

## Mediator concentrations in bronchoalveolar lavage fluid of patients with mild asymptomatic bronchial asthma

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**ABSTRACT:** Bronchoalveolar lavage (BAL) was performed on 11 atopic patients with mild asymptomatic bronchial asthma and 11 healthy nonasthmatic volunteers. All asthmatic subjects had evidence of bronchial hyper-responsiveness to inhaled carbachol.

The concentrations of leukotriene (LT) C<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>, LTB<sub>4</sub>, prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), platelet-activating factor (PAF) and histamine in BAL fluid measured. The leukotrienes were measured by radioimmunoassay following reverse phase high performance liquid chromatography. PGD<sub>2</sub> concentrations were determined by radio immunoassay after Amprep C<sub>2</sub> extraction. PAF was quantitated by means of *in vitro* aggregation of rabbit platelets and histamine content was measured using a single isotope radio-enzymatic assay.

There was an increase in the levels of PGD<sub>2</sub> and a decrease in the concentration of LTB<sub>4</sub> in asthmatic lavage samples.

There were no significant differences in the lavage concentrations of LTC<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> and histamine between the two groups of individuals. PAF was undetectable.

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Arachidonic acid released from membrane phospholipids during cellular activation can be oxidatively metabolized by the enzymes of the cyclooxygenase or the lipoxygenase pathways [1]. Arachidonic acid is metabolized by the cyclooxygenase pathway to prostaglandin (PG) G<sub>2</sub> and PGH<sub>2</sub>. These cyclic endoperoxides are metabolized by specific synthetases to PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub> (prostacyclin), and thromboxane (TxA<sub>2</sub>). PGD<sub>2</sub> is the major prostanoid metabolite produced by activated mast cells [2, 3] and it is a potent bronchoconstricting mediator [4]. The 5-lipoxygenase pathway, which has a more limited distribution than the cyclooxygenase cascade, generates 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) from arachidonic acid. 5-HPETE is reduced to 5-hydroxy-eicosatetraenoic acid (5-HETE) or is converted enzymatically to leukotriene (LT) A<sub>4</sub>. LTA<sub>4</sub> is processed enzymatically to LTB<sub>4</sub> or to LTC<sub>4</sub>. LTC<sub>4</sub> is cleaved by gamma-glutamyl transpeptidase to LTD<sub>4</sub> and by dipeptidase to LTE<sub>4</sub>. LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> comprise the activity previously recognized as slow-reacting substance of anaphylaxis.

Platelet activating factor (PAF) was initially described as generated by rabbit basophils [5]. Phospholipase A<sub>2</sub> generates the immediate precursor lyso-paf from ether phospholipids. Lyso-paf

is then acetylated by acetyl transferase to PAF. PAF is metabolized by acetyl hydrolase back to lyso-paf. PAF is a potent pro-inflammatory agent with potent chemotactic activities towards human eosinophils [6]. It has also been reported to augment bronchial hyperresponsiveness [7].

The contribution of lipid mediators and histamine to the pathophysiology of bronchial asthma is suggested by their potent biological properties [8, 9], their identification in the bronchoalveolar lavage fluid of asthmatic subjects, both at rest and following localised bronchial provocation with antigen in sensitised subjects [10-12], and the increased excretion of LTE<sub>4</sub> into the urine in acute asthma [13]. The inhibition of exercise induced asthma by receptor antagonists for LTC<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub> [14] and histamine [15] supports the role of these mediators in asthma.

Previous work on measurement of mediator levels in BAL fluid has focused on symptomatic asthmatic subjects [6-8, 16] and has only quantitated limited numbers of mediators. Thus there is a paucity of data on the relative concentrations of the different agents in BAL fluid. In the present study, we have measured the concentrations of the sulphidopeptide leukotrienes, LTB<sub>4</sub>, PGD<sub>2</sub>, PAF and histamine in the same asthmatic individuals,



when they were asymptomatic but still hyper-reactive to carbachol, and compared the results with those in 11 normal individuals.

