

## Increased $LTB_4$ metabolites and $PGD_2$ in BAL fluid after methacholine challenge in asthmatic subjects

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*Increased  $LTB_4$  metabolites and  $PGD_2$  in BAL fluid after methacholine challenge in asthmatic subjects. D. Nowak, F. Grimminger, R. Jörres, M. Oldigs, K.F. Rabe, W. Seeger, H. Magnussen. ©ERS Journals Ltd 1993.*

**ABSTRACT:** The bronchoconstrictor potency of inhaled methacholine is widely used to assess airway responsiveness. However, evidence has accumulated that methacholine inhalation challenge may lead to an inflammatory response in the lower respiratory tract. We therefore compared cellular, leukotriene and prostanoid profiles in bronchoalveolar lavages (BAL) obtained five hours after methacholine challenge to control lavages without prior challenge.

Eight subjects with asymptomatic to mild bronchial asthma and nine nonatopic healthy controls were enrolled in the study.

Without prior challenge, the percentage of BAL eosinophils was higher in the asthmatic subjects ((mean±SD),  $1.1±0.9\%$ ) than in the control subjects ( $0.1±0.1\%$ ). Leukotriene  $B_4$  ( $LTB_4$ ), and its omega-oxidation products (20-OH- $LTB_4$  and 20-COOH- $LTB_4$ ) were the only leukotrienes detectable in the baseline BAL fluids in five of the eight asthmatic patients. After methacholine challenge, no change in BAL cell profile occurred, but in the asthmatic patients, the total amounts of  $LTB_4$  and its omega-oxidation products rose from  $0.52±0.50$  ng·ml<sup>-1</sup> (pre-challenge) to  $1.55±1.32$  ng·ml<sup>-1</sup> (post-challenge), and prostaglandin  $D_2$  ( $PGD_2$ ) rose from  $49.1±15.7$  (pre-challenge) to  $94.4±25.4$  pg·ml<sup>-1</sup> (post-challenge), with no change in 6-keto-PGF<sub>1 $\alpha$</sub>  thromboxane  $B_2$  (TXB<sub>2</sub>), and prostaglandins F<sub>2 $\alpha$</sub>  and E<sub>2</sub> (PGF<sub>2 $\alpha$</sub>  and PGE<sub>2</sub>). In the healthy controls, no consistent change in BAL cell profile and mediators occurred after methacholine provocation.

We conclude that inhaled methacholine stimulates  $LTB_4$  and  $PGD_2$  release in asthmatics, but not in healthy controls, without affecting the number of inflammatory cells in BAL fluid.

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In bronchial asthma, airway hyperresponsiveness, as assessed by inhalation of methacholine or histamine, has been reported to increase with allergen exposure [1] and after inhalation of toluene diisocyanate (TDI) [2], or ozone [3]. Studies using bronchoscopy, bronchial biopsies and bronchoalveolar lavage (BAL) as a tool to investigate asthmatic airways have demonstrated that allergen [4], TDI [2], or ozone [3, 5] exposure is followed by an inflammatory airway response. Therefore, it has been suggested that inflammation of asthmatic airways is functionally reflected by airway hyperresponsiveness [6].

The usefulness of histamine or methacholine challenges as markers of airway inflammation depends on the ability of these compounds to constrict airways without concomitant effects on inflammatory reactions within the airway wall and lumen. Since an inflammatory airway response is most often observed several hours after application of an appropriate stimulus, we measured the pattern of cells and arachidonic acid metabolites within the BAL recovered from mild asthmatics and control subjects

5 h after methacholine challenge and compared the results to baseline values.

### Methods

#### Subjects

Seventeen subjects volunteered for these studies. Eight had mild allergic bronchial asthma (4 male, 4 female, mean±SD age  $23±3$  yrs), and nine healthy individuals served as controls (5 male, 4 female, mean age  $28±4$  yrs).

The criteria for selection of subjects with bronchial asthma were: 1) positive history of mild [7] seasonal or perennial allergic bronchial asthma; 2) positive skin-prick tests to at least one allergen; 3) treatment with beta-sympathomimetic agents on demand only, no treatment with corticosteroids, antihistamines, sodium cromoglycate, nedocromil sodium or theophylline for at least 3 months prior to the study; 4) baseline forced expiratory volume



in one second ( $FEV_1$ ) greater than 70% of the lower level of predicted value; and 5) hyperresponsiveness to inhaled methacholine with a provocative concentration causing a 20% fall in  $FEV_1$  ( $PC_{20}FEV_1$ ) below  $4 \text{ mg}\cdot\text{ml}^{-1}$ .

