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Effect of salmeterol compared with beclomethasone on allergen-induced asthmatic and inflammatory responses

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ABSTRACT: Salmeterol is a selective long-acting β_2-agonist bronchodilator considered to have added anti-inflammatory effects, but this is controversial. We investigated the effects of a single dose of salmeterol, 100 µg, on the physiological and inflammatory responses to inhaled allergen and compared these with the effects of a single dose of beclomethasone, 500 µg, and of placebo.

Eight atopic adults with mild stable asthma, treated only with inhaled short-acting β_2-agonist when needed, attended the laboratory sequentially for screening tests, two single-blind control inhalation tests preceded 30 min by placebo or salmeterol and three allergen inhalation tests preceded by placebo, salmeterol or beclomethasone double-blind in random order. Airway responsiveness to methacholine (assessed as the provocative concentration of methacholine producing 20% fall in forced expiratory volume in one second (PC_{20})), induced sputum eosinophils, blood eosinophils and serum eosinophil cationic protein (ECP) were examined before and 7–48 h after treatment. The statistical power to detect twofold changes in blood and sputum parameters was ≥90%.

Salmeterol inhaled before allergen challenge completely prevented the early asthmatic response, late asthmatic response and fall in methacholine PC_{20} at 24 h, and produced additional bronchodilatation. These effects were similar to those obtained by the inhalation of a single dose of salmeterol before the control inhalation test, and significantly better than those observed after a single dose of beclomethasone inhaled before the allergen test. Beclomethasone had no effect on the early asthmatic response or on the fall in methacholine PC_{20} at 24 h but partially inhibited the late asthmatic response. Neither salmeterol nor beclomethasone had any significant effect on sputum or blood inflammatory changes 7–48h after allergen inhalation.

In conclusion, whilst salmeterol had no demonstrable anti-inflammatory action in sputum after allergen challenge in asthma, neither did a single dose of the positive anti-inflammatory control, beclomethasone. The latter result excludes a more positive judgement on the possible anti-inflammatory action of salmeterol. However, the results do indicate that potent functional effects of a single dose of salmeterol can mask the airway inflammatory cell influx caused by inhaled allergen.

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Salmeterol is a selective long-acting β_2-agonist bronchodilator. *In vitro*, salmeterol is approximately four to sixfold more potent than salbutamol and its maximal bronchodilatation lasts twice as long [1]. In asthmatic subjects, a single inhaled dose causes prolonged bronchodilatation [2] and inhibits bronchoconstriction caused by methacholine [3], histamine [4], exercise [5] or hyperventilation of cold air [6] for at least 12 h.

Salmeterol may also have anti-inflammatory properties. *In vitro*, it inhibits the release of histamine, leukotrienes, prostaglandin D_2 [7], and thromboxane B_2 [8]. In guinea-pigs, it prevents lung neutrophil accumulation by lipopolysaccharide and eosinophil accumulation by platelet-activating factor [9]. In asthmatics, it prevents the allergen-induced early asthmatic response and attenuates the allergen-induced late asthmatic response and heightening of nonallergic airway responsiveness [10–13]. The effects on the late response and airway responsiveness were reported by TWENTYMAN *et al.* [10] to provide indirect evidence of its anti-inflammatory properties. However, these responses depend upon airway muscle constriction, and this interpretation was criticized because of inadequate control for functional changes in forced expiratory volume in one second (FEV_1) [14]. Subsequent studies, using blood and urinary markers to assess indirectly the effects of a single dose of salmeterol on inflammatory

Table 1. – Characteristics of subjects at screening period

	Baseline measurements					Screening period				
Sub No.	Age yrs	Sex	FEV_1 % pred	FEV_1/VC	$PC_{20}M$ $mg \cdot mL^{-1}$	Allergen/dilution		EAR %	LAR %	$PC_{20}M^*$ $mg \cdot mL^{-1}$
1	25	F	95	74	9.4	Ragweed	1:1024	-7.9	-18.2	1.0
2	26	F	94	82	12.0	Cat	1:16	-19.1	-25.8	2.9
3	23	F	82	73	0.8	*D. farinae*	1:512	-23.3	-19.0	0.2
4	41	F	110	70	7.7	*D. farinae*	1:16	-16.7	-26.4	1.1
5	58	F	89	70	6.7	*D. pteronyssinus*	1:16	-21.6	-21.6	2.9
6	21	F	88	70	6.2	*D. farinae*	1:32	-28.5	-16.0	1.9
7	24	F	87	85	2.3	*D. farinae*	1:16	-19.2	-17.6	0.5
8	27	M	81	70	2.2	Cat	1:32	-22.7	-25.8	0.7

