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Effects of salmeterol on mucosal inflammation in asthma: a placebo-controlled study

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ABSTRACT: Although the anti-inflammatory effects of inhaled corticosteroids in the treatment of asthma are established, the effects of long-acting β_2-adrenergic receptor agonists on inflammation are the subject of debate. The aim of the present study was to determine the effect of salmeterol on the numbers of inflammatory cells in biopsy samples of distinct immunophenotype and those expressing the genes for interleukin-4 and -5, regulatory cytokines particularly relevant to asthma.

Twenty patients (aged 18–55 yrs) with mild stable asthma were randomised in a three-way crossover study to 6 weeks of treatment with: 1) salmeterol (50 µg *b.d.*; SM50); 2) fluticasone propionate (250 µg *b.d.*; FP250), or 3) placebo.

Compared with placebo, SM50 significantly reduced the numbers of neutrophils in bronchial biopsy samples and the concentrations of myeloperoxidase and soluble E-selectin in serum, each of which reflect neutrophil involvement. Compared with FP250, SM50 reduced neutrophil number and human neutrophil lipocalin level in bronchial lavage fluid and intercellular adhesion molecule-1 level in bronchoalveolar lavage fluid. Compared with placebo, FP250 significantly reduced the numbers of (CD3+) T-lymphocytes, (CD4+) T-helper cells, (CD45RO+) activated T-helper cells and eosinophils in the biopsy samples; it also reduced the percentage of eosinophils and soluble intercellular adhesion molecule-1 in serum. The percentage of symptom-free days and nights and airways hyperresponsiveness improved significantly after SM50 compared to both placebo and FP250.

In conclusion, a novel antineutrophilic effect of the inhaled long-acting β_2-adrenergic receptor agonist, salmeterol, in mild asthma is reported.
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Asthma is a chronic potentially life-threatening condition that is increasing in both prevalence and severity. In mild-to-moderate asthma, there is persistent bronchial inflammation involving primarily lymphocytic and eosinophilic infiltration associated with remodelling of the airway wall [1, 2]. Activated T-lymphocytes of the T-helper-2 phenotype are generally considered to orchestrate the eosinophilic inflammation *via* secretion of pro-inflammatory regulatory cytokines that include interleukin (IL)-4 and -5 [3]. Neutrophil numbers are increased in severe asthma, occupational asthma and in association with exacerbations [4–7].

β_2-Selective short-acting β-agonists are highly effective bronchodilators but there has been some uncertainty as to the effects of their regular use in asthmatics who fail to comply with their prescribed inhaled glucocorticosteroid [8]. Reports have emerged suggesting that short-acting β_2-agonists might even be pro-inflammatory in this group [8, 9]. However, the introduction of inhaled long-acting β_2-selective agonists, such as salmeterol and formoterol, has shown that, in addition to their relatively long-lasting bronchodilator action, they can also attenuate both the early and late phase responses to experimental allergen challenge, the latter suggestive of anti-inflammatory activity [10, 11].

An early study of salmeterol in mild asthma demonstrated no pro-inflammatory effect on either bronchoalveolar lavage (BAL) fluid differential cell counts or inflammatory cell numbers in biopsy samples [12]. A study of formoterol, another long-acting β_2-agonist, in asthmatics, even demonstrated a reduction in the number of biopsy mast cells [13]. Moreover, addition of inhaled salmeterol to the treatment of asthmatics who continued to show symptoms despite current treatment with inhaled corticosteroids resulted in a reduction in airway tissue eosinophil number compared to using an increased dose of inhaled steroid alone [14]. Another study demonstrated that treatment with salmeterol was associated with the increased allergen-induced recruitment of biopsy CD45, CD45RO and mast cells [15]. The results of these studies might appear to be conflicting.

The aim of the present study was to investigate the effects of the long-acting β_2-agonist salmeterol on airway inflammation in mild stable asthma by conducting a placebo-controlled trial to determine

the numbers of immunohistochemically distinct inflammatory cells and cells expressing the genes encoding IL-2, -4 and -5 and interferon gamma (IFN-γ) in bronchial biopsy samples. As a positive comparator, a corticosteroid arm was included, in which a reduction in the numbers of selected inflammatory cells (*i.e.* T-helper lymphocytes, eosinophils and mast cells), as reported previously by the present authors and others, was expected. The concentrations of inflammatory mediators in blood and airway lavage fluid were also determined as these are complementary compartments involved in the inflammatory response.

Subjects and methods

Subjects

The study received ethical approval from the University Hospital (Gentofte, Denmark) and conformed to the Declaration of Helsinki. All patients gave written informed consent. A total of 20 nonsmoking asthmatic patients receiving treatment with inhaled short-acting β_2-agonist alone were randomised to the study. All subjects were recruited as outpatients from the city of Copenhagen. Of these 20 patients, one withdrew for reasons unrelated to the study. All of the remaining 19 patients (median age 27 yrs (range 20–55 yrs), median forced expiratory volume in one second (FEV_1) 74.4% of the predicted value (range 41–112% pred), median provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) 0.15 mg·mL^{-1} (range 0.3–0.71 mg·mL^{-1})) completed the trial and underwent bronchoscopy on three occasions. The characteristics of the subjects are shown in table 1.