The criteria for selection of healthy controls were: 1) absence of any disease; 2) negative skin-prick tests to inhalation allergens; 3) normal serum immunoglobulin E (IgE); 4) normal baseline spirometry and body plethysmography; and 5) a  $PC_{20}FEV_1$  for methacholine above  $16 \text{ mg}\cdot\text{ml}^{-1}$ .

A complete medical history was recorded and physical examination was performed in all subjects. Atopy was assessed by skin-prick tests against grass pollen, tree pollen, feathers, household pets, mould, house dust and storage mites, and cockroaches. A positive reaction was recorded if the reaction was greater than that induced by histamine control. All subjects had peak flow measured four times daily, over a period of one week (control subjects), or two weeks (patients), and the diurnal variation was calculated as mean maximum minus mean minimum divided by mean maximum value. All subjects were non-smokers or ex-smokers for at least 1 yr, and had no respiratory tract infection for at least 6 weeks prior to the study. The characteristics of patients and control subjects are given in table 1. Four patients took inhaled salbutamol, one to four puffs per month. Patients were younger ( $p<0.01$ ) and had a higher peak flow variation ( $p<0.005$ ) than controls, but lung function was similar in both groups.

The study was approved by the regional Ethics Committee, and written informed consent was given by all subjects.

#### Methacholine provocation challenge

Bronchial challenges with methacholine were performed according to the protocol proposed by CHAI *et al.* [8]. Briefly, we used a DeVilbiss nebulizer (No. 646, DeVilbiss Co., Somerset, PA, USA), triggered by a breath-synchronized solenoid valve that remained open for 0.6 s to allow generation of an aerosol by pressurized oxygen at 20 psi. The output of the nebulizer was evaluated by measuring the weight before and after 10 nebulizations; the mean $\pm$ SD output was found to be  $0.011\pm 0.002 \text{ ml}$  per nebulization. Different concentrations of methacholine (Sigma Chemie, Taufkirchen, Germany) were prepared from crystalline material, using normal saline. The subjects were instructed to take five breaths, starting from functional residual capacity (FRC) and inhaling to total lung capacity, at a constant inspiratory flow of about  $2 \text{ l}\cdot\text{s}^{-1}$ . After measuring baseline values and the airway response to the diluent, increasing concentrations of methacholine were given. Lung function parameters were assessed 3 min after each inhalation. Each provocation step, consisting of inhalation and measurement, lasted 7 min. Provocation was stopped when  $FEV_1$

Table 1. — Clinical features of: a) the patients with bronchial asthma; and b) the control subjects

Pt no.	Age yrs	Sex	Height cm	Therapy	$FEV_1$ l	$FEV_1$ %	PEF var. %	$PC_{20}FEV_1$ $\text{mg}\cdot\text{ml}^{-1}$	IgE $\text{IU}\cdot\text{ml}^{-1}$
<b>a) Patients with bronchial asthma</b>									
1	23	M	185	S <sup>4</sup>	4.7	90	8	2.09	60
2	20	M	184	S <sup>1</sup>	4.4	86	22	0.39	23
3	21	F	172	-	3.9	103	23	1.70	523
4	23	M	189	-	3.9	71	10	0.31	20
5	28	M	180	S <sup>4</sup>	4.5	94	9	0.16	41
6	22	F	176	-	3.7	82	8	1.65	669
7	21	F	176	-	3.8	87	22	0.24	157
8	22	F	167	S <sup>1</sup>	2.9	85	18	0.47	309
Mean	23		179		4.0	87	14	0.58	225
SD	3		9		0.6	9	6	2.70*	251
<b>b) Control subjects</b>									
1	27	M	180	-	4.5	94	4	>16	41
2	31	F	172	-	4.0	111	4	>16	10
3	25	M	183	-	4.4	88	6	>16	47
4	28	F	176	-	3.6	90	6	>16	15
5	35	M	172	-	2.5	78	7	>16	110
6	23	M	173	-	3.7	88	5	>16	10
7	31	F	171	-	3.7	103	4	>16	10
8	25	M	187	-	4.6	85	5	>16	75
9	24	F	165	-	3.5	97	5	>16	42
Mean	28		175		3.8	93	5		40
SD	4		7		0.6	10	1		34

S: salbutamol, superscript indicates puffs-month<sup>-1</sup>; \*: geometric mean/\* (divided by, multiplied with) variability factor;  $FEV_1$ : forced expiratory volume in one second; PEF var.: peak expiratory flow variation;  $PC_{20}FEV_1$ : provocative concentration producing a 20% fall in  $FEV_1$ ; IgE: immunoglobulin E.



dropped by 20% as compared to baseline values, or after a maximum methacholine concentration of 16 mg·ml<sup>-1</sup>. Dose-response curves were constructed by plotting FEV<sub>1</sub> against increasing doses of methacholine, and the PC<sub>20</sub>FEV<sub>1</sub> was defined as the methacholine concentration necessary to decrease post-saline FEV<sub>1</sub> by 20%.

