Chapter 2

Leukotrienes C_4 and D_4 Sensitize Human Neutrophils for Hyperreactivity to Chemoattractants

2.1 Introduction:

Although primarily involved in the immunopathogenesis of bronchial asthma and other atopic disorders, cysteinyl leukotrienes (CysLTs) are being increasingly implicated in the aetiology of acute and chronic inflammatory diseases of nonallergic origin, including cardiovascular diseases, autoimmune diseases, and certain malignancies (Peters-Golden & Henderson, 2007). The spectrum of proinflammatory activities of CysLTs may therefore extend beyond eosinophils, monocytes/macrophages, type 2 helper T (Th2) lymphocytes, and airway smooth muscle cells, to other types of inflammatory cells such as neutrophils. Human neutrophils do not possess the enzyme LTC_4 synthase and are therefore unable to generate CysLTs (Peters-Golden & Henderson, 2007). Interestingly, however, these cells do possess G protein/ phospholipase C-coupled membrane receptors for LTC_4 and LTD_4 (Akgul & Edwards, 2003; Andina *et al,* 2009; Heimbürger & Palmblad, 1996), with ligand/receptor binding resulting in modest activation of the cells, specifically Ca^{2+} mobilization and generation of nitric oxide (Heimbürger & Palmblad, 1996; Andina *et al,* 2009). On the other hand, relatively little is known about the potential of CysLTs to sensitize/"prime" neutrophils for enhanced reactivity to conventional activators, such as chemoattractants, as well as the potential of antagonists of type 1 CysLT receptors ($CysLT_1$ Rs) such as montelukast to attenuate these interactions.

In the current study, the effects of pretreatment of human neutrophils with the CysLTs, C_4 and D_4, on production of superoxide and release of elastase on subsequent activation of the cells with the chemoattractant, FMLP, as well as the effects of montelukast on superoxide generation, have been investigated.

2.2 Materials and methods

2.2.1 Leukotrienes

Leukotrienes C_4 and D_4 (50 $\mu g.ml^{-1}$ and 55 $\mu g.ml^{-1}$ respectively, in 70% aqueous methanol) were purchased from Sigma-Aldrich, St Louis, MO, USA, and used at a final concentration range of 50–300 $nmol.L^{-1}$. Montelukast sodium,2-[1[[1-[3-[2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-2[1-hydroxy-1-methyl-ethyl)phenyl]-propyl] sulfanyl-methyl]propyl] cyclopropyl] ethanoic acid, was kindly provided by Merck Research Laboratories, Rahway, NJ, USA, dissolved in dimethyl sulphoxide (DMSO) to a stock concentration of 10 $mmol.L^{-1}$, and used at a fixed, final concentration of 100 $nmol.L^{-1}$, which is a therapeutically relevant concentration of this agent as measured in blood after oral administration.(Cheng *et al*, 1996; Knorr *et al,* 2001). Unless indicated, all other chemicals and reagents were purchased from Sigma-Aldrich. Appropriate solvent controls were used in all of the assays described below.

2.2.2 Preparation of neutrophils

The study was approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria, and prior informed consent was obtained from all blood donors. Neutrophils were isolated from heparinized venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Health assessments were done by qualified nursing sisters and the donors were medication free as determined by questionnaires. Neutrophils were separated from mononuclear leucocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400 x *g* for 25 min at room temperature. The resultant pellets were suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine to remove most of the erythrocytes. Following centrifugation (280 x *g* at 4°C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), determined by flow cytometric procedures, were re-suspended to 1 x 10^7 ml^{-1} in PBS and held on ice until used. Contaminating cells were not regularly analized but were predominatly mononuclear cells (<5%).

