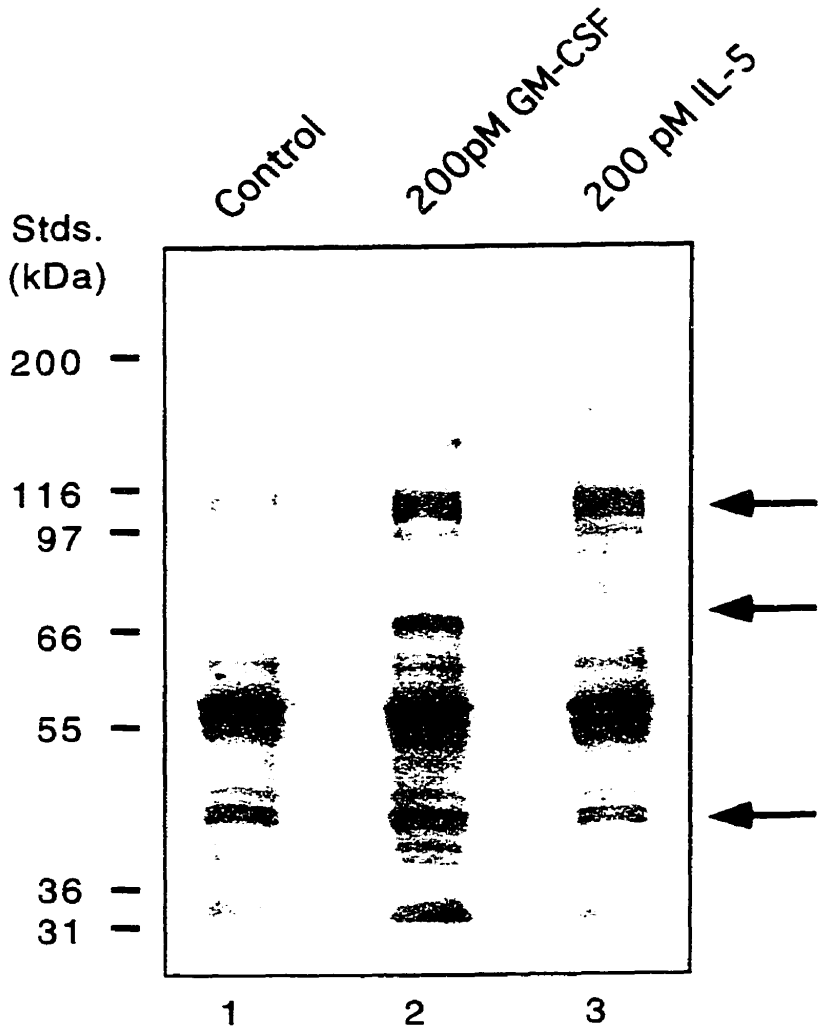

Control
200pM GM-CSF
200 pM IL-5
Stds.
(kDa)
200
116
97
66
55
36
31
1
2
3

Figure 9

5. MANUSCRIPT B

Regulation of Leukotriene-Biosynthetic Enzymes During Differentiation of Myelocytic HL-60 Cells to Eosinophilic or Neutrophilic Cells

Eur. J. Biochem. 239: 572-578 (1996)

In the previous manuscript we noticed that LTC_4 synthase activity and leukotriene production increased as HL-60 cells were differentiated towards eosinophilic cells. Thus, in this manuscript we investigated the regulation of leukotriene biosynthetic enzymes during myeloid cell differentiation towards eosinophils or neutrophils. This paper presents evidence that the mechanisms of regulation of leukotriene biosynthesis during myelocyte differentiation appear similar up to the point where the leukotriene biosynthetic pathway diverges. At this stage LTA_4 hydrolase is upregulated in neutrophils, while, LTC_4 synthase is upregulated in eosinophils.

REGULATION OF LEUKOTRIENE BIOSYNTHETIC ENZYMES DURING MYELOCYTIC CELLULAR DIFFERENTIATION TO EOSINOPHILIC VERSUS NEUTROPHILIC HL-60 CELLS

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ABBREVIATIONS

LT	leukotriene
LTA_4	5,6-oxido-7,9,11,14-eicosatetraenoic acid
LTB_4	5(S), 12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid
LTC_4	5(S)-hydroxy-6(R)-S-glutathionyl-7,9,trans-11,14-cis eicosatetraenoic acid
LTD_4	5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTE_4	5(S)-hydroxy-6(R)-S-cysteinyl-7,9-trans-11,14-cis eicosatetraenoic acid
$cPLA_2$	cytosolic phospholipase A_2
5-LO	5-lipoxygenase
FLAP	5-lipoxygenase-activating protein
ECL	enhanced chemiluminescence
BA	*n*-butyric acid
DMSO	dimethyl sulfoxide

SUMMARY

Leukotrienes (LTs) are potent mediators of bronchial inflammation and are predominantly produced by myeloid cells. As myelocytic cells differentiate towards either eosinophils or neutrophils, the profile of leukotrienes they produce upon stimulation diverges. Eosinophils produce mainly cysteinyl leukotrienes whereas neutrophils predominantly synthesize LTB_4. The mechanism by which this change in leukotriene composition occurs is unknown. In this study, we investigated the control of leukotriene biosynthetic enzymes during myeloid cell differentiation. Western blot analyses of myelocytic leukemia cell lines, HL-60#7 and HL-60, differentiated towards eosinophiiic or neutrophilic cell types, respectively, demonstrated that as myelocytic cells differentiate towards eosinophils or neutrophils, the protein levels of cytosolic phospholipase A_2 ($cPLA_2$) remain constant whereas 5-lipoxygenase (5-LO) and 5-lipoxygenase-activating protein (FLAP) levels are simultaneously elevated. As myelocytic cells become more eosinophil-like, the LTC_4 synthase activity and expression of both the protein and messenger RNA in the cells are dramatically increased (approximately 75-fold) while the LTC_4 synthase level and activity in neutrophil-like cells remain constant at very low levels. In contrast, in neutrophilic cells, the amount of LTA_4 hydrolase was elevated approximately 100-fold greater than the increase in LTA_4 hydrolase from eosinophilic cells. These results indicate that as a myeloid cell differentiates towards a granulocyte, similar mechanisms of regulation may be applied to the leukotriene biosynthetic pathway up to the point at which the pathway diverges. At the stage in the leukotriene biosynthetic pathway where LTA_4 may be converted to either LTC_4 or to LTB_4, specific regulators of transcription may become activated as a myelocytic cell differentiates, thereby causing increased LTA_4 hydrolase or LTC_4 synthase expression.

KEY WORDS

cellular differentiation, HL-60, leukotriene biosynthetic enzymes, genomic regulation, myeloid cells

INTRODUCTION

Leukotrienes are potent mediators of inflammatory and allergic immediate hypersensitivity reactions and are predominantly produced by myeloid cells through the 5-lipoxygenase pathway of arachidonic acid metabolism (1). Arachidonic acid can be liberated from the *sn*-2 position of membrane phospholipids by a cytosolic phospholipase A_2 (2,3), an arachidonate-selective PLA_2, and is then converted to the unstable epoxide leukotriene, LTA_4, in a two step mechanism catalyzed by 5-lipoxygenase (1) along with the membrane-bound 5-lipoxygenase-activating protein (4,5). At this stage, leukotriene biosynthesis can follow one of two pathways to produce biologically active leukotrienes. LTA_4 may be stereoselectively hydrolysed to LTB_4 by LTA_4 hydrolase or it may be conjugated to reduced glutathione by LTC_4 synthase to synthesize LTC_4. LTC_4 can be further metabolised to form LTD_4 and LTE_4 by consecutive removal of L-glutamic acid and glycine. The cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, collectively comprise the slow-reacting substance of anaphylaxis (6) and are predominantly produced by eosinophils and mast cells (7,8,9). These leukotrienes interact with specific G-protein coupled receptors resulting in bronchoconstriction, mucus hypersecretion, vasoconstriction, and vascular permeability changes, important characteristics of bronchial asthma (1,10). LTB_4 is also a potent mediator of inflammation, preferentially synthesized by neutrophils (7), affecting both lymphocyte and leukocyte (myelocyte) functions (11,12).

Although the effects of leukotrienes have been well characterized, the mechanisms responsible for the regulation of leukotriene biosynthesis remain largely unknown. Regulation may occur at the level of the protein through post translational modifications or by phosphorylation / dephosphorylation mechanisms as was demonstrated for $cPLA_2$ (13) and recently, LTC_4 synthase (14,15,16,17). Control of leukotriene biosynthesis may also occur at the level of the gene through transcription/translation

mechanisms. Messenger RNA encoding LTC_4 synthase has recently been shown to be present in human eosinophils, but not in neutrophils (18). In the present study, two cell lines were used to investigate the mechanism of regulation of leukotriene biosynthesis during myelocytic cellular differentiation. These were the human pro-myelocytic cell line, HL-60, which has been well characterized to increase its neutrophilic phenotype when cultured with DMSO (19,20,21), and the pro-eosinophilic HL-60#7 cell line in which butyric acid induces an eosinophilic phenotype (22,23).

MATERIALS AND METHODS

Materials

HL-60 cells (CCL 240) were obtained from the American Type Culture Collection (Rockville, MD). Cell culture media, antibiotics, *n*-butyric acid, bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), Hepes, and DL-dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Aprotinin, leupeptin, and pepstatin A were from Boehringer Mannheim GmbH, (Mannheim, Germany). Tris base was also from Boehringer Mannheim Corporation (Indianapolis, IN). Taurocholic acid, sodium salt was obtained from Calbiochem Corporation (La Jolla, CA). LTA_4-methyl ester was synthesized by the Department of Medicinal Chemistry at the Merck Frosst Centre for Therapeutic Research. Novapak C_{18} HPLC columns were from Waters Chromatography (Milford, MA). HPLC solvents were from BDH (Toronto, Ontario). The Department of Biochemistry and Molecular Biology at the Merck Frosst Centre for Therapeutic Research provided the specific polyclonal antibodies to the enzymes in the leukotriene (LT) biosynthetic pathway (24,25,26,27) and to the eosinophilic marker, major basic protein (MBP). The peptide antibodies for LTC_4 synthase and MBP were raised to LTC_4 synthase amino acid sequence 37-51 and to mature human MBP amino acid sequence 62-76, respectively. Renaissance western blot chemiluminescence reagent (ECL) was purchased from DuPont NEN Research Products (Boston, MA). Horseradish peroxidase-linked donkey anti-rabbit antibody was obtained from Amersham Life Sciences (Buckinghamshire, England). Blotting grade blocker non-fat dry milk and Tween-20 were from Bio-Rad Laboratories (Hercules, CA). Fetal bovine serum, sodium dodecyl sulfate, and total RNA isolation (Trizol) reagent were from Gibco BRL Life Technologies (Grand Island, NY and Gaithersburg, MD, respectively). Oligotex-dT poly A+ mRNA purification kit, QIAquick PCR purification kit, and QIAquick gel extraction kit were purchased from QIAGEN (Chatsworth, CA). Sheared DNA was

obtained from Research Genetics (Huntsville, AL). Dimethyl sulfoxide (DMSO), potassium hydroxide and sodium hydroxide were from A & C American Chemicals (St. Laurent, Quebec). All other reagents were of analytical grade and were purchased from Sigma.

Growth and Differentiation of HL-60#7 and HL-60 Cell Lines

The HL-60/MF211#7 cell line (abbreviated HL-60#7) is a pro-eosinophilic substrain of the human pro-myelocytic cell line, HL-60, that has been subcultured under alkaline conditions with the continuous selection of subtypes displaying eosinophilic properties when cultured with butyric acid (22,23,28,29). This pro-eosinophilic cell line was propagated as described previously (22) by subculturing at a seed density of 0.2 x 10^6 cells / mL into fresh sterile RPMI 1640 (supplemented with 0.2% (w/v) sodium bicarbonate and 0.03% (w/v) L-glutamine) containing 50 U / mL penicillin and 50 µg / mL streptomycin, and 10% (v/v) fetal bovine serum (not heat-inactivated). Cultures were grown at 37°C in a humidified atmosphere containing 6% CO_2 in 175-cm^2 culture flasks. To differentiate the HL-60#7 cell line into eosinophilic cells, cultures were seeded at 0.2 x 10^6 cells / mL in fresh medium (described above) and supplemented with 0.4 mM *n*-butyric acid (added from a 150 mM stock in ethanol). The cells were grown without further subculturing for up to 7 days.

The HL-60 cell line was subcultured using identical conditions for propagating the HL-60#7 cell line. To differentiate the HL-60 cells into neutrophilic cells, cultures were seeded at 0.2 x 10^6 cells / mL in fresh medium (described above) and supplemented with 1.2% (v/v) dimethyl sulfoxide (DMSO). Two days following the initial addition of 1.2% (v/v) DMSO, cultures were supplemented with 0.6% (v/v) DMSO and the cells were grown without further subculturing for up to 7 days.

Reverse time course experiments for both cell lines (HL-60#7 and HL-60) were set up by seeding cultures at 0.2 x 10^6 cells / mL. The appropriate differentiation agent (butyric acid or dimethyl sulfoxide) was added

immediately to the day 7 cultures and day 5 cultures were supplemented two days later and so on for day 3, day 2, day 1, and day 0 cultures. Cells were harvested by centrifugation at 500 x g for 15 min at 4°C. The resulting pellet was washed by resuspending the cells in Dulbecco's phosphate buffered saline (dPBS) and re-sedimenting them at 500 x g for 15 min.

Western Blot Analysis of Leukotriene Biosynthetic Enzyme Levels

HL-60 and HL-60#7 reverse time course cultures were harvested by centrifugation, washed with dPBS, and stored at -80°C. Frozen cell pellets at 5 x 10^6 cells / tube from reverse time course cultures (as described above) were resuspended in 500 µL of cold lysis buffer containing 10 mM Hepes/KOH (pH 7.4), 2 mM EDTA, 2% (w/v) taurocholate, with freshly added DTT (5 mM), PMSF (1 mM, from a fresh 200 mM stock in EtOH), leupeptin (20 µg / mL), pepstatin A (10 µg / mL, from a fresh 1 mg / mL stock in EtOH), and aprotinin (10 µg / mL). Samples were shaken for 20 min at 4°C and centrifuged for 10 min at 1000 x g. Bradford (30) protein assays (Bio-Rad) were performed on the supernatants and the protein levels were adjusted to equivalency using lysis buffer. SDS-containing sample buffer (31) was added to each sample which was subsequently heated for 5 min at 95°C and frozen in liquid nitrogen. Equivalent amounts of cellular protein from frozen lysates were thawed, electrophoresed through SDS/polyacrylamide gels (Novex) and electroblotted onto nitrocellulose. The transfer efficiency was visualized by Ponceau S staining. Blots were soaked for 1 h at 25°C in 5% Bio-Rad Blotting Grade Blocker Non-Fat Dry Milk (w/v) in Tris-buffered saline containing Tween 20 (20 mM Tris/HCl (pH 7.5), 0.5 M NaCl, 0.1% (v/v) Tween 20)(0.1%T TBS). The membrane was then washed twice with 0.1%T TBS for 5 min each at 25°C, and treated with a specific leukotriene biosynthetic enzyme polyclonal antibody (dilution 1:1 000) in 0.05%T TBS containing 5% dry milk (w/v) for 1 h at 25°C. After 3 x 5 min washes with 0.1%T TBS, the blot was exposed to a horseradish peroxidase-linked donkey anti-rabbit antibody (dilution 1:3 000) in 0.05%T TBS containing 1% dry milk

(w/v) for 1 h at 25°C. The blots were washed 3 x 5 min with 0.3%T TBS and 3 x 5 min with 0.1%T TBS and subsequently developed using enhanced chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent, DuPont NEN), according to the manufacturer's instructions.

Measurement of LTC_4 Synthase Enzymatic Activity

HL-60#7 cells were cultured for varying number of days with 0.4 mM butyric acid, harvested by centrifugation, and washed with dPBS. 5×10^5 cell equivalents were resuspended in 0.1 M KPi buffer (pH 7.4) with 1% (w/v) taurocholate for 15 min at 4°C. LTC_4 synthase activity was subsequently assayed as described previously (32) by measuring the amount of LTC_4 produced in incubations at 25°C with reduced glutathione (10 mM) and 40 µM LTA_4 (free acid) stabilized with 5 mg / mL BSA in the presence of serine borate (50 mM), phosphatidyl choline (0.2 mg / mL), and $MgCl_2$ (20 mM) to a final volume of 150 µL. The reaction was stopped after 15 min with an equivalent volume of acetonitrile:methanol:acetic acid at 50:50:1 and the amount of LTC_4 synthesized was determined by isocratic reverse-phase HPLC. Similar experiments were performed using HL-60 cells cultured for varying number of days in 1.2% (v/v) dimethyl sulfoxide. LTC_4 was resolved by isocratic reverse-phase HPLC on a Waters Associates Novapak C_{18} column (3.9 x 150 mm) in the mobile phase (acetonitrile:methanol:water:acetic acid at 28:14:54:1, pH 5.6) at a flow rate of 1.0 mL/min. LTC_4 was quantified by on-line measurement of the absorbance at 280 nm and the LTC_4 peak was identified by retention time matching a synthetic standard.

Isolation of Polyadenylated RNA From Reverse Time Course Cultures

Total RNA was prepared from 10×10^6 cells from reverse time course cultures (Days 0, 1, 2, 3, 5, and 7 as described above) using Trizol reagent (Total RNA Isolation Reagent, Gibco BRL) according to the manufacturer's instructions. Poly(A)+ RNA was then isolated from total RNA using the

Oligotex-dT poly A+ mRNA Purification Kit (Qiagen) as per the manufacturer's directions.

Production of LTC_4 Synthase cDNA by RT-PCR

Reverse transcription-PCR was performed on Day 5 HL-60#7/BA poly(A)+ RNA using Murine Leukemia Virus (MuLV) Reverse Transcriptase (Perkin Elmer). Primers 5' TCA CAC ACA GCC CGT GCC ACC ACA CCG 3', 5' CAG CTG GAG GCT CCG GCT CTT CTT TCC 3' and nested primers 5' CCG CTC GAG CGG CCG CGG ATC CAC CAT GAA GGA CGA GGT AGC TCT ACT GGC TGC 3', 5' GCT CTA GAG CGG CCG CGG ATC CTA TTA GGC CCA CGG CAG CAG CGT CCG GAG CCG TC 3' that were designed based on the published cDNA sequence for human LTC_4 synthase (GenBank accession nos. U09353 and U11552) were used in two rounds of PCR amplification, respectively, in order to obtain the LTC_4 synthase cDNA. Primary PCR cycles of amplification were 96°C for 30 sec, 50°C for 30 sec, and 72°C for 90 sec, for 25 cycles using the initial primers. The PCR product was purified to remove primers using the QIAquick PCR Purification Kit followed by gel purification using the QIAquick Gel Extraction Kit (QIAGEN). Secondary amplification cycles consisted of 96°C for 30 sec, 65°C for 30 sec, and 72°C for 90 sec, for 25 cycles with the nested primers. These cycles were performed using the Expand Long Template PCR System (Boehringer Mannheim). The RT-PCR products were gel purified, cloned into BlueScript II (SK+) vector and sequenced. The identity of the cDNA clone was also confirmed by producing radiolabeled LTC_4 synthase using TNT Coupled Reticulocyte Lysate System (Promega) followed by immunoprecipitating the radiolabeled LTC_4 synthase with a specific polyclonal antibody against LTC_4 synthase (data not shown).