### Bronchoscopy

Bronchoscopy followed the guidelines published in 1991 [9]. All subjects had a venous access and were pre-medicated subcutaneously with atropine, 0.5 mg, 30 min prior to bronchoscopy, which was performed at approximately 1.00 p.m. Immediately prior to bronchoscopy, all subjects inhaled 0.1 mg of fenoterol. Topical anaesthesia of the nose and pharynx was achieved by 0.5% lidocaine, and additional anaesthesia of the nose by 2% lidocaine gel. During bronchoscopy, 100% oxygen was delivered through a nasal cannula at a rate of 5 l·min<sup>-1</sup>. Bronchoscopy was performed with an Olympus BF20-D flexible bronchoscope (Olympus, Tokyo, Japan) by one experienced bronchoscopist (D.N.). The instrument was introduced transnasally, with additional 0.5% lidocaine applied through the channel to the larynx, vocal cords and trachea. After endobronchial examination and local anaesthesia of the middle lobe (first bronchoscopy), or lingula bronchus (second bronchoscopy), the bronchoscope was wedged into a respective segment. Bronchoalveolar lavage (BAL) was performed using 5×20 ml aliquots of sterile saline at 37°C from glass flasks. The fluid was immediately aspirated into siliconized glassware, and transported on ice to the laboratory. After initial centrifugation (15 min, 4°C, 1,000 rpm), the cells were transferred to the cytocentrifuge, and the supernatant was further processed on extraction columns.

### Cellular examination of the BAL fluid

Cytological examination of 500 BAL cells was performed by one investigator (M.O.) on cytocentrifuge slides stained with May-Grünwald-Giemsa stain.

### Reagents

Leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>, LTB<sub>4</sub>), the omega-oxidation products of LTB<sub>4</sub> (20-OH-LTB<sub>4</sub> and 20-COOH-LTB<sub>4</sub>), and the synthetic LTA<sub>4</sub>-methyl ester were a gift from J. Rokach, Merck Frosst, Canada. 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE) as well as 5S,12S-diHETE, 5,15-diHETE, and 12-hydroxyheptadecatrienoic acid were obtained from Paesel AG, Frankfurt, Germany. All leukotrienes were checked for purity and quantified spectrophotometrically prior to use, as described previously [10]. Tritiated leukotrienes, used as internal standards, were obtained from Amersham Buchler, Braunschweig, Germany. Chromatographic supplies included high-pressure liquid chromatography (HPLC)-grade solvents, distilled in glass (Fluka, Heidelberg, Germany), octadecylsilyl (5 µm; Hypersil),

and silical gel column packing material (5 µm; Machery-Nagel, Düren, Germany), and C-18 Sep-pak cartridges (Waters, Milford, Massachusetts, USA). Anti-LTB<sub>4</sub> antiserum was received from J. Salmon (Wellcome Research Laboratories, Beckenham, UK), and anti-LTC<sub>4</sub> anti-serum was obtained from NEN. All other biochemicals were obtained from Merck (Darmstadt, Germany) in p.a. quality.

### Analytical procedures

Leukotrienes and HETEs were extracted from BAL fluid by octadecylsilyl solid-phase (SP) extraction columns, immediately after removal of cells as described previously [11]. Material was mailed on dry ice from Grosshansdorf to Giessen. Reverse-phase (RP) HPLC of non-methylated compounds was carried out on octadecylsilyl columns (5 µm particles; Hypersil), with a mobile phase on methanol-water-acetic acid (72:28:0.16, pH 4.9) [10]. In addition to the conventional UV detection at 270 nm (leukotrienes) and 237 nm (HETEs), a photodiode array detector (model 990; Waters), which provided full UV spectra (190–600 nm) of eluting compounds and allowed checking for peak purity and subtraction of possible co-eluting material, was used. For further verification, samples were collected in 15 s fractions and subjected to post-HPLC radio-immunoassay (RIA) with anti-LTC<sub>4</sub> and anti-LTB<sub>4</sub>, as described previously [10]. Conversion into methyl esters was performed by the addition of freshly prepared diazomethane to ice-cold diethyl ether. RP-HPLC of methylated compounds was performed isocratically (72:28:0.16, pH 4.9) for 5 min, followed by a linear gradient to 90:10:0.16 over 10 min (model 250; Gynkotheek gradient former). Straight-phase HPLC was carried out by using a modification of the method described previously [12]. Isocratic elution with hexane-isopropanol-acetate (97:3:0.1; 12 min) was followed by a linear gradient to 86:14:0.1 over 10 min. All data obtained by the different analytical procedures were corrected for the individual recoveries of the overall analytical procedure, as assessed by the addition of 0.125 µCi of (<sup>3</sup>H)LTB<sub>4</sub> and 0.05 µCi of (<sup>3</sup>H)LTE<sub>4</sub> as internal standards in each lavage sample.

Thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and 6-keto-prostaglandin F<sub>1α</sub> (PGF<sub>1α</sub>) were assayed by RIA as described previously [13]. Prostaglandins (PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2α</sub>) were measured by commercially available RIA kits (Amersham, Braunschweig, Germany).

The recoveries of the overall analytical procedures ranged between 25–55%. All arachidonic acid metabolites are given in ng·ml<sup>-1</sup> lavage fluid. Detection limits of the different analytical procedures ranged between 0.3–0.6 ng·ml<sup>-1</sup> for all metabolites. Correcting values for the amounts of lavage fluid did not alter the results.

### Study design

Each subject was studied on two separate days, being at least 1 week apart, with a maximum interval of 3



weeks. On one of the study days, the methacholine provocation challenge was performed at 8.00 a.m., followed by bronchoscopy at 1.00 p.m. On the second day, bronchoscopy was performed at 1.00 p.m. without preceding methacholine challenge. Physical examination and spirometry were performed immediately prior to bronchoscopy and 2 h after the procedure. Study sessions with and without methacholine challenge were randomized.

#### Statistical evaluation

Non-parametric statistical tests were used in order to avoid assumptions on data distribution [14]. Comparison of data between groups was performed by the Mann-Whitney two sample test, and pre- versus post-challenge values were compared by the Wilcoxon test for matched pairs. Correlations were tested by Spearman's rank correlation test. All data are given as mean $\pm$ SD.

### Results

#### Tolerance of bronchoscopy

Bronchoscopy was well-tolerated by all subjects. In one of the patients (No. 7 in table 1a), visible narrowing of the bronchial lumen occurred during the first and second bronchoscopy, and despite application of fenoterol *via* the bronchoscope after the BAL procedure, bilateral wheezing was heard upon auscultation. After extubation and application of two further puffs of fenoterol, clinical status and lung function normalized within 10 min.

#### Spirometry

In the asthmatic subjects, mean $\pm$ SD vital capacity and FEV<sub>1</sub> 2 h after the BAL procedure were 4.4 $\pm$ 1.3 and 3.8 $\pm$ 0.9 l, respectively and, therefore, not significantly different from the values prior to bronchoscopy (table 1a). Similarly, in the control subjects, mean $\pm$ SD vital capacity and FEV<sub>1</sub> 2 h after the BAL procedure were 4.4 $\pm$ 0.8 and 3.6 $\pm$ 0.6 l, respectively, and, therefore, not significantly different from the values prior to bronchoscopy (table 1b).

#### Bronchoscopic findings

Mild oedema and swelling of the mucosa was present in three of the asthmatics (Nos 1, 2 and 5, table 1a) without and after methacholine challenge, and in one of the control subjects (No. 1, table 1b) after methacholine challenge. Bronchoscopy revealed normal findings in the other subjects.

#### BAL cellular profiles

Baseline BAL composition of the asthmatic patients revealed higher numbers of lymphocytes and eosinophils as compared to healthy control subjects. The difference was significant for eosinophils only ( $p < 0.05$ ). Mast cells were present in four patients with bronchial asthma, but in none of the controls. No significant correlation was found between any BAL cell population and the level of bronchial responsiveness in our asthmatic subjects.

No significant changes in cell composition were seen 5 h after methacholine provocation challenge in patients or controls (table 2).

#### BAL mediator profiles

**Leukotrienes and HETEs.** The RP-HPLC and SP-HPLC chromatograms of the BAL fluid from a typical experiment (patient No. 6, table 1a) are given in figure 1.

Sulphopeptide leukotrienes (0.46 ng·ml<sup>-1</sup> LTE<sub>4</sub>) were detected in only one of the asthmatics in the post-methacholine sample (No. 3, table 1a), and in none of the controls; HETEs were not detected at all.