2.2.3 Spectrofluorimetric measurement of cytosolic Ca^{2+}

Fura-2/AM was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments (Grynkiewicz, Poenie & Tsien, 1985). Neutrophils (1 × 10^7.ml^{-1}) were incubated with fura-2/AM (0.5 µmol.L^{-1}) for 30 min at 37°C in PBS, washed and re-suspended in indicator-free Hanks balanced salt solution (HBSS, pH 7.4), containing 1.25 mmol.L^{-1} $CaCl_2$. The fura-2-loaded cells (2 × 10^6.ml^{-1}) were then

pre-incubated for 8 min at 37°C with montelukast (100 nmol.L^{-1}) or an equivalent volume of DMSO in control systems, after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Perkin Elmer LS45 luminescence spectrometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (± 1 min), the neutrophils were activated by the addition of either LTC_4 or LTD_4 (100 nmol.L^{-1}), or the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µmol.L^{-1}, final), and alterations in fluorescence intensity monitored over a 1–3 min time course. The following were investigated in additional experiments: i) the effects of pre-treatment of neutrophils with montelukast (100 nmol.L^{-1}) on cytosolic Ca^{2+} fluxes in neutrophils activated with the CysLTs; and ii) the effects of pre-treatment of the cells with either LTC_4 or LTD_4 (both at 100 nmol.L^{-1}) on the FMLP-activated alterations in cytosolic Ca^{2+} (the CysLTs were added to the cells 10 sec before FMLP).

2.2.4 Measurement of superoxide production

This was measured using a lucigenin (bis-*N*-methyl-acridinium nitrate)-based chemiluminescence procedure which detects superoxide (Minkenberg & Ferber, 1984). Briefly, neutrophils (1×10^6) were preincubated for 10 min at 37°C without and with montelukast (100 nmol.L^{-1}) in 900 µl of HBSS containing lucigenin (0.2 mmol.L^{-1}), followed by addition of either LTC_4 or LTD_4 (100 nmol.L^{-1}) and 10 sec later by HBSS (unstimulated control systems) or FMLP (1 µmol.L-1) and chemiluminescence responses recorded using a Lumac Biocounter (Model 2010,

Lumac Systems). The final volume in each vial was 1 ml, and the results, which are expressed in relative light units (rlu), are the peak values for FMLP-activated systems which were reached 40-50 sec after addition of the stimulant. FMLP was used at 1 $\mu mol.L^{-1}$ because this is the concentration which is optimal in our hands for NADPH oxidase activation and superoxide production.

2.2.5 Elastase and MMP-8 release

Neutrophil degranulation was measured according to the extent of release of elastase and MMP-8 from the primary and specific granules, respectively. In the case of elastase release, neutrophils were incubated at a concentration of $2 \times 10^6.ml^{-1}$ in HBSS for 10 min at 37°C followed by successive addition at 10 sec intervals of either LTC_4 or LTD_4 (50–300 $nmol.L^{-1}$) and FMLP (1 $\mu mol.L^{-1}$) in combination with a submaximal concentration of cytochalasin B (CB; 0.5 $\mu mol.L^{-1}$, final) and the cells incubated for 15 min at 37°C. Neutrophil–free supernatants were assayed for elastase using a micromodification of a standard colourimetric procedure (Beatty et al, 1982). Briefly, 125 ml of supernatant were added to the elastase substrate, N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide [3 mmol•L-1 in dimethylsulphoxide (DMSO)] in 0.05 M Tris-HCl (pH 8.0), and elastase activity was monitored spectrophoto-metrically at a wavelength of 405 nm.

In the case of MMP-8 release, the experimental design was essentially similar, the only exceptions being that FMLP (1 $\mu mol.L^{-1}$) was used without added CB and the

CysLTs were used at a fixed, final concentration of 100 nmol.L^{-1}. MMP-8 in the supernatants of the cells was assayed using an ELISA procedure (Quantikine, R&D Systems, Inc. Minneapolis) and the results expressed as ng.ml^{-1}.

2.2.6 Expression and statistical analysis of results

With the exception of the results of the fura-2 fluorescence experiments, some of which are presented as the traces from representative experiments, the results of each series of experiments are presented as the mean values ± the standard error of the mean (SEM). Levels of statistical significance were determined by the Mann-Whitney U-test where appropriate by comparing LT-treated systems with the corresponding control system.

