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Effect of a 4-week treatment with theophylline on sputum eosinophilia and sputum eosinophil chemotactic activity in steroid-naive asthmatics

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Abstract:

Background The precise mechanism of action of theophylline in asthma is not fully understood but recent data have drawn attention to its potential anti-inflammatory effect.

Objective The purpose of this study was to assess the effect of theophylline on sputum eosinophilia and sputum eosinophil chemotactic activity in steroid-naive asthmatics.

Method We performed a 4-week randomized double-blind, placebo-controlled, parallel group study in 21 mild to moderate steroid-naive asthmatics whose sputum eosinophilia was found twice > 5% during the run in period. Eleven subjects received 600 mg/24h theophylline for the first 2 weeks and 900mg/24h for the last 2 weeks while 10 subjects took a placebo for 4 weeks. Sputum was induced after 2 and 4 weeks of treatment and 1 week after stopping the treatment. The sputum samples were compared for their cell counts, eosinophil cationic protein (ECP) levels and eosinophil chemotactic activity using micro-Boyden chambers.

Results Serum theophylline concentrations reached 7 and 11 μg/mL at V3 and V4, respectively. Intragroup comparisons showed that theophylline, but not placebo, caused a significant reduction in sputum eosinophil counts at V3 (62 ± 10% from baseline, $P < 0.01$) and a strong trend at V4 (67 ± 16% from baseline, $P = 0.07$) when compared to baseline. The intergroup difference obtained after comparing the area under the curve over the 4 week treatment period only approached the statistical significance ($P = 0.08$). At baseline the fluid phase of the sputum contained a significant eosinophil chemotactic activity which was inhibited after a 4-week treatment by theophylline ($P< 0.01$) but not by placebo. The mean sputum theophylline levels after 4 weeks of treament (1.7µg/mL) was lower than that required to cause significant inhibition of eosinophil chemotaxis *in vitro.*

Conclusion Theophylline decreases the natural sputum eosinophil chemotactic activity present in asthmatics. However, when using a small sample size, the 35% reduction in sputum eosinophilia achieved by theophylline failed to reach statistical significance when compared to that seen after placebo.

Keywords*:* Asthma, theophylline, sputum, eosinophils, chemotaxis.

Introduction

Theophylline is one of the most widely used drugs in the therapy of bronchial asthma and chronic obstructive pulmonary disease [1]. The therapeutic activity of theophylline has been classically related to its bronchodilator effect [2] and to its ability to improve diaphragmatic contractility [3]. It is now being increasingly recognized that theophylline is able to suppress *in vitro* several functions of inflammatory cells including lymphocytes and eosinophils [4].

Persistent airways inflammation is a classical feature of asthma [5]. Eosinophils are considered to play an important role in the pathogenesis of this disease since both their number and their state of activation have been repeatedly found to be increased in the airways of asthmatics. In addition eosinophils release a variety of toxic and inflammatory mediators which are thought to contribute to bronchial epithelial damage, airway wall oedema and bronchoconstric-tion seen in asthmatic airways [6].

Studies looking at the anti-inflammatory effect of theophylline *in vivo* in asthma are still sparse [7]. In the model of allergenic bronchial challenge theophylline was reported to attenuate the late phase reaction, its associated bronchial hyperreactivity [8-10] and local recruitment and activation of lymphocytes and eosinophils [11,12]. Further evidence for an anti-inflammatory role of theophylline in asthma came from studies showing that

withdrawal from a regular treatment with theophylline resulted in an increase in CD4 and CD8 lymphocytes within the bronchial mucosa [13] while giving theophylline to naive patients caused a reduction in the number of CD8 lymphocytes within the bronchial biopsies [14].

The technique of induced sputum has recently been developed to investigate airway inflammation in asthmatics [15,16]. Because of being non-invasive, relatively safe and reproducible, this technique is now considered to be suitable to perform serial assessment of airway inflammation in asthmatics [17]. Not only can the sputum analysis be useful to monitor airway inflammation but it also provides the opportunity to get insight into the pathophysiological mechanisms leading to airway eosinophilia. For instance we have previously shown that the fluid phase of sputum from asthmatics, but not that from healthy subjects, contained an eosinophil chemotactic activity [18].

The purpose of this study was to see whether oral theophylline can affect the extent of sputum eosinophilia and the sputum eosinophil chemotactic activity in mild to moderate steroid naive asthmatics selected on the basis of a naturally occurring sputum eosinophil count >5%.