Northern Blot Analysis of LTC_4 Synthase

LTC_4 synthase cDNA was radiolabeled using Random Primed DNA Labeling Kit (Boehringer Mannheim) and purified on G-50 sephadex

columns to be used as a probe during Northern blot analyses. Approximately 1 μg poly(A)+ RNA was electrophoresed through a 1.2% (v/v) formaldehyde/agarose gel and transblotted onto a nylon membrane. The blot was incubated at 55°C for 2 h in pre-hybridizing solution containing 6 x SSC, 2 x Denhart's solution, 0.1% SDS (v/v), and 100 μg / mL sheared DNA. The membrane was subsequently hybridized with the radiolabeled LTC_4 synthase cDNA probe at 1 x 10^6 cpm / mL under high stringency conditions (wash temperature, 65°C and 1 x SSC, 0.5% SDS) and exposed to film for 2 weeks. The above conditions were also used to probe the membrane with ß-actin.

RESULTS

Two myelocytic cell lines, HL-60 and HL-60#7, were used as tools to study the mechanisms underlying the predominant production of LTB_4 by neutrophils versus the preferential formation of cysteinyl leukotrienes by eosinophils. In order to try to understand the mechanism resulting in this differential profile of leukotrienes produced by neutrophils and eosinophils, we initially investigated the protein levels of the leukotriene biosynthetic enzymes present during myelocytic cellular differentiation to neutrophils or to eosinophils. HL-60 and HL-60#7 cells were cultured for varying number of days with DMSO or butyric acid, respectively, and the presence of the leukotriene biosynthetic enzymes throughout culturing were compared using specific polyclonal antibodies (Figures 1, 2, and 3).

Biosynthetic enzyme levels for LTB_4 formation are selectively up-regulated during differentiation to neutrophils

HL-60 cells were differentiated towards neutrophilic cells by culturing for varying number of days with DMSO. The presence and quantities of the leukotriene biosynthetic enzymes were subsequently compared during this time course of differentiation using specific polyclonal antibodies (Figure 1). Cytosolic phospholipase A_2 ($cPLA_2$) protein levels remained constant throughout culturing with 1.2% DMSO whereas both 5-lipoxygenase-activating protein (FLAP) and 5-lipoxygenase (5-LO) levels were elevated during neutrophilic differentiation. As predicted, LTA_4 hydrolase levels increased significantly from 0 to 7 days in culture with DMSO and the quantity of LTC_4 synthase remained very low. This increase in the amount of LTA_4 hydrolase was nearly 100-fold greater than the enhancement of LTA_4 hydrolase levels in eosinophilic differentiated HL-60#7 cells (Figure 2). These results suggest that as myelocytes differentiate towards neutrophils, FLAP, 5-LO, and principally LTA_4 hydrolase protein expression are up-regulated.

Biosynthetic enzyme levels for cysteinyl leukotriene formation are selectively up-regulated during differentiation to eosinophils

Leukotriene biosynthetic enzyme levels were monitored, using specific polyclonal antibodies, during differentiation of HL-60#7 cells to eosinophilic cells by culturing for varying number of days with butyric acid (Figure 2). Cytosolic PLA_2 protein levels remained constant throughout culturing with 0.4 mM butyric acid whereas both FLAP and 5-LO levels increased. This increase in FLAP and 5-LO, however, was not as marked as in DMSO differentiated HL-60 cells (Figure 1). As predicted, LTA_4 hydrolase levels remained relatively constant during eosinophilic differentiation while LTC_4 synthase levels increased dramatically (approximately 75-fold). Accordingly, these results indicate that the expression of FLAP, 5-LO, and particularly LTC_4 synthase proteins are elevated during myelocytic differentiation towards an eosinophil.

Direct comparison of leukotriene biosynthetic enzyme levels present as myelocytic cells differentiate towards neutrophils versus eosinophils

In the previous studies, the protein levels of each enzyme could only be compared within each particular cell line. Thus, similar experiments were performed using cellular lysates from undifferentiated and fully differentiated HL-60 and HL-60#7 cell lines in order to directly compare the leukotriene biosynthetic enzyme levels present in HL-60 cells to those in HL-60#7 cells over the course of neutrophilic or eosinophilic differentiation. These experiments confirmed the results obtained in the above investigations and provided information about the relative quantities of expression of the leukotriene biosynthetic enzymes found in HL-60 and HL-60#7 cell lines (Figure 3). Again, $cPLA_2$ levels remained constant at day 0 and day 7 of neutrophilic and eosinophilic differentiation, however, HL-60 cells contained approximately 2-fold more $cPLA_2$ than did HL-60#7 cells. As previously determined, both FLAP and 5-LO levels increased by culturing with either DMSO or butyric acid from day 0 to day 7. DMSO differentiated

HL-60 cells again contained higher levels of both FLAP and 5-LO than did HL-60#7 cells following differentiation. LTA_4 hydrolase levels dramatically increased during differentiation to neutrophils whereas, during eosinophilic differentiation of HL-60#7 cells, LTA_4 hydrolase levels only doubled. LTC_4 synthase was virtually undetectable in undifferentiated HL-60 and HL-60#7 cells and in neutrophilic HL-60 cells but, was substantially abundant in eosinophilic HL-60#7 cells. The eosinophil marker, MBP, was also only detected in HL-60#7 cells that had been cultured for 7 days with butyric acid.

Elevation of LTC_4 synthase biosynthetic activity and poly(A)+ RNA in eosinophilic differentiated HL-60#7 cells corresponds to increased protein levels

As myeloid cells differentiate to eosinophils the enzymatic activity of LTC_4 synthase increases substantially (approximately 75-fold), whereas in HL-60 cells differentiated to neutrophils the LTC_4 synthase activity remains almost undetectable (Figure 4). This enzymatic activity correlates well with the protein levels of the enzyme in both of these cell types.

Since LTC_4 synthase protein levels and enzymatic activity increase as HL-60#7 cells differentiate towards eosinophilic cells, we wanted to determine if this increase occurred in parallel with LTC_4 synthase messenger RNA expression. Poly(A)+ RNA was isolated from reverse time course cultures of HL-60#7 cells cultured for varying number of days with butyric acid (as described in Materials and Methods) and LTC_4 synthase messenger RNA was detected using a radiolabeled LTC_4 synthase cDNA probe. LTC_4 synthase message levels were equated to ß-actin message levels and were determined to increase as HL-60#7 cells were cultured for increasing days with butyric acid (Figure 5).

DISCUSSION

As myelocytic cells differentiate into granulocytes, the profile of leukotrienes that these cells produce diverges. Neutrophils predominantly produce LTB_4 whereas eosinophils preferentially synthesize the cysteinyl leukotrienes. To try to understand the molecular mechanisms involved in the synthesis of specific leukotrienes from these cell types, the enzymes of the leukotriene biosynthetic pathway were investigated during differentiation of HL-60 cells to neutrophils by induction with DMSO or during differentiation of HL-60#7 cells to eosinophils by treatment with butyric acid.

The mechanisms regulating leukotriene biosynthesis during myelocytic differentiation to neutrophilic or eosinophilic cells appeared to be similar up to the point where the leukotriene biosynthetic pathway diverges. The levels of $cPLA_2$ remained constant and both FLAP and 5-LO levels increased during differentiation towards neutrophilic or eosinophilic cells. These common mechanisms of regulation were anticipated as these steps lead to the shared substrate, LTA_4, that is required for both LTB_4 and LTC_4 formation. As well, these cells originated from a common predecessor, a pro-myelocytic cell, and may therefore retain many of its metabolic processes. The fact that $cPLA_2$ protein expression was uniform throughout differentiation indicates that it is not a point of genomic regulation for leukotriene biosynthesis. Recently, low level constitutive expression of $cPLA_2$ was determined to be controlled by a 27 bp element in the 5'-flanking region of the human $cPLA_2$ gene. This constitutive expression coincides with the concept that $cPLA_2$ is modulated post-translationally (33) and not at the level of transcription. The essential role of $cPLA_2$ in the production of leukotrienes is also controversial. The $cPLA_2$ and 5-LO genes have been shown to be differentially regulated at the level of transcription indicating that their cellular functions may not be coupled (34). Increases in FLAP and 5-LO levels during differentiation suggest that these proteins are regulated for the production of leukotrienes in these cell types.

differentiate to a committed cell type. The eosinophilic granule proteins are notable examples of genes that are specifically transcribed as myeloid cells differentiate to eosinophils. Similarly, myeloperoxidase (MPO) expression is specifically shut down as myeloid cells become eosinophilic and at the same time eosinophil peroxidase (EPO) expression is induced. These two proteins are very similar at both the nucleotide and amino acid levels, however, they have very little identity in their putative promoter regions which may explain the tissue-specific expression of the MPO gene in neutrophils and the EPO gene in eosinophils (39). A minimal promoter sequence on the FLAP gene has also been associated with cell-specific expression and myeloid cell-specific nuclear factors were identified that bind to the FLAP promoter and appear to be necessary for the tissue specific expression of FLAP (40 and Kennedy *et al.*, unpublished results). In the promoter region of LTA_4 hydrolase several putative regulatory elements have also been detected, however, a functional role for these transcription-factor-binding sites has not been determined (41). A family of transcription factors termed GATAs have been postulated to be specific regulators of gene expression for proteins that are characteristic of eosinophils (42). Among the GATA-binding proteins, GATA-1 is the most likely candidate for LTC_4 synthase regulation as it's expression is up-regulated during butyric acid differentiation of HL-60(c15) cells (42) and is expressed in eosinophils and mast cells but not in neutrophils (43).

In summary, the regulation mechanisms that control leukotriene biosynthesis during myeloid cell differentiation to eosinophils or neutrophils are analogous up to the point where the leukotriene biosynthetic pathway diverges. At this point, the expression of LTA_4 hydrolase is up-regulated in neutrophils, whereas the activity and expression of the protein and messenger RNA for LTC_4 synthase are elevated in eosinophils. These regulatory differences may result from the activation of specific transcription factors upon stimulation to differentiate. These regulatory elements may alter the expression of distinct leukotriene biosynthetic

enzymes, thus promoting the synthesis of diverse leukotrienes. Further studies are needed to confirm this assumption which may lead to the ability to control the regulation of leukotriene biosynthesis in inflammatory diseases such as asthma.

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FIGURE LEGENDS

Figure 1. Western blot analyses of leukotriene biosynthetic enzyme levels during neutrophilic differentiation of HL-60 cells by culturing with dimethyl sulfoxide. Equivalent amounts of cellular protein (0.20 or 0.37 μg / μL protein) from lysed HL-60 cells differentiated for the indicated number of days with 1.2% (v/v) DMSO were resolved by SDS/polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose. Western blot analyses were carried out, as described in Materials and Methods, using specific polyclonal antibodies to the enzymes in the leukotriene biosynthetic pathway and detected using enhanced chemiluminescence.

Figure 2. Western blot analyses of leukotriene biosynthetic enzyme levels during eosinophilic differentiation of HL-60#7 cells by culturing with butyric acid. Equivalent amounts (0.72 μg / μL protein) of cellular lysates from HL-60#7 cells differentiated for the indicated number of days with 0.4 mM butyric acid were electrophoresed through SDS/polyacrylamide gels, electroblotted to nitrocellulose, immunoblotted using polyclonal antibodies specific to the enzymes in the leukotriene biosynthetic pathway and detected using enhanced chemiluminescence as described in Materials and Methods.

Figure 3. Direct comparison of leukotriene biosynthetic enzyme levels in undifferentiated versus differentiated HL-60#7 and HL-60 cells. Equivalent amounts of cellular lysates (0.56 μg / μL protein) from HL-60 and HL-60#7 cells cultured for 0 and 7 days with 1.2% (v/v) DMSO or 0.4 mM butyric acid, respectively, were electrophoresed through SDS/polyacrylamide gels and electroblotted onto nitrocellulose. Western blot analyses were carried out, as described in Materials and Methods, using specific polyclonal antibodies to the enzymes in the leukotriene biosynthetic pathway and detected using enhanced chemiluminescence.

Figure 4. LTC_4 synthase biosynthetic activity is up-regulated in eosinophilic differentiated HL-60#7 cells. HL-60#7 (○) or HL-60 (●) cells were cultured for varying number of days with 0.4 mM butyric acid or 1.2% (v/v) DMSO, respectively, harvested by centrifugation, and washed with dPBS. 5 x 10^5 cell equivalents resuspended in 0.1 M KPi buffer (pH 7.4) with 1% taurocholate for 15 min at 4°C were assayed for LTC_4 synthase activity by measuring the amount of LTC_4 produced in incubations at 25°C with reduced glutathione (10 mM) and 40 μM LTA_4 (free acid) stabilized with 5 mg/mL BSA in the presence of serine borate (50 mM), phosphatidyl choline (0.2 mg/mL), and $MgCl_2$ (20 mM) as determined by isocratic reverse-phase HPLC.

Figure 5. LTC_4 synthase messenger RNA levels increase during differentiation of HL-60#7 cells to eosinophilic cells. Approximately 1 μg of poly(A)+ RNA from reverse time course cultures was electrophoresed through a 1.2% formaldehyde-containing-agarose gel and transblotted to a nylon membrane. The membrane was incubated with a random-primed radiolabeled LTC_4 synthase (upper panel) or ß-actin (lower panel) probe under stringent conditions (wash temperature, 60°C, with 1 x SSC;0.1% SDS) and subsequently exposed to x-ray film.

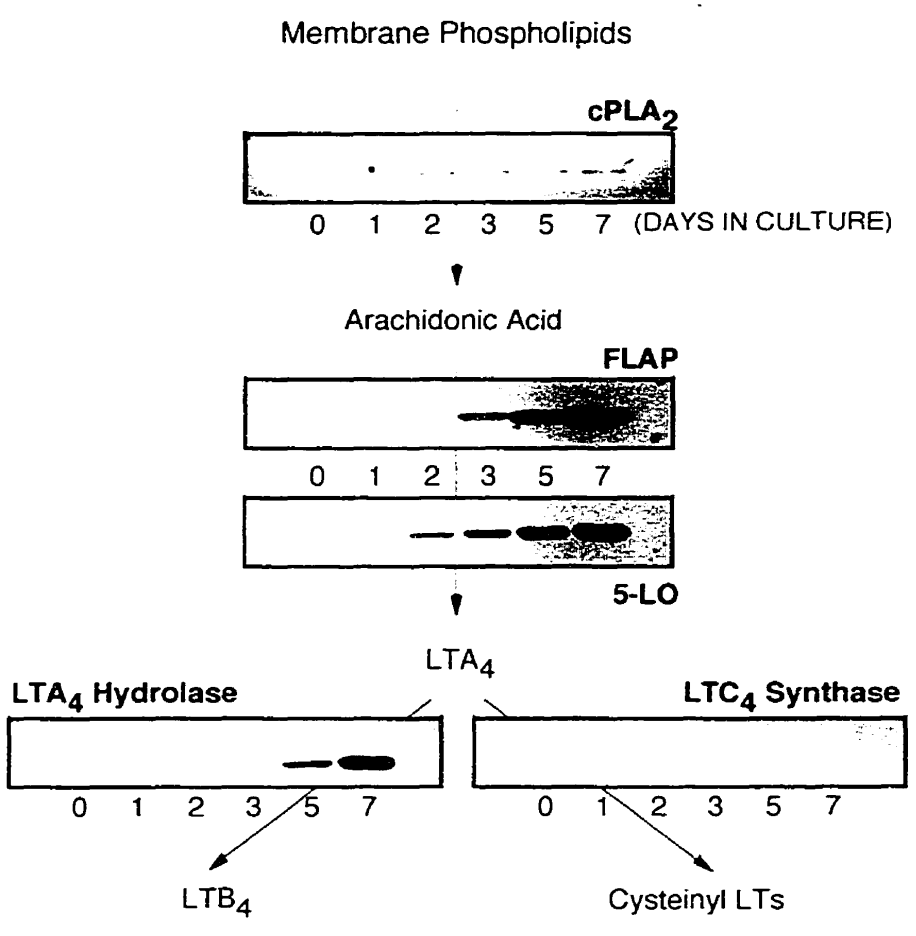
Membrane Phospholipids
cPLA2
0 1 2 3 5 7 (DAYS IN CULTURE)
Arachidonic Acid
FLAP
0 1 2 3 5 7
5-LO
LTA4
LTA4 Hydrolase
LTC4 Synthase
0 1 2 3 5 7
0 1 2 3 5 7
LTB4
Cysteinyl LTs

Figure 1

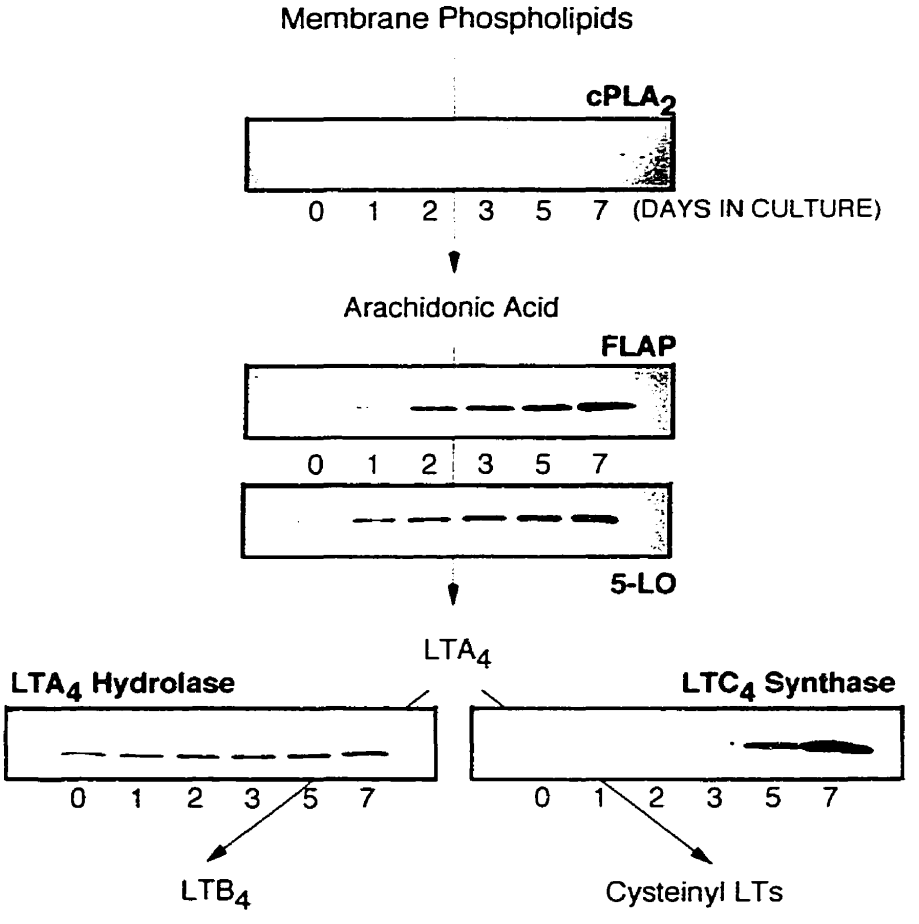
Membrane Phospholipids
cPLA2
0 1 2 3 5 7 (DAYS IN CULTURE)
Arachidonic Acid
FLAP
0 1 2 3 5 7
5-LO
LTA4
LTA4 Hydrolase
LTC4 Synthase
0 1 2 3 5 7
0 1 2 3 5 7
LTB4
Cysteinyl LTs

