

Effect of salmeterol on allergen-induced airway inflammation in mild allergic asthma

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Summary

Background A previous study suggested that the long-acting β_2 -adrenergic agonist salmeterol (SM) had inhibitory effects on bronchial mucosal inflammation 6 hours after allergen exposure.

Objective To further evaluate the influence of SM on allergen-induced airway inflammation.

Method We studied, in a randomized, double-blind, cross-over trial, 16 mild asthmatic patients who had a dual asthmatic response to allergen inhalation. Subjects received 50 μ g of SM or placebo (P), twice daily for 1 week each, separated by a 2-week wash-out period. At the end of each treatment period, after withholding SM for 24 h, they had a methacholine inhalation test (medication was resumed after the test), followed 24 h later by an AC with the concentration of allergen that had induced a LAR at baseline. Airway inflammation was assessed 24 h after the AC by bronchoalveolar lavage (BAL) ($n = 16$) and bronchial biopsies ($n = 13$).

Results As expected, SM improved baseline FEV₁ and PC₂₀ ($P \leq 0.009$) and decreased the allergen-induced early bronchoconstrictive response. There were no significant differences in BAL cell counts after the two treatments. On bronchial biopsies, numbers (median, mm²) of submucosal CD45 (P: 43 ± 23 ; SM: 161 ± 43 , $P = 0.031$), CD45Ro (P: 37 ± 19 ; SM: 126 ± 41 , $P = 0.047$) and AA1 positive cells (P: 38 ± 6 , SM: 65 ± 17 , $P = 0.006$) were significantly higher after SM than P treatment. The numbers of CD4 (P: 11 ± 10 ; SM: 32 ± 7 , $P = 0.085$), HLA-DR (P: 65 ± 30 ; SM: 116 ± 36 , $P = 0.079$) and EG2 positive cells (P: 25 ± 15 ; SM: 38 ± 26 , $P = 0.09$) tended to increase with SM treatment.

Conclusion In summary, compared to placebo, 1 week of regular use of SM is associated with an increase in bronchial inflammatory cells 24 h after AC. This is in keeping with the recommendation that salmeterol should only be used with an anti-inflammatory agent, particularly in the context of significant allergen exposure.

Keywords: SM, allergen-induced asthma, airway inflammation, long-acting β_2 -adrenergic agonists

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Introduction

Beta₂-adrenergic agonists are the most commonly used drugs for the treatment of asthma symptoms. Although

short-acting β_2 -adrenergic agonists are considered to have no significant anti-inflammatory effect, there are still discrepancies in the findings of studies looking at the potential influence of long-acting β_2 agonists on airway inflammation [1–5].

Long-acting β_2 -adrenergic agonists can modify some aspects of the inflammatory process, such as mediator

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Table 1. Study design**Screening**

Day -16	Baseline tests: including PC ₂₀ methacholine challenge
Day -15	Documentation of LAR after allergen challenge (8 a.m.)

Run-in

Days -14 to 0	Control day with saline challenge at the end of the run-in (8:00 am). Randomization to treatment (first dose at night day 0)
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Treatment period

Days 0-6	<i>Treatment A</i> (Salmeterol 50 µg BID or placebo)
Day 7	PC ₂₀ methacholine 8 a.m. (wash-out of 24 hours, treatment A taken after MIT and at night)
Day 8	Allergen challenge 8 a.m. (treatment A taken 1 hour before the test and at night on day 8)
Day 9	Blood sampling, bronchoscopy with BAL and bronchial biopsies 8 a.m. (treatment A taken 1 hour before the tests)
Days 9-21	Wash-out period (treatment B starts at night day 21)
Days 21-27	<i>Treatment B</i> (Salmeterol 50 µg BID or placebo)
Day 28	PC ₂₀ methacholine 8 a.m. (wash-out of 24 hours, treatment B taken after MIT and at night)
Day 29	Allergen challenge 8 a.m. (treatment B taken 1 hour before the test and at night on day 8)
Day 30	Blood sampling, bronchoscopy with BAL and bronchial biopsies 8 a.m. (treatment B taken 1 hour before the tests)