Nonsmokers aged 18–55 yrs with a mean weight of 75±18 kg were included. They had to have had a history of asthma, $\geqslant$15% reversibility in FEV_1 (measured 15 min after inhalation of 400 μg salbutamol at the first visit) or a documented history of reversibility of 15% after inhaled β-agonist within 3 months prior to the start of the study. Bronchial reactivity, as assessed by measurement of PC_{20}, had to be <4 mg·mL^{-1}. Patients were excluded if they had a respiratory tract infection or asthma exacerbation within 4 weeks of entry into the study and if they had received any asthma medication within the last 2 weeks (apart from inhaled short-acting β_2-agonist). Patients were also excluded if they had received inhaled steroids during the last 2 months or oral steroids during the 3 months prior to the study. Females who were pregnant or lactating were excluded. Patients who were skin-prick test-positive to a seasonal allergen could only take part in the trial if it was outside the season of their allergy.

Study design

The study was of randomised, placebo-controlled, double-blind, three-way crossover design. There were three 6-week treatment periods, preceded by a 2-week run-in period and ending with a 2-week follow-up. Each 6-week treatment period directly followed the previous one. After the run-in period, eligible patients were randomised to receive: 1) salmeterol 50 μg powder *b.d.* (SM_{50}); 2) fluticasone propionate 250 μg powder *b.d.* (FP_{250}); or 3) placebo (lactose) powder *b.d. via* Diskhaler (GlaxoSmithKline, UK). Patients were instructed not to use their regular study medication on the day of the clinic visit. At the first visit, all β-adrenoreceptor agonists were withdrawn and replaced with salbutamol Rotadisk (GlaxoSmithKline) for use on an "as required" basis for symptomatic relief throughout the run-in and

Table 1. – Characteristics of study subjects

Subject	Sex	Age yrs	Smoking history	FEV_1 L	FEV_1 % pred	PC_{20} mg·mL^{-1}
1	F	40	Non	2.41	66.5	0.26
2	M	20	Non	4.10	83.0	0.71
3	F	35	Non	2.58	80.5	0.18
4	F	55	Non	1.94	77.9	0.06
5	M	22	Ex[#]	4.36	88.4	0.17
6	F	21	Non	2.82	77.6	0.37
7	M	53	Non	2.61	64.4	0.62
8	F	23	Non	2.29	60.5	0.05
9	M	48	Non	2.96	77.9	0.25
10	M	22	Non	6.00	112.2	0.09
11	F	21	Non	2.60	73.7	0.28
12	F	25	Non	2.83	74.4	0.03
13	M	40	Non	1.80	41.2	0.05
14	F	27	Non	2.55	65.6	0.06
15	M	21	Non	3.00	62.6	0.11
16	F	30	Non	3.50	106.1	0.67
17	F	30	Non	3.13	88.4	0.14
18	F	42	Non	2.41	67.4	0.07
19	F	20	Non	2.55	68.4	0.20
Mean		31.3		3.00	75.6	0.29

FEV_1: forced expiratory volume in one second; PC_{20}: provocative concentration of methacholine causing a 20% fall in FEV_1; % pred: percentage of the predicted value; F: female; M: male; Non: nonsmoker; Ex: exsmoker. [#]: for 5 yrs.

treatment periods of the study. During the run-in and treatment periods, patients kept a daily record of their morning and evening peak expiratory flow (PEF), symptoms and rescue salbutamol usage. FEV_1 and forced vital capacity (FVC) were recorded at each clinic visit (Vitalograph model S; Spiropharma, Klampenborg, Denmark). Methacholine provocation tests were performed as described previously [16] during the run-in period and once at the end of each of the three treatment periods. Bronchoscopy was performed at the end of each treatment period and at least four endobronchial biopsy samples per bronchoscopic examination were taken unilaterally from second or third order bronchi; previous biopsy sites were avoided. The safety and tolerability of either active or placebo treatments were assessed by monitoring adverse events, blood pressure and the electrocardiogram. Chest radiography was performed at the first visit; all results were normal.

Fibreoptic bronchoscopy

Diazepam (10 mg orally) was given 1.5 h, atropine (0.5 mg intramuscularly) 0.5 h and midazolam (1–5 mg intravenously) immediately before bronchoscopy. The bronchoscopy was performed under local anaesthesia by application of 4 mL lidocaine gel ($40\ mg \cdot mL^{-1}$) in the chosen nostril. The bronchoscope (BF type 1T20; Olympus, Albertslund, Denmark) was introduced through the nose or mouth, and 3.0 ± 1.5 mL lidocaine ($40\ mg \cdot mL^{-1}$) was instilled through the working channel of the bronchoscope over and through the rima glottidis into the trachea. An additional 1.5 mL was instilled into the trachea and the two main bronchi to avoid cough during bronchoscopy. Biopsy samples were taken using fenestrated spoon forceps. During bronchoscopy, the airways were assessed for visual evidence of inflammation applying a previously validated inflammatory index [17] in which the airways were graded according to the severity of airway oedema, erythema, friability and secretions.

Biopsy samples

Biopsy samples were fixed immediately on removal from the patient. Two specimens were fixed in freshly prepared 2% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 2 h at 4°C, which was replaced with 15% sucrose in PBS as cryoprotectant containing 0.01% sodium azide as preservative, and then snap-frozen and stored at -80°C prior to immunohistochemistical and molecular analyses.