Levels of LTB<sub>4</sub>, 20-OH-LTB<sub>4</sub> and 20-COOH-LTB<sub>4</sub> in the BAL fluid of patients before and after methacholine challenge are presented in figure 2. Baseline BAL in patients revealed detectable levels of LTB<sub>4</sub> in two, 20-OH-LTB<sub>4</sub> in five and 20-COOH-LTB<sub>4</sub> in two. After methacholine challenge, there was an increase in mean $\pm$ SD LTB<sub>4</sub> from 0.08 $\pm$ 0.15 to 0.41 $\pm$ 0.48 (NS), accompanied by an increase in 20-OH-LTB<sub>4</sub> from 0.31 $\pm$ 0.28 to 0.76 $\pm$ 0.62 ( $p < 0.05$ ) and 20-COOH-LTB<sub>4</sub> from 0.13 $\pm$ 0.23 to 0.38 $\pm$ 0.41 ng·ml<sup>-1</sup> (NS). The total sum of LTB<sub>4</sub> and metabolites showed a threefold increase after methacholine challenge ( $p < 0.05$ ) (table 3).

Table 2. - BAL cells without and 5 h after methacholine challenge

	Bronchial asthma		Control subjects	
	Without	5 h after	Without	5 h after
Recovery %	67 $\pm$ 11	68 $\pm$ 7	71 $\pm$ 5	68 $\pm$ 7
Cells $\times 10^4$ ·ml <sup>-1</sup>	8.2 $\pm$ 2.9	8.8 $\pm$ 3.7	9.9 $\pm$ 2.6	7.4 $\pm$ 3.9
Macrophages	86.1 $\pm$ 5.8	87.4 $\pm$ 8.6	89.0 $\pm$ 6.0	91.8 $\pm$ 4.5
Lymphocytes	8.0 $\pm$ 5.2	5.0 $\pm$ 4.3	5.8 $\pm$ 3.0	4.4 $\pm$ 4.0
Neutrophils	0.7 $\pm$ 0.9	0.3 $\pm$ 0.3	0.6 $\pm$ 0.6	0.6 $\pm$ 0.4
Eosinophils	1.1 $\pm$ 0.9*	1.3 $\pm$ 2.0	0.1 $\pm$ 0.1	0.2 $\pm$ 0.2
Mast cells	0.2 $\pm$ 0.3	0.1 $\pm$ 0.2	0	0.1 $\pm$ 0.2
Epithelial cells	3.7 $\pm$ 1.6	5.8 $\pm$ 3.9	4.3 $\pm$ 4.8	2.8 $\pm$ 0.9

Data are presented as mean $\pm$ SD. BAL: bronchoalveolar lavages; \*:  $p < 0.05$  versus pre-challenge % eosinophils in control.