Figure 2

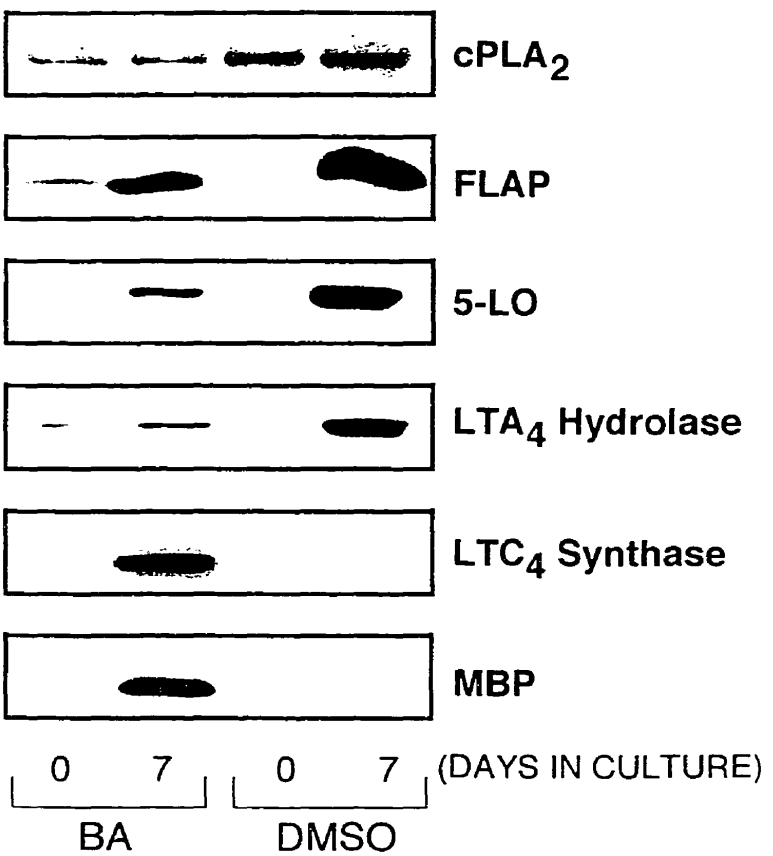
cPLA2
FLAP
5-LO
LTA4 Hydrolase
LTC4 Synthase
MBP
0
7
0
7
(DAYS IN CULTURE)
BA
DMSO

Figure 3

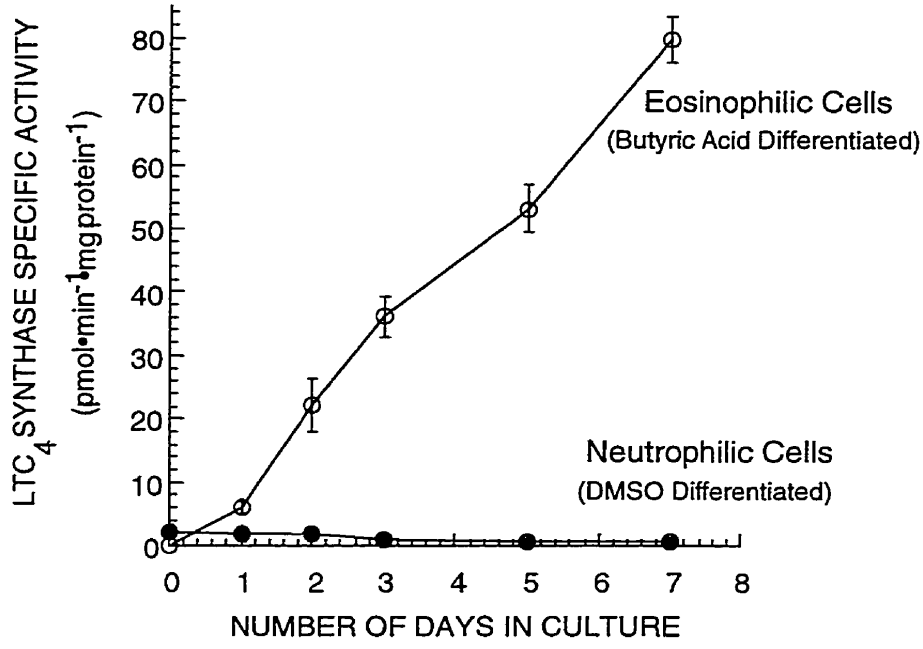
LTC4 SYNTHASE SPECIFIC ACTIVITY
(pmol•min-1•mg protein-1)
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
NUMBER OF DAYS IN CULTURE
Eosinophilic Cells
(Butyric Acid Differentiated)
Neutrophilic Cells
(DMSO Differentiated)

Figure 4

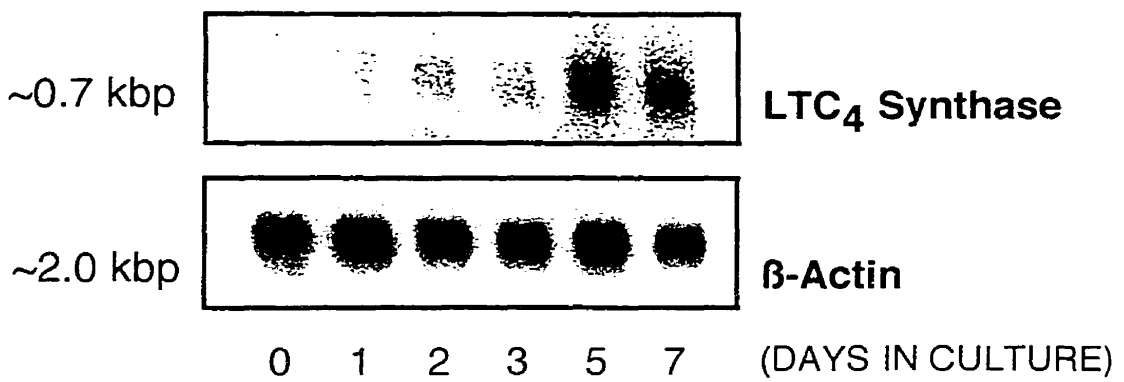

Figure 5

6. MANUSCRIPT C

Production of Leukotriene C_4 in Different Human Tissues is Attributable to Distinct Membrane Bound Biosynthetic Enzymes

J. Biol. Chem. 272: 10182-10187 (1997)

Recently, a novel membrane bound LTC_4 synthesizing enzyme, microsomal GST-II, was discovered. Since this enzyme was able to efficiently produce LTC_4 we wanted to determine the importance of this enzyme versus LTC_4 synthase for the production of the cysteinyl leukotrienes. This paper presents evidence that microsomal GST-II is the predominant enzyme responsible for LTC_4 formation in liver tissue and endothelial cells, whereas, LTC_4 synthase is the main enzyme capable of forming LTC_4 in lung tissue and platelets.

PRODUCTION OF LEUKOTRIENE C_4 IN DIFFERENT HUMAN TISSUES IS ATTRIBUTABLE TO DISTINCT MEMBRANE BOUND BIOSYNTHETIC ENZYMES

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ABBREVIATIONS

LT	leukotriene
LTA_4	5,6-oxido-7,9,11,14-eicosatetraenoic acid
LTC_4	5(S)-hydroxy-6(R)-S-glutathionyl-7,9,trans-11,14-cis eicosatetraenoic acid
LTD_4	5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis eicosatetraenoic acid
LTE_4	5(S)-hydroxy-6(R)-S-cysteinyl-7,9-trans-11,14-cis eicosatetraenoic acid
5-LO	5-lipoxygenase
FLAP	5-lipoxygenase activating protein
ECL	enhanced chemiluminescence
GST	glutathione S-transferase
GSH	glutathione
MGST-I	microsomal glutathione S-transferase-I
MGST-II	microsomal glutathione S-transferase-II

SUMMARY

Microsomal glutathione S-transferase-II (GST-II), has recently been discovered and characterized as a member of the 5-lipoxygenase-activating protein (FLAP)/leukotriene (LT)C_4 synthase gene family which also includes microsomal glutathione S-transferase-I (GST-I) as a distant member of this gene family. This new enzyme is unique as it is the only member of this family capable of efficiently conjugating reduced glutathione to both LTA_4 and 1-chloro-2,4-dinitrobenzene. Although microsomal GST-II has been demonstrated to display both general glutathione S-transferase (GST) and specific LTC_4 synthase activities, its biological function remains unknown. In this study, we investigated the physiological location of microsomal GST-II as well as the relative importance of this enzyme versus LTC_4 synthase for the production of LTC_4 in various human tissues and cells that have been previously demonstrated to possess LTC_4 synthase activity. As determined by Western-blot, microsomal GST-II was predominantly expressed in human liver microsomes, human endothelial cell membranes, and sparsely detected in human lung membranes. In contrast, LTC_4 synthase was prevalent in human lung membranes, human platelet homogenates, and human kidney tissue. Concomitant to the formation of LTC_4, microsomal GST-II also produces a new metabolite of LTA_4, a postulated LTC_4 isomer. This isomer was used to distinguish between microsomal GST-II and LTC_4 synthase activities involved in the biosynthesis of LTC_4. Based on the relative production of LTC_4 to the LTC_4 isomer, microsomal GST-II was demonstrated to be the principal enzyme responsible for LTC_4 production in human liver microsomes and human endothelial cells and played a minor role in the formation of LTC_4 in human lung membranes. In comparison, LTC_4 synthase was the main enzyme capable of catalyzing the conjugation of reduced glutathione to LTA_4 in human lung membranes and human platelet homogenates. Therefore, microsomal GST-II appears to be an

integral component in the detoxification of biological systems due to its marked presence in human liver, in accordance with its known GST activity. Microsomal GST-II, however, may also be pivotal for cysteinyl leukotriene formation in endothelial cells and this could change our current understanding of the regulation of leukotriene biosynthesis in inflammatory disorders such as asthma.

INTRODUCTION

Microsomal GST-II is the newest member of the FLAP/LTC_4 synthase gene family discovered to date. This novel enzyme displays 33% identity to FLAP, 44% identity to LTC_4 synthase, and limited sequence identity to microsomal GST-I at the amino acid level (1). These four proteins may also have similar structural configurations based on analogous hydrophobicity plots. Microsomal GST-II is a 16.6 kDa protein with a calculated pI of 10.4, and possesses both LTC_4 synthase as well as conventional glutathione S-transferase (GST) activities as shown through its ability to conjugate reduced glutathione with both LTA_4 and 1-chloro-2,4-dinitrobenzene, and was consequently characterized as a member of the membrane bound GSTs (1). This new enzyme, accordingly, may have important roles in both leukotriene biosynthesis and in cellular detoxification by glutathione S-transferase activity.

GSTs are a family of enzymes that catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophilic substrates. The GSTs belong to a gene superfamily in which four different gene families encode the cytosolic GSTs (α, μ, π, and θ), and two encoding the microsomal forms of the enzyme (1,2). Biologically, these enzymes are responsible for detoxification of xenobiotics by catalyzing GSH conjugation to generated metabolites and for protection from endogenous hydroperoxides produced during oxidative stress via their GSH-dependent peroxidase activity (3).

LTC_4 synthase is a unique membrane bound enzyme that has been distinguished from all previously known cytosolic and microsomal GSTs (4,5) through its narrow substrate specificity for LTA_4. LTC_4 synthase and FLAP are important components of the leukotriene biosynthetic pathway. FLAP is required for cellular LTA_4 formation possibly through the presentation of arachidonate to 5-lipoxygenase (6-9). LTC_4 synthase is the first committed enzyme for the conversion of LTA_4 to the cysteinyl leukotrienes, LTC_4, LTD_4,

and LTE_4, which have significant roles in immediate hypersensitivity reactions (see reviews 10-15).

Prior to the molecular characterization of LTC_4 synthase and microsomal GST-II a number of studies have described LTC_4 synthase activity in various cell types and tissues (15-24). The present study addresses the relative importance of these two enzymes in the synthesis of LTC_4 in various human tissues.

EXPERIMENTAL PROCEDURES

Materials

Human venous endothelial frozen cell pellet was purchased from Cell Systems (Kirkland, WA). Human liver and lung tissues were obtained from the International Institute for the Advancement of Medicine (IIAM) (Exton, PA). Protein medleys were from Clontech (Palo Alto, CA). Bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), Hepes, and DL-dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Aprotinin, leupeptin, and pepstatin A were from Boehringer Mannheim GmbH, (Mannheim, Germany). Tris base was also from Boehringer Mannheim Corporation (Indianapolis, IN). Taurocholic acid, sodium salt was obtained from Calbiochem Corporation (La Jolla, CA). LTA_4-methyl ester, 2-(2-(1-(4-chlorobenzyl)-4-methyl-6-((5-phenylpyridin-2-yl)-methoxy)-4,5-dihydro-1*H*-thiopyrano(2,3,4-*c,d*)indol-2-yl)ethoxy)butanoic acid (L-699 333), and 3-(1-(*p*-chlorophenyl)-5-isopropyl-3-*tert*-butylthio-1*H*-indol-2-yl)-2,2-dimethylpropanoic acid (MK-886) were synthesized by the Department of Medicinal Chemistry at the Merck Frosst Centre for Therapeutic Research. Novapak C_{18} HPLC columns were from Waters Chromatography (Milford, MA). HPLC solvents were from BDH (Toronto, Ontario). The Department of Biochemistry and Molecular Biology at the Merck Frosst Centre for Therapeutic Research provided the specific polyclonal antibodies to 5-lipoxygenase activating protein (FLAP), LTC_4 synthase, and microsomal GST-II (25, 26). The peptide antibody for microsomal GST-II was raised to microsomal GST-II amino acid sequence 42-57 (note: ^{57}Asp was replaced with His). Microsomal GST-I polyclonal antibody was a kind gift from Dr. John Hayes and purified microsomal GST-I was a kind gift from Dr. Ralf Morgenstern. Partially purified (137-fold) LTC_4 synthase from THP-1 cell extracts, HiLoad Q anion exchange fraction, was prepared as previously described (27). Renaissance western blot chemiluminescence reagent (ECL) was purchased from DuPont NEN

Research Products (Boston, MA). Horseradish peroxidase-linked donkey anti-rabbit antibody was obtained from Amersham Life Sciences (Buckinghamshire, England). Blotting grade blocker non-fat dry milk and Tween-20 were from Bio-Rad Laboratories (Hercules, CA). Sodium dodecyl sulfate and phosphate buffered saline without calcium and magnesium (PBS) were from Gibco BRL Life Technologies (Grand Island, NY and Gaithersburg, MD, respectively). All other reagents were of analytical grade and were purchased from Sigma.

Isolation of human peripheral blood platelets and preparation of homogenates

Human peripheral blood was collected from healthy volunteers who had not taken any medication for the previous 7 days. One tenth the volume of 3.8% (w/v) trisodium citrate, in 0.9% (w/v) saline, was immediately added to the collected blood as anticoagulant and the mixture was centrifuged at 200 x g for 10 min at 22°C. The platelet-rich plasma (PRP) was transferred to a tube containing 50% (v/v) Tris-buffered saline (TBS), containing 6.2 mM EDTA, and 30% (v/v) of 3.8% (w/v) trisodium citrate. After gentle mixing by inversion, the diluted PRP was centrifuged at 650 x g for 10 min at 22°C. The pellet was washed twice with TBS containing 6.2 mM EDTA and cells were counted using a coulter counter. The final concentration was adjusted to 1.0 x 10^8 platelets/mL in cold lysis buffer containing 10 mM Hepes/KOH (pH 7.4), 2 mM EDTA, 2% (w/v) taurocholate, with freshly added DTT (5 mM), PMSF (1 mM, from a fresh 200 mM stock in EtOH), leupeptin (20 µg / mL), pepstatin A (10 µg / mL, from a fresh 1 mg / mL stock in EtOH), and aprotinin (10 µg / mL). Lysates were shaken for 20 min at 4°C, sonicated for 5 s at 0°C, frozen in liquid nitrogen and stored at -80°C. Bradford (28) protein assay (Bio-Rad) was performed on the homogenate.

Preparation of human endothelial cell, liver, and lung membranes

A frozen human venous endothelial cell pellet containing 1 x 10^7 cells was thawed on ice and resuspended in PBS containing 2 mM EDTA and 2 mM PMSF. The cells were sonicated three times for 15 s each on ice and subjected to differential centrifugation at 1 000 x g for 15 min and 100 000 x g for 1 h, both at 4°C. The pellets were resuspended in 50 µL PBS containing 2 mM EDTA, frozen in liquid nitrogen, and stored at -80°C.

Subcellular fractions of frozen human liver and lung tissues were prepared according to standard procedures (29, 30, respectively).

Western-blot analyses of microsomal GST-I, microsomal GST-II, LTC_4 synthase, and FLAP expression

Western-blot analyses were performed similarly to those described previously (26). Briefly, SDS-containing sample buffer (31) was added to all samples. The samples were subsequently heated for 5 min at 95°C, electrophoresed through SDS/polyacrylamide gels (Novex), and electroblotted onto nitrocellulose. Ponceau S staining was used to visualize the efficiency of transfer. Membranes were then soaked for 1 h at 25°C in Tris-buffered saline containing 0.1% (v/v) Tween 20 (0.1% T TBS; 20 mM Tris/HCl, pH 7.5, 0.5 M NaCl) containing 5% (w/v) Bio-Rad Blotting Grade Blocker non-fat dry milk. Blots were washed twice for 5 min each with 0.1% T TBS and subsequently treated for 1 h at 25°C with the indicated specific primary polyclonal antibody (dilution 1:500) in 0.05% T TBS containing 5% dry milk. After washing the blots 3 times for 5 min each with 0.1% T TBS the membranes were incubated for 1 h at 25°C with a horseradish-peroxidase-linked donkey anti-rabbit antibody (dilution 1:3 000) in 0.05% T TBS containing 1% dry milk. The blots were washed 3 times for 5 min each with 0.3% T TBS and then 3 times for 5 min each with 0.1% T TBS and subsequently developed using enhanced chemiluminescence (Renaissance Western Blot Chemiluminescence Reagent, DuPont NEN), according to the manufacturer's instructions.