Immunohistochemistry

Biopsy cryosections were immunostained using commercially available validated antibodies directed against CD45 (catalogue number M701), CD3 (M835), CD4 (M716), CD45RO (M742; all DakoCytomation, Ely, UK), CD25 (20143; Becton Dickinson, Oxford, UK), eosinophil granule (EG)1 and EG2 (both Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), mast cell tryptase (Dako M7052), CD68 (Dako M876), neutrophil elastase (Dako M752), IL-4 and -5 (Genzyme Diagnostics, West Malling, UK), and IFN-γ (R&D Systems Europe Ltd, Abingdon, UK). Because of the probable presence of eosinophil-derived endogenous peroxidase, the alkaline phosphatase/antialkaline phosphatase immunostaining method, which reduces background staining, was applied. Nonspecific antibody or PBS was used to replace the primary antibody in negative controls, both of these resulting in minimal background staining. Tonsil tissue and biopsy samples previously obtained for use in other studies were used as positive controls.

In situ *hybridisation.* The isotopic method of HAMID *et al.* [18], with which the present authors have previous experience, was used to detect the presence of intracellular messenger ribonucleic acid (mRNA) specific to IL-2, -4 and -5 and IFN-γ gene expression. Radioactively labelled antisense (complementary RNA) and sense (mRNA transcripts of the complementary deoxyribonucleic acid of interest) were synthesised in the presence of adenosine triphosphate, guanosine triphosphate, cytidine triphosphate, ^{35}S-uridine triphosphate and SP-6 or T-7 polymerase. The "sense" probe was used as negative control and resulted in minimal staining. Previously validated tissues with confirmed expression of these regulatory cytokines were used as positive controls.

Positively immunostained or radiolabelled cells were counted using an eyepiece graticule in a tissue zone 100 μm deep at the external limit of the epithelial reticular basement membrane (referred to as the lamina propria). Adjacent nonoverlapping fields were used for counts until all of the available lamina propria had been analysed. The length of the reticular basement membrane was measured with the aid of an image analysis package, and the results expressed as the number of cells per millimetre of reticular basement membrane.

Bronchial and bronchoalveolar lavage

Fractional BAL was performed with the bronchoscope wedged in the middle lobe bronchus. Sterile isotonic saline at 37°C was sequentially instilled and immediately aspirated (at 80–100 cmH_2O) as three separate aliquots of 50 mL each, collected in sterile glass bottles and kept on ice. Bronchial lavage was performed by infusion of 20 mL saline followed by immediate aspiration and collection in a separate bottle. The aspirated fluid was filtered to remove mucus, the recovered volume measured and the result expressed as a percentage of the infused volume. Aspirated lavage cells were pelleted by centrifugation and the supernatant collected and stored at -70°C until analysis. Total and differential cell counts and the levels of the following inflammatory markers were measured: eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil chemotactic factor, myeloperoxidase (MPO), human neutrophil lipocalin

(HNL), granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-2, -4 and -5, and IL-2 receptor.

Blood

Blood for biochemical/haematological analysis was taken 2–3 days before bronchoscopy, depending on which day of the week biopsy was performed. This was carried out at the same time as the urine analysis, electrocardiography, chest radiography and measurement of FEV_1, FVC and PC_{20}. Venous blood samples for analysis of inflammatory mediator levels were taken ~2 h before bronchoscopy and before premedication.

Levels of the inflammatory markers, ECP, EPO, HNL and MPO, and soluble adhesion molecules, soluble E-selectin (sE-selectin), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1), were measured in serum and plasma. ECP and MPO were measured by radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. The detection limits of these assays were 2 and 8 $\mu g \cdot L^{-1}$, respectively. The soluble adhesion molecules were all assayed by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA) and according to the manufacturer's instructions. The detection limits were 0.1 $ng \cdot L^{-1}$ for sE-selectin, 0.35 $ng \cdot L^{-1}$ for sICAM-1 and 2 $ng \cdot L^{-1}$ for sVCAM-1. EPO and HNL were assayed by radioimmunoassay as described previously, and with detection limits of 0.5 and 3.6 $\mu g \cdot L^{-1}$ [19, 20]. The total imprecision of all assays was <10% (coefficient of variation).

Statistics

For the histopathological analysis, all tissue slides were coded prior to examination such that the patient and treatment were unknown to the observer. Based on previously reported power calculations, 20 eligible patients were considered sufficient to provide adequate power for the study [21].

Analyses of variance were applied to the normally distributed data (*e.g.* measurements of lung function). A nonparametric approach was adopted if log transformation of the data did not yield a normal distribution (*e.g.* cell counts) and the analysis proceeded *via* a series of Wilcoxon rank sum tests for a two-period crossover design adapted for a three-period crossover design. For each pairwise comparison, patients were stratified into six groups according to the periods in which the two treatments of interest were administered (irrespective of the order in which the treatments were administered). The groups were paired if the treatments were administered in the same periods and the data analysed within each stratum using the method of KOCH [22] for a two-period crossover design. The overall test between two treatments was formed by pooling the three individual Wilcoxon statistics to derive an overall Wilcoxon statistic. All statistical tests were two-tailed and a p-value of <0.05 was considered significant.

Each 6-week treatment period directly followed the previous one. The following approach was adopted to test for carryover effects. For each patient, the parameter values for placebo, FP_{250} and SM_{50} were summed to give a total value. In the absence of carryover, it would be expected that all patients would have similar total values. The Kruskal-Wallis test was used to determine whether or not these total values were, on average, different between the six sequences in which the different treatments were administered.