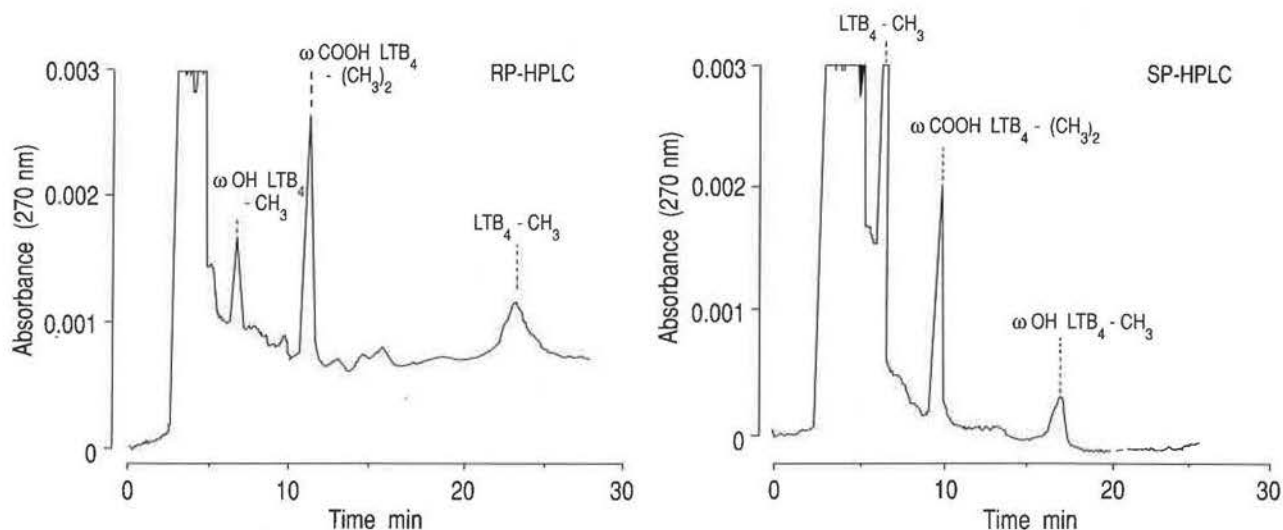


Fig. 1. - Analysis of leukotrienes extracted from the bronchoalveolar lavage fluid of patient No. 6 (table 1a) after methacholine challenge. In addition to the conventional RP-HPLC system,  $LTB_4$  and its omega-oxidation products ( $\omega$ OH- $LTB_4$  and  $\omega$ COOH- $LTB_4$ ) are separated as methylated derivatives isocratically (SP-HPLC) and with gradient-elution (RP-HPLC). RP-HPLC: reverse-phase high pressure liquid chromatography (HPLC); SP-HPLC: solid-phase HPLC;  $LTB_4$ : leukotriene  $B_4$ .

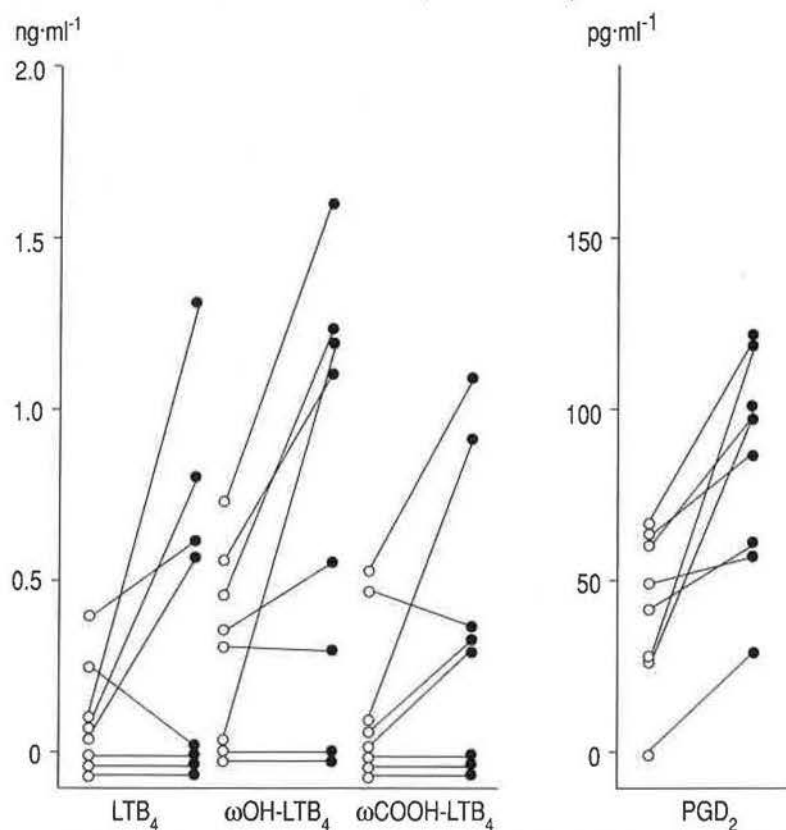


Fig. 2. - Bronchoalveolar lavage leukotriene  $B_4$  and its omega oxidation products ( $LTB_4$ ,  $\omega$ OH- $LTB_4$  and  $\omega$ COOH- $LTB_4$ ) and prostaglandin  $D_2$  ( $PGD_2$ ) levels in asthmatic patients without (open circles) and 5 h after methacholine challenge (closed circles).

In the control subjects, only three baseline BAL samples and three post-methacholine BAL samples contained detectable 20-OH- $LTB_4$  levels, with no significant difference between  $LTB_4$  and its metabolites before and after challenge (table 3).

With respect to baseline BAL samples, there was no significant difference in the content in  $LTB_4$  and its metabolites between patients and controls.

**Prostanoids.** In all asthmatic patients, there was a

significant increase in  $PGD_2$  from baseline to post-challenge values (fig. 2). No significant differences between baseline and post-challenge values were detected with respect to 6-keto  $PGF_{1\alpha}$ ,  $TXB_2$ ,  $PGF_{2\alpha}$  or  $PGE_2$  (table 3).

In the control subjects, no significant differences between baseline and post-challenge values were found with respect to any prostanoid mediator tested.