Measurement of LTC_4 synthase and microsomal GST-II enzymatic activities by reverse-phase HPLC

LTC_4 synthase and microsomal GST-II activities were assayed as previously described (1) by measuring the amount of LTC_4 and an isomer of LTC_4 produced in incubations at 25°C from various samples in 0.1 M potassium phosphate (Kpi) buffer pH 7.4 containing reduced glutathione (5 mM) and 60 μM LTA_4 (free acid) stabilized by the presence of 0.05% (w/v) BSA in a final volume of 100 μL. After 15 min the reaction was terminated by the addition of an equivalent volume of acetonitrile/methanol/acetic acid at 50:50:1 and the precipitated proteins were removed by centrifugation at 16 000 x g for 15 min at 4°C. The amount of LTC_4 and an isomer of LTC_4 synthesized were resolved by isocratic reverse-phase HPLC on a Waters Associates Novapak C_{18} column (3.0 mm x 150 mm, 4 μm particle size) with the mobile phase (acetonitrile/methanol/water/acetic acid at 28:14:54:1, pH 5.6) at a flow rate of 1.2 mL/min. Quantification of the amount of products formed was based on the measurement of the peak absorbance at 280 nm from known amounts of injected LTC_4. The LTC_4 and the LTC_4 isomer peaks were identified by comparison to retention time of synthetic LTC_4 and on-line analysis of the UV absorbance spectra of the eluted compounds using a Waters 991 diode-array spectrophotometer.

Preparation of microsomal GST-II, LTC_4 synthase, and FLAP membranes from baculovirus infected Sf9 cells

Microsomal GST-II, LTC_4 synthase, and FLAP proteins were obtained from baculovirus infected Sf9 cell membranes as described previously (1, 8, respectively). Briefly, Sf9 cells (Invitrogen) were infected with recombinant or wild type virus and cultured for 72 h at 28°C in Grace's insect media supplemented with 10% FBS (v/v), gentamycin (50 μg / mL), and fungizone (2.5 μg / mL). The cells were then harvested, washed, resuspended in PBS, and sonicated three times for 10 s each on ice. The sonicates were

subsequently subjected to differential centrifugation at 500 x g for 10 min and 100 000 x g for 1 h, both at 4°C. The pellets were resuspended in PBS and stored at -80°C. Coomasie protein (Pierce) assay was performed according to the manufacturer's instructions.

Microsomal GST-I, microsomal GST-II, and FLAP polyclonal antibodies are specific, whereas, the LTC_4 synthase antibody displays cross reactivity.

Polyclonal antibodies were used as tools to determine the relative contribution of LTC_4 synthase versus microsomal GST-II for the production of LTC_4 in biological systems. In order to determine the presence of either of these proteins, the specificity of these antibodies with respect to one another and to the other members of this family, FLAP and microsomal GST-1, were tested. The antibodies used to detect microsomal GST-II, LTC_4 synthase, and FLAP had been raised against peptides in the region displaying the highest identity to one another, the FERV region (Fig. 1). The microsomal GST-I polyclonal antibody was raised against the full length protein, which also contains a similar region of homology to the other peptides. As the different peptides contain homologous regions, the four antibodies were tested for possible cross reactivity by Western-blot analyses. Figure 2 demonstrates that microsomal GST-I, microsomal GST-II, and FLAP antibodies were specific. However, the LTC_4 synthase antibody displayed some cross reactivity by detecting microsomal GST-II and FLAP proteins. These results indicate that we have specific polyclonal antibodies for the detection of microsomal GST-I, microsomal GST-II, and FLAP, and a polyclonal antibody that cannot distinguish between LTC_4 synthase and microsomal GST-II.

Expression of microsomal GST-I, microsomal GST-II, LTC_4 synthase, and FLAP in various human tissues.

After establishing the specificity of the above polyclonal antibodies, they were subsequently used to search for their respective protein targets in various human tissues (Fig. 3). As expected, microsomal GST-I was markedly detected in liver tissue, as well as in adrenal gland, kidney and to a lesser extent mammary gland tissues. Microsomal GST-II was expressed

highly in liver (Fig. 3) and in lower amounts in adrenal gland, kidney, pancreas, and thymus tissues (after prolonged exposure, data not shown). The LTC_4 synthase antibody detected ~17 kDa proteins intensely in kidney, adrenal gland, liver, lymph, and weakly in skeletal, and stomach samples, whereas, the FLAP antibody displayed strong ~18 kDa bands in lymph and thymus tissues and weak bands in adrenal gland, liver, and skeletal tissues. These results have identified many important human tissues in which these proteins may be found.

Expression of LTC_4 producing enzymes in human liver, lung, endothelial cells, and platelets

Figure 3 demonstrates that microsomal GST-II was primarily present in human liver tissue. To confirm and extend this finding, the subcellular localization of microsomal GST-II was investigated in human liver tissue by probing liver samples prepared by differential centrifugation with the microsomal GST-II and LTC_4 synthase polyclonal antibodies (Fig. 4). Both microsomal GST-II and LTC_4 synthase antibodies displayed a ~17 kDa band in the liver homogenate, the 10 000xg supernatant, and the 100 000xg pellet fractions. The intensity of detection increased with increasing fractionation of the membrane samples and no bands were observed in the liver 100 000xg supernatant fraction. These results indicate that microsomal GST-II is present in human liver as a membrane bound protein.

Also, the presence of microsomal GST-II versus LTC_4 synthase was investigated in human lung, platelets and endothelial cells. The LTC_4 synthase antibody recognized a ~17 kDa band in the lung 10 000xg pellet and a more intense band in the 100 000xg pellet as well as a ~17 kDa band in platelet homogenates. The LTC_4 synthase antibody also displayed a band in endothelial cell membranes after a prolonged exposure time (20 min, data not shown). In contrast, microsomal GST-II was only slightly detected in lung membranes, not observed in platelet homogenates, and detected in endothelial cell membranes. These results indicate that LTC_4

synthase is the predominant enzyme present in human lung tissue and human platelets, whereas, microsomal GST-II appears to be the principal enzyme found in human endothelial cell membranes.

LTC_4 synthase and microsomal GST-II biosynthetic activities in human tissues can be distinguished by product profiles.

Microsomal GST-II catalyzes the formation of LTC_4 and a possible LTC_4 isomer from LTA_4 and glutathione. LTC_4 and the putative LTC_4 isomer have retention times of 9.0 min and 7.6 min, respectively, and distinct maximum UV absorbencies of 281 nm and 283 nm, respectively (1) (Fig. 5). LTC_4 synthase, however, mainly catalyzes the synthesis of LTC_4. Therefore, the ratio of the production of LTC_4 to the postulated LTC_4 isomer was used to distinguish between the presence of microsomal GST-II activity and LTC_4 synthase activity (Table 1). In order to confirm, as well as discern, the presence of either microsomal GST-II or LTC_4 synthase, as demonstrated by Western-blot (Fig. 4), activity assays were performed. Lung 100 000xg pellet, platelet homogenates, and LTC_4 synthase derived from both THP-1 cell extracts and Sf9 cell membranes all displayed an LTC_4: LTC_4 isomer ratio of >50. In contrast, liver 100 000xg pellet, endothelial 100 000xg pellet, and microsomal GST-II from Sf9 cell membranes, all demonstrated ratios of <5. These ratios indicate that LTC_4 synthase activity is mainly in human lung and platelets, whereas, microsomal GST-II activity resides predominantly in human liver and endothelial cells.

Due to the similarities in structure between microsomal GST-II, LTC_4 synthase, and FLAP, and their involvement in leukotriene biosynthesis, activity assays were also performed on microsomal GST-II in the presence of known inhibitors of leukotriene biosynthesis. MK-886 is a potent inhibitor of FLAP (nanomolar range), while both L-699 333 and MK-886 are capable of inhibiting LTC_4 synthase activity in the micromolar range. L-699 333 and MK-886, were both found to inhibit LTC_4 formation by microsomal GST-II expressed in Sf9 cell membranes with IC_{50} values in the 10 μM range (data

not shown). The inhibition of microsomal GST-II activity by these leukotriene biosynthesis inhibitors suggests a similar active site for these three proteins.

DISCUSSION

Recently, a novel membrane bound protein, microsomal GST-II, was discovered that displayed homology at the amino acid level to FLAP, LTC_4 synthase, and to a lesser extent microsomal GST-I (1). These proteins all display similar hydrophobicity patterns and consequently may have similar structures. Due to the above similarities these four proteins appear to be members of a gene family that encodes membrane bound proteins important for either leukotriene production or cellular detoxification by GSH conjugation. Microsomal GST-II is a unique member of this family due to its ability to efficiently conjugate reduced GSH to both LTA_4 and to 1-chloro-2,4-dinitrobenzene (1). Hence, microsomal GST-II maybe more important than LTC_4 synthase as a catalyst for the formation of LTC_4 in certain tissues. To try to understand the biological function (importance) of this unique enzyme, its location in human cells and tissues compared to other family members was determined by Western-blot. In addition, a new method was developed for distinguishing between previously determined LTC_4 synthase activity and what may actually be microsomal GST-II activity.

Microsomal GST-I, microsomal GST-II, and FLAP polyclonal antibodies were determined to be specific for the recognition of their respective proteins, even though the peptides used to raise these antibodies contained regions of homology. Only the LTC_4 synthase antibody displayed cross reactivity by recognizing FLAP, microsomal GST-II, and LTC_4 synthase. This non-specific association of the LTC_4 synthase antibody to FLAP may be explained by a combination of high antibody concentration (1:500 dilution) and high expression of FLAP from baculovirus infected Sf9 cells resulting in its high abundance on the blot. The recognition of FLAP by the LTC_4 synthase antibody could be discerned from LTC_4 synthase and microsomal GST-II due to FLAP's higher molecular weight and was therefore not a problem in determining the presence of FLAP in various tissues. This antibody, however, could not discriminate between LTC_4 synthase and

microsomal GST-II, thus, the presence of LTC_4 synthase was inferred based on the absence of specific microsomal GST-II recognition by the microsomal GST-II polyclonal antibody in identical tissues.

Interestingly, microsomal GST-II expressed from Sf9 cells was detected as a doublet, which may indicate the phosphorylation of this enzyme or a downstream degradation product. Phosphorylation of microsomal GST-II could be a possible regulatory mechanism for this enzyme as it has also been postulated for LTC_4 synthase (27). LTC_4 synthase has two potential protein kinase C phosphorylation sites which are not however present in microsomal GST-II. Microsomal GST-II from Sf9 cells was also inhibited by leukotriene biosynthesis inhibitors with a similar IC_{50} to their effect on LTC_4 synthase (32). As these inhibitors have been shown to inhibit FLAP and LTC_4 synthase, these proteins may have a similar active site. FLAP amino acids in the region ^{41}Ser to ^{61}Val have been shown to be critical for binding of leukotriene biosynthesis inhibitors (33). This region is the most homologous region between the members of this family including microsomal GST-I and is therefore postulated to be the lipid binding site of these proteins.

As expected, human microsomal GST-I is mainly expressed in human liver tissue in accordance with previous findings of location and its function as a phase II detoxifying enzyme (3). The significant detection of microsomal GST-I in the adrenal gland and kidney substantiates previous findings of microsomal GST activity, protein expression, or mRNA expression, and probably serves to protect these extrahepatic tissues from endogenous or exogenous toxicants (34, 35). Microsomal GST-II is also primarily expressed in human liver membranes and to a lesser extent in endothelial cells and minimally in lung membranes, displaying a very narrow tissue distribution. The marked detection of microsomal GST-II in the liver along with its glutathione S-transferase activity suggests that it may have a similar biological function to microsomal GST-1. Microsomal GST-II also appears to be post-transcriptionally regulated since the protein was not

significantly detected in many of the tissues that exhibit mRNA expression (1). However, this lack of protein detection may also be a matter of sensitivity in the Western-blot analysis. FLAP and LTC_4 synthase were widely distributed with expression in overlapping tissues as might be anticipated for two proteins involved in the biosynthesis of cysteinyl leukotrienes. However, there were also tissues (kidney, stomach, and thymus) that only expressed either FLAP or LTC_4 synthase. Consequently, these tissues demonstrate either the requirement of transcellular metabolism for leukotriene biosynthesis or in the case of those tissues that only express FLAP may solely produce LTB_4. These observations may also indicate the recognition of other putative members of this gene family by the cross-reactive LTC_4 synthase antibody. Contamination of tissue preparations by infiltrating peripheral blood cells could also explain any discrepancies in tissue distributions. FLAP was distinctly found in human lymph and thymus tissues, confirming previous findings of FLAP in B- and T-lymphocytes (36), whereas, LTC_4 synthase is significantly detected in human kidney, lung, platelets, and to a lesser extent in skeletal tissue, identical to earlier observations of mouse LTC_4 synthase mRNA expression (37) and human LTC_4 synthase protein detection (38). The LTC_4 synthase antibody appears to detect a 17 kDa band in the liver with an equivalent intensity to that detected by the microsomal GST-II antibody. These band intensities are most likely saturated and have reached a plateau due to overexposure of the blots. Therefore, the band intensities displayed in the liver by different antibodies cannot be quantitatively compared.

Microsomal GST-II possesses both generalized glutathione S-transferase and LTC_4 synthase activities thereby contributing to the formation of LTC_4 in biological systems. Microsomal GST-II concomitantly produces LTC_4 and a new LTA_4 metabolite, a possible isomer of LTC_4, that can be separated from LTC_4 by RP-HPLC. LTC_4 synthase stereoselectively produces LTC_4, thus, the production ratio of LTC_4 to the LTC_4 isomer clearly differentiates microsomal GST-II activity versus LTC_4 synthase activity.

Identification of microsomal GST-II activity corresponds directly to the detection of microsomal GST-II in Western-blot analyses. There is significant microsomal GST-II activity in liver 100 000xg membrane samples. Microsomal GST-II is accordingly the predominant membrane bound enzyme responsible for LTC_4 formation in human liver and may subsequently be a fundamental enzyme responsible for detoxification of lipid epoxides. Based on the above data, however, the possibility that LTC_4 synthase still exists in the liver in an inactive form cannot be excluded.

LTC_4 was selectively synthesized by lung membranes with a minor production of the LTC_4 isomer, demonstrating LTC_4 synthase as the favoured enzyme for LTC_4 production in human lung tissue with a minimal contribution from microsomal GST-II. In the lung, microsomal GST-II may play a minor part in LTC_4 production, however, it may have another function such as protection from oxidative stress and inhaled xenobiotics.

The limited formation of LTC_4 from liver cytosol most likely represents the ability of cytosolic GSTs (mostly μ family GSTs) to conjugate reduced GSH to LTA_4 (39).

Platelet homogenates produced significant amounts of LTC_4 in the absence of notable quantities of the LTC_4 isomer, which together with the Western-blot data indicate that the LTC_4 synthase enzyme predominates for LTC_4 production in platelets. In contrast, microsomal GST-II was detected in endothelial cell membranes by Western-blot and activity assays and may be the enzyme responsible for converting exogenous LTA_4 to LTC_4 during transcellular metabolism in these cells. The presence of microsomal GST-II as the main enzyme for LTC_4 production in endothelial cells could explain the findings of Habib and Maclouf (40). In these studies, endothelial cells and platelets were both demonstrated to produce LTC_4 from exogenously added LTA_4, however, platelets were more efficient at this conversion. Endothelial cells displayed a higher apparent K_m for LTA_4 in comparison to platelets. Similarly, microsomal GST-II has a higher K_m for LTA_4 than LTC_4 synthase and is less stereoselective for the production of

LTC_4 (1). Analogous to our results, the HPLC chromatogram demonstrating LTC_4 production by endothelial cells (40), also contains an extra peak where the LTC_4 isomer would be predicted to appear. Such a peak is absent or less pronounced in the chromatogram from platelets. These observations may support the present findings, that LTC_4 production is catalyzed by microsomal GST-II in endothelial cells and by LTC_4 synthase in platelets.

Overall, microsomal GST-II is an integral membrane bound protein in human liver tissue that is potentially involved in detoxification of biological systems. Importantly, we have developed a method to distinguish microsomal GST-II activity from LTC_4 synthase activity. This has lead to the discovery that microsomal GST-II is predominantly responsible for LTC_4 production in liver membranes and may be a key enzyme in the transcellular metabolism of LTA_4 into cysteinyl leukotrienes in certain cells that lack all of the enzymes required for *de novo* leukotriene biosynthesis.

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FIGURE LEGENDS

Figure 1. Comparison of homology in the complete amino acid sequences of the members of the FLAP/LTC_4 synthase family. Amino acids that are homologous in two or more members of the FLAP/LTC_4 synthase family are indicated by bold capital letters. The numbering of the amino acids coincides to the amino acid sequence of microsomal GST-I. The peptides used to raise the corresponding polyclonal antibodies are underlined. *The peptide used to produce the microsomal GST-II polyclonal antibody contained a histidine residue instead of an asparagine as the last amino acid in the peptide sequence.

Figure 2. Microsomal GST-I, microsomal GST-II, and FLAP polyclonal antibodies are specific, whereas, the LTC_4 synthase antibody cannot distinguish between microsomal GST-II and LTC_4 synthase proteins. Purified microsomal GST-I (0.26 μg and 0.026 μg protein), microsomal GST-II from baculovirus infected Sf9 cell membranes (5 μg and 0.5 μg protein), FLAP from baculovirus infected Sf9 cell membranes (50 μg and 5 μg protein), partially purified LTC_4 synthase from THP-1 cell extracts (0.013 μg LTC_4 synthase protein), and wild type baculovirus infected Sf9 cell membranes (50 μg protein) were resolved by SDS/polyacryamide gel electrophoreses and electroblotted onto nitrocellulose. Western-blot analyses were performed as described in Experimental Procedures, using the polyclonal antibodies that were raised against the various peptides in Figure 1, and detected using enhanced chemiluminescence. The different blots: αMGST-I, αMGST-II, αLTC_4 synthase, and αFLAP were exposed to film for 1 min, 15 sec, 15 sec, and 1 sec, respectively.