## Methods

### Patients

Eleven asthmatic patients (age range 20–35 yrs; 7 males) and eleven healthy non-asthmatic volunteers (age range 18–31 yrs; 5 males) have been studied. All asthmatic subjects showed airway hyperresponsiveness to inhaled carbachol. The cumulative provoking dose (PD) of carbachol which produced a two-fold increase in airways resistance was  $0.4 \pm 0.1$  mg (mean  $\pm$  SEM). All non-asthmatic patients had a PD carbachol  $>1$  mg. All the asthmatic subjects and none of the non-asthmatic subjects were atopic on the basis of one or more positive single skin prick test responses to five common inhalant allergens: *Dermatophagoides pteronyssinus*, mixed grass pollen, moulds, cat fur, and dog hair (Bencard, Brentford, Middlesex UK). A positive skin test was defined by the presence of a wheal, the size of which was 4 mm greater than the diluent control. In addition, all patients had raised radioallergosorbent test (RAST) titres of 3 or 4 for at least one of the above allergens, as determined by enzyme-linked immunosorbent assay (ELISA) (Pharmacia, Sweden).

The asthmatic patients gave a history of pollen-induced asthma. All had very mild disease with a history of intermittent symptoms of wheezing, and none had required treatment with inhaled or oral corticosteroids.

All subjects were asymptomatic at the time of study. All subjects were nonsmokers and there was no history of an upper respiratory tract infection during the 4 weeks before the study. Approval was obtained from the Ethics Committee of the Augusta Teaching Hospital, Bochum, FRG and informed consent was obtained from each subject. The resting forced expiratory volume in one second  $FEV_1$  was  $94.5 \pm 4.3\%$  predicted (mean  $\pm$  SEM) in the asthmatic patients and  $101.5 \pm 3.2\%$  predicted (mean  $\pm$  SEM) in the non-asthmatic subjects.

### Fibreoptic bronchoscopy and lavage

Fibreoptic bronchoscopy was performed with a Olympus BFP 20 D bronchoscope after premedication with 5 mg diazepam given intravenously. Topical anaesthesia was achieved with inhalation of 4 ml 4% lidocaine through the mouth. The bronchoscope was coated with anaesthetic gel and inserted transnasally to the vocal cords, which were then anaesthetized with up to 6 ml 2% lignocaine in 2 ml aliquots. Local anaesthesia of the tracheobronchial tree was achieved with 2 ml aliquots of 2% lignocaine up to a maximum of 14 mls. The bronchoscope was then wedged into a segmental bronchus of the lingular lobe. BAL was

performed with 100 ml physiological saline in 20 ml aliquots that had been pre-warmed to 30°C. The fluid was gently aspirated after each instillation into a silicon-lined glass container which was kept in ice. The recovery of fluid was  $60 \pm 6\%$  (mean  $\pm$  SEM). Bronchoscopy and lavage were carried out according to the American Thoracic Society's guidelines [17]. Carbachol challenge was performed approximately one month before bronchoscopy.

### Sample processing

Lavage fluid was filtered twice through surgical gauze and the total cell count in the lavage fluid was estimated with a Turk chamber. The lavage fluid was then centrifuged at  $250 \times g$  at room temperature for 5 min. The supernatant was stored at  $-70^\circ\text{C}$  until analysis. The cell pellet was washed three times in Hanks buffered salt solution (pH 7.4) and was resuspended in 10 ml phosphate buffered saline. Cytochrome preparations were made and the differential cell counts were analysed following May-Grunwald-Giemsa staining.