Sub: subject; F: female; M: male; FEV_1: forced expiratory volume in one second, expressed as a percentage of predicted values (% pred) [15]; VC: vital capacity; $PC_{20}M$: provocative concentration of methacholine causing a 20% fall in FEV_1; EAR: early asthmatic response; LAR: late asthmatic response; Allergen dilution: to provoke early and late asthmatic responses; $PC_{20}M^*$: PC_{20} methacholine 24 h after allergen test; *D. farinae*: *Dermatophagoides farinae*; *D. pteronyssinus*: *Dermatophagoides pteronyssinus*.

changes induced by allergen inhalation, were unable to demonstrate any significant anti-inflammatory effects [12, 13].

We have investigated the protective effects of a single dose of salmeterol on the physiological and inflammatory responses to inhaled allergen in a double-blind cross-over study. There were two novel features in the study design. The first was to include a comparison with the effect of beclomethasone as a positive anti-inflammatory control in addition to placebo. The second was to assess the anti-inflammatory effects of the drugs directly by measurement of the proportion of eosinophils in induced sputum (as well as on peripheral blood eosinophil count and serum eosinophil cationic protein (ECP) concentration).

Subjects and methods

Subjects

Eight adults with asthma were recruited from the Firestone Regional Chest and Allergy Clinic and from responders to an advertisement (table 1). The asthma was mild, as indicated by little or no symptoms, treatment only with inhaled β_2-agonist when needed, an FEV_1 of at least 70% predicted, and methacholine airway responsiveness within the moderately increased to borderline normal range. The asthma was stable; there had been no exacerbations or need for other treatment for 1 month. All subjects were studied out of their allergen season and none had had a respiratory infection for 1 month. Two subjects were ex-smokers. The study was approved by the hospital Research Committee and all subjects gave written informed consent.

Study design

Subjects attended the laboratory sequentially for a screening period to select appropriate individuals, two control inhalation test periods preceded by placebo and salmeterol 100 µg given single blind and not randomized, and then three allergen inhalation test periods preceded by placebo, salmeterol, 100 µg, and beclomethasone, 500 µg, given in a randomized, double-blind, cross-over design (fig. 1). The time between the screening allergen test and