Table 3. – BAL mediators without and 5 h after methacholine challenge

	Bronchial asthma			Control subjects			
	Without	5 h after	P	Without	5 h after	P	
LTB <sub>4</sub> +20-OH-LTB <sub>4</sub> + 20-COOH-LTB <sub>4</sub>	ng·ml <sup>-1</sup>	0.52±0.50	1.55±1.32	<0.05	0.12±0.19	0.15±0.23	NS
6-keto-PGF <sub>1α</sub>	pg·ml <sup>-1</sup>	154.9±21.1	169.9±68.4	NS	147.1±51.0	144.3±39.9	NS
TXB <sub>2</sub>	pg·ml <sup>-1</sup>	197.9±68.7	227.5±70.5	NS	169.5±85.1	173.7±88.1	NS
PGF <sub>2α</sub>	pg·ml <sup>-1</sup>	20.0±14.8	28.6±16.6	NS	28.0±15.8	25.5±14.2	NS
PGE <sub>2</sub>	pg·ml <sup>-1</sup>	198.4±350.7	154.8±329.9	NS	181.3±298.2	200.3±394.1	NS
PGD <sub>2</sub>	pg·ml <sup>-1</sup>	49.1±15.7	94.4±25.4	<0.02	46.1±48.2	54.3±49.0	NS

Data are presented as mean±SD. LTB<sub>4</sub>: leukotriene B<sub>4</sub>; 20-OH-LTB<sub>4</sub> and 20-COOH-LTB<sub>4</sub>: omega-oxidation products of LTB<sub>4</sub>; PG: prostaglandin; TXB<sub>2</sub>: thromboxane B<sub>2</sub>; BAL: bronchoalveolar lavage; NS: nonsignificant.

### Discussion

In the present study, in patients with mild bronchial asthma, no inflammatory cell response within the BAL fluid was detectable 5 h after a methacholine inhalation challenge; however, there was a significant increase in LTB<sub>4</sub> and its metabolites and in PGD<sub>2</sub>.

Methacholine is a synthetic analogue of acetylcholine, which produces bronchoconstriction by direct stimulation of muscarinic cholinergic receptors on bronchial smooth muscle. Few investigators have addressed the question of whether methacholine may induce an inflammatory response in addition to its bronchoconstrictor ability. BAL has been collected 10 min [15, 16] and 1 h [17] after methacholine challenge, with no effect on the cellular pattern [17] and mediator profile [15, 16]. However, BEASLEY *et al.* [18] observed neutrophilic inflammation in the bronchial wall in asthmatic subjects 18 h after methacholine challenge. These findings were in line with experiments in guinea-pigs, where methacholine challenge [19, 20] or infusion [21], caused an influx of inflammatory cells into the airway lumen. We wondered whether this phenomenon might be relevant in man and whether it is accompanied by a change in the respective mediator profile. In the present study, we performed bronchoscopy 5 h after methacholine challenge, since an inflammatory cell reaction is expected to occur later than 1 h after an appropriate inhalation challenge.

Additionally, we confirmed several aspects of bronchoscopy and BAL in patients with mild bronchial asthma. Firstly, the procedure is safe and does not cause deterioration of lung function [22]. Secondly, a slight increase in eosinophils is the only consistent abnormality in the cellular BAL profile of asthmatic patients [23]. Thirdly, the presence of LTB<sub>4</sub> in BAL fluid of asthmatics is in accordance with previous observations [24–27]. In asthmatic subjects, WARDLAW *et al.* [26] found BAL LTB<sub>4</sub> to be higher than in healthy controls, whereas ZEHR *et al.* [27] found no difference between asthmatics and controls, and CREA *et al.* [28] found even higher values in normals. These authors, however, did not study the omega-metabolites of LTB<sub>4</sub>, which in our study were higher than LTB<sub>4</sub> itself in the majority of patients. The prostanoid concentrations detected in our baseline lavage samples are well within the range published [16, 29].

The interpretation of mediator concentrations in BAL fluid is always difficult, since the fluid might have different access to the lung in asthmatic and healthy subjects. However, upon the start of bronchoscopy, spirometric values were within ±5% of baseline values measured before methacholine challenge, and none of the patients had signs of hypersecretion or mucous pluggings. It is, therefore, likely that BAL fluid had a comparable access to the bronchoalveolar space of asthmatics and controls.

The number and composition of BAL cells were not altered 5 h after methacholine challenge in either the healthy volunteers or the asthmatic patients. The cholinergic agent, thus, clearly differs from allergen [1, 17], ozone [3, 30], and TDI [2, 31], all reported to cause substantial inflammatory cell influx. Therefore, the characteristic feature in our study was an increase in LTB<sub>4</sub> and its metabolites, and in PGD<sub>2</sub>, without a detectable change of any cell population. The following aspects may help to explain this finding.