### Analysis for mediator concentrations in BAL fluid

**Leukotrienes.** These were extracted from 10 ml BAL fluid and purified as reported previously [18–20] with minor modifications. Stored BAL samples were thawed and centrifuged at  $1,800 \times g$  for 30 min at  $0^\circ\text{C}$ . The supernatants were removed. Fifty  $\mu\text{l}$  of a solution containing [ $^3\text{H}$ ]LTC<sub>4</sub> ( $51 \text{ Ci}\cdot\text{mmol}^{-1}$  Amersham, Cardiff, Wales UK), [ $^3\text{H}$ ]LTD<sub>4</sub> ( $192 \text{ Ci}\cdot\text{mmol}^{-1}$ ) and [ $^3\text{H}$ ]LTE<sub>4</sub> ( $224 \text{ Ci}\cdot\text{mmol}^{-1}$ ) were added to 10 ml of BAL to give 1,200 cpm for each leukotriene. Fifty  $\mu\text{l}$  of glacial acetic acid was then added to the mixture to bring the pH to 3.5–3.8. The sample was immediately loaded onto a C18 Amprep cartridge (Amersham) which had been pre-conditioned with 10 ml methanol, followed by 10 ml high performance liquid chromatography (HPLC) grade water and finally with 5 ml 0.1% EDTA. The cartridge was washed with 10 ml each of water and 20% methanol/water (v/v). The leukotrienes were then eluted in 10 ml of 90% methanol and collected. The methanol-containing eluate was roto-evaporated under vacuum at  $23^\circ\text{C}$  until there was 500  $\mu\text{l}$  of a aqueous solution remaining in the tube. Two hundred  $\mu\text{l}$  methanol was then added, the sample was mixed and the whole mixture was subjected to reverse phase-HPLC (RP-HPLC). RP-HPLC was performed on an Ultrasil-ODS analytical column (5 mm  $250 \times 4.6$  mm, Beckman, Fullerton, CA, USA) protected by an ultrasil-ODS guard column (10  $\mu\text{m}$ ,  $45 \times 3.2$  mm, Beckman) and equilibrated in 64% methanol/35% water/0.1% acetic acid/0.1% EDTA (v/v), pH 5.6. The leukotrienes were eluted at a flow rate of  $1 \text{ ml}\cdot\text{min}^{-1}$ . Ultraviolet absorbance at 280 was continuously monitored with a Waters 990 diode array detector (Water-Millijou, Milford, MA,



USA). One ml fractions were collected. The column was calibrated for the retention times of synthetic LTC<sub>4</sub>, LTB<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> (12.4±0.2 min, 17.0±0.2 min, 19.0±0.2 min and 23.0±0.2 min (mean±SEM, n=12), respectively). Following RP-HPLC, 300 µl of each fraction was counted in a beta liquid scintillation counter and the peaks of radioactivity containing the tritiated internal standards were defined. Three hundred µl of fractions 3 and 4, 300µl of the two fractions eluting before and after the peaks of internal standards and 300 µl of each of the fractions containing the [<sup>3</sup>H] LTC<sub>4</sub>, [<sup>3</sup>H] LTD<sub>4</sub> and [<sup>3</sup>H] LTE<sub>4</sub> were dried under vacuum at 23°C and resuspended in 200 µl of 10 mM tris-HCl buffer, pH 7.4 containing 0.15 M NaCl and 0.1% gelatin (Isogel tris buffer). The concentrations of immunoreactive peptidoleukotrienes and LTB<sub>4</sub> in the fractions were assessed by radioimmunoassay (RIA) as described previously [18–20]. The antibody used for peptido-leukotrienes has a sensitivity of 0.01 ng for LTC<sub>4</sub>, with 44% cross-reactivity for LTD<sub>4</sub> and 57% cross-reactivity for LTE<sub>4</sub>. Fifty percent inhibition of binding (IC<sub>50</sub>) of LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> occurred 0.08, 0.18 and 0.14 ng, respectively. The cross-reactivity was <0.1% for LTB<sub>4</sub>, 5-HETE and PGE<sub>2</sub>. Fractions 3 and 4 and the fractions eluting on either side of the radioactive peaks of internal standards were used to calculate a mean background immunoreactivity.