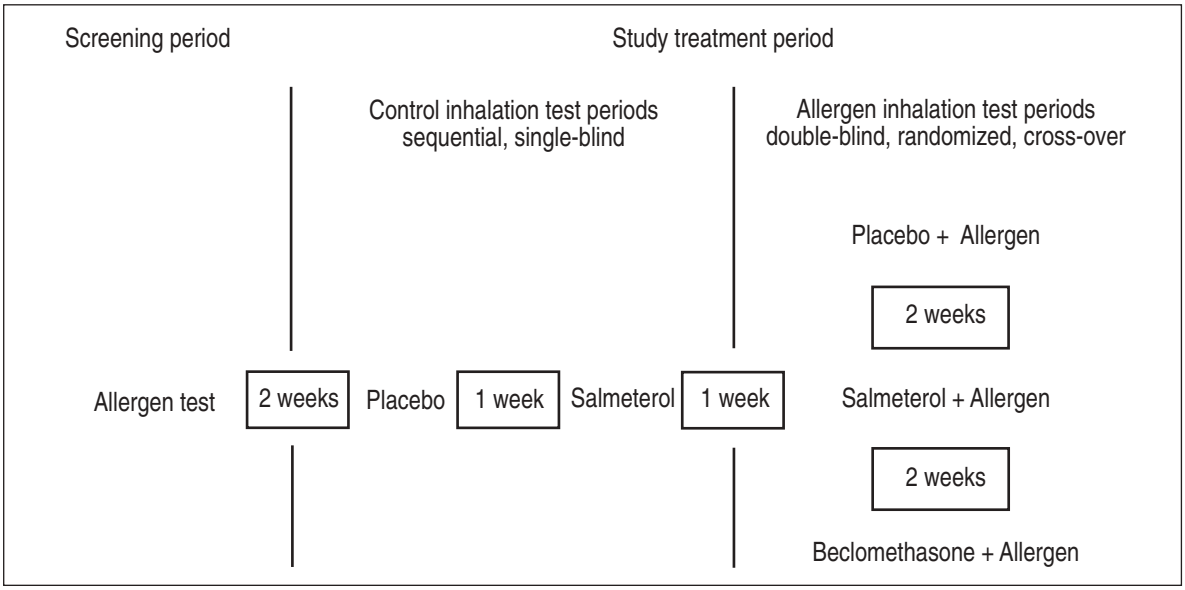

Fig. 1. – Study design.

the first control test and between each allergen test was at least 14 days or as long as necessary for the provocative concentration of the methacholine producing a 20% fall in FEV_1 (PC_{20}) to return to within a onefold dilution of the baseline measurement. The time between the second control test and the first allergen period was at least a week.

In the screening period, subjects were seen on 3 days. On the first day, a questionnaire of symptoms, methacholine inhalation test, and allergy skin prick tests were performed. On the second day, an allergen inhalation test was carried out to identify the dose of allergen required to cause early and late asthmatic responses. On the third day, a methacholine inhalation test was performed 24 h after the allergen test. Each of the next five periods consisted of 4 days. On the first, third and fourth day, a methacholine inhalation test was performed between 7 and 9 a.m., and peripheral blood was obtained for total and differential white cell count and ECP level. Sputum was then induced with hypertonic saline, and cell counts were examined blind to the airway measurements. On the second day of the two control periods, the subjects inhaled placebo (two puffs) in the first period, and salmeterol in the second period, 30 min before each saline control inhalation test. All medications were inhaled through a Nebuhaler® (A.B. Draco, Lund, Sweden) to minimize unblinding by taste. On the second day of the final three periods, subjects inhaled either salmeterol, beclomethasone or placebo from pressurized metered-dose inhalers 30 min before allergen inhalation tests. Each puff was inhaled from residual volume, slowly over a minimum of 5 s to near total lung capacity (TLC) and the breath was held for 10 s. On the second day, sputum was also induced after 7 h.

Airways measurements, provocation tests and sputum induction

Spirometry was performed with a dry, rolling-seal spirometer (PK 131 Morgan Spiroflow Spirometer; Roxon Medi-Tech, Rexdale, Ontario, Canada). Baseline measurements of FEV_1 and vital capacity (VC) were made according to American Thoracic Society (ATS) criteria [16], and reference values were taken from CRAPO *et al.* [17]. Methacholine inhalation tests were carried out using the method described by JUNIPER *et al.* [18], and the results were expressed as the PC_{20} in noncumulative units. Allergy skin tests were performed using the modified prick technique [19] with 17 common allergen extracts. Twofold dilutions of the extract selected for inhalation tests were also used to identify the lowest concentration to cause a 2 mm wheal. This concentration together with the methacholine PC_{20} was used to estimate the allergen PC_{20} and the starting concentration for allergen inhalation was fourfold concentrations below this [20]. Control and allergen inhalation tests were performed, in an exposure chamber exausted to the outside, by the method described by COCKCROFT *et al.* [20] with modifications [21]. Sputum induction was performed with an aerosol of hypertonic saline as described by PIN *et al.* [22].

Sputum examination. The collected sputum samples were examined within 2 h as described by POPOV *et al.* [23]. One cytospin was fixed by methanol and stained by the Wright method for an overall differential cell count on at least 200 nonsquamous cells. Two other cytospins were fixed in Carnoy's fixative and stained with toluidine blue and 1,500 nonsquamous cells were counted for metachromatic cells.

