HYPERRESPONSIVENESS of the airways to nonspecific stimuli is a characteristic feature of asthma. Airway responsiveness is usually characterized in terms of the position and shape of the dose–response curve to methacholine (MDR). In the study we have investigated the influence of fluticasone propionate (FP), a topically active glucocorticoid, on arachidonic acid (AA) metabolites in broncho-alveolar lavage (BAL) fluid (i.e. TxB_2, PGE_2, PGD_2, $6kPGF_{1\alpha}$ and LTC_4) on the one hand and MDR curves on the other hand. The effect of FP was studied in a randomized, double-blind, placebo-controlled design in 33 stable non-smoking asthmatics; 16 patients received FP (500 µg b.i.d.) whereas 17 patients were treated with placebo. We found that the forced expiratory volume in 1 s (FEV_1 % predicted) increased, the $\log_2 PC_{20}$ methacholine increased and the plateau value (% fall in FEV_1) decreased after a 12 week treatment period. No changes in AA-metabolites could be determined after treatment except for PGD_2 which decreased nearly significantly ($p = 0.058$) within the FP treated group, whereas the change of PGD_2 differed significantly ($p = 0.05$) in the FP treated group from placebo. The levels of the other AA metabolites (i.e. TxB_2, PGE_2, $6kPGF_{1\alpha}$ and LTC_4) remained unchanged after treatment and were not significantly different from the placebo group. Our results support the hypothesis that although FP strongly influences the position, the shape and also the maximum response plateau of the MDR curve, this effect is not mainly achieved by influence on the level of AA metabolites. Other pro-inflammatory factors may be of more importance for the shape of the MDR curve. It is suggested that these pro-inflammatory factors are downregulated by FP.

Effects of fluticasone propionate on arachidonic acid metabolites in BAL-fluid and methacholine dose-response curves in non-smoking atopic asthmatics

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Introduction

Bronchial hyperresponsiveness (BHR), a prominent feature of asthma, can be demonstrated by generating dose–response curves through inhalation of histamine or methacholine. Usually these curves are sigmoid in shape, with a distinct threshold, a linear slope in the midpart and a maximum response.[1] The provocative concentration producing a fall of 20% in the FEV_1 (PC_{20}) is called the sensitivity, whereas the slope in the midpart is defined as reactivity. The plateau value of the curve reflects maximal airway narrowing.

Asthmatics not only show a leftward shift of the dose–response curve, but also higher or even unmeasurable plateau values as compared with normal subjects.[1] Activation of inflammatory

cells and release of mediators, such as arachidonic acid (AA) metabolites in bronchoalveolar lavage (BAL) fluid, may be present in asthma and may influence the shape of the methacholine dose–response (MDR) curve by enhancing BHR.[2–5]

Although it is known that anti-inflammatory therapy with inhaled corticosteroids (ICS) shifts the dose–response curve to the right[6] and reduces the maximum response,[7] data concerning the influence of inflammatory mediators such as AA metabolites are scarce.[4] One may expect that inhibition of their release by ICS may lead to a change in the shape of the dose–response curve. Therefore, in asthmatics, we tested the hypothesis that fluticasone propionate (FP), a new topically active ICS, downregulates AA

metabolites in BAL fluid on the one hand, and influences the different characteristics of the MDR curves on the other hand. We also investigated the relation between the various parameters studied.

Methods

Subjects: Thirty-three nonsmoking atopic asthmatics (23 male, median age 26 years, range 18 to 56 years) fulfilled the following criteria: PC_{20} histamine $\leq$ 8 mg/ml and $\geq$ 9% reversibility in forced expiratory volume in 1 s (FEV_1), relative to baseline, following inhalation of 1 000 µg terbutaline sulphate. Atopy was defined by at least one positive skin-prick test to a panel of 16 common aero-allergens in the presence of a positive histamine and negative control.

In the month preceding the run-in period, patients were only allowed to take inhaled short-acting β_2-agonists, on an as needed basis. All other medication was stopped. Patients with a history suggesting respiratory infection or exacerbation of asthma in the month prior to the study were excluded. All subjects gave informed written consent to the study, which was approved by the local ethics committee.

Study design: The study was of a randomized, double-blind, placebo-controlled design. After a run-in period of 2 weeks there was a 12 week treatment period. During the 2 week run-in period patients discontinued use of their usual inhaled bronchodilator which was replaced with salbutamol 400 µg as dry powder via the Diskhaler four times daily. Up to four additional doses were allowed as needed. Baseline data were obtained on two visits with an interval of 2 weeks. At each baseline visit that consisted of two morning or afternoon sessions, a flow volume curve was constructed, bronchodilator response was measured and a provocation test was carried out. At the second baseline visit, intradermal skin testing was performed. When patients fulfilled the mentioned criteria, bronchoalveolar lavage (BAL) was performed 1 week after the last baseline visit. Following the BAL, the patients were randomized to treatment with either inhaled 500 µg FP or placebo, both given twice daily as dry powder via the Diskhaler. Patients continued salbutamol 400 µg four times a day, but could take up to four additional doses as needed for symptomatic relief.