2.3 Results

2.3.1 Fura-2 fluorescence response

The results shown in Figure 2.1 (page 65) are representative traces from one experiment (6 in the series all of which showed similar effects) which depict the fura-2 fluorescence responses of neutrophils following exposure to 100 nmol.L^{-1} LTD_4 or LTC_4. Addition of these CysLTs to neutrophils was accompanied by an abrupt, albeit moderate, increase in fura-2 fluorescence, compatible with an elevation in the cytosolic concentration of Ca^{2+}, with LTD_4 being more potent than LTC_4. FMLP (1 µmol.L^{-1}) was included for comparison, and was found to be a considerably more potent activator of Ca^{2+} mobilization than either LTC_4 or LTD_4

(Figure 2.1). Pre-treatment of neutrophils with LTD_4 (100 $nmol.L^{-1}$) did not affect the FMLP-activated alterations in cytosolic Ca^{2+} (Figure 2.2, page 66), compatible with lack of effect of LTD_4 on either phospholipase C activation and store-operated Ca^{2+} influx in FMLP-activated neutrophils. These responses of FMLP-activated cells were also unaffected by exposure to LTC_4 (results not shown). Comparable results were obtained when FMLP was used at a final concentration of 100 $nmol.L^{-1}$ (not shown). As shown in Figure 2.3 (page 67) pre-treatment of neutrophils with 100 $nmol.L^{-1}$ montelukast attenuated the increase in cytosolic Ca^{2+} following exposure of the cells to LTD_4.

2.3.2 Superoxide production

The effects of addition of LTC_4 or LTD_4 individually on superoxide production by neutrophils, as well as their sensitizing effects on oxidant generation by FMLP-activated neutrophils, are shown in Figure 2.4 (page 68). Addition of either LTC_4 or LTD_4 at a concentration of 100 $nmol.L^{-1}$ to neutrophils had no significant effects on superoxide generation. However, addition of either of the CysLTs to the cells 10 sec prior to FMLP (1 $\mu mol.L^{-1}$) resulted in significant augmentation of the chemoattractant-activated responses. These observations demonstrate that LTC_4 and LTD_4 sensitize/“prime” neutrophils for enhanced oxidant production following activation with FMLP. The “priming” of the FMLP-activated superoxide responses of neutrophils are shown in Figure 2.5 (page 69). Pre-treatment of neutrophils with montelukast resulted in partial, but statistically significant ($P < 0.05$) antagonism of the sensitizing effects of the CysLTs on FMLP-activated superoxide generation.

Importantly, montelukast at the concentration used (100 nmol.L^{-1}) did not affect superoxide production by cells activated with FMLP in the absence of the CysLTs (data not shown).

2.3.3 Elastase and MMP-8 release

Exposure of neutrophils to either LTC_4 or LTD_4 did not result in release of either elastase or MMP-8 from neutrophils (results not shown). However, as shown in Figure 2.6 (page 70), addition of LTC_4 or LTD_4 (50–300 nmol.L^{-1}) to neutrophils prior to FMLP/CB, resulted in augmentation of elastase release in comparison with the responses of cells exposed to FMLP/CB only. As shown in Table 2.1 (page 71), similar effects were observed with respect to release of MMP-8 from cells activated with FMLP (without CB) in the presence of the CysLTs at a fixed concentration of 100 nmol.L^{-1}.