release by mast cells, vascular permeability and neurogenic inflammation [6–8]. Salmeterol (SM) has been reported to reduce bronchoalveolar lavage (BAL) fluid eosinophil cationic protein concentration and inhibit macrophage function in asthmatic subjects [2]. In studies looking at the influence of SM on allergen response, SM prevented the increase in serum eosinophil cationic proteins but not in blood eosinophil count [4]. We previously showed, in a pilot-study in mild asthmatic atopic subjects, that the use of SM over a 2-month period was associated with lower numbers of bronchial HLA-DR, AA1 and CD45Ro-positive cells 6 hours after allergen challenge compared to placebo [3]. This suggests that although SM does not influence most baseline inflammatory features, it can prevent some inflammatory effects of allergen exposure. However, as at 6 hours post-allergen-challenge, the inflammatory response is only at its onset, and as the number of subjects in the previous study was small, we decided to further evaluate the effects of SM on allergen-induced airway inflammation. The aims of this study were therefore to better determine the effects of SM on allergen-induced airway inflammation, 24 h after allergen challenge.

Methods*Subjects*

Sixteen atopic subjects, 10 M, 6 F, > 18 years of age, with a diagnosis of asthma as defined by the American Thoracic Society, were studied [9]. The diagnosis had been made at least 6 months prior to enrolment. All subjects had stable asthma for at least 1 month, had baseline forced expiratory

volume in 1 s (FEV₁) > 70% of the predicted, and had at least one positive response to a battery of common aeroallergens. To be included in the study, subjects also had to present a late asthmatic response (LAR), defined as a 15% fall in FEV₁ between 2 and 8 hours after the allergen inhalation challenge.

Patients were not eligible for the study if they met any of the following exclusion criteria: respiratory infection or significant allergen-induced reactions in the 2 weeks prior to the study, significant concomitant disease, hospital admission for reversible airways disease within 4 weeks prior to the first visit, ECG abnormalities, history of any drug allergy, or contraindications to inhalation challenges. Pregnant or breastfeeding women and current smokers were also excluded.

Subjects had not received any oral or inhaled corticosteroid or any other drug with anti-inflammatory properties within at least 1 month preceding the study. All subjects provided written informed consent before their participation. Except for study medication and rescue salbutamol, no other medications were allowed during the study.

Study design

This study was a randomized, double-blind cross-over trial (Table 1). On the first visit, subjects had measurements of airway responsiveness and allergy skin-prick tests with a battery of common allergens, including animal danders, dust mites, pollens and moulds (Bayer, Ontario, Canada) (Table 1). A skin-test titration was performed with the allergen chosen for challenge, and the results were used to estimate the specific dose of that allergen which when

inhaled would induce a $> 20\%$ fall in FEV_1 . An inhalation allergen challenge was then performed to document the occurrence of EAR and LAR.

After a 2-week run-in period, without medication other than a rescue β_2 -adrenergic agonist (not used regularly), a challenge with saline solution was done on the day before randomization.

Patients were evaluated after a week of treatment with SM 50 μg daily and after 1 week of inhaled placebo given in random order. Inhaled salbutamol was provided as rescue medication to be used on an as-required basis for the relief of asthma symptoms, and its use was recorded on the diary card.

A 2-week wash-out period separated the two treatment weeks. Parameters measured included asthma symptoms, expiratory flows, airway response to methacholine before and at the end of each treatment, 24 h after withholding the study medication (SM was resumed after the test), and airway inflammatory features on BAL and bronchial biopsies 24 h following an allergen challenge, done with the same allergen dose determined on the screening test, before the run-in period. Each allergen challenge was preceded the day before by a methacholine inhalation test.

Spirometry and methacholine challenge

Spirometry and responsiveness to inhaled methacholine were measured according to standardized procedures [10,11]. Concentrations of methacholine up to 16 mg/mL were used. Responses were expressed as the provocative concentration of methacholine inducing a 20% fall in FEV_1 (PC_{20}), obtained from the log dose-response curve.

The study treatment and salbutamol were withheld for periods of 24 and 8 h, respectively, before the methacholine test to avoid the influence of bronchodilatation on this measurement.

Allergen inhalation challenges

Allergen challenges were performed in a provocation chamber according to standard methods [12]. Doubling concentrations of allergen aerosolized by a Wright's nebulizer (output 0.13 mL/min, 50 PSI) were inhaled for 2 minutes using a face mask, every 10 min, followed by measurement of the FEV_1 at 1 and 10 min after the end of the inhalation. When a $> 15\%$ fall in FEV_1 was observed or the maximal concentration dilution given, the test was stopped and FEV_1 measured at 1, 5, 10, 20, 30, 40, 50, 60, 90 min, then hourly for up to 8 hours. Salmeterol or placebo were given 1 hour before the test and in the evening (see Table 1).