Methods

Subjects

Patients with a clinical history of asthma (intermittent or mild to moderate) were recruited by advertisement from the hospital staff members as well from the outpatient clinic. They were taking no drug except inhaled short-acting β2-agonists occasionally and had had no clinical history of upper airway respiratory infection over the past 4 weeks. A total of 62 patients volunteered for the study and were screened for their percentage of sputum eosinophils. Twenty-seven patients out of the 62 had sputum eosinophils > 5% at the screening visit. Four out of the 27 patients were subsequently excluded during the Run-in period because of a sputum eosinophilia < 5% at V1 or V2. Finally 23 patients were randomized and only 21 subjects completed the study protocol. These were eight males and three females whose mean (range) age was 26 years (20-45) in the theophylline group and seven males and three females whose age was 23 years (18-32) in the placebo group. Mean ±SEM baseline FEV_1 were 98±5% predicted and 96±5% predicted while geometric mean (range) PC20 methacholine were 0.78 mg/ mL (0.07-16) and 0.41mg/mL (0.2-2.4) in the theophylline and the placebo group, respectively.

Study design

This was a randomized double-blind parallel study, comparing the effect of a sustained-release oral theophylline (Unair R 300 mg/capsule, 3 M Health Care Ltd, Loughborough, UK) vs placebo on sputum cytology in asthmatics. The design planned five visits at the hospital for sputum induction, bronchial methacholine challenge (PC20M) and blood sampling (serum theophylline) (Fig. 1). There were two run-in visits separated by 7-9 days (V1, V2), two visits after 14 and 28 days of treatment (V3,V4), and finally one run-out visit 7-9 days after stopping the medication (V5). At V1 a blood sample was taken and a sputum induction was performed in the morning. Six hours later the subjects underwent a methacholine bronchial challenge. At V2 a second sputum induction was performed and the patients were randomized to Unair or placebo if the sputum eosino-philia was found to be>5% at V1 and V2. They were instructed to take the caps once a day at night for one month. The daily dose was two capsules (600 mg Unair or placebo) for the first 2 weeks and 3 capsules (900 mg Unair or placebo) for the next 2 weeks. At V3, V4 and V5, the patients had a sputum induction and a blood sampling while a methacholine bronchial challenge was performed at V4 and V5. The volunteers were asked to come in the morning, at the same hour of the day for each visit, and to refrain from smoking, drinking coffee and taking β2-agonists during the 10h preceding the visit. The subjects were included in the study only after having been thoroughly informed about the goal and the protocol of the study and having given written consent. The protocol of the study was approved by the Ethics Committee of the Liège Medical School.

Sputum induction

The subjects were premedicated with 400 µg inhaled salbuta-mol. Hypertonic saline (4.5%) was aerosolized by ultrasonic nebulizer (Devilbiss, PA, USA), with output set at 1.5 mL/ min. The subjects wore a nose clip and quietly inhaled aerosol through a mouth piece for up to four 5-min periods. After each inhalation, the subjects rinsed their mouth with water and dried it with tissue paper to minimize contamination with saliva. Then they coughed up sputum into a Petri dish which was immediately placed on ice until processing. Peak expiratory flow rate was measured after each 5 min inhalation period (Mini-Wright) and if>250L/min, the challenge was continued. After challenge, the subjects were supervised for at least 1 h and PEF monitored regularly.

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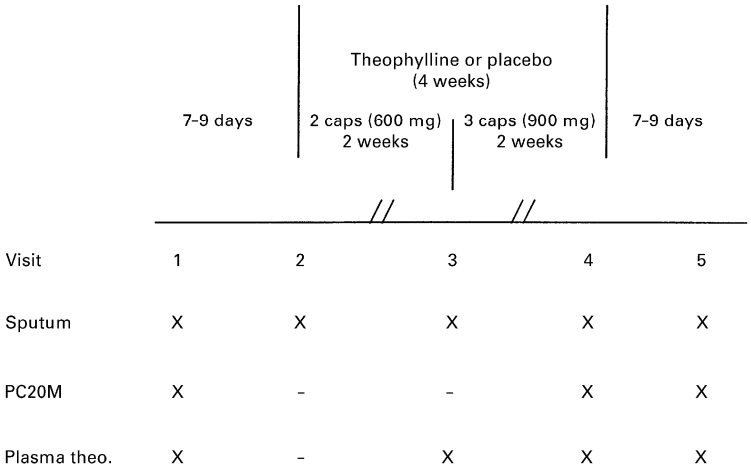
Fig. 1. *Scheme of the study*



Sputum processing

The sputum was processed as previously described [18]. The whole sputum was transferred into 50-mL polypropylene tubes (Becton Dickinson, Abingdon, UK), weighed and an equal weight of 0.01M dithioerythritol (DTE, Flluka, Gillingham, Dorset, UK) solution added as a mucolytic.