2.4 Discussion

CysLTs exert their effects through cysteinyl leukotriene receptors. These receptors are seven transmembrane-spanning receptors that couple to G proteins and activate intracellular signaling pathways in response to agonist binding (Capra, 2004; Peters-Golden & Henderson, 2007). Neutrophils have been shown to possess G protein/phospholipase C (PLC)-coupled membrane receptors for LTC_4 and LTD_4 (Heimbürger & Palmblad, 1996; Lärfars *et al,* 1999; Zhu *et al,* 2005), although they are unable to produce these leukotrienes (Peters-Golden &

Henderson, 2007). Two CysLT receptors, $CysLT_1$ and $CysLT_2$ have been identified, cloned, and characterized (Kanaoka & Boyce, 2004; Peters-Golden & Henderson, 2007). The CysLT1R has nanomolar affinity for LTD_4 and couples to the heterotrimeric G protein Gq to promote calcium mobilization. LTC_4 is also a full agonist of the $CysLT_1R$, but is 10 times less potent (Naik *et al,* 2005). The rank order of potency of agonist activation for the CysLT1R is $LTD_4 > LTC_4 > LTE_4$, while for the $CysLT_2R$ it is $LTC_4 = LTD_4 > LTE_4$ (Capra, 2004).

In the current study, addition of either LTC_4 or LTD_4, at a fixed concentration of 100 $nmol.L^{-1}$ to human neutrophils, cause rapid, transient elevations in cytosolic Ca^{2+}, which were of lesser magnitude than those observed following activation of the cells with FMLP (1 $\mu mol.L^{-1}$). LTD_4 was found to be a more potent activator of Ca^{2+} mobilization than LTC_4. In another study, Heimbürger and Palmblad reported that exposure of neutrophils to LTD_4 at 100 $nmol.L^{-1}$, but not LTC_4, caused transient elevations in cytosolic Ca^{2+}, which were of lesser magnitude than those observed following activation of the cells with FMLP (100 nmol.L-1) (Heimbürger & Palmblad, 1996). As observed in the present study with montelukast, these authors observed that pretreatment of neutrophils with the $CysLT_1R$ antagonist, SKF104353, abrogated the LTD_4-mediated elevation in cytosolic Ca^{2+}. In apparent contrast, Bautz *et al*. reported that LTD_4-activated Ca^{2+} fluxes in neutrophils were not affected by pretreatment of the cells with MK571, a selective leukotriene D_4 receptor antagonist, suggesting the involvement of other types of receptors, such as CysLT2 (Bautz *et al,* 2001). Mobilization of both stored and extracellular Ca^{2+} normally precedes, and is a prerequisite, for membrane receptor- mediated

activation of the proinflammatory activities of human neutrophils, including generation of superoxide, degranulation, and adhesion of the cells to vascular endothelium. Previous studies have reported that exposure of neutrophils to either LTC_4 or LTD_4 results in activation of nitric oxide synthase and mobilization of secondary granules, while, in keeping with the current study, NADPH oxidase and primary granule release are unaffected (Lärfars *et al,* 1999; Lew *et al,* 1987).

However, CysLT-mediated sensitization of neutrophil NADPH oxidase activity and mobilization of primary granules on exposure of the cells to a second, more potent stimulus, although potentially important, has not to our knowledge been addressed in previous studies. Importantly, we observed that brief, pretreatment of neutrophils with either of the CysLTs, sensitized the cells for enhanced generation of superoxide and release of elastase and MMP-8 on subsequent exposure to the chemoattractant, FMLP. Interestingly, the CysLTs were equipotent with respect to their priming interactions with neutrophils, which were attenuated in part by montelukast, compatible with involvement of $CysLT_1Rs$. Because of concerns about alternative mechanisms of montelukast-mediated anti-inflammatory activity, such as inhibition of 5-lipoxygenase (Ramires *et al,* 2004), which are unrelated to $CysLT_1R$ antagonism, reservations were evident about using the drug at concentrations in excess of 100 $nmol.L^{-1}$.

Although the precise mechanisms involved in the priming process are poorly understood, two lines of evidence appear to exclude the involvement of Ca^{2+} in CysLT-mediated priming of superoxide production by FMLP-activated neutrophils,

as well as elastase release. These are: i) LTD_4 is more effective than LTC_4 with respect to Ca^{2+} mobilization, however, the priming potencies of the two CysLTs are comparable; and ii) the fura-2 responses of FMLP-activated neutrophils are unaffected by pretreatment of the cells with either of the CysLTs. Alternative possibilities include alterations in the activities of various intracellular kinases involve in signal transduction, including tyrosine kinase, PI3K and MAPK family members (Hallett & Lloyds, 1995; Dang *et al,* 2006).