Results

Clinical indices

There was a significant difference in the number of symptom-free nights and days between SM_{50} and placebo in favour of SM_{50} ($p<0.05$; data not shown). There were no significant differences between the two active and placebo treatments in respect of FEV_1, FVC or PEF (morning, evening and diurnal variation). Compared to placebo alone, salmeterol caused a significant improvement in the total inflammatory index (score of 5 reduced to 3; $p<0.05$). FP_{250} significantly reduced ($p<0.05$) and salmeterol showed a trend towards reduction ($p=0.06$) of the score for erythema (data not shown). Analysis of PC_{20} demonstrated that SM_{50} significantly reduced airway hyperresponsiveness compared with placebo and FP_{250} ($p=0.01$ and $p<0.05$, respectively). Prior to treatment, the geometric mean PC_{20} was 0.29 $mg \cdot mL^{-1}$; after 6 weeks of treatment with placebo, SM_{50} or FP_{250}, the values were 0.44, 1.26 and 0.55 $mg \cdot mL^{-1}$, respectively.

Biopsy

The results of the counts of inflammatory cells are shown in table 2. SM_{50} significantly reduced the numbers of neutrophil elastase-positive cells (*i.e.* neutrophils) compared to treatment with placebo

Table 2. – Effect of treatment on numbers of inflammatory cells in bronchial biopsy samples

	Placebo	SM_{50}	FP_{250}
Neutrophils	21.5 (0–127)	14.3 (0–47)*	18.4 (2–120)
EG1	11.8 (1–110)	7.0 (0–187)	5.5 (0–80)*
EG2	8.6 (2–197)	6.6 (0–139)	4.8 (0–40)
Mast cells	19.3 (0–136)	16.6 (0–191)	13.7 (0–99)
CD3	77.9 (6–206)	52.7 (27–140)	56.5 (12–105)*
CD4	36.4 (5–94)	30.2 (2–114)	17.5 (3–47)*,**
CD45RO	41.2 (3–162)	32.3 (9–75)	26.7 (3–63)*

Data are presented as median (range) number of cells per millimetre of reticular basement membrane. Treatment with salmeterol 50 μg powder *b.d.* (SM_{50}) significantly reduced the numbers of neutrophils, whereas fluticasone propionate 250 μg powder *b.d.* (FP_{250}) reduced numbers of eosinophils and lymphocytes compared with the placebo-treated group. EG: eosinophil granule. *: $p<0.05$ *versus* placebo; **: $p<0.01$ *versus* SM_{50}.

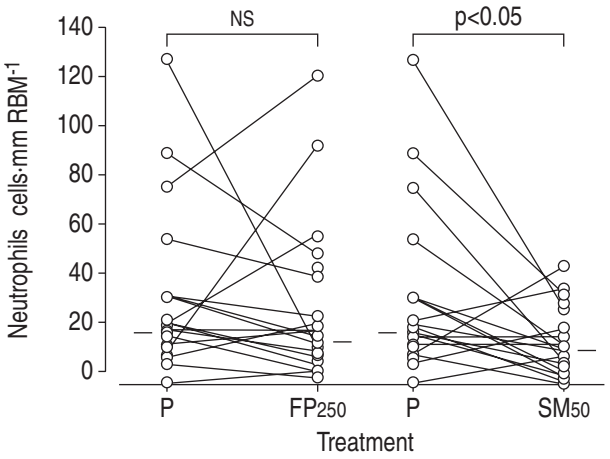

Fig. 1.–Numbers of neutrophils in bronchial biopsy samples. Treatment with salmeterol 50 μg powder *b.d.* (SM50) resulted in a significant decrease in numbers of neutrophils compared with the placebo (P)-treated group. The horizontal bars indicate medians. RBM: reticular basement membrane; FP250: fluticasone propionate 250 μg powder *b.d.*

(overall 34% reduction in median value; fig. 1). Compared to the placebo group, treatment with FP250 significantly reduced the numbers of CD3+, CD4+, CD45RO+ and EG1+ cells by 27, 50, 37 and 50%, respectively ($p<0.05$; fig. 2). The numbers of CD4+ cells were also significantly reduced after FP250 compared with the period in which SM50 was given. The numbers of cells expressing the gene for IL-4 were significantly lower in the SM50 group than in the FP250 group ($p<0.05$; fig. 3). There were no significant effects of FP250 on the number of cells expressing the genes for IL-5 or -2 or IFN-γ.

Lavage

In the bronchial lavage fluid, the percentage of neutrophils and level of HNL were lower in the SM50 group compared with the FP250 group (neutrophils: 1 (0–89) *versus* 4 (0–74)%; HNL: 33.5 (4–286) *versus* 60.1 (11–1437) $\mu g \cdot L^{-1}$; $p<0.05$ for both) but the SM50 values were not significantly different to those of placebo.

In BAL fluid, the concentration of each of the mediators was low and they were often undetectable. Treatment with SM50 was associated with reduced concentrations of sICAM-1 compared with FP250 (7.8 (20–110) *versus* 65.6 (24–144) $ng \cdot L^{-1}$; $p<0.05$) and a tendency to lower concentrations of HNL compared with placebo ($p=0.08$). In the SM50 group, there was a significant increase in the percentage of CD4+ T-cells compared with placebo (47 (30–82) *versus* 41 (21–76); $p<0.01$). FP250 was associated with a reduction in eosinophil chemotactic factor concentration compared with placebo (11 (1–28) *versus* 19 (1–56) units ($p<0.05$)).