This was vortexed for 10 s, rocked for 30min at room temperature, and again vortexed for 10 s. The samples were then filtered through a 70-µm strainer (Becton Dickinson) and the collected fluid centrifuged at 400*g* for 10 min at 4 °C. The supernatants were removed and stored at -20 °C. The cell pellets were resuspended in 1 mL of PBS without Ca + + and Mg + + and cells counted in a manual haemo-cytometer. The squamous cell count was determined under the haemocytometer. It averaged 12.9 ± 10% (mean ± SD) and ranged from 0 to 52% for all the samples collected in this study (*n*=105). The differential cell count was performed on cytospins stained with Diff Quick° by one technician blind to the protocol after counting 600 cells (excluding squamous).

ECP and theophylline immunoassays

ECP was measured with the UNICAP system (Pharmacia, Uppsala, Sweden) with a sensitivity of 2 ng/mL according to the manufacturer's instructions. To ensure that ECP recovery was adequate in the sputum samples treated with DTE a curve for standard concentrations of ECP (1-100 ng/mL) was performed in a pool of sputum samples. The added ECP concentrations to sputum were 1, 2.5, 7.5, 50 and 100 ng/mL and the recovery reached 4.4, 6, 11.3, 49.3 and 97.3 ng/mL, respectively. Serum and sputum theophylline concentrations were measured by fluorescence polarization immunoassay (FPIA-Abbott) with a sensitivity of 0.8 μg/mL [19]. Sputum recovery of theophylline was satisfactory since spiking a pool of naive samples with 2.83 μg/mL and 17.93 μg/mL gave measured concentrations of 2.17μg/mL and 17.87μg/mL, respectively.

Methacholine bronchial challenges

Methacholine chloride (Biochemicals) solutions were prepared in saline, stored at 4 °C and used within 14 days after preparation. Bronchial responsiveness was assessed according to the method described by Cockroft *et al.* [20] and using a compressed air nebulizer, the characteristics of which were described previously [21]. Provided forced expiratory volume in one second (FEV_1) did not fall by more than 10% of baseline after saline, doubling concentrations of methacholine (starting at 0.03 mg/mL and reaching maximally 16mg/mL) were inhaled every 5 min until a 20% fall in FEV_1 had occurred. FEV_1 was measured 30 s after each concentration and the best of three curves was recorded (Flowscreen Jaeger). The provocative concentration that produced a 20% fall in FEV_1 (PC20) was read from the log dose-response curve by linear interpolation.

Eosinophil chemotaxis

Chemotaxis assays for eosinophils were performed using micro-Boyden chambers using >99% pure eosinophils obtained from peripheral blood of the same non treated atopic donor by immunomagnetic cell separation according to Hatzelmann and coworkers [22]. Experiments were performed in triplicate in a 48-well microchemotaxis Boyden chamber incubated in 5% $C0_2$ at 37 °C for 60min. Aliquots of 25 μL of sputum fluid were placed in the lower chambers and 50μL of eosinophil suspension (10^6 cells/mL) were placed in the upper chambers. In experiments investigating the effect of theophylline *in vitro* on sputum eosinophil chemotactic activity the drug was placed both in the lower and the upper chambers. The two chambers were separated by a polyvinyl-pyrrolidone (PVP)-free filter (Nucleopore polycarbamate PC, Costar, High Wycombe, UK). The controls consisted of a solution of Hank's balanced salt solution (HBSS) with Ca + + and Mg + + (PH 7.4) and containing 20mM HEPES and 0.2% BSA. The filters were fixed in methanol and stained with Diff-Quick. Migrated cells adherent to the lower surface were counted in 10 fields in each well at a × 600 magnification. The results were expressed as the number of eosinophils having migrated/ l0hpf.

Statistical analysis

Results were expressed as mean ± SEM except for sputum ECP levels and PC20M which were expressed as geometric mean (range). The baseline values for sputum cell counts (total and differential) were computed for each patient by taking the mean of the first two visits. The sputum variables were log transformed before being subjected to statistical analyses. A generalized linear mixed model (GLMM) for repeated measures (ANOVA) was applied for each variable. In each model, the effects of treatment, time and their interaction were studied. The variations in sputum eosinophilia were further analysed by expressing the change as a percentage of baseline and calculating the area under the curve (AUC) over the 4-week treatment period. Comparison between theophylline and placebo was then performed by an unpaired Student's *t*-test on the AUC values. Variations in FEV_1, PEFR and PC20M within the groups were analysed by a repeated measure ANOVA. Intergroup comparisons were performed by unpaired *t*-test. Correlations between change in functional parameters and change in sputum eosinophilia were performed by calculating the Spearman coefficient of correlation. Changes in sputum eosinophil chemotactic activity between V2 and V4 were assessed by a paired Student's *t*-test. Similarly a paired *t*-test was performed to assess the antichemotaxis effect of theophylline *in vitro.* Statistical calculations were carried out using the SAS package (SAS Institute Inc., Cary, North Carolina, 1996, USA). All results were considered to be significant at the 5% critical level (P<0.05).