After 6 and 12 weeks of treatment patients attended the clinic on which occasion a MDR curve (see below) was performed. After 13 weeks, 1 week after the last dose–response curve was obtained, the BAL procedure was repeated.

Long function testing:

Inclusion measurements. FEV_1 was derived from a maximal expiratory flow volume curve, using a pneumotachometer (Jaeger, Würzburg, Germany). Reversibility was tested 20 min after four separate inhalations of 250 µg of terbutaline sulphate. A histamine provocation test was performed with a 2 min tidal breathing method[8] using a nose clip. Reference values of lung function measurements are according to Quanjer *et al*[9]

Study measurements. Methacholine was administered according to a standardized tidal breathing method.[10] Dose–response curves were obtained after inhalation of doubling concentrations of acetyl-β-methylcholine bromide (0.03–256 mg ml^{-1} in normal saline). Methacholine and not histamine was chosen as bronchoconstrictor stimulus during the study, because it produces less systemic side effects when given in high doses.[11] Solutions of methacholine were stored at 4°C and administered at room temperature. The aerosols were generated by a De Vilbiss 646 nebulizer (output 0.13 ml min^{-1}) and inhaled by tidal breathing for 2 min. The response to methacholine was measured as change in FEV_1 expressed as percentage of initial value and related to log_2 dose. A test was interrupted if the FEV_1 fell by more than 60%, or if unpleasant side effects or dyspnoea compelled the patient to stop.

A recently developed and validated sigmoid Cumulative Gaussian Distribution (CGD) function was fitted to the data.[12] Although the sensitivity (log_2PC_{20}) was obtained by linear interpolation of two successive log_2 concentration values,[8] the plateau value and the reactivity (defined as slope in the 50% point of the CGD function) were obtained as best fit parameters. Hence, reactivity denotes the percentual change from baseline FEV_1 per doubling dose (%/dd) in the steepest point of the CGD function. Details of the fit procedure and validation of the CGD fit are according to Aerts *et al*[12]

Bronchoalveolar lavage: Fibre-optic bronchoscopy was performed according to guidelines of the American Thoracic Society.[13] After premedication with inhaled terbutaline and atropine i.m., the bronchoscope (Olympus B1 IT 10, Tokyo, Japan) was introduced into the lateral segment of the middle lobe under local anaesthesia and placed in wedge position. BAL was performed with four 50-ml aliquots of sterile phosphate buffered saline (PBS) warmed to 37°C. The fluid was then immediately aspir-

ated by gently suctioning with –40 cm H_2O into a siliconized specimen trap placed on melting ice and transported to the laboratory for processing and analysis.

The BAL fluid was centrifuged at 400 × g for 5 min at 4°C. The supernatants were decanted and stored. The cell pellets were then washed in PBS supplemented with 0.5% heat-inactivated bovine serum albumin (BSA). For total leukocyte numbers in BAL fluid, cell suspensions were counted in a Coulter Counter and viability was assessed by cellular exclusion of trypan blue. Cytospin preparations were stained with May–Grünwald–Giemsa stain, and the differential counts were performed by counting at least 500 cells.

Determination of AA metabolites: Immediately after the BAL procedure, 20 ml of supernatant was processed on C18 SepPak cartridges (Millipore, Bedford, USA) as described previously,[14] eluated with 2.5 ml methanol and stored at –80°C until analysis. Samples of 200 µl BAL eluted fluid were pipetted into polypropylene tubes and dried with a Savant sample concentrator. After dissolving in 300 µl assay buffer, levels of thromboxane B_2 (TxB_2) were determined by means of a [³H] RIA with antisera from Advanced Magnetics Inc. (Cambridge, MA) and [³H] labelled compounds from Amersham International (Buckinghamshire, UK). Levels of prostaglandin PGD_2 and PGE_2 were determined with commercially available [³H] kits (Amersham, UK) and 6kPGF$_{1\alpha}$ with a [¹²⁵I] RIA kit (Du Pont de Nemours, Dreieich, Germany), according to the manufacturer's instructions. Leukotriene $C_4/D_4/E_4$ (LTC_4) was measured at room temperature in a microtitre enzyme immunoassay according to protocol (Biotrak, Amersham, UK).