Blood

Compared with placebo, SM50 caused significant reductions in the concentrations of both MPO (fig. 4) and sE-selectin in blood ($p<0.05$ for both). FP250, but not SM50, caused a significant reduction in the percentage of blood eosinophils ($p<0.05$; fig. 5). The results of analyses of serum for inflammatory markers of eosinophils (*i.e.* ECP) and neutrophils (MPO) and relevant soluble adhesion molecules, sICAM-1, sVCAM-1 and sE-selectin, are shown in table 3. FP250 caused a significant reduction in the level of sICAM-1 compared to placebo ($p=0.01$). There were no significant differences in the concentrations of EPO or HNL between groups (data not shown).

Carryover effect

The results of 105 tests for "carryover effect" showed that only two results were significantly different: the numbers of monocytes found in bronchial lavage fluid differential cell counts ($p=0.02$) and the number of macrophages in bronchial biopsy samples ($p=0.05$). Given that such a large number of such tests at the $p=0.05$ level would be expected to yield approximately five significantly different results in the absence of true carryover for any of the parameters tested, it was concluded that these were due not to carryover but to chance.

Discussion

The present study determined the effects of the inhaled long-acting β_2-agonist, salmeterol xinafoate (SM50), on the numbers of immunohistochemically distinct inflammatory cells in the bronchial mucosa of asthmatics and on cells and mediators in blood and bronchial lumina. The effects of SM50 treatment were compared with those of placebo or fluticasone propionate (FP250). As expected, FP250 given over the 6-week period significantly reduced numbers of (CD3+) T-lymphocytes, (CD4+) T-helper cells, (CD45RO+) antigen-primed T-cells and (EG1+) eosinophils. These results are in keeping with the already reported effects of inhaled corticosteroids used in the treatment of mild asthma. However, the antineutrophilic effects of SM50 are both novel and interesting, particularly as the asthmatics studied were stable and mild and exhibited relatively low levels of neutrophils and associated markers.

Neutrophil numbers increase in association with the early response to allergen challenge [4], in nocturnal asthma [6], during acute exacerbations [5, 23], and in occupational asthma [24], severe steroid-dependent persistent asthma [7, 25] and sudden fatal asthma [26]. There is also a report of increased neutrophil activation in asthma and a negative correlation of neutrophil numbers and PC20 [27], a finding investigated but not confirmed in the present study. Although inhaled corticosteroids are clearly effective in reducing eosinophil activation and numbers in asthma, they appear to have little effect on neutrophil activation [28]. Indeed, inhaled corticosteroids are reported to increase neutrophil longevity and numbers (*in vitro*) by inhibition of neutrophil apoptosis [29].