Leukotriene concentrations were expressed as ng leukotriene·ml<sup>-1</sup> recovered BAL fluid and were calculated from measured immunoreactivity minus the mean background level, overall recovery of each tritiated leukotriene internal standard and volume of BAL sample extracted. The recoveries for LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> were 76±3.2%, 80±3.9%, 65±2.5% (mean±SEM, n=18) respectively.

The antibody used for LTB<sub>4</sub> (donated by Merck Frosst) has a sensitivity of 0.01 ng. Fifty percent inhibition of binding (IC<sub>50</sub>) occurred by 0.06 ng. The cross-reactivity was <0.1% for LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>, HETEs and PGE<sub>2</sub>. The coefficient of variation for duplicate samples was 12%.

**Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>).** PGD<sub>2</sub> concentrations in BAL were determined after Amprep C<sub>2</sub> extraction, using commercial RIA (Amersham). Fifty µl of 1 M HCl was added to 5 ml BAL to bring the pH to 3.5. The sample was immediately loaded onto a C<sub>2</sub> Amprep cartridge, which had been preconditioned with 10 ml methanol, followed by 10 ml water and 5 ml 0.2% EDTA. The cartridge was washed with 5 ml of water, 5 ml 10% ethanol and 5 ml Hexane. PGD<sub>2</sub> was then eluted with 5 ml methylformate and collected. Recovery for PGD<sub>2</sub> was >90% using this extraction procedure. The methylformate containing fraction was evaporated under argon; the extract was redissolved in 0.5 ml RIA buffer prior to RIA. The RIA assay for PGD<sub>2</sub> has a sensitivity of 6.2 pg and 50% inhibition of binding (IC<sub>50</sub>) occurred at 20 pg. The cross-reactivity was 7% for PGI<sub>2</sub>, 0.3% for thromboxane B<sub>2</sub>

(TxB<sub>2</sub>) and <0.1% for all other prostaglandins. The coefficient of variation for duplicate samples was 10%.

**Histamine.** BAL histamine was measured using a single isotope radio-enzymatic assay based upon the method of GUILLOUX *et al* [21] and CHURCH *et al* [22]. Histamine-N-methyl transferase containing 12.4 mg protein·ml<sup>-1</sup> was obtained from rat kidney and [<sup>3</sup>H]-S-adenosylmethionine was purchased from Amersham Laboratories. The assay was modified as reported from BELCHER *et al.* [23] by the addition of 5 µl of diamine oxidase (1 I.U.·ml<sup>-1</sup> [Sigma]) to 50 µl of each sample. These were run in parallel with non-digested samples to correct variations in nonspecific background methylation. The sensitivity of the assay was 0.05 ng·ml<sup>-1</sup>. The coefficient of variation for duplicate samples was 15%.

**Platelet-activating factor (PAF).** PAF was quantitated by means of *in vitro* aggregation of rabbit platelets [24, 25]. Rabbit platelets were prepared from platelet-rich plasma that was harvested from arterial blood and anticoagulated with 0.2mol·l<sup>-1</sup> of EDTA. The sample of whole blood was centrifuged at 400 × g for 20 min at 20°C; the platelet-rich plasma was aspirated and then centrifuged at 800 × g for 15 min at 20°C. The pelleted platelets were washed once in Tyrode's buffer containing 0.25% gelatin (Merck Frosst Laboratories, Darmstadt, W. Germany), 0.2 mmol·l<sup>-1</sup> of ethylene glycol-bis-(beta-aminoethyl ether)N, N, N' N'-tetraacetic acid (Sigma Chemical Co.), pH 6.5, treated with 0.1 mmol·l<sup>-1</sup> of aspirin (Aspegic; Egic-Jouille Laboratories, Paris, France) for 15–60 min at 20°C, and then washed. The platelets were resuspended at densities of 4.5 × 10<sup>9</sup> cells·ml<sup>-1</sup> in Tyrode's buffer containing 0.25% gelatin and 0.2 mmol·l<sup>-1</sup> of EDTA. The assay of PAF was performed in a dual-channel platelet aggregometer (Coulter Electronics Ltd, Beds, UK) in 450 µl of Tyrode's buffer containing 1 mmol·l<sup>-1</sup> of CaCl<sub>2</sub>·2H<sub>2</sub>O (Sigma Chemical Co.) and the adenosine diphosphate scavenger complex, 1 mmol·l<sup>-1</sup> of creatine phosphate per 10 U·ml<sup>-1</sup> of creatine phosphokinase (both Sigma Chemical Co.) at a platelet density in BAL of 2.25 × 10<sup>8</sup> cells per 450 µl. The quantity of PAF was interpolated from the linear portion of the calibration curve from 5 to 50 pg of authentic synthetic PAF. The coefficient of variation for duplicate samples was 20%.