Statistical analysis: The paired *t*-test was used to analyse within-treatment changes in FEV_1 and bronchial hyperresponsiveness. The unpaired *t*-test was used for comparisons between groups. In case of non-normally distributed variables non-parametric tests were used (paired and unpaired Wilcoxon test). A *p*-value smaller than 0.05 was supposed to indicate statistical significance. Spearman–Rank correlations (r_s) were used for testing intercorrelation between outcome variables within the groups. Values of $|r_s| > 0.6$ were considered relevant only if they reached a significance level ($p < 0.01$). Group means and standard error of the mean ($\pm$ S.E.M.) at the various time points were calculated.

Results

Sixteen patients (12 men) were randomized into the FP group and 17 patients (11 men) into the placebo group. Baseline values such as FEV_1, reversibility and PC_{20} histamine were not significantly different between the groups on entry to the study (Table 1). Thirty-one of the 33 subjects completed the study. One patient receiving placebo and one receiving FP were withdrawn after experiencing a pulmonary exacerbation. Data of these two patients have not been included in the analysis.

Mean values for FEV_1 (as % predicted), sensitivity (log_2PC_{20}methacholine), reactivity (%/dd) and plateau (% fall in FEV_1) before and after treatment are shown in Table 2. No statistical significant differences were present between the indices before treatment. In the FP group significant changes occurred after 12 weeks with respect to means of PC_{20} (an increase of 3.6 doubling doses), plateau value (from 58.8% at randomization to 36.5% fall in FEV_1) and baseline FEV_1 (from 82.9% to 91.0%) in contrast to the placebo group. Changes for reactivity were less marked.

Mean values for AA metabolites and total cell recovery before and after treatment are presented in Table 3. Values were comparable in both treatment groups on entry to the study. Significant results were found only for PGD_2; it decreased nearly significantly in the FP group from 25.8 (S.E.M. $\pm$ 10.9) to 7.3 (S.E.M. $\pm$ 1.8)($p = 0.059$), whereas its change in the FP group differed significantly from that in the placebo group ($p = 0.05$) after 12 weeks of treatment.

We also determined if there were correlations

Table 1. Baseline characteristics of the study patients

Characteristics	Fluticasone	Placebo
n	16	17
Sex M/F	12/4	11/6
Age (years)	28.4 (10.8)	34.5 (13.5)
FEV_1 (% predicted)	84.2 (15.4)	86.0 (17.6)
Reversibility*	15.5 (7.8)	18.1 (14.3)
$PC_{20}H^\dagger$ mg/ml	0.71 (0.59)	0.88 (0.87)
$log_2PC_{20}H^\dagger$	–1.12 (1.62)	–0.96 (1.70)
Plateau value‡	58.8 (19.8)	50.8 (12.7)
Reactivity (%/doubling dose)	11.6 (5.3)	9.4 (3.7)
Total IgE (IU/ml)	311 (288)	299 (289)

All values are expressed as mean $\pm$ standard deviation (between brackets).
*Reversibility: change in FEV_1 expressed as % baseline, after 1 000 µg terbutaline sulphate.
$^\dagger PC_{20}H = PC_{20}$ histamine; $log_2PC_{20}H$ - log_2PC_{20} histamine.
‡Plateau value expressed as % fall in FEV_1.
No statistical significant differences were present between the treatment groups.

Table 2. MDR-curve indices of the study patients

Lung function parameters	Fluticasone		Placebo	
	Before treatment	After 12 weeks	Before treatment	After 12 weeks
FEV_1 (% predicted)	82.9 (4.1)	91.0 (3.8)*,**	82.9 (4.2)	80.1 (5.1)
$\log_2 PC_{20}M^\dagger$	0.18 (0.56)	3.77 (0.72)*,**	-0.05 (0.74)	0.26 (0.46)
reactivity (%/doubling dose)	11.6 (1.3)	9.0 (1.8)	9.4 (0.9)	10.8 (1.0)
plateau (% fall in FEV_1)	58.8 (4.9)	36.5 (4.1)*,**	50.8 (3.2)	48.8 (3.6)

All values are expressed as mean ± S.E.M. (between brackets).
$^\dagger\log_2 PC_{20}M = \log_2 PC_{20}$ methacholine.
No statistical significant differences were present between the indices before treatment. Significance of changes during treatment are indicated (*$p < 0.05$). Also significant differences in changes between the treatment groups are indicated (**$p < 0.01$).