In contrast, the results of the present study

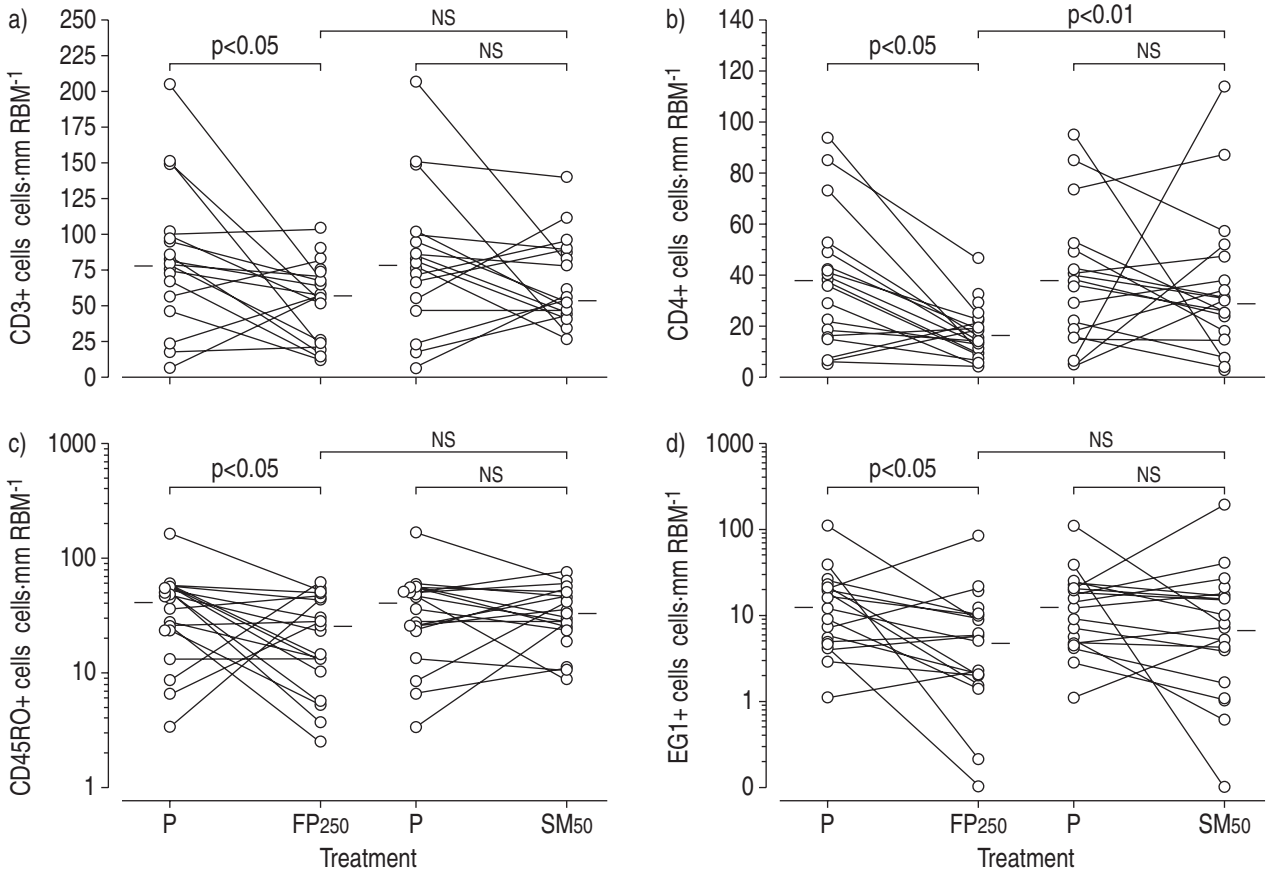

Fig. 2. – Numbers of: a) CD3+; b) CD4+; c) CD45RO+; and d) eosinophil granule (EG)1+ cells in bronchial biopsy samples. Treatment with fluticasone propionate 250 μg powder *b.d.* (FP_{250}) resulted in a significant decrease in the numbers of all of these cell types compared with the placebo (P)-treated group. The horizontal bars indicate medians. RBM: reticular basement membrane; SM_{50}: salmeterol 50 μg powder *b.d.*

demonstrate that salmeterol reduces the numbers of tissue neutrophils as well as the concentrations of blood-borne markers and mediators associated with neutrophil recruitment. Salmeterol has also been reported to reduce levels of IL-8, a key neutrophil chemoattractant, in the BAL fluid of asthmatics [30]; this provides one possible mechanism for the observed

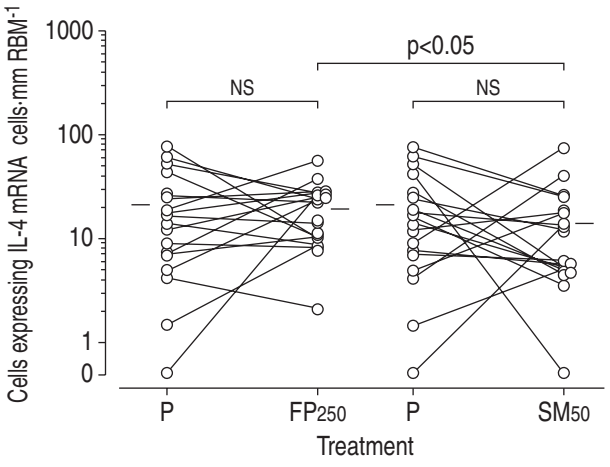

Fig. 3. – Numbers of cells expressing the gene encoding interleukin (IL)-4 in bronchial biopsy samples. Compared with fluticasone propionate 250 μg powder *b.d.* (FP_{250}), the number of cells containing IL-4 messenger ribonucleic acid (mRNA) was significantly lower after treatment with salmeterol 50 μg powder *b.d.* (SM_{50}). The horizontal bars indicate medians. RBM: reticular basement membrane; P: placebo.

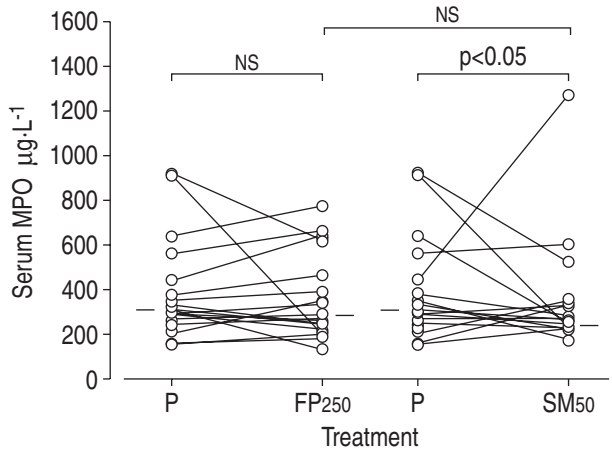

Fig. 4. – Neutrophil myeloperoxidase (MPO) concentration in serum, which reflects the number of neutrophils in blood. Treatment with salmeterol 50 μg powder *b.d.* (SM_{50}) resulted in a significant decrease in MPO concentration compared with placebo (P). The horizontal bars indicate medians. FP_{250}: fluticasone propionate 250 μg powder *b.d.*

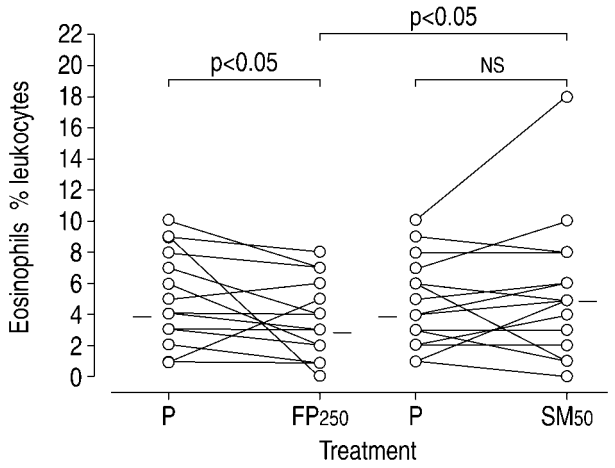

Fig. 5. – Numbers of eosinophils in blood relative to total leucocytes. Treatment with fluticasone propionate 250 μg powder *b.d.* (FP_{250}) resulted in a significant decrease in the percentage blood eosinophils compared with the placebo (P) or salmeterol 50 μg powder *b.d.* (SM_{50})-treated groups ($p<0.05$ for both). The horizontal bars indicate medians.

reduction in neutrophilia in the present study. *In vitro* and animal experimental studies provide further supportive evidence for the antineutrophilic effects of salmeterol. Salmeterol has been shown to induce apoptosis in neutrophils obtained from asthmatics [31], an effect that would be expected to result in reduced numbers of these cells. Salmeterol has also been shown to inhibit lipopolysaccharide-induced neutrophil accumulation in the lung of experimental animals [32]. Some of these effects are suggested to be due to mechanisms other than its β-agonist bronchodilator property [33, 34].