Results

(1) Compliance and tolerance to the treatment

Initially 23 subjects were included in the study (11 placebo, 12 theophylline). There were two dropouts (one in each group) for non compliance to the protocol (no attendance of the scheduled visit). As expected, theophylline was undetectable in all the samples taken from the 10 volunteers receiving placebo while mean serum theophylline reached 7.3 ± 1.1μg/mL at V3 and 10.6 ± 1.6μg/mL at V4 in the subjects treated with Unair®. At V5 theophylline was no longer detectable in the serum of patients except in two subjects whose levels were 7 and 13μg/mL, respectively. Unair® was well-tolerated since only two patients reported some nervousness and insomnia.

(2) Effect of theophylline vs placebo on sputum parameters

The weight of sputum samples and the total and differential sputum cell counts were not significantly different between the two groups at V1 and V2 (Table 1). The squamous cell counts were lower in the placebo group at V2 (P<0.05).

In the placebo group, intragroup comparisons failed to reveal any significant change in total and differential cell counts at V3, V4 and V5 by comparison to the mean values of V1 and V2 (Table 1). In contrast, asthmatics treated by theophylline (Unair®) displayed a significant decrease in their sputum eosinophil counts at V3 (P<0.01) and V4 (P<0.01) when compared to baseline (Table 1). Intergroup comparisons showed a trend for lower sputum eosinophil counts at V3 and V4 in the theophylline group (P = 0.09 and P=0.11, respectively). There was no significant change regarding the other cell types (Table 1).

When the variations in sputum eosinophils were expressed as percent of baseline, theophylline, but not placebo, caused a significant decrease in sputum eosinophils at V3 (62 ± 10% of baseline, P<0.01) and a trend at V4 (67

± 16% of baseline, $P = 0.07$) (Fig. 2). Intergroup comparison by analysing the AUC for the 4 week treatment period showed a trend towards a greater reduction in sputum eosinophils in the theophylline group (1337 ± 229 U vs 1991 ± 274 U, $P = 0.08$). One week after stopping the treatment (V5), the change in sputum eosinophils in the theophylline group was no longer significant (72 ± 13% of baseline, $P>0.05$). This was even more obvious after excluding the two patients in whom theophylline was still detectable in the serum at V5 (80 ± 15% of baseline, $P>0.05$).

Sputum ECP levels were not significantly different between the two groups at V1 and V2 (Table 1). Both intragroup and intergroup comparisons showed that asthmatics treated with theophylline had no significant change in their sputum ECP levels at V3, V4 and V5 (Table 1) even if there was a trend for ECP levels to be lower in the theophylline group than in the placebo group at V4 ($P = 0.13$).

Table 1. *Effect of theophylline vs placebo on sputum cell counts and sputum ECP levels*

	Baseline		**Treatment**		**Run-out**
	V1	**V2**	**V3**	**V4**	**V5**
Placebo (n=10)					
Weight (g)	6.28 ± 1.3	6.21 ± 1.3	5.82 ±1.3	5.68 ±1.4	6.08 ± 1
Squamous (%)	8.4 ±1.6	9.4 ±1.7	9.4 ± 2.9	12.3 ±3.9	7.9 ±2
Total non squamous (10^6/g)	1.86 ±0.41	1.29 ±0.41	1.67 ±0.39	2.38 ±1.07	2.13 ±0.63
Macrophages (%)	40.8 ± 7.2	42 ± 8.2	38 ±7.2	42.2 ± 8.1	41.6 ±8.9
Lymphocytes (%)	0.6 ± 0.2	0.8 ± 0.2	1.1 ±0.4	0.6 ± 0.2	0.5 ±0.1
Neutrophils (%)	25.7 ± 7.4	23.8 ± 8.9	22.9 ± 8	23.8 ± 8.8	26.4 ± 9.9
Eosinophils (%)	23.7 ± 5.6	24.4 ± 5	25.1 ±6.7	25 ± 6.6	21 ±6
Epithelial cells (%)	8.9 ± 2.5	7.8 ±2	11.6 ±2.9	8.3 ±1.9	9.6 ±3.6
ECP (ng/ml)	81 (110-665)	51 (6-731)	59(11-1380)	107 (12-2000)	50(11-1130)
Theophylline (n=11)					
Weight (g)	5.9 ±1.2	5 ±0.4	6.3 ± 0.7	6.7 ±1	6 ±0.8
Squamous (%)	15.1 ±4.1	15.4 ±2	14.6 ±2	18.2 ±3	17.4 ± 2.6
Total non squamous (10^6/g)	2.27 ± 13.4	11.5 ±2.7	9.8 ±2.3	11.3 ±2.8	10.9 ± 2.3
Macrophages (%)	45.2 ± 5.8	48 ± 4.8	52.2 ± 5.9	54.5 ± 4.4	55.7 ± 4.8
Lymphocytes (%)	1.2 ±0.5	0.4 ±0.1	0.4 ±0.1	1 ±0.3	0.3 ±0.1
Neutrophils (%)	21 ±4.5	19 ± 4.7	15.7 ±4	16.3 ±3.5	17.5 ± 4.3
Eosinophils (%)	20.2 ± 3.7	20.3 ± 4.3	12.7±4.2**†	14.5 ±3.9**	13.7 ±3.3*
Epithelial cells (%)	12.3 ± 3.7	12 ±3.3	18.7 ±4.2	13.7 ±3.3	12.7 ± 3
ECP (ng/ml)	38 (6-200)	35 (6-246)	41 (2-488)	24 (9-143)	30 (6-136)