Figure 3. Comparison of human tissue distributions of microsomal GST-I, microsomal GST-II, LTC_4 synthase, and FLAP. Equivalent amounts of various human tissue homogenates (75 μg protein), LTC_4 synthase and microsomal

GST-II from baculovirus infected Sf9 cell membranes (50 μg and 5 μg protein, respectively) were electrophoresed through polyacrylamide gels and electroblotted onto nitrocellulose. Western-blot analyses were performed using the polyclonal antibodies raised against the peptides in figure 1 and detected using enhanced chemiluminescence as described in Experimental Procedures. The various blots: αMGST-I, αMGST-II, αLTC$_4$ synthase, and αFLAP had exposure times of 15 sec, 5 min, 5 min, and 1 min, respectively.

Figure 4. Human liver and endothelial cell membranes principally express microsomal GST-II, while, human lung and platelet homogenates primarily express LTC$_4$ synthase. Differential centrifugation fractions from human liver (75 μg protein), human lung membrane fractions (75 μg protein), human endothelial cell membranes (10 μL of 1 x 10^7 cell equivalents), human platelet homogenates (75 μg protein), microsomal GST-II from baculovirus infected Sf9 cell membranes (5 μg protein), and partially purified LTC$_4$ synthase from THP-1 cell extracts (7.35 μg protein) were electrophoresed through polyacrylamide gels, electroblotted onto nitrocellulose, and immunoblotted using the polyclonal antibodies to microsomal GST-II or LTC$_4$ synthase and detected using enhanced chemiluminescence as described in Experimental Procedures. The liver, lung, and control blots were exposed to film for 15 sec, whereas, the endothelial and platelet blots had an exposure time of 1 min.

Figure 5. Comparison of LTA$_4$ metabolites produced by microsomal GST-II, versus LTC$_4$ synthase in human liver, lung, and platelets. This RP-HPLC chromatogram demonstrates the representative LTA$_4$ metabolites obtained in Table 1 due to the presence of either microsomal GST-II activity or LTC$_4$ synthase activity. The scale of the different chromatograms were in all cases normalized to the LTC$_4$ peak. The LTC$_4$ peak displayed a retention time of 9.0 min and a maximum UV absorbance at 281 nm, while the

postulated LTC_4 isomer peak had a retention time of 7.6 min and a maximum UV absorbance at 283 nm.

Table 1. Microsomal GST-II activity is predominant in human liver membranes, whereas, LTC_4 synthase activity is primarily in human lung and platelets. Differential centrifugation fractions from human liver (0.4 mg protein), human endothelial 100 000xg membrane fractions (0.4 mg protein), human lung 100 000xg membrane fractions (0.4 mg protein), human platelet homogenates (0.2 mg protein), partially purified LTC_4 synthase from THP-1 cell extracts (2.45 μg protein), microsomal GST-II from baculovirus infected Sf9 cell membranes (0.05 mg protein), LTC_4 synthase from baculovirus infected Sf9 cell membranes (0.05 mg protein), and wild type baculovirus infected Sf9 cell membranes (0.05 mg protein) were prepared as described in Experimental Procedures. Microsomal GST-II or LTC_4 synthase activity was assayed in the above samples by measuring the amount of LTC_4 and a postulated LTC_4 isomer produced in 15 min incubations at 25°C in 0.1 M Kpi, pH 7.4, containing reduced glutathione (5 mM) and 60 μM LTA_4 (free acid) stabilized by the presence of 0.05% (w/v) BSA, as resolved by isocratic reverse-phase HPLC. The relative production of LTC_4 to the postulated LTC_4 isomer was used to define microsomal GST-II activity versus LTC_4 synthase activity. The liver 100 000xg pellet samples represent 6 different human livers (n=6) including one sample representing the mean of a triplicate. The endothelial 100 000xg membrane fraction is a representative experiment of n=2. All other samples were performed in triplicate ± standard error of the mean.

[a] At the limit of detection, define limit as 15 pmol in assay

[b] Ratios not performed due to limit of detection of both LTC_4 and the LTC_4 isomer

Sample	LTC_4 formed (pmol • mg protein^{-1})	Isomer formed (pmol • mg protein^{-1})	Ratio (LTC_4 : isomer)
Liver 100 000xg sup	<50	a	b
Liver 100 000xg pellet	833 ± 147	199 ± 25	<5
Endothelial 100 000xg pellet	200	64	<5
Lung 100 000xg pellet	5270 ± 146	105 ± 3.5	>50
Platelet homogenate	507 ± 10	a	>50
LTC_4 synthase/THP-1 partially purified	129000 ± 1880	a	>50
LTC_4 synthase/Sf9 100 000xg pellet	1280 ± 365	a	>50
MGST-II/Sf9 100 000xg pellet	5430 ± 29	2170 ± 16	<5
wt Bac/Sf9 100 000xg pellet	a	a	b

Table 1

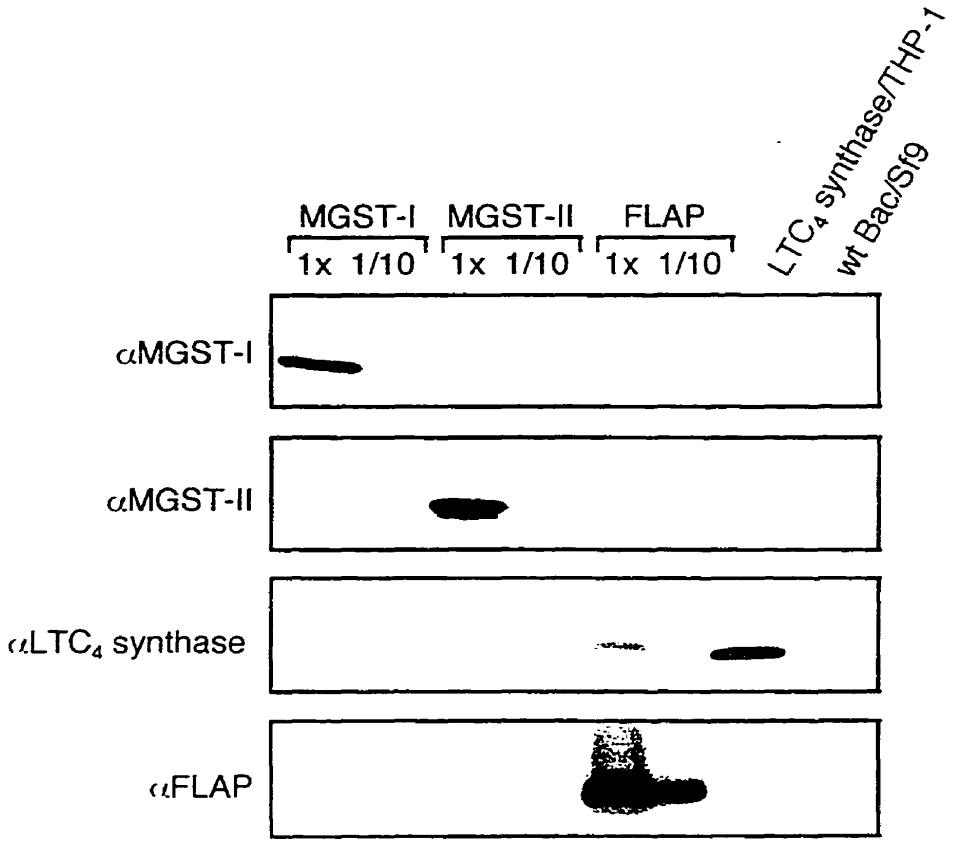

MGST-I
MGST-II
FLAP
1x 1/10
1x 1/10
1x 1/10
LTC4 synthase/THP-1
wt Bac/Sf9
αMGST-I
αMGST-II
αLTC4 synthase
αFLAP

Figure 2

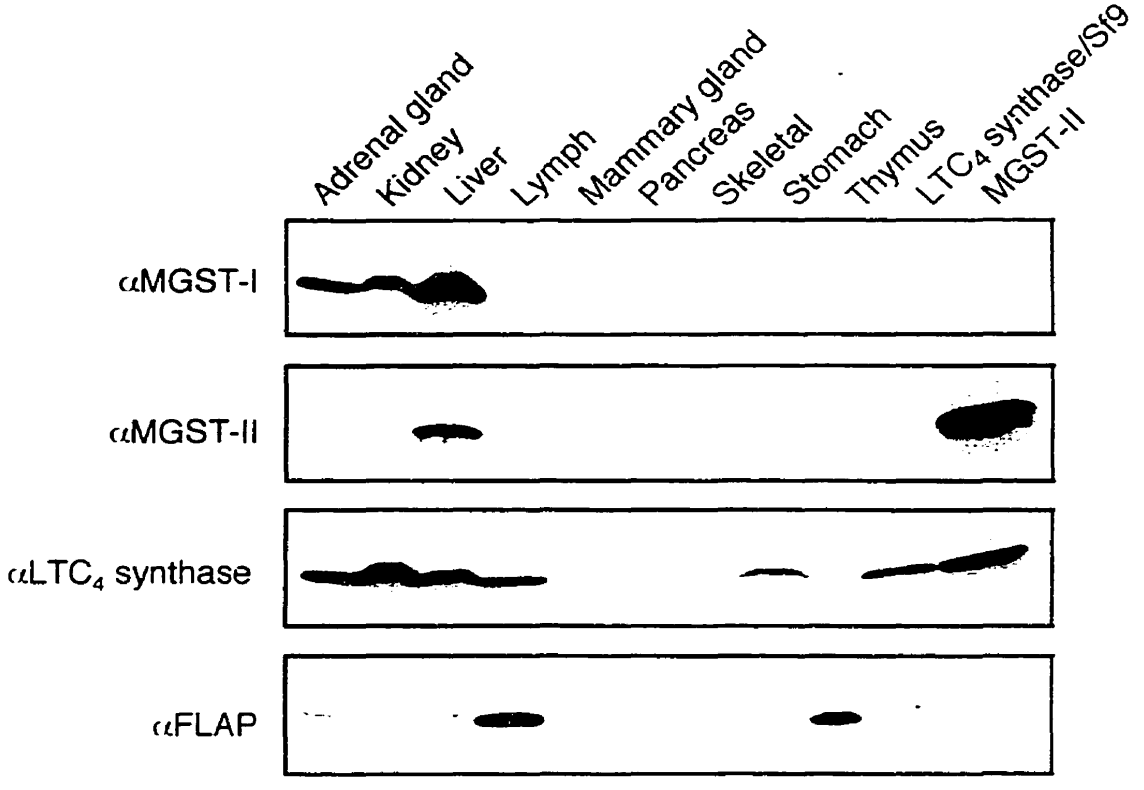

Adrenal gland
Kidney
Liver
Lymph
Mammary gland
Pancreas
Skeletal
Stomach
Thymus
LTC4 synthase/Sf9
MGST-II
αMGST-I
αMGST-II
αLTC4 synthase
αFLAP

Figure 3

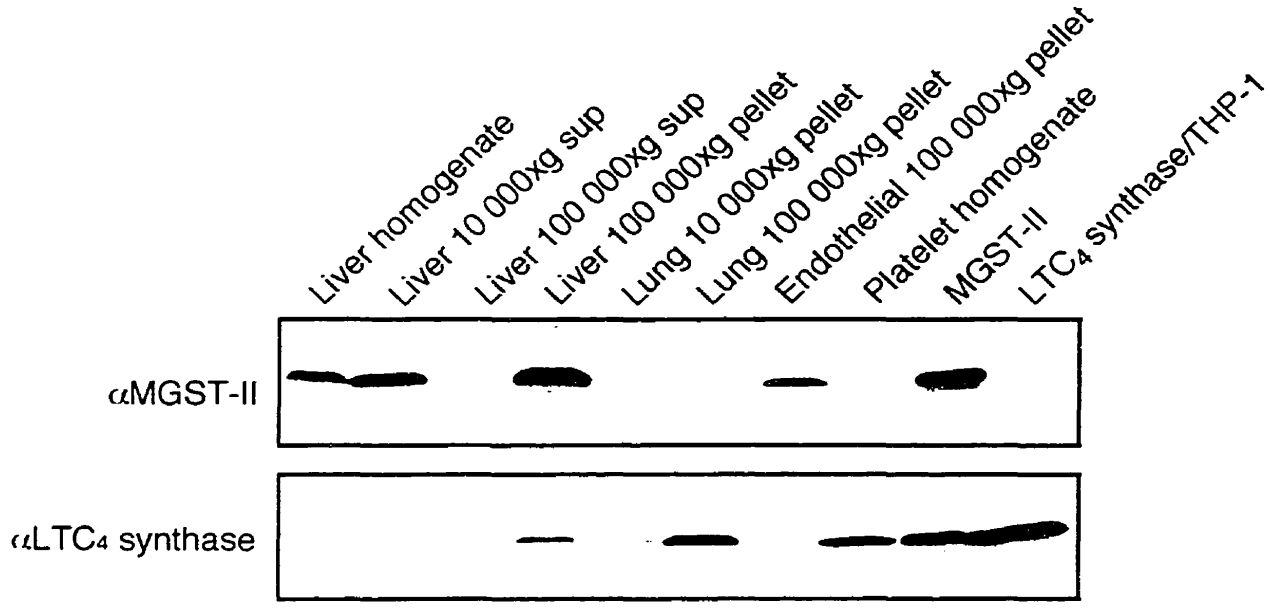
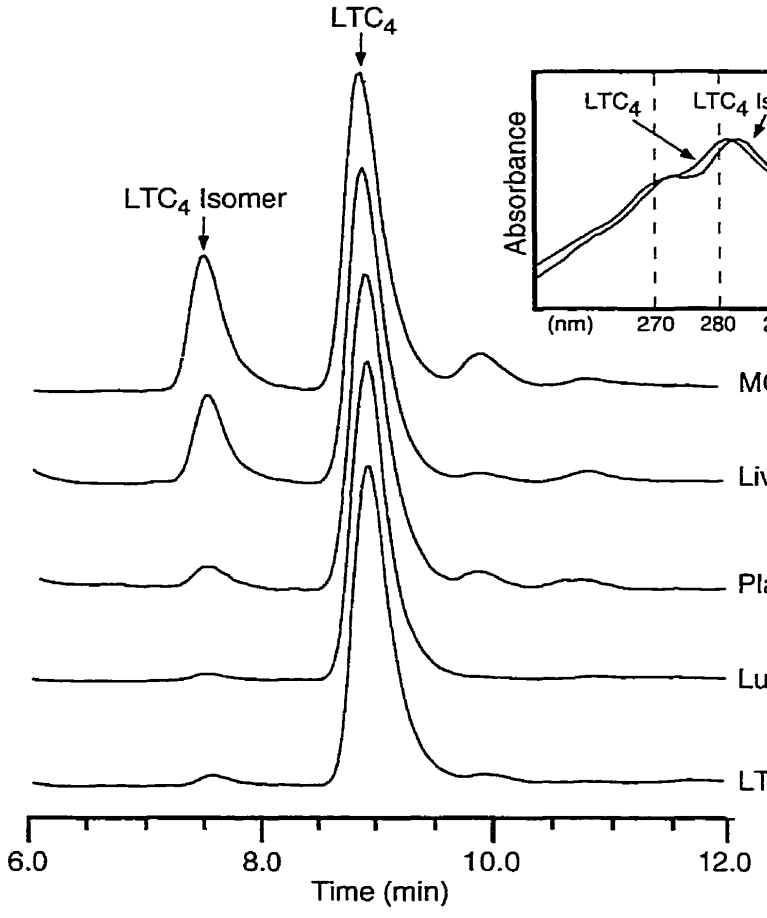

Liver homogenate
Liver 10 000xg sup
Liver 100 000xg sup
Liver 100 000xg pellet
Lung 10 000xg pellet
Lung 100 000xg pellet
Endothelial 100 000xg pellet
Platelet homogenate
MGST-II
LTC4 synthase/THP-1
αMGST-II
αLTC4 synthase

Figure 4



Ratio
LTC_4 : Isomer

< 5

> 50

Figure 5

7. DISCUSSION

Leukotrienes are potent mediators of bronchial inflammation and are predominantly produced by myeloid cells. The regulatory mechanisms governing leukotriene formation by these cells, however, are largely unknown. Leukotriene biosynthetic enzymes have been shown to be governed either by direct phosphorylation mechanisms or by gene regulatory mechanisms that alter gene transcription and/or translation. To further our understanding of the regulation of leukotriene biosynthesis, we investigated the cytokine regulation of leukotriene formation, the regulation of leukotriene biosynthetic enzymes during myeloid cell differentiation, and the relative importance of a novel membrane bound enzyme, microsomal GST-II, versus LTC_4 synthase in the production of LTC_4.

7.1 Cytokine regulation of leukotriene biosynthesis

Eosinophils are major producers of cysteinyl leukotrienes in the lung and have been associated with the deleterious effects that occur in the late phase of asthma. Thus, understanding the control mechanisms for accumulation and activation of eosinophils may be of therapeutic value. Cytokines have been demonstrated to prime eosinophils, enhancing their migration capacity and their production of cysteinyl leukotrienes upon activation. Cytokines are also capable of stimulating both phosphorylation events and gene regulation activities and may therefore control leukotriene production through cytokine signal transduction mechanisms. To elucidate how cytokines alter leukotriene formation in eosinophils, the effects of GM-CSF and IL-5 on leukotriene biosynthesis in an eosinophilic cell line (HL-60#7/BA also referred to as eosinophilic HL-60 cells) were examined.

HL-60#7 cells cultured with butyric acid resemble eosinophils both morphologically and functionally (chapter 4 (ref. 272), 124). These cells

contain eosin-staining granules that comprise major basic protein and eosinophil peroxidase. Eosinophilic HL-60 cells have also been demonstrated to respond chemotactically to mediators with an analogous rank order of potency as eosinophils. Importantly, these cells express cytokine and leukotriene receptors as well as the cysteinyl leukotriene biosynthetic enzymes. As large quantities of pure eosinophils are difficult to obtain, this cell line was required to examine the mechanism of cytokine regulation of leukotriene biosynthesis. Characteristic of eosinophils, these cells predominantly produce the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, and very low amounts of LTB_4. Also in agreement with previous studies using peripheral blood eosinophils, GM-CSF dose-dependently primed leukotriene biosynthesis by approximately 45% in eosinophilic HL-60 cells upon calcium ionophore challenge. Interestingly, asthmatics also display an enhanced leukotriene synthetic capacity as compared to normals, which is speculated to be caused by the presence of GM-CSF, IL-3, and IL-5 found only in the circulation of asthmatics (261). This enhancement in leukotriene biosynthesis by eosinophilic HL-60 cells was the result of an increase in the rate of their formation and not an increase in the total amount of leukotrienes formed, however, high concentrations of A23187 may have masked later effects. A23187 is cytotoxic and may inhibit cellular functions at high doses or after extended periods of time. Upon activation, leukotrienes are rapidly released, therefore, any increase in their rate of formation will result in a greater quantity of mediators available for immediate release. This enhanced release of leukotrienes to surrounding cells and tissues may partially explain the contribution of cytokines to the pathology of asthma.