Bronchoscopy, bronchoalveolar lavage and bronchial biopsies

SM or placebo was taken as usual on the day of the challenge and the morning of the bronchoscopy. Before bronchoscopy, a 40- μg dose of ipratropium (Boehringer Ingelheim) was given using a metered-dose inhaler. Spirometry was done before and after the procedure and patients were observed for at least 1 hour after the bronchoscopy. The bronchoscopy technique used in our laboratory has been previously reported [13]. The BAL was performed by injecting two boli of 50 cc of saline solution at 37 °C in the middle lobe or the lingula. After gauze filtration, the lavage fluid was kept on ice and pooled and the total number of cells was measured on a haemocytometer (Reichert Scientific Instruments, Division of Warner-Lambert Technologies Inc., Buffalo, New York). Cell differentials were obtained by counting at least 300 cells on glass-cover preparations of pooled lavage fluid, after staining with Diff-Quik (Baxter Healthcare Corporation, Scientific Product Division, McGraw Park, Illinois) and non-specific esterase stains. Immediately after the BAL, for evaluation of inflammatory parameters, four to six bronchial biopsies were taken from the carinae of the lobar and segmental bronchi of the same lung as the BAL using conventional forceps. Specimens were processed for immunohistochemistry. Samples, BAL and bronchial biopsies were taken in different lung sides after each of the two treatments.

Immunohistochemistry and quantification of immunohistochemical staining

Bronchial biopsies were immediately placed in acetone antiprotease solution (-20°C) and embedded in GMA resin the same day [14]. Samples were stored at -20°C in an airtight container containing a desiccant. Two micrometer sections were cut on an ultramicrotome (Leica Reichert Ultracut S, Austria), then floated on water containing 0.02% ammonia until fixed on poly L-lysine-coated slides. The following monoclonal antibodies were used: HLA-DR, CD25, CD3, CD4, CD8, CD45RO, AA1 (Dako Diagnostics Canada Inc, Mississauga, Ontario, Canada), EG1, EG2 (Kabi Pharmacia diagnostics, Baie D'Urfé, Canada); immunostaining was performed as previously described [15]. Sample peroxidase activity background was blocked by incubation with 0.3% H_2O_2 0.1% sodium azide in Tris Buffer Solution (TBS) for 30 min and normal serum for 30 min. The samples were then incubated overnight with primary antibodies (4°C), for 1 h with a biotinylated secondary antibody (23°C) and 40 min with the ABC-HRP complex (23°C). The slides were washed three times with TBS between steps. Bound

Table 2. Subjects' characteristics

Subjects	Sex	Age	Atopic score*	Baseline				
				FEV ₁ L (% pred.)	FVC L (% pred.)	Challenge fall in FEV ₁ (%)		PC ₂₀ (mg/ml)
						Early	Late	
1	M	24	7	2.84 (72)	4.09 (87)	28	33	0.59
2	F	25	10	3.07 (98)	3.33 (91)	20	22	2.30
3	M	22	10	4.63 (94)	5.81 (102)	33	24	0.91
4	M	28	16	4.11 (88)	5.08 (92)	35	17	1.70
5	M	26	5	4.83 (106)	6.18 (114)	20	18	6.80
6	F	28	6	3.29 (115)	3.69 (112)	29	28	9.60
7	M	23	7	3.59 (86)	4.90 (101)	17	18	0.36
8	M	19	7	4.44 (102)	5.43 (108)	36	23	2.48
9	F	29	6	2.93 (94)	3.98 (108)	22	20	0.87
10	M	23	21	3.54 (75)	5.25 (96)	29	24	0.76
11	M	28	3	3.72 (90)	4.89 (100)	28	28	1.13
12	F	23	11	3.29 (108)	3.77 (107)	43	45	0.34
13	M	29	6	4.07 (102)	5.24 (107)	22	24	2.93
14	F	23	7	3.02 (104)	3.43 (104)	33	33	2.70
15	M	22	3	3.89 (87)	5.06 (97)	34	22	4.68
16	F	32	7	3.03 (106)	3.52 (105)	31	17	0.80
Mean		25.2	8.2	3.64 (95)	4.60 (102)	29	25	1.53
± SEM		± 0.9	± 1.3	± 0.16 (± 3)	± 0.91 (± 2)	± 2	± 2	± 1.28

*Number of positive responses to 26 airborne allergens.

antibodies were detected with amino-ethylcarbazole (AEC) substrate until staining was visible on the microscope. Reaction was stopped by washing in water and Mayer haematoxylin was used for counter-staining. Slides were mounted with Crystal mount (Biomedex, Foster City, CA, USA). Irrelevant isotypic antibodies served as negative controls.