Irrespective of the molecular/biochemical mechanisms of CysLT-mediated priming of neutrophils, the observations reported in the current study clearly identify a previously unrecognized proinflammatory interaction of CysLTs with human neutrophils, which results in the exaggerated production of superoxide and release of the granule proteases, elastase and MMP-8. All of these have been implicated in the pathogenesis of airway hyperresponsiveness, damage and remodelling (Suzuki *et al,* 1996; Prikk *et al,* 2002; Hiraguchi *et al,* 2008; Sugiura & Ichinose, 2008;). If operative in vivo, this mechanism may explain the therapeutic activity of $CysLT_1R$ antagonists, such as montelukast, in diseases such as COPD (Rubinstein, Kumar & Schriever, 2004; Celik *et al,* 2005) and certain types of bronchial asthma, in which the neutrophil is thought to be the primary offender (Barnes, 2007).

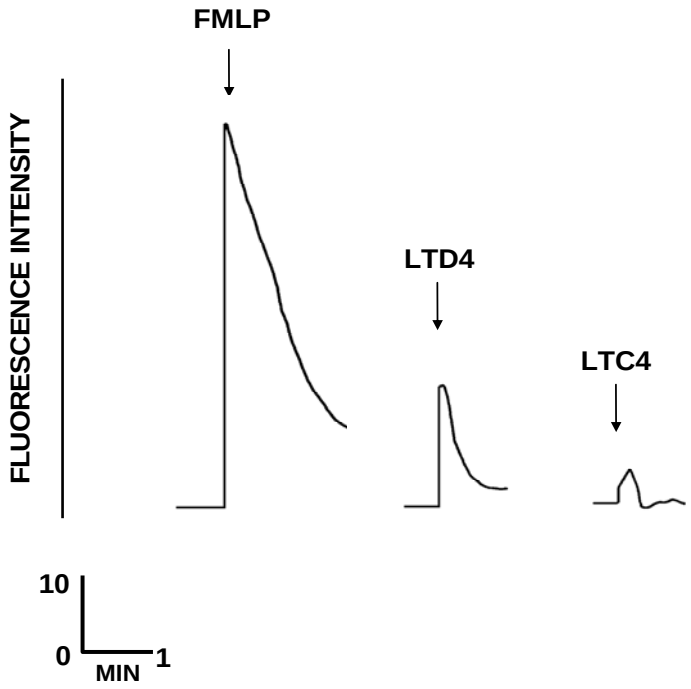

Figure 2.1: Fura-2 fluorescence responses of neutrophils activated with FMLP (1 µmol.L^{-1}), LTC_4 or LTD_4 (100 nmol.L^{-1}). FMLP and the CysLTs were added as indicated (↓) when a stable baseline was obtained. These are typical traces of 6 different experiments.