To the present authors' knowledge, the current report is the first demonstration of a reductive effect of salmeterol on neutrophil numbers in asthma. The experimental findings and results lead the present authors to speculate that the combination of a corticosteroid and a long-acting β_2-agonist might provide the added benefit of reducing both the eosinophilic and neutrophilic components of the inflammatory response in asthma, particularly the reported increase in neutrophil numbers associated with exacerbations and which develops in severe disease. The clinical benefits of such combination therapy are now accepted and include reduction in exacerbation frequency [35] and severity [36] and improvements in both lung function [37] and symptoms [38].

In conclusion, the present results demonstrate that the long-acting β_2-agonist, salmeterol, shows novel reductive effects on the numbers of neutrophils in mild asthma. The present authors speculate that this anti-inflammatory effect of salmeterol could usefully complement the effects of steroids in the treatment of asthma. Additionally, the use of long-acting β_2-agonists could also be considered for the prevention and treatment of inflammatory and obstructive conditions more often associated with tissue neutrophilia such as chronic obstructive pulmonary disease and cystic fibrosis. Further clinical studies are required to test these hypotheses.

Table 3. – Effects of treatment on concentrations of inflammatory markers in blood

	Placebo	SM_{50}	FP_{250}
MPO	302.0	249.0	286.0
$\mu g \cdot L^{-1}$	(149–915)	(166–1274)*	(134–759)
ECP	17.2	13.7	14.1
$\mu g \cdot L^{-1}$	(6–56)	(7–47)	(4–35)
sE-selectin	47.4	39.5	39.1
$ng \cdot L^{-1}$	(18–147)	(17–93)*	(19–103)
sICAM-1	260	261	248
$ng \cdot L^{-1}$	(176–452)	(195–469)	(187–534)*
sVCAM-1	565	533	518
$ng \cdot L^{-1}$	(389–681)	(345–766)	(355–798)$^{\#}$

Data are presented as median (range). Treatment with salmeterol 50 μg powder *b.d.* (SM_{50}) significantly reduced a neutrophil-related marker and relevant adhesion molecule, whereas fluticasone propionate 250 μg powder *b.d.* (FP_{250}) reduced levels of adhesion molecules important to the tissue accumulation of eosinophils. MPO: neutrophil myeloperoxidase; ECP: eosinophil cationic protein; sE-selectin: soluble E-selectin; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular cell adhesion molecule-1. *: $p<0.05$ *versus* placebo; $^{\#}$: $p<0.05$ *versus* SM_{50}.

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References

1. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000; 161: 1720–1745.
2. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001; 164: S28–S38.
3. Azzawi M, Bradley B, Jeffery PK, *et al.* Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis* 1990; 142: 1407–1413.
4. De Monchy JGR, Kauffman HF, Venge P, *et al.* Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am Rev Respir Dis* 1985; 131: 373–376.
5. Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995; 95: 843–852.
6. Martin RJ, Cicutto LC, Smith HR, Ballard RD, Szefler SJ. Airways inflammation in nocturnal asthma. *Am Rev Respir Dis* 1991; 143: 351–357.
7. Wenzel SE, Szefler SJ, Leung DYM, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high