#### Statistics

Statistical analysis was performed by non-paired Student's t-test.

#### Results

There was a significant increase in the total numbers of lavage cells in asthmatic subjects. This was reflected in significant increases in macrophages,



Table 1. — The total cell numbers (mean±SEM) and numbers of the different cell types in the bronchoalveolar lavage sample obtained from asthmatic and normal patients

Subjects	n	Total cell nos × 10 <sup>3</sup> ·ml <sup>-1</sup>	No. of cells × 10 <sup>3</sup> ·ml <sup>-1</sup>			
			Mac	Lym	Neut	Eos
Asthmatic	11	230±44	184±2.3	20±2	10±1	16±4
Normal	11	134±72	125±8	4±1	5±0.3	0
p		<0.05	<0.001	<0.0001	NS	<0.0002

Mac: macrophages; Lym: lymphocytes; Neut: neutrophils; Eos: eosinophils; ns: not significant.

Table 2. — The concentrations (ng·ml<sup>-1</sup>; mean±SEM) of the leukotrienes, PGD<sub>2</sub>, PAF and histamine (HIST) in the bronchoalveolar lavage sample of asthmatic and normal subjects

Subjects	n	LTC <sub>4</sub>	LTD <sub>4</sub>	LTE <sub>4</sub>	LTB <sub>4</sub>	PGD <sub>2</sub>	PAF	HIST
Asthmatic	11	0.4±0.08	0.7±0.08	1.7±0.4	0.1±0.01	0.6±0.07	ND	1.7±0.8
Normal	11	0.8±0.2	1.8±0.9	1.8±0.3	1.3±0.2	0.3±0.03	ND	0.4±0.2
p		NS	NS	NS	<0.0001	=0.004		NS

Each sample was measured in duplicate and the results were averaged. LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>, LTB<sub>4</sub>: leukotrienes C<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub> and B<sub>4</sub>, respectively; PGD<sub>2</sub>: prostaglandin D<sub>2</sub>; PAF: platelet-activating factor; ND: not detected; NS: not significant.

lymphocytes and eosinophils (table 1). The sulphidopeptide leukotrienes were present in the most abundant quantities in BAL fluid as compared to the other mediators. There was no significant difference in the concentrations of LTC<sub>4</sub>, LTD<sub>4</sub> or LTE<sub>4</sub> between asthmatic and non-asthmatic subjects (table 2). LTE<sub>4</sub> was the major sulphidopeptide leukotriene detected in asthmatic subjects, whereas LTD<sub>4</sub> and LTE<sub>4</sub> were present in equal quantities in normal individuals. There was a significantly lower level of LTB<sub>4</sub> in the asthmatic lavage than in the non-asthmatic samples (table 2). In contrast, there was a two-fold increase in the levels of PGD<sub>2</sub> (p=0.004). PAF was not detected in any of the samples. There was a greater level of histamine in the asthmatic samples compared to the non-asthmatic lavages, but this difference did not reach statistical significance.