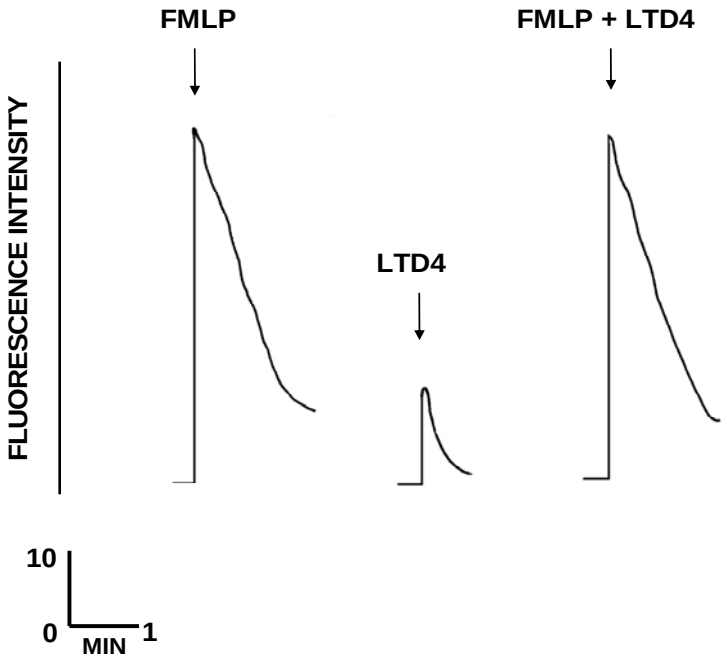

Figure 2.2: Fura-2 fluorescence responses of neutrophils activated with FMLP (1 µmol.L^{-1}) or LTD4 (100 nmol.L^{-1}) individually or in combination. LTD_4 was added 10 sec prior to FMLP. FMLP and LTD_4 were added as indicated (↓) when a stable base-line was obtained. These are typical traces of 3 experiments.

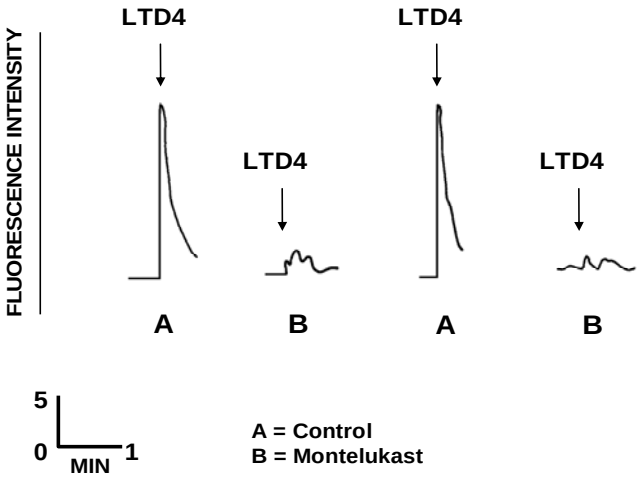

Figure 2.3: LTD_4-activated fura-2 fluorescence responses of control and montelukast (100 $nmol.L^{-1}$)-treated neutrophils. LTD_4 was added as indicated (↓) when a stable base-line was obtained. These are typical traces of 3 experiments.