Blood tests. Peripheral blood samples were collected into a 5.0 mL ethylenediamine tetra-acetic acid (EDTA) (K_3 Vacutainer BD, Rutherford, NJ, USA). A differential white cell count was obtained using Coulter STKS (Coulter Corp. Hialeah, Fl, USA). Serum was collected after blood coagulation for 1 h at room temperature. It was centrifuged at 20°C at 1,500 rpm for 10 min and stored at -20°C until analysis. Serum ECP levels were determined by radioimmunoassay using the procedure described by the manufacturer (Pharmacia, Sweden).

Data analysis

Sample size was estimated for an alpha specification of 0.05 and a beta specification of 0.10 (90% power). Based on our data on repeatability of blood and sputum eosinophils and serum ECP in the baseline period, these had 90% power to detect a twofold change in sputum eosinophils and 93% power to detect a twofold change in blood absolute eosinophil counts and serum ECP level. Descriptive statistics were used to summarize clinical and demographic characteristics of the sample. Grouped data were reported as the arithmetic mean±SEM. PC_{20} data were log-transformed and reported as geometric mean (GM)± geometric standard deviation (GSD). Dependent variables with non-normal distribution were log (blood eosinophils and ECP) or square root (sputum eosinophils) transformed before analysis and reported as GM±GSD. Physiological measurements and inflammatory indices in sputum and blood were compared between salmeterol (S)/control (C) salmeterol/allergen (A) periods and between placebo (P)/allergen *versus* beclomethasone (B)/allergen periods. Physiological indices were also compared between salmeterol/control *versus* placebo/control and between salmeterol/allergen *versus* placebo/allergen periods. Repeated measures analysis of variance (ANOVA) was used in a model to analyse the effects of two independent variables (time and treatment) on dependent variables (FEV_1, methacholine PC_{20} and inflammatory indices). Significance was accepted at the level of 95% and the source of significant variation was identified by the Student-Neuwman Kuels procedure [24]. Paired t-tests were used for within period comparisons from baseline to early and late and asthmatic response. The intraclass correlation coefficient (ICC) was used as the measure of reliability [25].

Results

Effects on FEV_1

Salmeterol inhaled before the control inhalation test caused a mean±SD increase in FEV_1 from baseline of

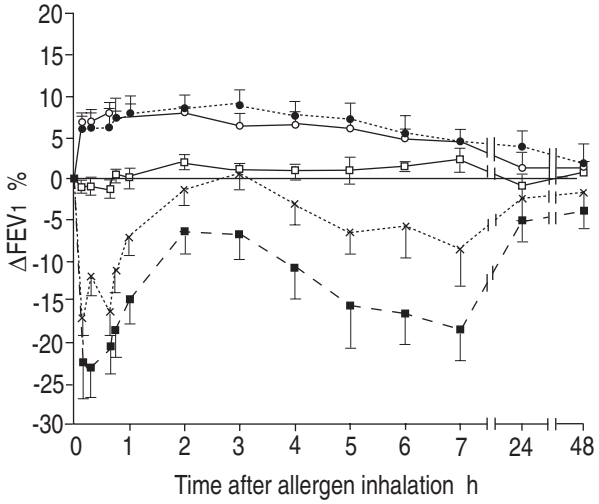

Fig. 2. – Changes in FEV_1 (ΔFEV_1) from baseline after control (C) or allergen (A) inhalation preceded by placebo (P), salmeterol (S) or beclomethasone (B). Salmeterol completely inhibited the early and late asthmatic responses and caused bronchodilation. Beclomethasone inhibited the late asthmatic response. Values are presented as mean±SEM. : S/A; : S/C; : P/C; : B/A; : P/A. FEV_1: forced expiratory volume in one second.