Immunostained submucosal cells present were enumerated by an observer unaware of the treatment group. Counts were expressed as the number of positive cells/mm² of connective tissue, excluding mucus glands and smooth muscle. The area of submucosa was measured with calibrated image analysis (Mocha image analysis software, Jandel Scientific, San Raphael, CA, USA).

Analysis and sample size calculation

It was determined that with 12 subjects per group, it was possible to detect a significant difference in the studied parameters [16]. The biopsy parameters obtained pre- and postallergen challenge were analysed using a multivariate Anova with a paired T²-test and with Student's paired-*t*-tests for each parameter. All reported *P*-values are

two-sided. When appropriate, results were analysed using nonparametric analysis. The representative measures used to locate the centre and the dispersion of the distribution of observations concerning subjects' characteristics, FEV₁ and airway responsiveness to methacholine are mean and SEM and median and range for bronchial biopsy inflammatory cell counts.

Results

Subject's characteristics

All 16 asthmatic subjects completed the study. In three subjects, the bronchial biopsy material was insufficient for analysis. No subject had an exacerbation of asthma during the study. Age, gender, atopic status, baseline FEV₁ and PC₂₀ of the 16 subjects are presented in Table 2. Mean baseline PC₂₀ (mg/mL) was 1.53 ± 1.28. Mean per cent decreases in FEV₁ after the screening allergen challenge were 28.7 ± 1.7% during EAR and 24.6 ± 1.8% during LAR.