It was initially speculated that GM-CSF may increase the rate of leukotriene formation by increasing LTC_4 synthase activity. LTC_4 synthase was considered a probable candidate since it is the first committed enzyme in the leukotriene biosynthetic pathway for the formation of LTC_4, LTD_4, and LTE_4, which eosinophils primarily produce. The predominant

production of cysteinyl leukotrienes by eosinophilic HL-60 cells is now known to be the result of an increase in the activity and expression of both mRNA and protein levels of LTC_4 synthase upon terminal differentiation towards an eosinophil (124, chapter 5 (ref. 273)). The gene for LTC_4 synthase has also recently been cloned and located on chromosome 5 next to a group of genes encoding growth factors, cytokines, and receptors that are involved in allergic and inflammatory diseases, including IL-3, IL-5, and GM-CSF itself (131, 132). Thus, GM-CSF may prime leukotriene biosynthesis by regulating LTC_4 synthase gene transcription and/or translation. The LTC_4 synthase gene contains several putative DNA-binding motifs including a STAT-binding site in the first intron (132). GM-CSF could, therefore, enhance LTC_4 synthase gene transcription via the STAT-recognition motif as was proposed for the ability of IL-3 to enhance LTC_4 synthase protein and steady-state mRNA levels in mouse bone marrow-derived mast cells differentiated with c-kit ligand and IL-10 (132). A GM-CSF pre-treatment period of 30 minutes is sufficient for early response genes to be expressed. Results measuring GM-CSF induction of FLAP mRNA steady-state levels were observed within a 30 min treatment period, although ^{35}S incorporation into the protein was only observed after 2 h (274). The stimulatory effect of GM-CSF on ^{35}S incorporation into 5-LO protein was most evident between 30 min and 1 h, however, *de novo* protein synthesis of 5-LO is thought to be regulated post-transcriptionally by GM-CSF (275) and not at the level of the gene.

LTC_4 synthase was also regarded as an ideal enzyme for GM-CSF modulation because it contains two consensus sequences for PKC phosphorylation sites (108, 125, 127) and its activity has been demonstrated to be negatively regulated by PKC activation (129, 130). GM-CSF may, therefore, be able to increase the rate of leukotriene formation by enhancing the activity of LTC_4 synthase through phosphorylation/dephosphorylation events via its PKC consensus sequences. Similarly, the activity of other enzymes in the leukotriene

biosynthetic pathway such as $cPLA_2$ and 5-LO have been demonstrated to be phosphoregulated (55, 276, 277)

Unexpectedly, neither GM-CSF nor IL-5 modulated the activity of LTC_4 synthase. The kinetic parameters, K_m and V_{max}, were unaltered by GM-CSF or IL-5 pre-treatment indicating that GM-CSF did not enhance the rate of leukotriene biosynthesis by upregulating the activity of LTC_4 synthase. GM-CSF most likely acts at an earlier catalytic event in the leukotriene biosynthetic pathway. Recently, GM-CSF and IL-3 treatment of cells was reported to enhance the expression of FLAP and 5-LO in monocytes (278) and FLAP and 5-LO expression, as well as PLA_2 activity, were all shown to be elevated by GM-CSF treatment of neutrophils (274, 275, 279, 280) (Figure 7.1). Activated MAP kinase can phosphorylate $cPLA_2$ resulting in an enhancement of $cPLA_2$ enzymatic activity (276, 277). Since GM-CSF can activate MAP kinase, subsequent activation of $cPLA_2$ may occur through phosphorylation mechanisms. 5-LO has also recently been demonstrated to be present in two molecular forms, a phosphorylated and a non-phosphorylated state, and is regulated by phosphorylation mechanisms (55). Tyrosine kinase activity modulates catalysis and translocation of cellular 5-LO. Inhibition of MAPK kinase blocks activation and redistribution of 5-LO in HL-60 cells (281). Since GM-CSF can signal through the Ras-MAP kinase pathway, GM-CSF may activate MAPK kinase which is required for 5-LO activation and translocation. MAPK kinase can then phosphorylate MAP kinase leading to the activation and translocation of $cPLA_2$. Additionally, GM-CSF priming may increase the amount of free arachidonic acid available for leukotriene formation (282, 283). Therefore, pre-treatment of ionophore-challenged eosinophilic HL-60 cells with GM-CSF appears to prime leukotriene production by upregulating the activity and expression of proteins that occur upstream of LTC_4 synthase in the leukotriene biosynthetic pathway.

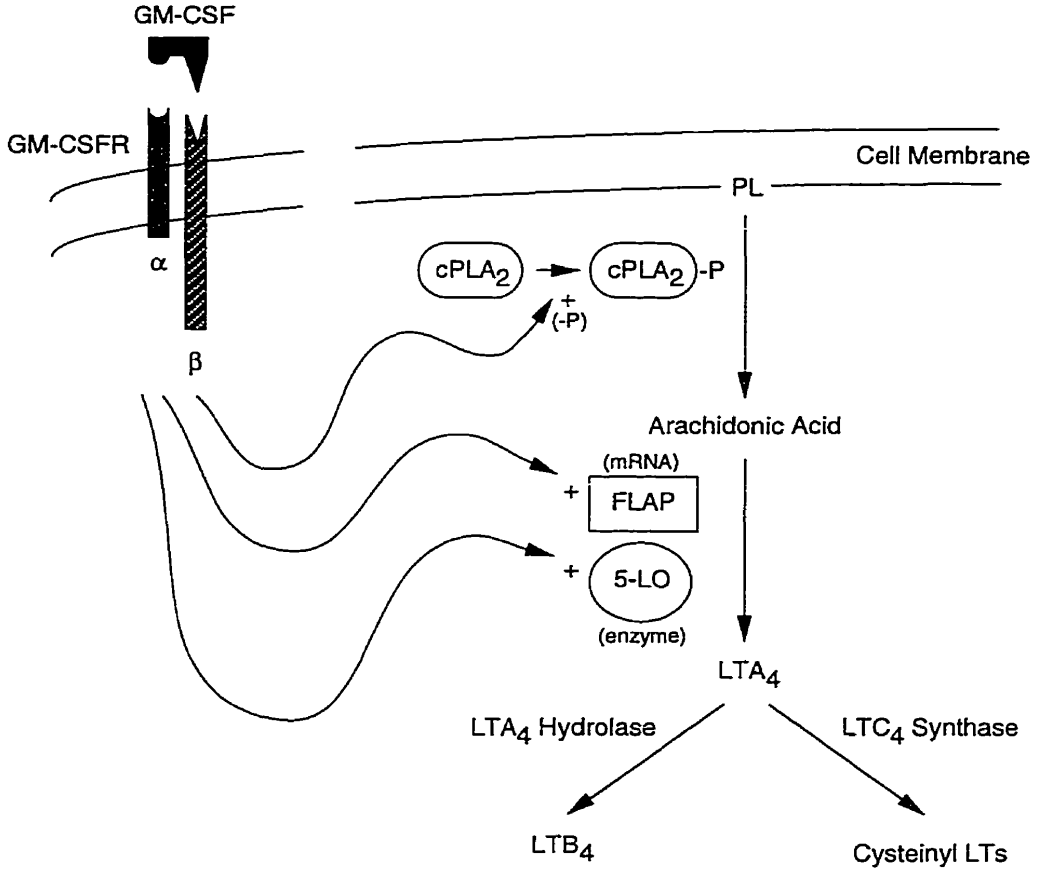


Figure 7.1. Established effects of GM-CSF on the leukotriene biosynthetic pathway

CSF and IL-5 are different, or that IL-5 is incapable of transducing a signal in this cell line. At least three evident bands were tyrosine phosphorylated by treatment with GM-CSF that were not apparent in IL-5-treated or control cells. Coinciding with these findings, GM-CSF and IL-3 have been reported to signal through tyrosine phosphorylation mechanisms, while IL-5 uses tyrosine dephosphorylation to mediate signal transduction (286). Since the ß-chain component of the receptor is common between these receptors, the α-subunit may be important for conferring distinct signalling pathways, in addition to, specific ligand binding (286, 287). Further studies have demonstrated that the cytoplasmic domain of the IL-5 receptor α-chain is essential for IL-5 signal transduction (226, 288, 289). In contrast, although the GM-CSF receptor α-chain is involved in GM-CSF mediated signal transduction it is not essential (224). ß-chain dimerization of the cytoplasmic domain may also be important for activating the IL-5 receptor complex and transducing intracellular growth signals (226, 290). Human IL-3 induces disulfide-linked IL-3 receptor α- and ß-chain heterodimerization, which is required for receptor activation but not high-affinity binding (291). Conceivably, in eosinophilic HL-60 cells, IL-5 may form a high-affinity receptor without disulfide bridges, resulting in the high-affinity binding of IL-5 with the inability to transduce a signal.

This unique cell line provides an excellent model for studying the distinct signal transduction pathways elicited by GM-CSF and IL-5. Further experiments need to be carried out to elucidate the different signal transduction pathways stimulated by these cytokines. Initially, the proteins phosphorylated by pre-treatment with either GM-CSF or IL-5 could be determined by immunoprecipitating specific proteins and then determining if these proteins are phosphorylated. Specific kinase and phosphatase inhibitors can also be used to delineate the various signalling pathways. Tyrosine kinase inhibitors have been shown to inhibit GM-CSF, but not IL-5 stimulated effects.

7.3 Regulation of leukotriene biosynthetic enzymes during myelocytic cellular differentiation

In the previous investigation leukotriene biosynthesis and LTC_4 synthase activity were upregulated as myelocytic cells were differentiated towards eosinophilic cells. It is well established that as myeloid cells differentiate towards granulocytes the profile of leukotrienes that they produce changes. Neutrophils predominantly produce LTB_4, whereas, eosinophils primarily form the cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4. The underlying mechanisms that cause these changes in the profile of leukotriene formation, however, are mostly unknown. The HL-60 and HL-60#7 myelocytic cell lines permit the opportunity to try to elucidate these mechanisms of specific leukotriene biosynthesis. These cell lines enable the examination of the leukotriene biosynthetic enzymes throughout the course of myelocytic differentiation towards either neutrophils or eosinophils (Figure 7.2). HL-60 cells can be differentiated towards neutrophilic cells by culturing with dimethylsulfoxide or HL-60#7 cells (a pro-eosinophilic substrain of HL-60 cells) can be differentiated towards eosinophilic cells by treatment with butyric acid.

The mechanisms of regulation of leukotriene biosynthesis appear to be similar throughout differentiation towards both neutrophilic and eosinophilic cells up to the point where the leukotriene biosynthetic pathway diverges (chapter 5 (ref. 273)). $cPLA_2$ protein levels remained constant, while both FLAP and 5-LO expression were upregulated. These analogous mechanisms of regulation were expected as these enzymes lead to the common substrate, LTA_4, that is required for both LTB_4 and LTC_4 formation. These cells also originated from a common predecessor, a pro-myelocytic cell, and may therefore conserve some of its common metabolic processes. The fact that $cPLA_2$ protein levels were unaltered suggests that this enzyme is not genomically controlled for leukotriene biosynthesis. Saturating levels of this enzyme may already be present in

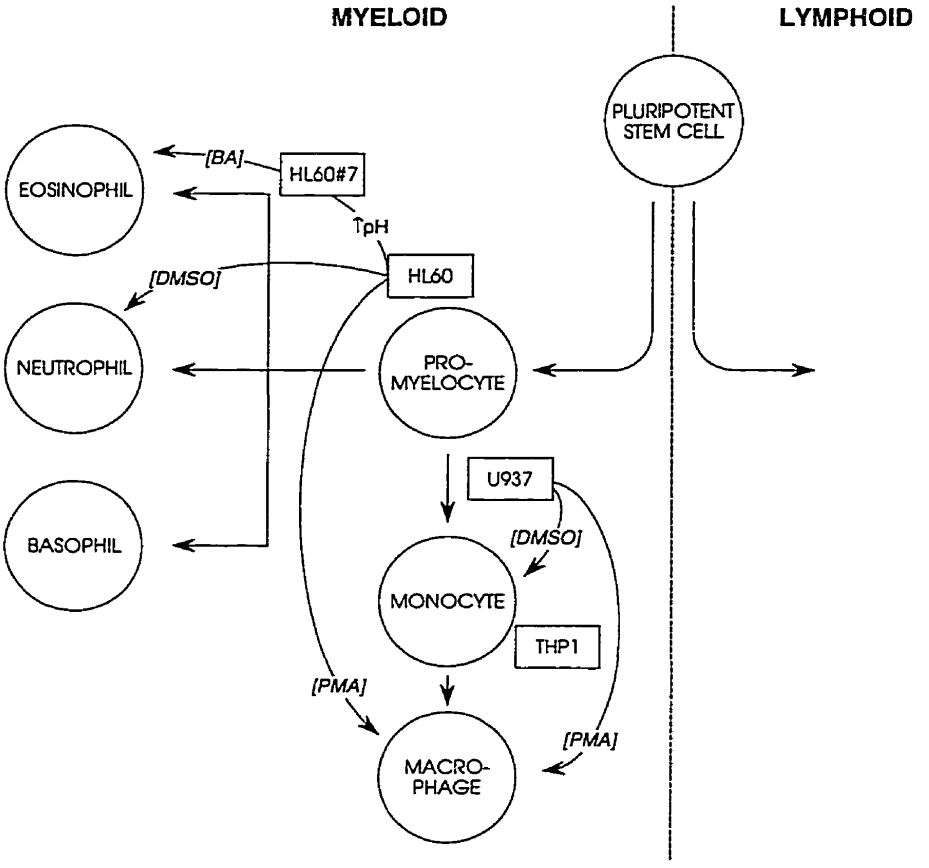


Figure 7.2. Differentiation pathways of myeloid cells and myelocytic leukemia cell lines (adapted from Nicholson, D. W., 1993, (ref. 33))

these cells. Similarly, $cPLA_2$ may not be the only PLA_2 responsible for arachidonate release in the formation of leukotrienes. The requirement of $cPLA_2$ in the formation of leukotrienes is controversial. Specific inhibition of the cell-associated type II 14 kDa PLA_2 in human monocytes completely abolished leukotriene formation and had no effect on prostaglandin production, whereas, the reverse was true for the specific inhibition of $cPLA_2$ (292). Also, the genes for $cPLA_2$ and COX-2 have both been localized to chromosome 1q25, denoting the possibility of co-ordinate regulation (23, 293, 294). In contrast, $cPLA_2$ and 5-LO genes were reported to be differentially regulated at the level of transcription, indicating that their cellular functions may not be linked (295). A 27 bp sequence in the 5'-flanking region of the $cPLA_2$ gene has been demonstrated to confer low level constitutive expression also suggesting that $cPLA_2$ is regulated post-translationally and not at the level of transcription (26). FLAP and 5-LO appear to be co-ordinately regulated for the production of leukotrienes during differentiation of these cell types. The concurrent increases in both FLAP and 5-LO expression during differentiation agree with previous findings (296, 297) of parallel induction of FLAP and 5-LO mRNA (38, 298). This expression of FLAP and 5-LO mRNA was due to a post-transcriptional event other than mRNA stabilization in HL-60 cells differentiated with DMSO (298). Although FLAP and 5-LO appear to be concurrently regulated in granulocytic HL-60 cells, discoordinate regulation of FLAP and 5-LO was demonstrated during differentiation of HL-60 cells to monocytes and macrophages, as well as, in five lymphoblastoid T-cell lines (39, 298).

After the formation of LTA_4, the leukotriene biosynthetic pathway diverges. LTA_4 can be converted to LTB_4 by LTA_4 hydrolase or LTA_4 can be conjugated to GSH by LTC_4 synthase to form LTC_4. LTA_4 hydrolase protein levels were markedly upregulated as HL-60 cells became more neutrophilic. Thus, the biosynthetic enzyme levels for LTB_4 formation are selectively upregulated during differentiation to neutrophils. FLAP, 5-LO, and notably, LTA_4 hydrolase protein quantities were dramatically

enhanced. These increases in the expression of the enzymes required for LTB_4 formation account for the predominant production of LTB_4 by neutrophils. Accordingly, the lack of expression of LTC_4 synthase explains the deficiency in production of cysteinyl leukotrienes by neutrophils.

LTA_4 hydrolase expression was only slightly increased (approximately 2-fold), as HL-60#7 cells became more eosinophilic, whereas, the amounts of the enzymes necessary for cysteinyl leukotriene biosynthesis were markedly enhanced. Significantly, LTC_4 synthase activity and mRNA expression were also dramatically elevated, consistent with previous findings that LTC_4 synthase mRNA is expressed in human eosinophils but not in neutrophils (125). These results explain the predominant formation of LTC_4 by eosinophils and the minor production of LTB_4 by HL-60#7/BA cells.

The regulation of LTC_4 synthase expression was independent of all of the other leukotriene biosynthetic enzymes. The elevation of LTC_4 synthase activity and mRNA expression in eosinophilic HL-60 cells correlates with its increased protein levels, implying that LTC_4 synthase may be regulated at the level of the gene. LTC_4 synthase expression may therefore be induced by specific transcription factors that are stimulated during differentiation to an eosinophil. Likewise, specific DNA-binding proteins may be provoked during differentiation to neutrophils that enhance the expression of LTA_4 hydrolase resulting in an increased production of LTB_4.

All of the genes that encode the leukotriene biosynthetic enzymes have now been cloned, structurally characterized, and their promoter regions analyzed. These genes contain several putative *cis*-acting elements in their promoter regions (Table 7.1). Although similar elements are present in various genes encoding the leukotriene biosynthetic enzymes, a simple explanation for the regulation of leukotriene formation during myeloid cell differentiation towards granulocytes cannot be concluded based on these known transcription-factor-binding consensus sequences. Further analyses are required to determine which DNA-binding motifs are functional. In the promoter region of the FLAP gene a limited

Table 7.1. Putative transcription factor binding sites in the promoter regions of the genes encoding the leukotriene biosynthetic enzymes

Enzyme	Putative cis-elements of promoter regions	Chromosomal location
$cPLA_2$	NF-κB, NF-IL-6, AP-1, AP-2, PEA3	1q25
5-LO	Sp1, AP-2, NF-κB	10
FLAP	TATA, AP-2, GRE	13
LTC_4 synthase	Sp1, AP-1, AP-2	5q35
LTA_4 hydrolase	XRE, AP-2	12

sequence has been associated with cell-specific expression and myeloid-cell-specific nuclear factors were recognized that bind to the FLAP promoter and appear to be required for the tissue specific expression of FLAP (64, Kennedy *et al.*, unpublished results). The LTA_4 hydrolase gene consists of several putative regulatory elements; however, a functional role for these transcription-factor-binding sites has not yet been determined (103). The LTC_4 synthase gene is the most recently cloned gene among the genes that encode proteins in the leukotriene biosynthetic pathway (131, 132). Surprisingly, the promoter region of the human LTC_4 synthase gene does not contain putative DNA-binding motifs that are typical of regulated genes with cell-specific expression (ie. a TATA or CAAT box). The LTC_4 synthase gene may therefore contain novel regulatory elements. Of interest, this gene contains putative AP-1 and AP-2 DNA-binding sites which is consistent with previous findings of enhanced LTC_4 synthase expression upon treatment with PMA (299, 300). The promoter region of this gene also contains a putative CREB-binding motif, as well as, a STAT-binding motif in the region of the first intron. The transcription factors that bind to these DNA sequences are known to be regulated by many proinflammatory cytokines, such as GM-CSF and IL-5. Thus, the localization of the LTC_4 synthase gene to the same region of chromosome 5 as many growth factors, cytokines, and receptors that are involved in inflammatory responses may explain the increased production of cysteinyl leukotrienes in diseases such as asthma.