6.9±2.4% within 10 min (fig. 2). The FEV_1 remained significantly greater than placebo before the control test for up to 5 h (p=0.04), but it was not significantly different at 24 h (2.3±4.5%) or 48 h (-0.3±4.5%). Allergen inhalation preceded by placebo caused a fall in FEV_1 during the early response of 22.3±11.9% (p=0.004) and during the late response of 18.5±10.7% (p=0.007). These results were similar to those in the screening allergen test (table 1), the coefficient of reliability (ICC) being 0.64 for FEV_1 at 10 min and 0.81 for FEV_1 at 7 h after antigen inhalation. Salmeterol before the same dose of allergen completely inhibited the early and the late asthmatic response (fig. 2), (S/A *vs* P/A, p=0.007 for both early and late responses) and caused additional bronchodilatation which was still present 24 h after allergen inhalation (S/C *vs* P/A, p=0.003 and S/A *vs* P/A, p=0.003, respectively). Even when compared with salmeterol before the control inhalation test, to adjust for its bronchodilator and other possible effects, salmeterol completely prevented the early and the late asthmatic response. Beclomethasone had no significant effect on the allergen-induced early response (fall in FEV_1, of 16.8±7.6%) but partially inhibited the late response, the fall in FEV_1 being 9.9±9.4% (B/A *vs* P/A, p=0.007).

Effects on methacholine responsiveness

The geometric mean±GSD baseline methacholine PC_{20} $mg \cdot mL^{-1}$ was not different between treatment periods (table 2). Salmeterol inhaled before the control inhalation test caused an increase in methacholine PC_{20} at 24 h of 2.5±1.7 fold (p=0.01) (fig. 3) and returned to 1.2±0.7 fold above baseline at 48 h (fig. 3). When placebo treatment was given before allergen inhalation, the methacholine

Table 2. – Effects of treatment on methacholine PC_{20} and on inflammatory indices in blood after control or allergen inhalation

	Baseline	24 h	48 h
Methacholine PC_{20} $mg \cdot mL^{-1}$			
Placebo/control	3.5±2.3	3.5±2.5	4.4±2.3
Salmeterol/control	3.3±2.3	7.6±1.3	4.6±1.4
Placebo/allergen	3.5±2.3	2.0±2.9	3.2±2.6
Salmeterol/allergen	3.6±2.6	9.3±2.6	5.2±2.6
Beclomethasone/allergen	3.5±1.9	2.2±2.5	3.7±2.1
Blood eosinophils $\times 10^6 \cdot L^{-1}$			
Placebo/control	282±1.2	247±1.2	245±1.2
Salmeterol/control	234±1.2	229±1.5	224±1.2
Placebo/allergen	316±1.3	550±1.3	457±1.3
Salmeterol/allergen	257±1.4	371±1.8	389±1.2
Beclomethasone/allergen	251±1.8	457±1.8	426±1.2
Serum ECP $\mu g \cdot mL^{-1}$			
Placebo/control	8.7±1.5	12.5±1.9	11.0±2.3
Salmeterol/control	6.8±1.7	9.5±2.1	8.5±2.3
Placebo/allergen	7.5±1.7	15.8±1.9	12.9±1.7
Salmeterol/allergen	6.8±1.5	16.9±1.9	17.8±2.2
Beclomethasone/allergen	7.9±1.7	15.1±1.7	13.8±1.9

Data are expressed as geometric mean±GSD. ECP: eosinophil cationic protein; PC_{20}: provocative concentration of methacholine causing a 20% fall in forced expiratory volume in one second.

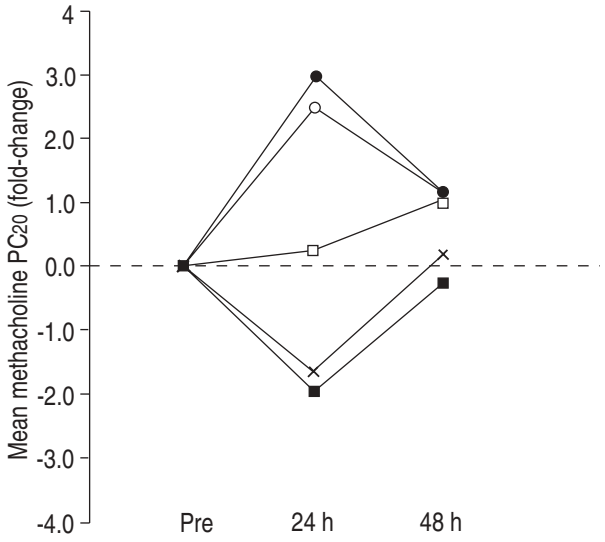

Fig. 3. – Geometric mean "fold" change in methacholine PC_{20} for each study period, standardized to baseline. Salmeterol completely inhibited the effect of allergen after 24 h, whilst beclomethasone had no effect. : S/A; : S/C; : P/C; : B/A; : P/A. PC_{20}: provocative concentration of methacholine causing a 20% fall in FEV_1. For further abbreviations see legend to figure 2.