Results are expressed as mean ± SEM except for ECP which is expressed as geometric mean (range). *P<0.05 and **P<0.01 vs baseline within the group; †P = 0.09 for intergroup comparison.

(3) Effect of theophylline vs placebo on lung function parameters

There was no significant difference at V1 and V2 between the two groups with respect to the PEFR, FEV1 or PC20M. Intragroup comparisons show that theophylline, but not placebo, slightly increases the FEV1 at V4 (106.3 ± 2.2% of baseline for theophylline, $P < 0.01$ vs. 99.7 ± 2.7% for the placebo, P>O.05) (Table 2). When compared to placebo the bronchodilation caused by theophylline at V4 approached the statistical significance (P = O.07). By contrast both theophylline and placebo failed to significantly improve PEFR and PC20 methacholine during the study (Table 2).

(4) Relationships between variations in sputum eosinophils and lung function

When pooling the two groups and including data from the visits 4 and 5 there was an inverse and significant relationship between the change in sputum eosinophil counts and the change in FEV1 expressed as a percentage of baseline (r_s= -0.43, P<0.01) (Fig. 3). This correlation was not driven by the effect of theophylline since the correlation between two parameters was weak and not significant when considering the 11 subjects treated by

theophylline at V4 (r_s = -0.27, P > 0.05). By applying the same analysis for the PC20M we found a positive relationship between the change in PC20M (expressed in doubling dilution) and that in sputum eosinophils (r_s = 0.34, P = 0.05). There was no relationship between the change in sputum eosinophil counts and that in PEFR (r_s=-0.11,P>0.05)

Fig. 2. *Variations in sputum eosinophil counts after administration of placebo (O, n = 10) or theophylline (●, n=11). Results are expressed as a percentage of baseline sputum eosinophils. Values represent mean ± SEM. **P<0.01 vs baseline and †P = 0.07 vs baseline (that is the mean of VI and V2). Comparison of the AUC (V3 and V4 included) between theophylline and placebo gave a P = 0.08.*

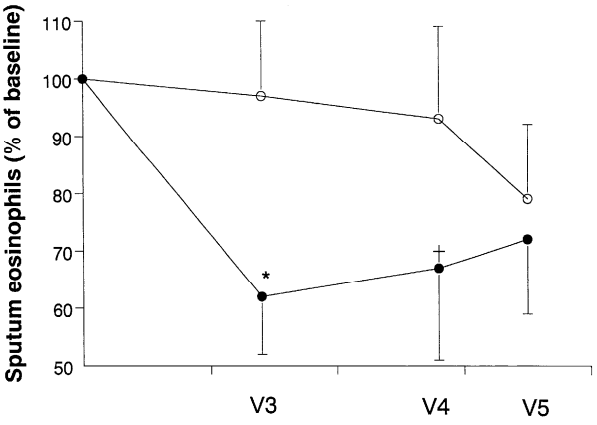


Table 2. Effect of theophylline vs placebo on lung function parameters

	Baseline			**Treatment**	**Run-out**
	V1	**V2**	**V3**	**V4**	**V5**
Placebo (*n*=10)					
FEV_1 (1)	3.85 ± 0.25	ND	ND	3.86 ± 0.29	4.01 ± 0.24
PEFR (l/min)	526 ± 26	489 ± 26	516 ± 26	512 ± 25	530 ± 27
PC20M (mg/ml)	0.41 (0.02-2.42)	ND	ND	0.77 (0.06-2.38)	0.55 (0.11-1.96)
Theophylline (*n*=11)					
FEV_1 (1)	3.92 ± 0.20	ND	ND	4.19 ± 0.25*	3.96 ± 0.25
PEFR (l/min)	552 ± 29	557 ± 25	574 ± 26	571 ± 29	554 ± 31
PC20M (mg/ml)	0.78 (0.07-16)	ND	ND	0.90 (0.06-3.34)	0.69 (0.05-3.45)