7.4 Relative importance of microsomal GST-II versus LTC_4 synthase for the production of LTC_4

Until recently, the only known enzyme capable of catalyzing efficient LTC_4 production was LTC_4 synthase. This membrane bound protein was determined to be distinct from all other previously known GSTs (105-110). In 1996, however, Jakobsson *et al.* discovered another membrane bound

however, could not discriminate between LTC_4 synthase and microsomal GST-II. The presence of LTC_4 synthase was consequently deduced based on the absence of specific microsomal GST-II recognition by the microsomal GST-II polyclonal antibody in identical tissues in which the LTC_4 synthase antibody detected a 17 kDa band.

Since the LTC_4 synthase antibody displays cross-reactivity with microsomal GST-II, Western-blot analyses as well as activity assays were initially performed to confirm that LTC_4 synthase and not microsomal GST-II was measured in the previous investigation concerning the regulation of leukotriene biosynthesis during myeloid cell differentiation towards an eosinophil (see Appendix B). LTC_4 synthase was verified to be upregulated during differentiation of HL-60#7 cells to eosinophilic cells. However, microsomal GST-II was also constitutively expressed at low levels during differentiation of these cells, consistent with the presence of microsomal GST-II mRNA in HL-60 cells (133).

Interestingly, the microsomal GST-II antibody detected microsomal GST-II expressed from Sf9 cells as a doublet. This finding may signify the presence of a downstream degradation product or that this enzyme is phosphorylated. Microsomal GST-II could potentially be controlled by phosphoregulatory mechanisms as has been demonstrated for other leukotriene biosynthetic enzymes such as $cPLA_2$, 5-LO, and most significantly, LTC_4 synthase (55, 129, 130, 276, 277). Microsomal GST-II, however, does not possess the two putative protein kinase C phosphorylation sites that are found on LTC_4 synthase (108, 125, 127). Microsomal GST-II expressed from Sf9 cells was also shown to be inhibited by leukotriene biosynthesis inhibitors with a similar IC_{50} value as for LTC_4 synthase (301). These inhibitors have been demonstrated to inhibit both FLAP and LTC_4 synthase, therefore, these proteins may have a similar active site. The amino acids Serine-41 to Valine-61are critical for binding FLAP to leukotriene biosynthesis inhibitors (302) and this region corresponds to the most homologous area between microsomal GST-I, microsomal GST-

II, LTC_4 synthase, and FLAP (133). Thus, this region is proposed to be the lipid binding site of these proteins.

Microsomal GST-I was mainly expressed in human liver tissue and in the adrenal gland and kidney in agreement with previous findings of microsomal GST activity, protein expression, or mRNA expression and its function as a phase II detoxifying enzyme (136, 303). Since this enzyme is fundamental for cellular detoxification, it probably serves to protect these extrahepatic tissues from endogenous and exogenous toxicants. Microsomal GST-II is also predominantly expressed in the liver, to a lesser extent in endothelial cells, and minimally in lung membranes. The limited tissue expression of microsomal GST-II was surprising since the mRNA transcript is widely distributed, suggesting that microsomal GST-II is post-transcriptionally regulated. However, this lack of protein detection may also be a circumstance of sensitivity in the Western-blot analyses.

Unexpectedly, the low abundance protein, LTC_4 synthase, was widely distributed in tissues that both, did and did not, express FLAP. Since LTC_4 synthase and FLAP are involved in the biosynthesis of cysteinyl leukotrienes this overlap in expression is expected. However, the isolated expression of FLAP or LTC_4 synthase in kidney, stomach, and thymus, indicates the possibility for transcellular metabolism for leukotriene biosynthesis or in the case of tissues that only express FLAP, the sole production of LTB_4. These observations may also indicate the recognition of other putative members of this gene family by the cross-reactive LTC_4 synthase antibody. Preliminary results have identified the existence of at least three other members of this gene family (Jakobsson *et al.*, unpublished data), however, subsequent experiments are necessary to determine if these novel proteins are recognized by the LTC_4 synthase antibody. Contamination of tissue preparations by infiltrating peripheral blood cells could also explain any discrepancies in tissue distributions.

FLAP was distinctly found in human lymph and thymus tissues, confirming previous findings of FLAP in B- and T-lymphocytes (39), whereas,

LTC_4 synthase is notably detected in human kidney, lung, platelets, and to lesser extent in skeletal tissue, identical to earlier observations of mouse LTC_4 synthase mRNA expression (126) and human LTC_4 synthase protein expression (113).

Product profiles that can distinguish LTC_4 synthase activity from microsomal GST-II activity correlated directly with protein detection by Western-blot analyses. Microsomal GST-II concomitantly produces LTC_4 and a new LTA_4 metabolite, a possible isomer of LTC_4, that can be separated from LTC_4 by RP-HPLC (Jakobsson, P.-J., Scoggan, K. A., Yergey, J., Mancini, J. A. and Ford-Hutchinson, A. W. (1997) manuscript submitted). LTC_4 synthase stereoselectively produces LTC_4, thus, the ratio of the production of LTC_4 to the LTC_4 isomer clearly differentiates microsomal GST-II activity versus LTC_4 synthase activity. Using these product profiles there is significant microsomal GST-II activity in liver 100 000xg membrane samples. Microsomal GST-II is accordingly the predominant membrane bound enzyme responsible for LTC_4 formation in human liver. However, the possibility that LTC_4 synthase still exists in the liver in an inactive form cannot be excluded. Although, there is evidence that cysteinyl leukotrienes are involved in the pathogenesis of hepatic injury (304-307), little is known about the physiological relevance of cysteinyl leukotriene production by liver cells. Similarly, the mechanism of cysteinyl leukotriene biosynthesis in the liver is ambiguous. Recent investigations, however, have started to define the *in vivo* process for cysteinyl leukotriene formation in the liver. Fukai *et al.* have demonstrated that transcellular cysteinyl leukotriene biosynthesis in the liver is physiologically feasible even though the donor cells (Kupffer cells) are not in direct contact with the recipient cells (hepatocytes) (158). Hepatocyte membranes display most of the LTC_4 synthesizing activity in rat liver, while, Kupffer cells are assumed to predominantly produce LTA_4 (308). *In vitro*, hepatocytes form LTC_4 in the presence of exogenous LTA_4, exogenous arachidonic acid, or in co-culture with Kupffer cells in response to A23187, but not with A23187 alone (158, 308, 309). Comparably, Kupffer

cells stimulated with A23187 only produced LTC_4 when co-incubated with isolated hepatocytes or in the presence of LTC_4 synthase and GSH (158). Additional experiments that determine the participation of specific leukotriene biosynthetic enzymes in these cells are needed to deduce the exact mechanism of cysteinyl leukotriene production in the liver. Microsomal GST-II appears to be the enzyme responsible for LTC_4 production in the liver, however, this is probably not its main function. The presence of microsomal GST-II in the liver is most likely to protect cells from endogenous and exogenous toxicants. Microsomal GST-II is not dedicated exclusively to the conjugation of GSH to LTA_4. This novel enzyme is also capable of conjugating GSH to 1-chloro-2,4-dinitrobenzene and further experiments may identify additional substrates. Moreover, microsomal GST-II has recently been demonstrated to possess GSH-dependent peroxidase activity, which is responsible for cellular protection from endogenous toxicants (Jakobsson, *et al.*, unpublished data). Microsomal GST-II is, therefore, predominantly expressed in the liver and displays both general GST and GSH-dependent peroxidase activities, similar to microsomal GST-I, a known phase II detoxifying enzyme. These analogies suggest that the main function of microsomal GST-II is cellular detoxification and not LTC_4 production. Thus, microsomal GST-II may subsequently be a fundamental enzyme responsible for detoxification of lipid epoxides. The limited formation of LTC_4 from liver cytosol most likely represents the ability of cytosolic GSTs (mostly μ family GSTs) to conjugate reduced GSH to LTA_4 (142).

LTC_4 was selectively synthesized by lung membranes with a minor production of the LTC_4 isomer, demonstrating LTC_4 synthase as the favoured enzyme for LTC_4 production in human lung tissue with a minimal contribution from microsomal GST-II. In the lung, microsomal GST-II may play a minor part in LTC_4 production, however, once again its main function may be protection from oxidative stress and inhaled xenobiotics.

Platelet homogenates produced significant amounts of LTC_4 in the

absence of notable quantities of the LTC_4 isomer, which together with the Western-blot data indicate that the LTC_4 synthase enzyme predominates for LTC_4 production in platelets. In contrast, microsomal GST-II was detected in endothelial cell membranes by Western-blot and activity assays and may be the enzyme responsible for converting exogenous LTA_4 to LTC_4 during transcellular metabolism in these cells. The presence of microsomal GST-II as the main enzyme for LTC_4 production in endothelial cells versus LTC_4 synthase as the primary enzyme in platelets could explain the differences observed by Habib and Maclouf (310). In these studies, the apparent K_m for LTA_4 and the HPLC chromatogram peak patterns between endothelial cells and platelets for the production of LTC_4 were different and support the present findings, that LTC_4 production is catalyzed by microsomal GST-II in endothelial cells and by LTC_4 synthase in platelets. Microsomal GST-II may, therefore, be a key enzyme in the transcellular metabolism of LTA_4 into cysteinyl leukotrienes in certain cells that lack all of the enzymes required for *de novo* leukotriene biosynthesis. Importantly, the development of product profiles that can distinguish microsomal GST-II versus LTC_4 synthase activities can be used to determine the relative contribution of these enzymes in the formation of LTC_4 in other cell types such as smooth muscle cells.

Overall, leukotriene biosynthesis appears to be a highly regulated process. Leukotrienes are potent lipid mediators involved in immediate hyperreactivity, thus, physiologically it is crucial that their synthesis is controlled by many different mechanisms. These mediators are required for normal homeostasis and immune functions, however, when control mechanisms are not performing properly these mediators may become involved in the pathogenesis of many inflammatory disorders, such as asthma. Understanding the regulation of leukotriene biosynthesis may lead to novel therapeutic targets and eventually enable the control of these mediators in such diseases. Cytokines can regulate leukotriene production

at the level of transcription, post-transcription, as well as post-translationally by possible phosphoregulatory mechanisms. These proteins have been implicated in the pathology of asthma and may be of significance in understanding its pathogenesis. Throughout cellular differentiation, leukotriene biosynthesis is regulated by the tissue specific expression of the enzymes in the leukotriene biosynthetic pathway. The interaction of various cells can also control leukotriene production by transcellular biosynthesis of either LTB_4 or the cysteinyl leukotrienes. Finally, novel enzymes are being detected that may also be important for leukotriene formation. The ability of microsomal GST-II to produce LTC_4 may change the therapeutic approach to inflammatory disorders that involve cysteinyl leukotrienes. An inhibitor that exclusively affects LTC_4 synthase or microsomal GST-II may only block cysteinyl leukotriene biosynthesis in certain cell types but not in others. An alternative to the inhibition of cysteinyl leukotriene biosynthesis would be the use of specific cysteinyl leukotriene receptor antagonists. These inhibitors block the effects of the final products in the pathway and would inhibit the actions of cysteinyl leukotrienes in all cells, despite their mechanism of formation. A further understanding of the regulatory mechanisms of leukotriene biosynthesis may eventually lead to new therapeutic targets.

8. ORIGINAL CONTRIBUTIONS TO THE LITERATURE

This thesis demonstrates for the first time that:

1. GM-CSF primes leukotriene biosynthesis by approximately 45% in a dose-dependent manner in eosinophilic HL-60#7 cells by enhancing the rate of leukotriene biosynthesis at an earlier catalytic event than LTC_4 synthase in the biosynthetic pathway, whereas IL-5 has no effect on leukotriene production in this cell line.

2. This differential priming effect was not merely due to any differences between GM-CSF or IL-5 high-affinity receptors on eosinophilic HL-60#7 cells.

3. Phosphotyrosine patterns produced by pre-treatment of HL-60#7 cells with either GM-CSF or IL-5 suggest that the differential priming effect between GM-CSF and IL-5 is due to these cytokines signalling differently.

4. During myeloid (HL-60) cell differentiation to granulocytes $cPLA_2$ expression is constitutive while 5-LO and FLAP expression are co-ordinately upregulated.

5. LTA_4 hydrolase expression is upregulated as myeloid (HL-60) cells differentiate towards neutrophils.

6. LTC_4 synthase activity and expression at both the protein and mRNA levels are upregulated throughout myelocytic (HL-60#7) cellular differentiation towards eosinophils.

7. Microsomal GST-II is primarily located in human liver microsomes, endothelial membranes, and to a lesser extent in human lung membranes and is the predominant enzyme responsible for LTC_4 production in human endothelial and liver microsomes.

8. LTC_4 synthase is mainly located in human lung membranes, platelet homogenates, eosinophilic HL-60 cells and is the principal enzyme capable of conjugating LTA_4 to glutathione in these samples.

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APPENDIX A-Additional data for chapter 4

This appendix describes data that was previously mentioned in the original manuscript (chapter 4 (ref. 270)) as data not shown.

IL-5 receptor α-subunit transcripts and the IL-5 receptor α- and ß-subunit cDNA sequences all resemble the natural human IL-5 receptor

The presence of an alternate IL-5 receptor in eosinophilic HL-60#7 cells may account for the lack of priming of leukotriene biosynthesis by IL-5 in this cell line. To determine if the IL-5 receptor on eosinophilic HL-60#7 cells varies from the natural human IL-5 receptor, the expression of eosinophilic IL-5 α-chain receptor transcripts were analyzed and α-and ß-receptor subunit cDNAs were cloned and sequenced. Northern blots containing 15 μg of total RNA (lanes 1-3) or 5 μg of poly(A)+ RNA (lanes 4-6) from butyric acid differentiated HL-60#7 cells, TF-1 cells, and U937 cells, respectively, were probed using (^{32}P) radiolabelled IL-5 receptor α-subunit cDNA (Figure 1). Two transcripts were identified, a predominant 1.4 kb and a minor 5.3 kb, in the lanes containing RNA from eosinophilic HL-60#7 and TF-1 cells that were absent in the lanes containing RNA isolated from U937 cells (negative control). These transcripts correlate to those found in human eosinophils. The more abundant 1.4 kb transcript encodes for a soluble form of the IL-5 receptor α-subunit, whereas the less abundant 5.3 kb transcript may encode for an anchored form of the IL-5 receptor α-subunit. As well, the IL-5 receptor α-and ß-subunit cDNA sequences cloned from butyric acid differentiated HL-60#7 cells were identical to those previously published from human eosinophils. Therefore, the IL-5 receptor present in this cell line resembles that found in human eosinophils and is unlikely to account for any differences observed in the ability of GM-CSF versus IL-5 to prime leukotriene biosynthesis in this eosinophilic cell line and offers an excellent system for studying any variation in the intracellular

signalling mechanisms between these two cytokines.

Isolation of Polyadenylated RNA From Eosinophilic HL-60 Cells, TF-1 Cells, and U937 Cells

Total RNA was prepared as described previously (chapter 5 (ref. 271)) from 125 x 10^6 cells from HL-60#7 cells cultured for 5 days with butyric acid, TF-1 cells cultured for 5 days with IL-3 (200 pM), and U937 cells using Trizol reagent (Total RNA Isolation Reagent, Gibco BRL) according to the manufacturer's instructions. Poly(A)+ RNA was then isolated from total RNA using the Oligotex-dT poly A+ mRNA Purification Kit (Qiagen) as per the manufacturer's directions.

Production of IL-5 Receptor α-Subunit cDNA by RT-PCR

Reverse transcription-PCR was performed on Day 5 HL-60#7/BA poly(A)+ RNA using Murine Leukemia Virus (MuLV) Reverse Transcriptase (Perkin Elmer). Primers 5' ATG ATC ATC GTG GCG CAT GTA TTA CTC 3' and 5' TCA AAA CAC AGA ATC CTC CAG GGT CTC 3' that were designed based on the published cDNA sequence for human IL-5 receptor α-subunit (GenBank accession no. M96652) were used in PCR amplification in order to obtain the IL-5 receptor α-subunit cDNA. PCR cycles of amplification were 94°C for 60 sec, 57.3°C for 30 sec, and 72°C for 90 sec, for 40 cycles. The PCR product was purified to remove primers using the QIAquick PCR Purification Kit followed by gel purification using the QIAquick Gel Extraction Kit (QIAGEN). The gel purified RT-PCR products were cloned into BlueScript II (SK+) vector and sequenced.

Production of IL-5 Receptor ß-Subunit cDNA by RT-PCR

Reverse transcription-PCR was performed on Day 5 HL-60#7/BA poly(A)+ RNA using Superscript/Vent Polymerase System (Perkin Elmer). The IL-5 receptor ß-subunit was amplified in two separate parts, a front half and

a rear half. IL-5 receptor ß-subunit front half primers 5' GCC TGC CTG TCC AGA GCT GAC CAG GGA G 3', 5' TCT TGC TGG GGT TGG GGA TCT TCT CCT C 3' and nested primers 5' CGC GGA TCC ACC ATG GTG CTG GCC CAG GGG CTG CTC TCC A 3', 5' CGC GGA TCC CAG CCT GTA CCC GTA GAT GCC ACA GAA G 3' and IL-5 receptor ß-subunit rear half primers 5' TAC TGG GCC AGG GTG AGG GTC AGG ACC T 3', 5' CCT CTA GCT ACC TTG ACA GGA GGC TGC C 3' and nested primers 5' CGC GGA TCC CAA CGG GAT CTG GAG CGA GTG GAG TGA G 3', 5' CGC GGA TCC CTA TCA ACA CAC CTC CCC AGG CTT GTT 3' that were designed based on the published cDNA sequence for human IL-5 receptor ß-subunit (GenBank accession nos. M59941 and M38275) were used in two rounds of PCR amplification, respectively, in order to obtain both parts of the IL-5 receptor ß-subunit cDNA. Primary PCR cycles of amplification were 94°C for 60 sec, 64°C for 60 sec, and 72°C for 90 sec (including a 30 sec ramp), for 35 cycles using the initial primers. The PCR product was purified to remove primers using the QIAquick PCR Purification Kit (QIAGEN). Secondary amplification cycles consisted of 94°C for 60 sec, 63°C for 60 sec, and 72°C for 90 sec (including a 30 sec ramp), for 30 cycles with the nested primers. These cycles were performed using Taq Polymerase (Boehringer Mannheim). The RT-PCR products were gel purified, cloned into pCR Vector using the TA Cloning System (Invitrogen), and sequenced.