1. *Quantity of mediator generation.* MARTIN *et al.* [32] instilled LTB<sub>4</sub> in the middle lobe of healthy volunteers, using quantities two to three orders of magnitude greater than those presently detected, and they observed a dramatic neutrophil influx into the bronchoalveolar lumen. EMERY *et al.* [33] found eosinophil accumulation up to 4 h after intratracheal instillation of PGD<sub>2</sub> in dogs [33]. Their concentration of PGD<sub>2</sub>, however, was fourfold greater than that observed in our subjects. Therefore, in the present study, the quantities of LTB<sub>4</sub> and PGD<sub>2</sub> released in response to methacholine challenge may be too low to induce significant cellular changes.

2. *Time course of mediator generation.* A 5 h interval between methacholine challenge and BAL analysis was chosen, since early asthmatic reactions are usually not explained by airway inflammation, and late asthmatic reactions occurring 4–7 h after the challenge [34] are associated with an inflammatory cell response in BAL fluid [23]. The 5 h interval would, thus, have been appropriate to detect significant cellular changes.

3. *Cellular origin and gradient of mediators.* The present study does not allow specification of the cellular origin



of LTB<sub>4</sub> and its metabolites and of PGD<sub>2</sub> detected in the BAL fluid. In addition to bronchial smooth muscle cells, endothelial [35] and epithelial cells [36], neutrophils [37], lymphocytes [38], mast cells [39], monocytes and macrophages [40] have been reported to possess muscarinic acetylcholine receptors. Among these, the macrophages have been established as predominant sources of LTB<sub>4</sub> generation [41, 42]. Such assignment of leukotriene generation to single cell types has, however, been questioned by the finding of considerable intercellular co-operation in lung arachidonic acid metabolism [43]. Neutrophils may thus be "indirectly" stimulated *via* interaction with methacholine-sensitive epithelial or mononuclear cells. The predominance of the omega-oxidation products of LTB<sub>4</sub>, indeed, strongly suggests involvement of granulocytes, which have the corresponding enzymatic equipment, whereas no significant omega-oxidation occurs in granulocyte-free lung tissue [11]. Depending on the cellular origin of methacholine-evoked LTB<sub>4</sub>, the climax of the gradient of this potent chemotactic agent may well be localized within the bronchial tissue, but not within the bronchoalveolar lumen. Interestingly, in the study of BEASLEY *et al.* [18], an increase in neutrophil numbers in bronchial biopsy specimens, but not in the lavage, was the only significant alteration in cellular profiles detected in asthmatics after methacholine challenge. Alternatively, in the asthmatic subjects, preactivated ("primed") granulocytes may have been stimulated by methacholine to release larger quantities of inflammatory lipid mediators, the number of inflammatory cells being unchanged. It remains open for discussion which of these mechanisms may underlie the present findings.

Prostaglandin D<sub>2</sub> is the major prostanoid metabolite produced by mast cells [44], and is a potent bronchoconstrictor [45]. The finding that BAL levels of PGD<sub>2</sub> are increased 5 h after methacholine challenge, but not 10 min after challenge [16], suggests that cellular, *e.g.* mast cell, activation might have occurred in the meantime. Allergen challenges have been demonstrated to cause a tenfold increase in PGD<sub>2</sub> if BAL is performed 5 min later [4]. In contrast to methacholine, allergen was also followed by an increase in TXB<sub>2</sub> and PGE<sub>2</sub> [4].

Our data do not allow us to distinguish whether the mediator response in BAL fluid is a direct effect of methacholine, or a consequence of the bronchoconstriction itself. The latter hypothesis could have been tested by inducing bronchoconstriction in the normal subjects similar to that seen in the asthmatic patients. In the normal subjects, however, the maximum methacholine concentration applied was 16 mg·ml<sup>-1</sup> which did not, according to the inclusion criteria, cause an obstructive airways response. Since this concentration did not induce a mediator response in the healthy controls, the increase in eicosanoid mediators cannot be directly attributed to the cholinergic agonist, regardless of the subjects' airway response.

In conclusion, the present study demonstrates that methacholine challenge provokes LTB<sub>4</sub> and PGD<sub>2</sub> generation in the BAL fluid of asthmatic patients, in addition to its established role as a bronchoconstricting agent. The absence of cellular changes in the post-methacholine BAL

samples suggests that the inflammatory response is too mild to question the role of methacholine as an appropriate and safe bronchoconstrictor for testing airway hyperresponsiveness in asthmatics. However, in studies where a methacholine challenge is performed prior to BAL, its effect on BAL mediator content has to be taken into account.

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