Results are expressed as mean ± SEM except PC20M which is expressed as geometric mean (range). *P < 0.05 vs baseline within the group.

(5) Effect of theophylline vs placebo on sputum eosinophil chemotactic activity

The fluid phase of the sputum collected at V2 contained a significant eosinophil chemotactic activity. In the theophylline group the fluid phases caused a mean migration of 34 ± 6.8 eosinophils vs. 17 ± 3.3 eosinophils with the buffer alone (P<0.05) while the mean migration after PAF 0.1 μM was 32 ± 6 cells. In the placebo group the number of eosinophils having migrated in response to the sputum and the buffer were 23 ± 3.1 and 15 ± 2 cells, respectively (P<0.05*)* while PAF 0.1 μM induced a mean migration of 27 ± 5 eosinophils. In the theophylline group the sputum-induced eosinophil migration was significantly reduced at V4 (33±10% inhibition, P<0.01) while it remained unchanged in the placebo group (6 ± 15% inhibition, P>0.05) (Fig. 4). Furthermore adding theophylline (30 μM to 1000 μM) into the fluid phase of the sputum samples collected at V2 resulted in a dose-dependent inhibition of the eosinophil chemotactic activity which becomes significant at 60 μM (approximately 10 μg/mL) (Fig. 5). In order to interpret the reduction in the sputum eosinophil chemotactic activity seen at V4 in patients treated by theophylline, the drug concentration was measured in the fluid phase of the samples collected at V4 and the theophylline levels were found to reach 1.7 ± 0.3μg/mL (approximately 10 μM).

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***Fig. 3**. Relationship between change in sputum eosinophils counts at V4 and V5 and the corresponding change in FEV_1. The changes are expressed as a percentage of baseline. (●) represent the patients treated by theophylline at V4. (O) represent the patients in the theophylline group at V5 and the patients in the placebo group at V4 and V5.*

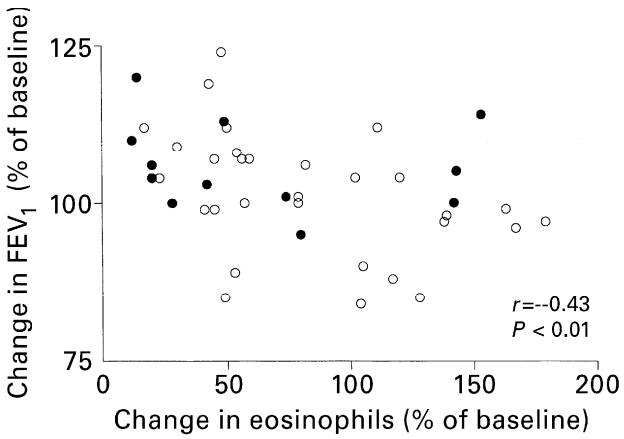
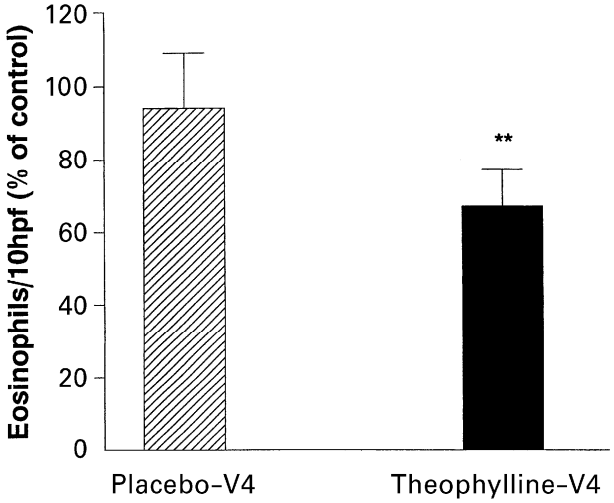


***Fig. 4.** Variations in sputum eosinophil chemotactic activity after a 4-week treatment with placebo (shaded bar) or theophylline (black bar). The results are expressed as mean ± SEM and as a percentage of baseline eosinophil migration which reached 23 ±3.1 cells and 34 ±6.8 cells/10hpf in the placebo and theophylline groups, respectively. **P<0.01 vs baseline.*