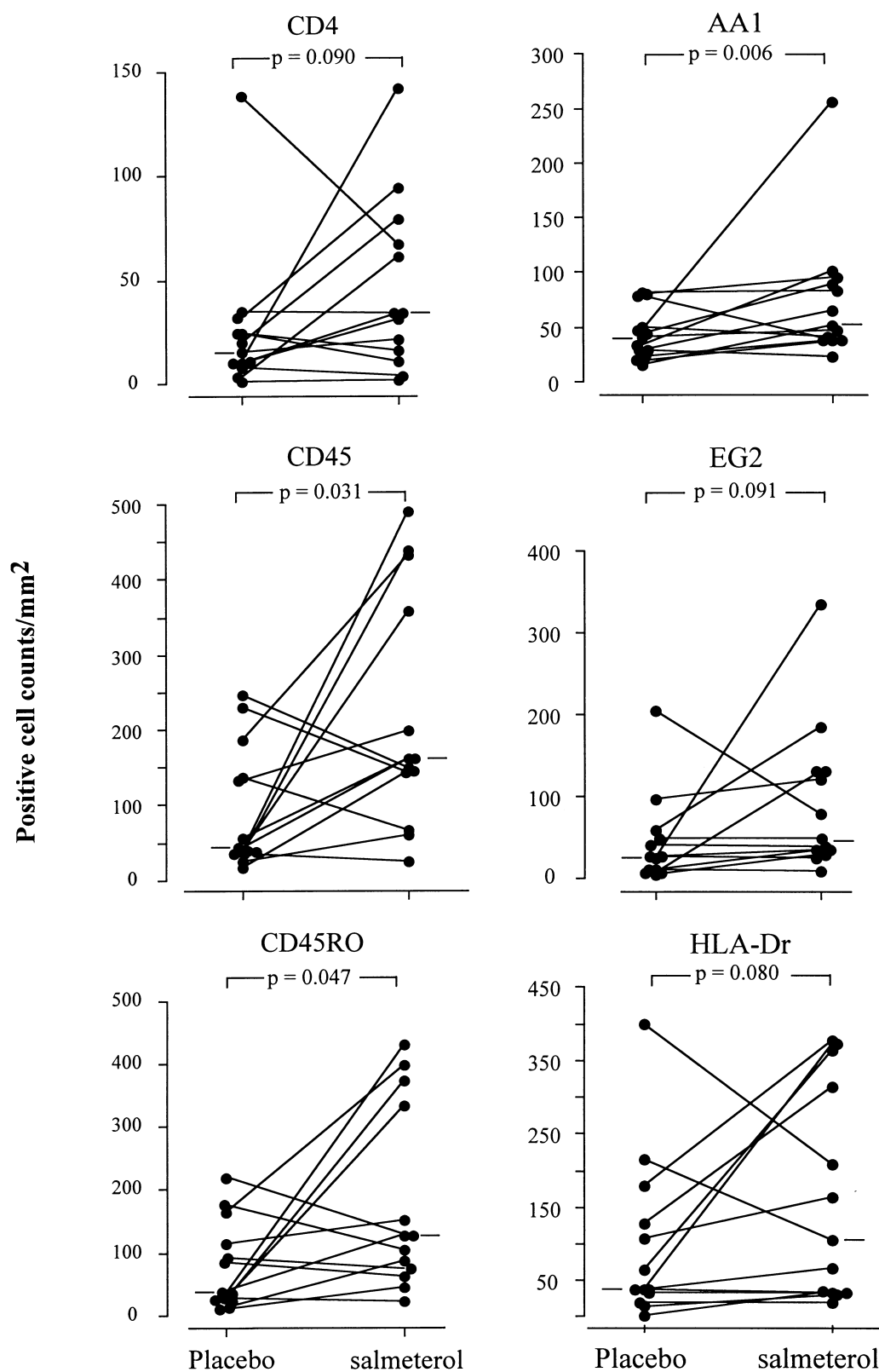


Fig. 1. Bronchial biopsy inflammatory cell counts/mm² of connective tissue for placebo and salmeterol groups. Bars represent medians.

Effect of treatments on airway responsiveness

The mean FEV₁ before methacholine challenge 24 h after the last dose of placebo and SM were 3.50 ± 0.15 and 3.64 ± 0.15 L ($P = 0.06$), respectively. Mean PC₂₀ was 1.15 ± 1.34 and 2.20 ± 1.31 ($P = 0.009$) after the placebo and SM treatments, respectively.

Effect of treatments on asthmatic response to allergen challenges

During the treatment period, in comparison with the screening value, FEV₁ measured before allergen challenge improved after SM but not placebo, with a mean FEV₁ of 3.87 ± 0.15 and 3.49 ± 0.16 , respectively ($P = 0.0005$). After allergen challenge, FEV₁ decreased, respectively, by $18.6 \pm 0.25\%$ and $9.8 \pm 1.4\%$ for the EAR and $16.1 \pm 1.96\%$ and $14.5 \pm 2.7\%$ for the LAR with placebo and SM treatments, respectively. SM significantly inhibited FEV₁ fall during EAR ($P = 0.004$) compared with placebo period, but LAR was similar after the two treatments.

Effects of treatments on bronchoalveolar lavage and bronchial biopsy cell counts

There were no significant differences in BAL macrophage, lymphocyte, neutrophil and eosinophil counts 24 h after the allergen challenge following the two treatments ($P > 0.05$). However, bronchial biopsies showed significant increases in the number of cells/mm² of connective tissue (median [range]) for CD45 + (P: 43 [16–246]; SM: 161 [25–489], $P = 0.03$), CD45Ro + (P: 37 [9–218]; SM: 126 [23–431], $P = 0.047$) and AA1 + cells (P: 38 [15–81], SM: 65 [23–255], $P = 0.006$) after SM compared with P treatment (Fig. 1). Moreover, CD4 + (P: 11 [1–138]; SM: 32 [2–94], $P = 0.09$), HLA-DR + (P: 65 [16–400]; SM: 116 [20–377], $P = 0.08$) and EG2 + (P: 25 [2–204]; SM: 38 [7–334], $P = 0.09$) cell counts tended to increase with SM treatment. Finally, CD3 + (P: 28 [4–236]; SM: 87 [7–238], $P = 0.14$), CD8 + (P: 14 [1–73]; SM: 31 [2–92], $P = 0.37$), CD25 + (P: 3 [0–41]; SM: 13 [0–54], $P = 0.30$) and EG1 + cells (P: 32 [2–178], SM: 33 [10–284], $P = 0.18$) cell counts were similar after P or SM treatment.

Discussion

This study showed an increase in inflammatory cells on bronchial biopsies 24 h after an allergen challenge in asthmatic subjects taking SM for 1 week as monotherapy, in comparison to a placebo. These data suggest that, while SM can improve baseline expiratory flows and reduce airway responsiveness to allergen, when used alone, it may

be associated with a mildly increased allergen-induced airway cellular response.