### Discussion

Previous studies on the measurement of immunoreactive sulphidopeptide leukotrienes in BAL fluid have generally measured a combination of LTC<sub>4</sub> and LTD<sub>4</sub> [12, 16], largely because the antisera lacked adequate specificity for LTE<sub>4</sub>. We elected to measure all three sulphidopeptide leukotrienes in the BAL samples to maximize the ability to quantitate accurately the total sulphidopeptide leukotrienes generated. Specificity was achieved by combining the inherent specificity of the radio-immunoassay with RP-HPLC resolution of the extracted sample. The antibody used in these experiments demonstrated substantial crossreactivity with all three sulphidopeptide

leukotrienes, therefore allowing satisfactory quantitation of each product. The inclusion of an internal standard allowed overall recovery for each sample to be calculated. There are limited data on the concentrations of immunoreactive sulphidopeptide leukotrienes in BAL fluid samples. The detection of small quantities of sulphidopeptide leukotrienes in BAL fluids obtained from normal healthy subjects in the present study confirms the results of previous work [19, 20]. The present finding that the concentration of sulphidopeptide leukotrienes in BAL fluids did not differ between healthy volunteers and patients with asthma suggests that either the biosynthesis of these leukotrienes was not enhanced in the patients studied or that, in the patients an increased generation of LTC<sub>4</sub>/D<sub>4</sub>/E<sub>4</sub> was masked by an enhanced metabolism of these compounds. These results differ from those of WARDLAW *et al.* [16] who studied symptomatic asthmatic subjects and demonstrated increased amounts of LTB<sub>4</sub> and LTC<sub>4</sub> in BAL fluid [16]. The differences between these two studies may relate to the selection of asymptomatic patients with only very mild asthma for this study.

LTB<sub>4</sub> concentrations in BAL were significantly less in the asthmatic group. This could be due as much to reduced biosynthesis as augmented omega-metabolism of LTB<sub>4</sub>, possibly due to the presence of increased numbers of inflammatory cells in asthmatic airways. The latter suggestion is supported by the previous demonstration of the presence of omega-metabolites of LTB<sub>4</sub> in the BAL obtained from asthmatic individuals [12] and the evidence that airways inflammation is marked even in patients with mild asthma [26].



PGD<sub>2</sub> was measured by radioimmunoassay following a simple Amprep C<sub>2</sub> extraction. The PGD<sub>2</sub> levels were significantly raised in the asthmatic group, compared to the normal individuals, confirming a previous report [27]. Since the major source of PGD<sub>2</sub> is mast cells [2], these results suggest that there may be an *in vivo* activation of airway mast cells for augmented prostanoid mediator release in mild asthma. This view is supported by the finding that the lavage histamine concentrations were on average four fold higher than those of normal subjects. However, because of the scatter of the data in histamine measurements, the difference in the histamine levels did not reach statistical significance. WENZEL *et al.* [28] and RANKIN *et al.* [29] also found no significant differences in lavage fluid histamine content between asthmatic and normal subjects, whereas the studies of CASALE *et al.* [30] and LU *et al.* [27] demonstrated increased histamine content in asthmatic lavage samples. The discordance is likely to be due to the selection of patients with different severities of asthma.

We were unable to detect any PAF in the samples. PAF was measured by a biological assay dependent upon the aggregation of rabbit platelets. The assay is very sensitive and is able to detect 5 pg of PAF. It is therefore unlikely that inability to detect PAF was due to lack of sensitivity of the assay. There are at least three possible reasons for the absence of PAF in the BAL fluid. PAF may not have been generated; PAF was generated but remained cell-associated and could not be detected in the BAL fluid; or PAF was generated but was rapidly metabolized to the inactive lyso-PAF. The use of a technique such as gas chromatography mass spectrometry to measure PAF and lyso-PAF may resolve this issue in the future.

The present results suggest that even in patients with very mild bronchial asthma, who are asymptomatic, there is mast cell activation resulting in PGD<sub>2</sub> release. PGD<sub>2</sub> may have a role to play in the resting airway inflammation in this group of individuals. There was no significant difference in the concentrations of the sulphidopeptide leukotrienes or PAF. The lower level of LTB<sub>4</sub> in the lavage sample from asthmatic subjects may be due to omega-metabolism of this mediator by the increased numbers of inflammatory cells in the asthmatic airways.

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