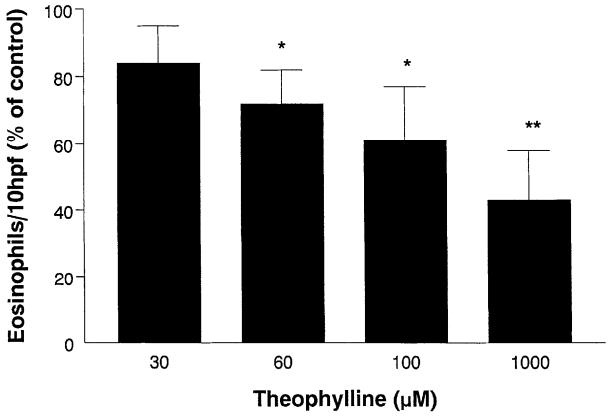
Discussion

Here we investigated for the first time in a double blind placebo controlled study the effects of a 4-week treatment with theophylline on sputum eosinophilia in steroid naive asthmatics. The reduction in sputum eosinophil count seen in the theophylline group tended to be significantly greater than that seen in the placebo group. This was accompanied by a suppression of the eosinophil chemotactic activity contained in the fluid phase of the sputum samples. The reduction in the sputum eosinophil counts was paralleled by an increase in airway calibre without change in methacho-line bronchial hyperresponsiveness.

In this study asthmatics treated with theophylline displayed a mean reduction in their sputum eosinophil count reaching approximately 35% of baseline. If the theophylline effect on sputum eosinophil count lacks of statistical significance in this study it is likely to result from the small sample size which obviously decreases the power to detect a real small effect (increase in the b error). As this was a pilot study it was difficult to predict the

magnitude of the effect of theophylline and consequently the sample size needed to give a sufficient statistical power to the study. Starting from a baseline eosinophilia reaching 20% and given the standard deviation (12%) of the sputum eosinophil percentage in those patients with sputum eosinophils >5%, our sample size had a 70% power to detect a 50% reduction in the baseline eosinophil count but a power of only 60% to detect a 35% reduction. Assuming that a 35% reduction in sputum eosinophil counts is the real effect of the drug, it would require to quadruple the sample size to give the study a power of 80%. This would certainly imply to conduct a multicentre study because of the difficulty in patient selection. By all means the effect of theophylline appears to be modest when compared to that of oral prednisone. Indeed using a similar design and a rather similar sample size and starting from a baseline sputum eosinophil counts of 15%, Claman *et al.* showed that a 6-day course of oral prednisone reduced the sputum eosinophil count by 90% [23]. From our data, the reduction in sputum eosinophilia caused by theophylline does not seem to be proportional to the serum drug level nor to the duration of the treatment since the decrease in sputum eosinophils was maximal with the low serum level (7μg/mL) and after only 2 weeks of treatment. In addition the effect seems to be short lasting since the reduction in sputum eosinophil count was no longer observed one week after stopping the drug.

Fig. 5. *In vitro effect of theophylline on eosinophil migration induced by the fluid phases of some sputum samples (n=10) collected at V2. The results are expressed as mean ± SEM and as a percentage of baseline eosinophil migration which reached 40 ±5.3 cells/1 0 hpf for these samples. *P<0.05 and **P<0.01 vs baseline.*



Although we did not perform a blood differential cell count in this study, a recent very large multicentre study has clearly indicated that regular treatment with theophylline, that gave blood levels between 8 and 15μg/mL, failed to reduce the blood eosinophil counts despite causing significant clinical improvement [24]. Taking this observation into account we could hypothesize that theophylline might reduce bronchial eosinophilia by interfering with the recruitment or the survival of eosinophils in the airways rather than by blunting the bone marrow production. We have previously shown that the fluid phase of the sputum samples from asthmatics contained chemotactic factors for eosinophils. This study confirmed and extended our previous finding by showing that the baseline eosinophil chemotactic activity contained in the sputum was reduced after a 4-week treatment by theophylline. In addition we found that theophylline was able to reduce eosinophil migration *in vitro* in response to chemotactic factors present in sputum. Interestingly this effect was already significant at 60 μM a concentration similar to that found in the serum of our subjects at V4 suggesting that theophylline may well inhibit *in vivo* the passage of eosinophils from the blood into the bronchial tissue. The mechanism by which theophylline can decrease eosinophil migration towards chemotactic agents *in vitro* is not entirely known but is likely to involve type IV phosphodiesterase inhibition within the eosinophils [25]. However our data suggest that theophylline also reduces eosinophil chemotaxis by an indirect mechanism. As the concentrations of theophylline measured in the fluid phase of our sputum samples were lower than those required to suppress the sputum chemotactic activity *in vitro* we assume that the suppression of this activity seen after treatment is not related to a direct effect of the drug on eosinophils but rather reflects a reduction in the amount of chemotactic factors generated *in vivo*. The nature of these chemotactic factors needs to be elucidated in future studies but one of those might be LTB4 the production of which has recently been shown to be suppressed by treatment with theophylline [26]. Obviously chemotaxis is not the only biological event leading to tissue eosinophilia and theophylline might also act by additional mechanisms such as an inhibition of eosinophil survival [27] possibly through suppression of IL-5 production [14].