Table 3. AA metabolites and cell levels in BAL fluid

AA metabolites	Fluticasone		Placebo	
	Before treatment	After 12 weeks	Before treatment	After 12 weeks
TxB_2	34.1 (4.2)	36.1 (4.2)	32.2 (4.8)	29.2 (3.8)
PGE_2	11.3 (0.6)	10.2 (0.8)	11.3 (0.6)	11.3 (0.9)
PGD_2	25.8 (10.9)	7.3 (1.8)	15.4 (4.0)	17.9 (4.5)
$6kPGF_{1\alpha}$	4.0 (0.4)	3.2 (0.2)	4.2 (0.4)	3.3 (0.5)
LTC_4	12.1 (3.8)	6.7 (2.0)	11.6 (4.1)	12.1 (3.0)
Total cell recovery $\times 10^6$	10.8 (0.08)	15.2 (0.11)	12.4 (0.09)	11.4 (0.06)

All values are expressed as mean ± S.E.M. (between brackets).
AA = arachidonic acid; TXB_2 = thromboxane B_2; PGE_2 = prostaglandin E_2; PGD_2 = prostaglandin D_2; $6kPGF_{1\alpha}$ = 6 keto-prostaglandin $F_{1\alpha}$; LTC_4 = leukotriene C_4. The decrease in PGD_2 within the fluticasone group was nearly significant ($p = 0.058$). With respect to the difference in changes between both groups only the change in PGD_2 differed significantly ($p = 0.05$) between the fluticasone and the placebo group.

between changes in either treatment group and investigated whether these correlations were different between the treatment groups. In neither of the treatment groups, however, relevant and significant correlations were found between the parameters investigated.

Discussion

We showed that after 12 weeks of treatment fluticasone propionate (FP) significantly decreased both sensitivity to methacholine and the maximal airway narrowing response, whereas it substantially decreased PGD_2 levels in BAL fluid. The change in PGD_2 level after treatment with FP was significantly larger than the change in the placebo group. We were unable, however, to demonstrate a correlation between these changes in sensitivity and plateau level with the change in PGD_2 or one of the other arachidonic acid (AA) metabolites.

To date, several studies have described the relation between airway inflammation, the subsequent release of AA metabolites and bronchial responsiveness. It has to be kept in mind, however, that the bronchial responsiveness in those studies has never been measured by the entire methacholine dose–response (MDR) curve. Bronchial responsiveness as determined

by the MDR curve is defined as the sensitivity of the airways to a wide variety of nonsensitizing bronchoconstricting stimuli. It has been demonstrated that the curves from asthmatics could be differentiatied from those of normal subjects by their position, slope and maximal response.[1] MDR curves in asthma have a steeper slope and a higher maximal response at high doses of methacholine as compared to normal subjects.[1] A leftward shift of the curve can be regarded as being the result of any augmentation of airway narrowing stimuli (i.e. prejunctional mechanisms) such as activation of inflammatory cells and release of mediators such as AA metabolites.[15-17] An upward movement of the plateau is the result of an increase in the response of the effector organ (i.e. postjunctional mechanisms) such as smooth muscle contraction and swelling of the airway wall. To our knowledge this is the first study that investigated the possible correlation of sensitivity, reactivity and plateau value of the MDR curve on the one hand and AA metabolites in BAL fluid on the other hand. However, as mentioned above, we could not demonstrate such a correlation, nor could we find a significant correlation between the FP-induced decrease in BHR and the levels of AA metabolites in BAL fluid. This suggests that in addition to AA metabolites other factors, such as epithelial damage, numbers

of eosinophils and neutrophils in BAL fluid, platelet activating factor, histamine and major basic protein, are important as was shown by other authors.[18-22] Our findings are in contrast with those of Oosterhoff *et al.* who demonstrated that the levels of PGD_2 in BAL fluid are inversely correlated with the PC_{20} histamine. A possible explanation for this observed discrepancy could be the difference in treatment period; they found a correlation between AA metabolite levels and PC_{20} histamine after long-term treatment (2.5 years) with ICS.

Prostaglandins D_2 and $F_{2\alpha}$, thromboxane B_2, and leukotrienes B_4, C_4 and D_4 are potent pro-inflammatory mediators with a wide variety of biological activities, including smooth muscle contraction, mucus hypersecretion and leukocyte activation.[23-25] In a previous study we demonstrated that the subclinical inflammation in smokers was associated with higher levels of $PGF_{2\alpha}$ and TxB_2 in BAL fluid as compared to non-smokers.[14] It is conceivable that the increased amounts of $PGF_{2\alpha}$ and TxB_2 are due to activation of alveolar macrophages in the airways of smokers. $PGF_{2\alpha}$ and TxB_2 induce airway secretion, constrict isolated human airways and increase the sensitivity to contractile stimuli.[26] In a study of Wardlaw *et al.* levels of leukotrienes (LTC_4 and LTB_4) were higher in BAL fluid of symptomatic asthmatics with bronchial hyperresponsiveness as compared to asymptomatics.[27] Although their methodology to measure AA metabolites differed from ours, which makes comparison of the results rather difficult, the levels of LTC_4 in our study appeared to be equal to those of the asymptomatics in their study. The fact that these levels were already low at the start of the study may explain why we were unable to demonstrate an effect of treatment.