PC_{20} at 24 h decreased 1.9±0.8 fold (p=0.01) and returned to baseline values after 48 h. Salmeterol inhalation before inhaled allergen not only inhibited the decrease in methacholine PC_{20} but also caused a mean increase at 24 h of 3.0±1.7 fold (p=0.007), which was similar to the increase caused by inhalation of salmeterol before the control inhalation test (2.5±1.2 fold). Beclomethasone had no effect on the allergen-induced fall in methacholine PC_{20} at 24 h of 1.7±1.9 fold.

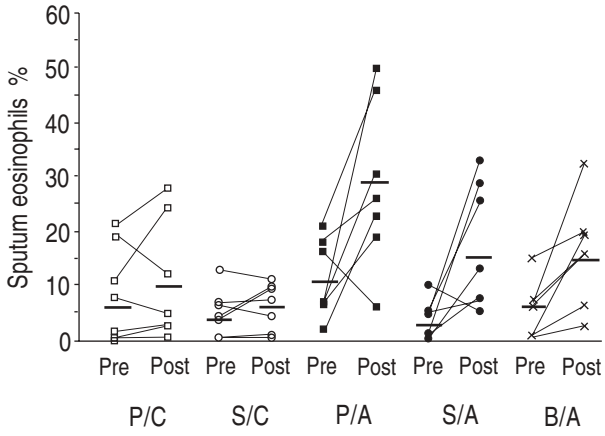

Fig. 4. – Individual values and means (horizontal bars) of sputum differential eosinophil count before and within 48 h of each treatment period. Comparison between periods showed an increase in sputum eosinophils after S/A in comparison with S/C (p=0.05), P/A *vs* P/C (p=0.04), and B/A *vs* P/C (p=0.06). For abbreviations see legend to figure 2.

Effects on inflammatory indices

Sputum was successfully obtained in 109 of 140 (78%) inductions. Seven of the eight subjects produced suitable sputum in all baseline periods. Two of these seven subjects failed to produce sputum at all times (7, 24 and 48 h). Therefore, examination of the changes was made between baseline and the highest value; 30 out of 35 of these values were at 24 h.

The mean baseline sputum eosinophil differential count was similar between the different study periods with the exception of the placebo allergen day when it was higher (fig. 4). When placebo was given before allergen, the eosinophil counts increased (from 10±1.3 to 26.6±2.4%; p=0.03) and this was not reduced by pretreatment with salmeterol (2.6±1.5 to 15.7±4.9%; p=0.02) or beclomethasone (4.0±1.3 to 15.1±4.8%; p=0.01) before allergen. The mean baseline sputum differential metachromatic cell count was similar in each period and did not change after the control inhalations tests (from 0.1±0.1 to 0.2±0.3% when preceded by placebo, and from 0.1±0.1 to 0.2±0.2% when preceded by salmeterol). When placebo was given before allergen the metachromatic cell counts increased (from 0.2±0.2 to 1.0±1.0%; p=0.01) and this was not reduced by pretreatment with salmeterol (0.3±0.4 to 1.1± 1.0%; p=0.01) or beclomethasone (0.1±0.1 to 0.8±0.8%; p=0.01). The sputum differential counts of neutrophils, macrophages and lymphocytes were not significantly changed by any of the inhalation tests.

The mean baseline blood absolute eosinophil count and serum ECP were similar in the different baseline periods (table 2). Placebo pretreatment with subsequent allergen increased both measurements at 24 h (p=0.008 for blood eosinophils and p=0.04 for serum ECP) and this increase was not reduced by pretreatment with salmeterol or beclomethasone before allergen. The blood eosinophils and serum ECP were still elevated after 48 h.