Although causing to some extent a fall in sputum eosinophil counts and being able to inhibit ECP release from eosinophils *in vitro* [22], theophylline failed to significantly reduce the sputum ECP levels. This might be explained by a slow clearance rate for this highly cationic molecule. Because of its positive charge, ECP tends to stick to the anionic glycoproteins of the bronchial mucus and the better the sputum is homogenized, the greater is the amount of ECP recovered from the supernatant of the sample [28,29]. Thus it would be reasonable to think that the secreted protein can persist longer in the airway lumen than the secretory cell itself but this requires confirmation with studies conducted over a longer period.

As far as the functional parameters are concerned, theophylline slightly increased the baseline airway caliber as reflected by a rise in FEV_1 which is confirmatory of previous data [1] and indicates that low concentration of theophylline can still cause a modest bronchodilation. However theophylline failed to improve methacholine bronchial hyperresponsiveness at all. This confirms previous data from Dutoit *et al.* [30] who failed to find any significant change in PC20 histamine after a 3-week treatment with theophylline and more recent data coming from the American Academy of Allergy, Asthma and Immunology Beclomethasone Dipropionate-Theophylline Study group showing that PC20 methacholine did not change over a 12-month treatment period [24]. Thus theophylline appears more potent at preventing allergen induced airway hyperresponsiveness [8] than at reversing the naturally present bronchial hyperresponsiveness in asthmatics. The absence of effect of theophylline on PC20 methacholine despite the fall in sputum eosinophilia may appear somewhat surprising.

Although several authors looking at BAL [31-33] or sputum [18] have shown a convincing relationship between the extent of airway eosinophilia, or the levels of their secretory products, and the degree of methacholine airway responsiveness in asthmatics, others failed to confirm that observation [34]. In addition there are some reports showing airway eosinophilia without marked airway hyperresponsiveness in patients with chronic cough [35] or inflammatory bowel disease [36]. It is worth noting that, in our study, sputum eosinophilia, although significantly reduced at the end of the treatment period, remains clearly high approaching 15%. It is therefore sensible to argue that the effect of theophylline on sputum eosinophilia was not strong enough to attenuate the methacholine bronchial hyperresponsiveness. On the other hand the fact that ECP levels were not decreased after the treatment with theophylline might partly explain the failure of the drug to down regulate the methacholine bronchial responsiveness since the cationic proteins were shown to contribute to bronchial hyperresponiveness [37]. The relationship between airway eosinophilia and methacholine bronchial responsiveness is further obscured by the surprising positive relationship we found in this study between the change in PC20M and those in sputum eosinophils. Clearly more longitudinal studies looking at the variations in responsiveness to direct or indirect stimuli in relation with those in airway eosinophilia in different clinical settings (natural or drug induced variations) are needed to shed further light on the precise relationship between airway inflammation and airway hyperresponsiveness. However by showing here a convincing inverse relationship between the variations in sputum eosinophils and those in FEV_1 over time we provide arguments to suggest that natural fluctuation in airway calibre might better reflect change in airways inflammation in asthmatics than would fluctuation in methacholine bronchial responsiveness. On this point our observation is in line with a recent study conducted by Fahy *et al.* [38]. Interestingly the inverse relationship between the change in FEV_1 and that in sputum eosinophils did not hold for the subjects treated by theophylline. This may certainly be interpreted as an indication that theophylline can improve airway calibre in an other way than by reducing airway inflammation and in particular by directly relaxing airway smooth muscle.

In conclusion our study shows that a one month treatment with theophylline achieving low plasma concentration can slightly decrease the extent of sputum eosinophilia and reduce the sputum chemotactic activity in mild to moderate steroid naive asthmatics. This potentially important antiinflammatory effect of theophylline in asthma should be confirmed on a large scale study. It remains also to see whether this anti-inflammatory effect of theophylline can contribute to the clinical improvement the subjects can show when using this drug.

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