Allergen exposure has been found to increase airway inflammation [17,18]. In a previous study on potential anti-inflammatory properties of long-acting β_2 agonists, we reported inhibitory effects of SM on allergen-induced airway inflammation, particularly of certain subsets of lymphocytes on bronchial biopsies taken 6 hours after allergen challenge [3]. SM intake has been associated with a decrease in the serum concentration of eosinophil cationic protein (ECP) during natural pollen exposure [15]. It also seemed able to reduce ECP but not inflammatory cells in bronchoalveolar lavage 24 h after allergen inhalation [2]. Moreover, Brick et al. reported that SM inhibited recruitment of eosinophils in bronchoalveolar lavage 24–48 h after segmental allergen challenge [19]. More recently, Dente et al. reported that a single dose of SM inhibited the 24 h allergen challenge induced sputum eosinophilia [20].

On the other hand, Pizzichini et al. showed that the late increase in airway eosinophils after allergen challenge was not prevented by SM, nor by beclomethasone given before repeated allergen challenges [5]. Finally, no effect of SM was found by Roberts et al. on baseline airway inflammation in asthma [21].

The influence of duration of salmeterol intake may possibly influence its effects on allergen-induced airway inflammation. In a study from Gardiner et al. looking at the effects of 8 weeks of treatment with SM on airway inflammation in asthmatic subjects receiving regular inhaled corticosteroid therapy, there was no significant change in BAL cell profile and no change in percentages of CD4 and CD8 lymphocytes or proportion of lymphocytes expressing HLA-DR after SM [22]. However, in this last study, the concomitant use of corticosteroids could have influenced the results. In our study, SM was withheld for 24 h before methacholine challenge but as this is a short time-interval and as at least two other doses of SM were given before the subsequent allergen challenge, the influence of regular use should still be present.

The variability of SM effects observed in previous studies, compared to ours, may be due to the methods used and parameters evaluated, but also to a possible change in its effects on the inflammatory process over time, although this remains to be studied.

Although SM reduced the EAR after 1 week of treatment, it did not modify the LAR. This can be partially explained by the difficulty of reproducing LAR even though its presence had been confirmed in all subjects prior to entry in the study. It may also be due to a possible reduction of inhibitory effects of long-acting β_2 agonists on some inflammatory mechanisms over time, as observed for physiological parameters [23]. This has however, to be

further evaluated as most studies looking at the effects of SM on inflammation were done after a single dose.

Our observations are also in keeping with a recent report showing an increase in induced-sputum eosinophils following weaning from corticosteroids before any significant reduction in expiratory flows may be observed in subjects using regularly SM [24]. Long-acting β_2 agonists, when associated with an inhaled corticosteroid, have been associated with a reduction in asthma exacerbations and an improvement in asthma control [24–27]. When used as monotherapy, however, in comparison with inhaled corticosteroids an increased frequency of asthma exacerbations has been reported with long-acting β_2 agonists [27,28].

As we showed no significant anti-inflammatory but rather a potential mild pro-inflammatory effect of SM on the allergen-induced cellular inflammatory process, our observations support the current asthma consensus recommendations to use long-acting β_2 agonists with concomitant anti-inflammatory medications such as corticosteroids. This could be particularly important for atopic asthmatic subjects who are currently exposed to significant concentrations of airborne allergens.

As inhaled corticosteroids are potent anti-inflammatory agents that can antagonize transient allergen-induced increases in airway inflammation and therefore probably prevent the effects of long-acting β_2 agonists on this process, it would be of interest to repeat this type of study, adding an inhaled corticosteroid to SM.

SM may possibly have beneficial effects on the allergen-induced airway inflammatory response that were not documented in this study, such as cytoprotection of the epithelium, increased mucociliary clearance or reduction in airway oedema. Our conclusions are therefore limited to the cellular aspect of airway inflammation. Furthermore, we cannot exclude the possibility that factors such as a possible reduction of allergen-induced increase in airway wall oedema in SM treated patients could have influenced the determination of numbers of cells per surface unit [6]. This number of cells may possibly be artificially lower for example in an oedematous airway compared to one with no increase in airway wall water content, for a similar cell infiltration.

In conclusion, we found that there was an increase in tryptase mast cell and total leucocyte counts in bronchial biopsies of sensitized subjects taking SM regularly for 1 week 24 h after an acute allergen exposure compared with those taking a placebo. Although the clinical significance of our observations is uncertain, they are in keeping with the recommendations that long-acting β_2 agonists should only be used with an anti-inflammatory agent, particularly for patients currently exposed to allergens to which they are sensitized. Further studies are, however, required to determine the long-term effects of allergen exposure in

subjects taking both anti-inflammatory and long-acting β_2 agonist agents.

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