Northern Blot Analysis of IL-5 Receptor α-Subunit Transcripts

IL-5 receptor α-subunit cDNA was radiolabelled using Random Primed DNA Labelling Kit (Boehringer Mannheim) and purified on G-50 sephadex columns to be used as a probe during Northern blot analyses. Approximately 15 µg of total RNA and 5 µg poly(A)+ RNA were electrophoresed through a 0.8% (v/v) formaldehyde/agarose gel and transblotted onto a nylon membrane. The blot was incubated at 55°C for 2 h in pre-hybridizing solution containing 6 x SSC, 2 x Denhart's solution, 0.1% SDS (v/v), and 100 µg / mL sheared DNA. The membrane was subsequently

hybridized with the radiolabelled IL-5 receptor α-subunit cDNA probe at 1 x 10^6 cpm / mL under high stringency conditions (wash temperature, 65°C and 1 x SSC, 0.1% SDS) and exposed to x-ray film overnight. The above conditions were also used to probe the membrane with ß-actin.

Figure 1. Northern blot analysis of IL-5 receptor α-subunit transcripts from eosinophilic HL-60, TF-1, and U937 cells. Approximately 15 µg of total RNA and 5 µg of poly(A)+ RNA from eosinophilic HL-60, TF-1, and U937 cell cultures were electrophoresed through a 0.8% formaldehyde-containing-agarose gel and transblotted to a nylon membrane. The membrane was incubated with a random-primed radiolabelled IL-5 receptor α-subunit cDNA probe under stringent conditions (wash temperature, 65°C, with 1 x SSC, 0.1% SDS) and subsequently exposed to x-ray film.

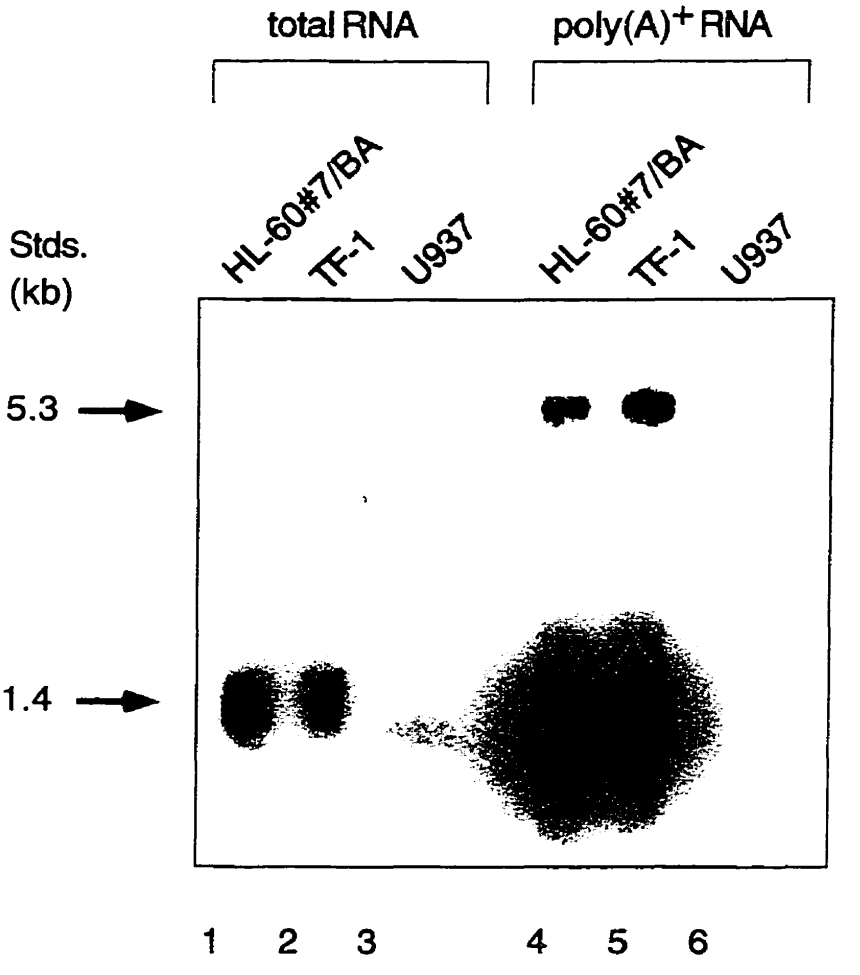

total RNA
poly(A)+ RNA
HL-60#7/BA
TF-1
U937
HL-60#7/BA
TF-1
U937
Stds.
(kb)
5.3
1.4
1
2
3
4
5
6

Figure 1

APPENDIX B-Supplemental data to chapters 5 and 6

This appendix verifies that LTC_4 synthase is the predominant enzyme responsible for LTC_4 production in eosinophilic HL-60 cells.

Relative Contribution of Microsomal GST-II versus LTC_4 Synthase in the Production of LTC_4 from Eosinophilic HL-60 Cells

Following our investigation of the regulation of leukotriene-biosynthetic enzymes during differentiation of myelocytic HL-60 cells to eosinophilic or neutrophilic cells, a novel LTC_4 synthesizing enzyme was discovered. This enzyme, termed *microsomal GST-II*, was recognised by the LTC_4 synthase polyclonal antibody that had been used in the previous study mentioned above. Thus, to confirm that in our earlier work we were measuring LTC_4 synthase and not microsomal GST-II, the relative contribution of microsomal GST-II versus LTC_4 synthase in the production of LTC_4 was investigated in eosinophilic HL-60 cells by Western-blot analyses and by product profiles from activity assays.

Western-blot analyses (Figure 1) using the LTC_4 synthase polyclonal antibody recognised a 17 kDa band in eosinophilic HL-60 cells. The intensity of this band increased with increasing days of differentiation with butyric acid. The microsomal GST-II antibody also detected a faint 17 kDa band in HL-60#7 cells differentiated for 0, 3, and 5 days with butyric acid. These results indicate that LTC_4 synthase levels are enhanced during differentiation of myeloid cells to eosinophilic HL-60 cells, whereas, microsomal GST-II is uniformly (constitutively) present at low levels.

The different product profiles obtained from activity assays were used to distinguish LTC_4 synthase activity from microsomal GST-II activity in eosinophilic HL-60 cells (Table 1). Microsomal GST-II produces LTC_4 as well as a new LTA_4 metabolite, a possible isomer of LTC_4. LTC_4 synthase produces LTC_4 stereoselectively thus, the ratio of the formation of LTC_4 to

the LTC_4 isomer can be used to distinguish microsomal GST-II from LTC_4 synthase activity. LTC_4 synthase displays a LTC_4 to LTC_4 isomer ratio of >50, while microsomal GST-II exhibits a ratio of <5. In HL-60#7 cells cultured for 0, 3, and 5 days with butyric acid the LTC_4 to LTC_4 isomer ratios were 16, 34, and 31, respectively. These results demonstrate that LTC_4 synthase activity is upregulated during myeloid cell differentiation to eosinophilic HL-60 cells and that LTC_4 synthase is the predominant enzyme responsible for LTC_4 formation in eosinophilic HL-60 cells.

LTC_4 synthase expression was upregulated during differentiation of myeloid cells to eosinophils as demonstrated by both Western-blot and activity assay product profiles. These results reveal that in our earlier work we were measuring LTC_4 synthase and confirm our previous investigation of the regulation of leukotriene biosynthesis during myeloid cell differentiation.

Preparation of HL-60#7 cell membranes

Reverse time course cultures for the HL-60#7 cell line were set up as previously described (chapter 5 (ref. 271)). The resulting pellets were resuspended in PBS containing 2 mM EDTA and 2 mM PMSF and mixed rapidly with a dounce using 'A' pestle on ice. The cells were ruptured by nitrogen cavitation with 800 psi for 20 min and subjected to differential centrifugation at 1 000 x g for 15 min, 10 000 x g for 20 min, and 100 000 x g for 1 h, all at 4°C. The pellets were resuspended to 1 x 10^9 cell equivalents/mL in PBS containing 2 mM EDTA, frozen in liquid nitrogen, and stored at -80°C.

Figure 1. HL-60#7 cells cultured for varying number of days with butyric acid primarily express LTC_4 synthase. 100 000xg membrane fractions from HL-60#7 cells cultured for 0, 3, and 5 days with butyric acid (75 μg of

protein), microsomal GST-II from baculovirus infected Sf9 cell membranes (5 μg of protein), and partially purified LTC_4 synthase from THP-1 cell extracts (7.35 μg of protein) were electrophoresed through polyacrylamide gels, electroblotted onto nitrocellulose, and immunoblotted using the polyclonal antibodies to microsomal GST-II or LTC_4 synthase and detected using enhanced chemiluminescence as described under Experimental Procedures (chapter 6 (ref. 299)).

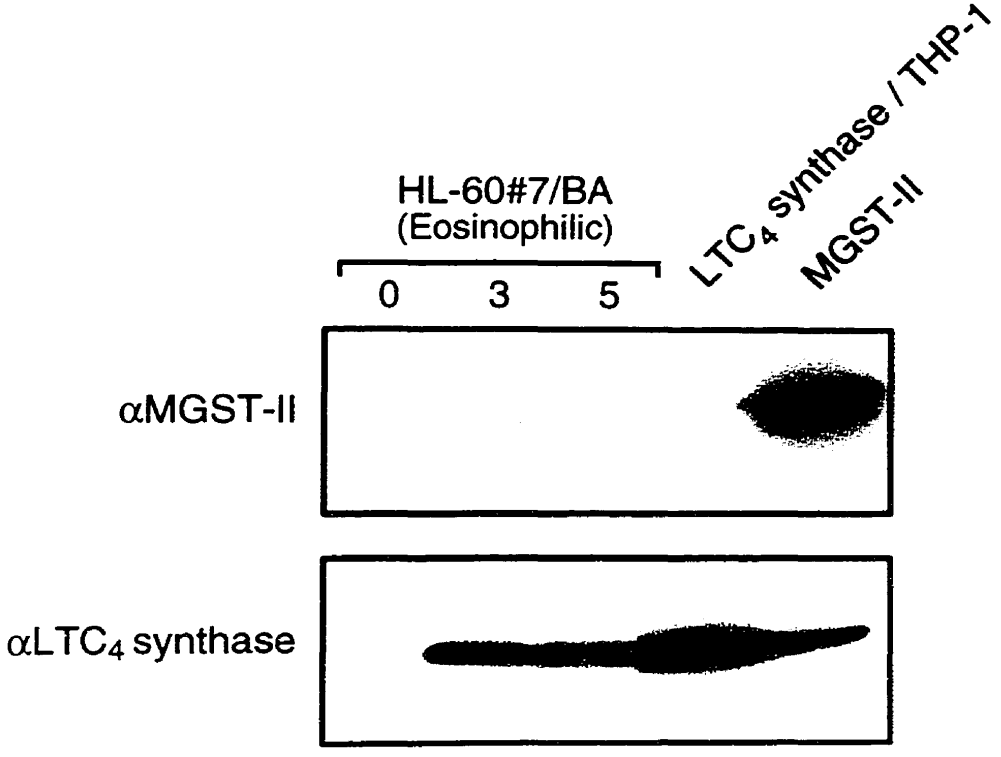

HL-60#7/BA
(Eosinophilic)
0
3
5
LTC4 synthase / THP-1
MGST-II
αMGST-II
αLTC4 synthase

Figure 1

Table 1.

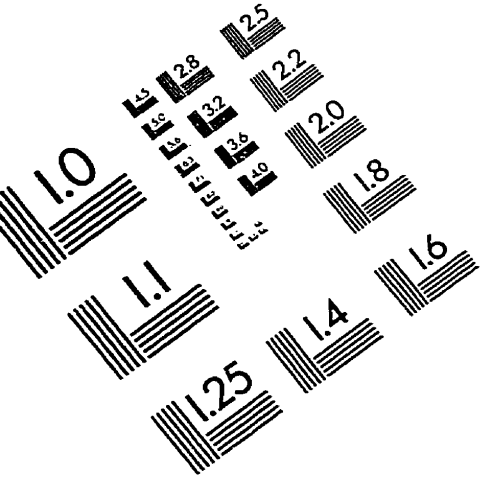
LTC_4 synthase activity is predominant in eosinophilic HL-60 cells.

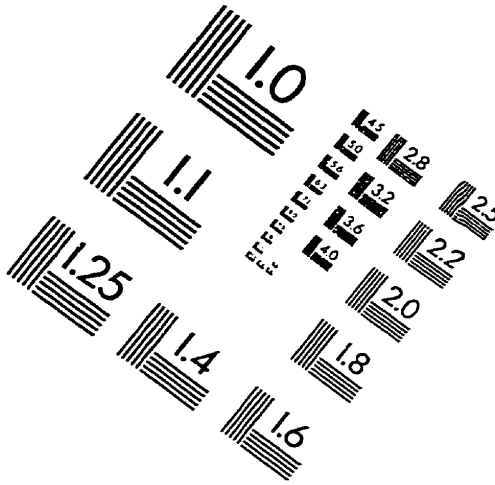
Sample **HL-60#7/BA**	LTC_4 formed (pmol • mg protein^{-1})	Isomer formed (pmol • mg protein^{-1})	Ratio (LTC_4 : isomer)
Day 0 100 000xg pellet	600	37	16
Day 3 100 000xg pellet	5998	177	34
Day 5 100 000xg pellet	6596	210	31

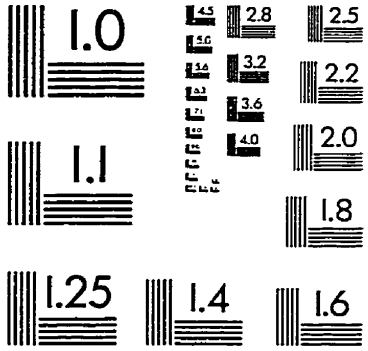
100 000xg membrane fractions from HL-60#7 cells cultured for 0, 3, and 5 days with butyric acid (0.4 mg og protein), partially purified LTC_4 synthase from THP-1 cell extracts (2.45 μg of protein), LTC_4 synthase from baculovirus infected Sf9 cell membranes (0.05 mg of protein), microsomal GST-II from baculovirus infected Sf9 cell membranes (0.05 mg of protein), and wild type baculovirus infected Sf9 cell membranes (0.05 mg of protein) were prepared as described under Experimental Procedures (chapter 6 (ref. 299)). Microsomal GST-II or LTC_4 synthase activity was assayed in the above samples by measuring the amount of LTC_4 and a postulated LTC_4 isomer produced in 15 min incubations at 25°C in 0.1 M Kpi, pH 7.4, containing reduced glutathione (5 mM) and 60 μM LTA_4 (free acid) stabilized by the presence of 0.05% (w/v) BSA, as resolved by isocratic reverse-phase HPLC. The relative production of LTC_4 to the postulated LTC_4 isomer was used to define microsomal GST-II activity versus LTC_4 synthase activity.

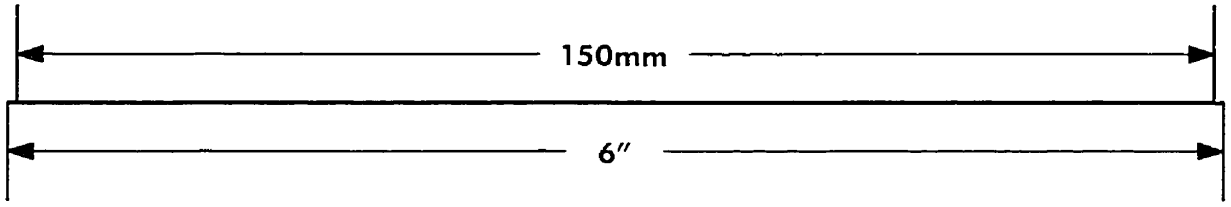
Table 1. LTC_4 synthase activity is predominant in eosinophilic HL-60 cells. 100 000xg membrane fractions from HL-60#7 cells cultured for 0, 3, and 5 days with butyric acid (0.4 mg og protein), partially purified LTC_4 synthase from THP-1 cell extracts (2.45 μg of protein), LTC_4 synthase from baculovirus infected Sf9 cell membranes (0.05 mg of protein), microsomal GST-II from baculovirus infected Sf9 cell membranes (0.05 mg of protein), and wild type baculovirus infected Sf9 cell membranes (0.05 mg of protein) were prepared as described under Experimental Procedures (chapter 6 (ref. 299)). Microsomal GST-II or LTC_4 synthase activity was assayed in the above samples by measuring the amount of LTC_4 and a postulated LTC_4 isomer produced in 15 min incubations at 25°C in 0.1 M Kpi, pH 7.4, containing reduced glutathione (5 mM) and 60 μM LTA_4 (free acid) stabilized by the presence of 0.05% (w/v) BSA, as resolved by isocratic reverse-phase HPLC. The relative production of LTC_4 to the postulated LTC_4 isomer was used to define microsomal GST-II activity versus LTC_4 synthase activity.

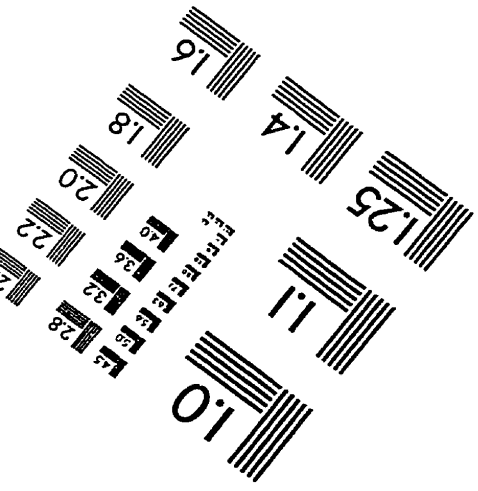
IMAGE EVALUATION
TEST TARGET (QA-3)

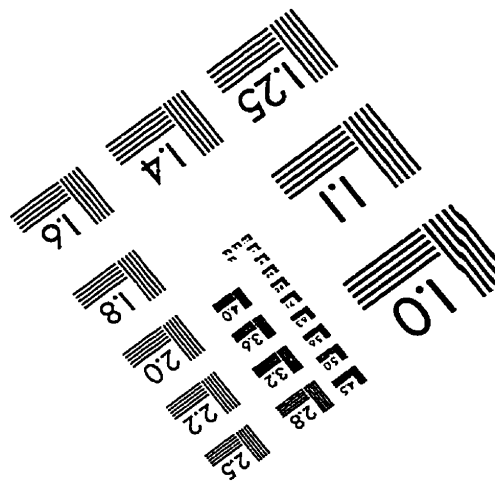












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