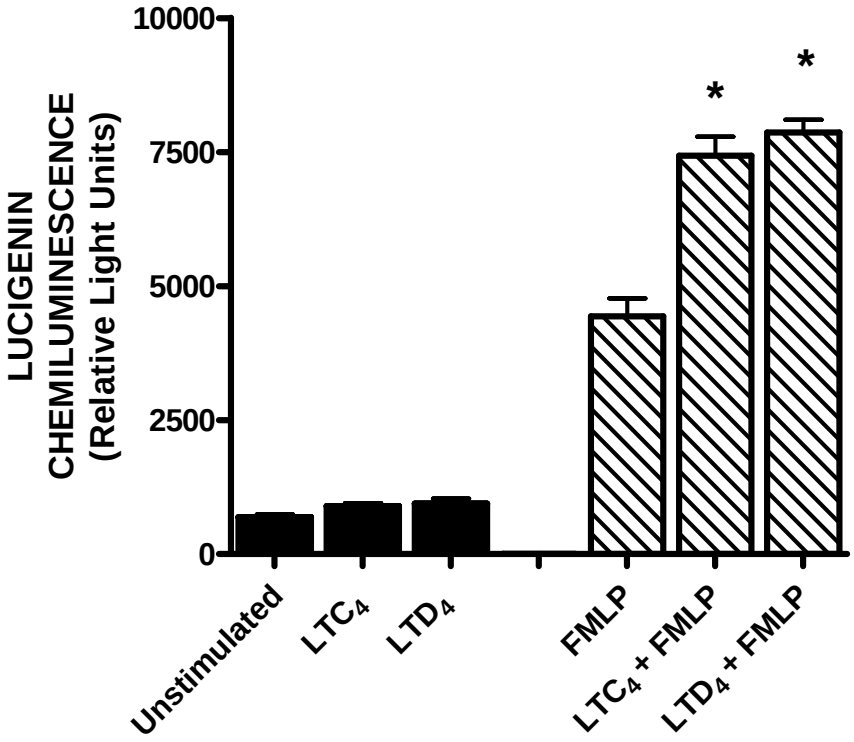

Figure 2.4: Effects of LTC_4 or LTD_4 (100 $nmol.L^{-1}$) on superoxide production by unstimulated cells and cells activated with FMLP (1 $\mu mol.L^{-1}$). The leukotrienes were added 10 sec prior to the FMLP. The results of 6 different experiments are presented as the mean peak lucigenin enhanced chemiluminescence values ± S.E.M.
* $P < 0.05$ for comparison with neutrophils activated with FMLP only.

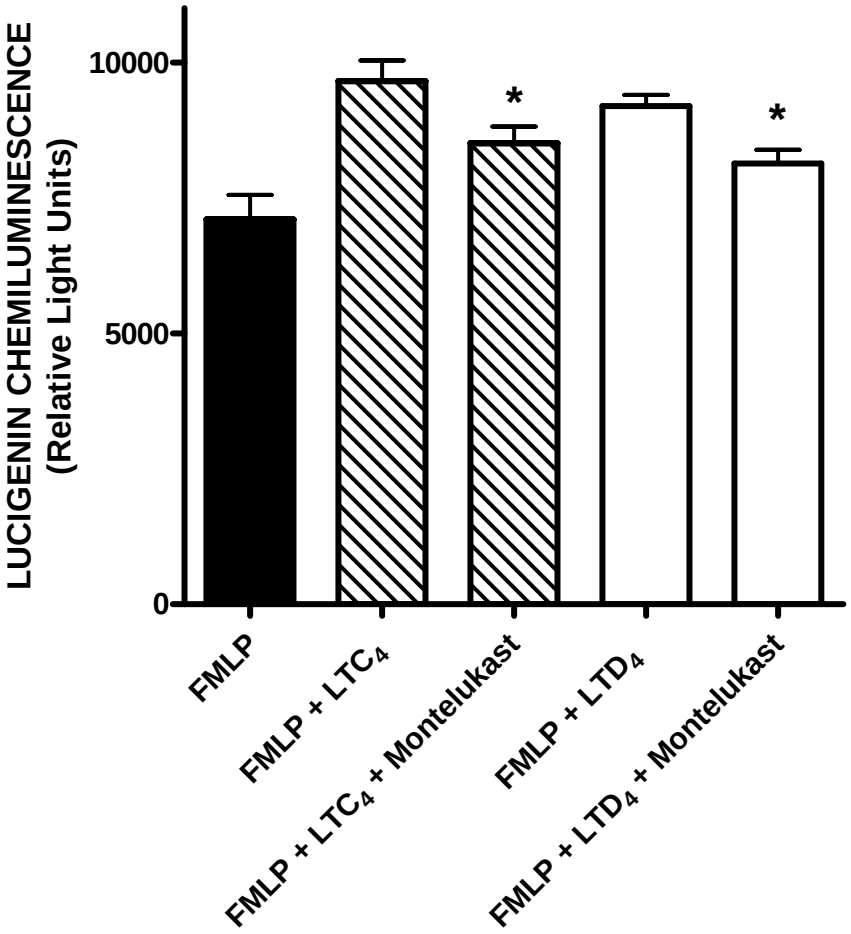

Figure 2.5: The effects of montelukast (100 nmol.L^{-1}) on superoxide production by neutrophils activated with a combination of LTC_4 or LTD_4 (100 nmol.L^{-1}) and FMLP (1 µmol.L^{-1}). The leukotrienes were added 10 sec prior to FMLP. The results of 5 different experiments are presented as the mean peak lucigenin-enhanced chemiluminescence values ± S.E.M.
* $P < 0.05$ for comparison with neutrophils activated by a combination of LTC_4/LTD_4 and FMLP in the absence of the drug.