dose glucocorticoids. *Am J Respir Crit Care Med* 1997; 156: 737–743.
8. Sears MR. Short-acting inhaled β-agonists: to be taken regularly or as needed? *Lancet* 2000; 355: 1658–1659.
9. Gauvreau GM, Jordana M, Watson RM, Cockcroft DW, O′Byrne PM. Effects of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects. *Am J Respir Crit Care Med* 1997; 156: 1738–1745.
10. Pedersen B, Dahl R, Larsen BB, Venge P. The effect of salmeterol on the early- and late-phase reaction to bronchial allergen and postchallenge variation in bronchial reactivity, blood eosinophils, serum eosinophil cationic protein, and serum eosinophil protein X. *Allergy* 1993; 48: 377–382.
11. Dente FL, Bancalari L, Bacci E, *et al.* Effect of a single dose of salmeterol on the increase in airway eosinophils induced by allergen challenge in asthmatic airways. *Thorax* 1999; 54: 622–624.
12. Roberts JA, Bradding P, Britten KM, *et al.* The long-acting β_2-agonist salmeterol xinafoate: effects on airway inflammation in asthma. *Eur Respir J* 1999; 14: 275–282.
13. Wallin A, Sandstrom T, Soderberg M, *et al.* The effects of regular inhaled formoterol, budesonide, and placebo on mucosal inflammation and clinical indices in mild asthma. *Am J Respir Crit Care Med* 1999; 159: 79–86.
14. Li X, Ward C, Thien F, *et al.* An antiinflammatory effect of salmeterol, a long-acting β2-agonist, assessed in airway biopsies and bronchoalveolar lavage in asthma. *Am J Respir Crit Care Med* 1999; 160: 1493–1499.
15. Boulet LP, Chakir J, Milot J, Boutet M, Laviolette M. Effect of salmeterol on allergen-induced airway inflammation in mild allergic asthma. *Clin Exp Allergy* 2001; 31: 430–437.
16. Juniper EF, Frith PA, Dunnet C, Cockcroft DW, Hargreave FE. Reproducibility and comparability of responses to inhaled histamine and methacholine. *Thorax* 1978; 33: 705–710.
17. Thompson AB, Daughton D, Robbins RA, Ghafouri MA, Oehlerking M, Rennard SI. Intraluminal airway inflammation in chronic bronchitis. Characterization and correlation with clinical parameters. *Am Rev Respir Dis* 1989; 140: 1527–1537.
18. Hamid QA, Corrin B, Dewar A, Hoefler H, Sheppard MN. Expression of gastrin-releasing peptide (human bombesin) gene in large cell undifferentiated carcinoma of the lung. *J Pathol* 1990; 161: 145–151.
19. Carlson MGC, Peterson CGB, Venge P. Human eosinophil peroxidase: purification and characterisation. *J Immunol* 1985; 134: 1875–1879.
20. Xu SY, Petersson CG, Carlson M, Venge P. The development of an assay for human neutrophil lipocalin (HNL) to be used as a specific marker of neutrophil activity *in vivo* and *vitro*. *J Immunol Methods* 1994; 171: 245–252.
21. Richmond I, Booth H, Ward C, Walters EH. Intrasubject variability in the airway inflammation in biopsies in mild to moderate stable asthma. *Am J Respir Crit Care Med* 1996; 153: 899–903.
22. Koch GG. The use of non-parametric methods in the statistical analysis of the two-period change-over design. *Biometrics* 1972; 28: 577–584.
23. Lamblin C, Gosset P, Tillie-Leblond I, *et al.* Bronchial neutrophilia in patients with noninfectious status asthmaticus. *Am J Respir Crit Care Med* 1998; 157: 394–402.
24. Fabbri LM, Boschetto P, Zocca E, *et al.* Bronchoalveolar neutrophilia during late asthmatic reactions induced by toluene diisocyanate. *Am Rev Respir Dis* 1987; 136: 36–42.
25. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999; 160: 1532–1539.
26. Sur S, Crotty TB, Kephart GM, *et al.* Sudden onset fatal asthma: a distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa? *Am Rev Respir Dis* 1993; 148: 713–719.
27. Kelly C, Ward C, Stenton CS, Bird G, Hendrick DJ, Walters EH. Number and activity of inflammatory cells in bronchoalveolar lavage fluid in asthma and their relation to airway responsiveness. *Thorax* 1988; 43: 684–692.
28. Schleimer RP, Freeland HS, Peters SP, Brown KE, Derse CP. An assessment of the effects of glucocorticoids on degranulation, chemotaxis, binding to vascular endothelium and formation of leukotriene B_4 by purified human neutrophils. *J Pharmacol Exp Ther* 1989; 250: 598–605.
29. Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J Immunol* 1995; 154: 4719–4725.
30. Ward C, Li X, Wang N, *et al.* Salmeterol reduces BAL IL-8 levels in asthmatics on low dose inhaled corticosteroids. *Eur Respir J* 1998; 12: Suppl. 28, s380.
31. Lee E, Smith J, Robertson T, Reynolds P, Opesan K, Kilfeather SA. Salmeterol and inhibitors of phosphodiesterase 4 (PDE4) induce apoptosis in neutrophils from asthmatics: β-adrenergic receptor-mediated salmeterol activity and additive effects with PDE4 inhibitors. *Am J Respir Crit Care Med* 1999; 159: A329.
32. Whelan CJ, Johnston M, Vardey CJ. Comparison of the anti-inflammatory properties of formoterol, salbutamol and salmeterol in guinea-pig skin and lung. *Br J Pharmacol* 1993; 110: 613–618.
33. Anderson R, Feldman C, Theron AJ, Ramafi G, Cole PJ, Wilson R. Ani-inflammatory, membrane-stabilizing interactions of salmeterol with human neutrophils *in vitro*. *Br J Pharmacol* 1996; 117: 1387–1394.
34. Bloemen PG, van den Tweed MC, Henricks PA, *et al.* Increased cAMP levels in stimulated neutrophils inhibit their adhesion to human bronchial epithelial cells. *Am J Physiol* 1997; 272: L580–L587.
35. Pauwels RA, Lofdahl C-G, Postma DS, *et al.* Effect of inhaled formoterol and budesonide on exacerbations of asthma. *N Engl J Med* 1997; 337: 1405–1411.
36. Tattersfield AE, Postma DS, Barnes PJ, *et al.* Exacerbations of asthma. *Am J Respir Crit Care Med* 1999; 160: 594–599.
37. Greening AP, Ind PW, Northfield M, Shaw G and on behalf of Allen & Hanburys Limited UK Study Group. Added salmeterol *versus* higher-dose corticosteroid in asthma patients with symptoms on existing inhaled corticosteroid. *Lancet* 1994; 344: 219–224.
38. Woolcock A, Lund J, Ringler DJ, Jacques M. Comparison of addition of salmeterol to inhaled steroids with doubling of the dose of inhaled steroid. *Am J Respir Crit Care Med* 1996; 153: 1481–1488.