Other workers carried out allergen challenge and measured BAL fluid and urinary levels of LTE_4, the end product of enzymatically converted LTC_4 and LTD_4. It was found in asthmatics that the basal levels of leukotrienes were not elevated, but increased *in vivo* after allergen challenge.[28-31] Christie *et al.* showed that children with atopic asthma who were resident at high altitude, exhibit a fall in FEV_1 and an increase in airway responsiveness to histamine upon visiting regions at sea level. This was associated with a three-fold increase in urinary LTE_4 excretion.[32] Thus it seems that in asymptomatic asthma patients or asthmatics with minor symptoms, the levels of leukotrienes in BAL fluid are not increased. Upon stimulation (tobacco smoke, allergen challenge or visit to sea level), however, leukotriene levels rapidly increase resulting in bronchial hyper-responsiveness.

In support of this hypothesis, Bel *et al.* demonstrated that inhaled LTD_4 not only caused a higher maximal response plateau than methacholine, but also increased the maximal response to methacholine for at least 3 days. These findings could be prevented by the administration of inhaled steroids.[33]

Tamaoki *et al.*[34] found that prednisone reduced the synthesis of eicosanoids by stimulated macrophage-rich BAL-fluid cells *in vitro*. However, Dworski *et al.* showed that oral prednisone reduced symptoms but had no significant effect on BAL fluid eicosanoid levels *in vivo*.[35] In line with this study, we also failed to find a reduction in AA metabolites in BAL fluid. Only PGD_2 appeared to be nearly significantly lower ($p = 0.058$) in FP treated subjects, whereas the change in PGD_2 before and after 12 weeks of treatment with FP was significantly larger ($p = 0.05$). It has been demonstrated in canine airway smooth muscle that PGD_2 prejunctionally augments the parasympathetic contractile response.[34] Stimulation of asthmatics with PGD_2 significantly increased the reactivity to histamine and methacholine.[2] It was suggested that enhanced cholinergic tone underlied these findings. Beasley *et al.* demonstrated that PGD_2 and its metabolite $9\alpha,11\beta$-PGF_2 caused a marked increase of methacholine induced bronchoconstriction that could only partially be prevented by an anticholinergic.[23] In addition, several studies showed that allergen challenge resulted in a marked increase in prostaglandin levels in BAL fluid.[36,37] This suggests that PGD_2 may augment the histamine or methacholine induced hyper-responsiveness. The effects of prostaglandins on reactivity or plateau value is unknown, because none of the above mentioned authors investigated the entire MDR curve and a possible relation of prostaglandins with reactivity or plateau value.

In our study PGD_2 levels in BAL fluid of asthmatics substantially decreased after treatment with FP. Since PGD_2 is a product derived from mast cells and to a lesser extent from alveolar macrophages,[38,39] it may be concluded from our results that ICS, particularly, downregulate mast cells to produce PGD_2 although the influence on alveolar macrophages is not excluded.

In conclusion, in our study we demonstrated that BHR as determined by the MDR curve is downregulated by FP. Although FP strongly influences the position, the shape and also the maximum response plateau of the MDR curve, it did not influence levels of AA metabolites in BAL fluid except for PGD_2. Our results indicate that the effect of FP on BHR is not achieved mainly by its influence on the level of the AA metabo-

lites. It is suggested that other pro-inflammatory factors are of more importance for the shape of the MDR curve.

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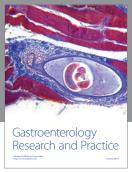22. Oosterhoff Y, Overbeek SE, Douma R, Noordhoek JA, Postma DS, Hoogsteden HC, Zijlstra FJ. Lower leukotriene C_4 levels in bronchoalveolar lavage fluid of asthmatic subjects after 2.5 years of inhaled corticosteroid therapy. *Mediators of Inflammation* 1995; **4**: 426–430.
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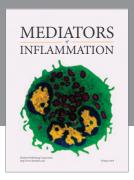31. Wenzel SE, Larsen GL, Johnston K, Voelkel NF, Westcott JY. Elevated levels of leukotriene C_4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge. *Am Rev Respir Dis* 1990; **142**: 112–119.
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