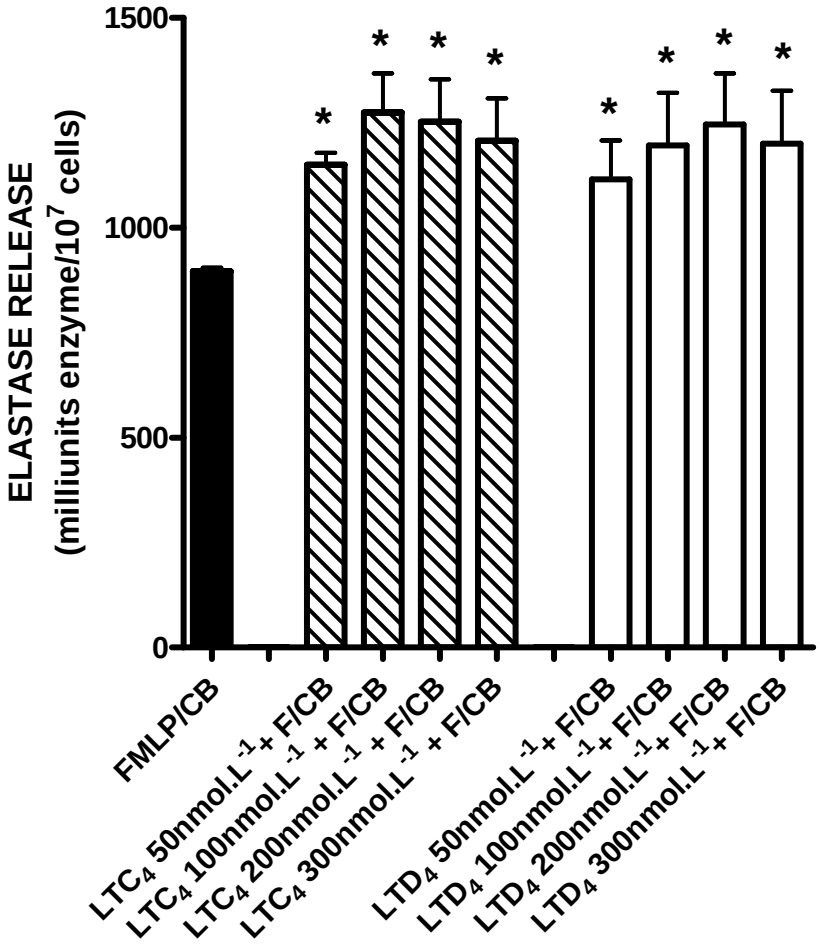

Figure 2.6: Effects of varying concentrations of LTC_4 or LTD_4 (50-300 $nmol.L^{-1}$) on elastase release by neutrophils activated with FMLP/CB. The leukotrienes were added 10 sec prior to FMLP. The results of 3 different experiments are presented as the mean values ± S.E.M for release of elastase.
* $P < 0.05$ for comparison with neutrophils activated by FMLP/CB only.

Table 2.1: Effects of LTC_4 or LTD_4 (100 $nmol.L^{-1}$) on MMP-8 release by cells activated with FMLP (1 $\mu mol.L^{-1}$).

System	MMP-8 Level ($ng.ml^{-1}$)	P-value
FMLP (1 $\mu mol.L^{-1}$)	86 ± 4	
LTC_4 + FMLP	108 ± 6	0.001*
LTD_4 + FMLP	116 ± 10	0.001*

The results of 4 experiments are expressed as the mean values ± S.E.M.

* P-value with respect to FMLP only control system. MMP-8 was undetectable in unstimulated cells.