Discussion

The results of this study demonstrate that a single dose of salmeterol (100 µg) inhaled before allergen challenge completely prevented the early asthmatic response, late asthmatic response and fall in methacholine PC_{20} at 24 h, and produced additional bronchodilatation. These effects were similar to those obtained by the inhalation of salmeterol before the control inhalation test, and significantly better than beclomethasone (500 µg) inhaled before the allergen test. Beclomethasone had no effect on the early asthmatic response or on the fall in methacholine PC_{20} at 24 h but partially inhibited the late asthmatic response. Neither salmeterol nor beclomethasone had any significant effect on blood or sputum inflammatory changes as measured by sputum eosinophils and metachromatic cells, blood eosinophils or serum ECP 24 h after antigen inhalation. Therefore, no anti-inflammatory action of salmeterol was demonstrated. These results indicate that the functional effects of a single dose of salmeterol can mask the airway cellular infiltration caused by inhaled allergen.

The complete protection by a single dose of salmeterol on the allergen-induced early asthmatic response was similar to earlier studies using a single dose of inhaled salmeterol [10, 13], or formoterol [21]. The complete protection on the late asthmatic response and the heightened methacholine airway responsiveness at 24 h, even when the bronchodilator effect was considered, was better than earlier studies. The reason for the better effect may be the twofold higher dose of salmeterol used in this study. In contrast, a single dose of beclomethasone, also twice as high as in earlier studies [21], was less effective than earlier studies in inhibiting the heightening of methacholine airway responsiveness. The explanation for this result is less obvious. It does not seem to be due to a difference in the severity of the inflammatory component as judged by the increase in sputum or peripheral blood eosinophils or serum ECP.

This is the second study to investigate the anti-inflammatory effect of drugs on allergen-induced airway responses by using examination of induced sputum. The use of induced sputum was not entirely successful, since adequate specimens could not be obtained on each occasion. Successful induction is partly dependent on aspects of the induction process and partly on methods of processing the sputum [23, 26]. In future studies, failed attempts at induction should be repeated within hours of the measurement in an effort to avoid missing data. The method of processing sputum that we use is still evolving. The method that we used in this study is the one evaluated by POPOV *et al.* [23] and, subsequent to the study, has been further developed to include fluid phase measurements, such as ECP [27]. The method is highly repeatable, responsive and valid for cell counts as performed in this study. In the study, sputum eosinophils and metachromatic cells increased in association with allergen-induced late asthmatic responses, as has been reported in earlier studies [15, 28]. However, salmeterol did not inhibit the allergen-induced increase in eosinophils, which is similar to the observation made by WONG *et al.* [21] in the previous study using formoterol.

We also examined the anti-inflammatory effects of salmeterol indirectly by changes in peripheral blood eosinophil count and serum ECP levels. Despite adequate statistical power our results failed to demonstrate any inhibition of the allergen-induced increase in eosinophils or ECP. This result was similar to the study by WEERSINK *et al*. [13], who examined the effect of salmeterol (50 µg), and the study by WONG *et al*. [21] with formoterol (24 µg). They partly differ from the results of PEDERSEN *et al*. [12], who found that salmeterol (50 and 100 µg) did not inhibit the allergen-induced rise in blood eosinophils but prevented the rise in serum ECP.

An unexpected observation in the present study was that beclomethasone (500 µg) before allergen inhalation failed to inhibit the allergen-induced rise in sputum eosinophils and metachromatic cells, blood eosinophils or serum ECP. This lack of inhibition by beclomethasone calls into question the significance of the lack of anti-inflammatory effect of salmeterol. However, when inhaled steroid is given regularly for 1 week or more it does inhibit the allergen-induced rise in airway and blood eosinophils [29]. Perhaps possible anti-inflammatory effects of long-acting β-agonists should similarly be studied after the drug has been given regularly for a number of days or, perhaps, by measurement of other inflammatory indices. In this regard DAHL *et al*. [30] compared the effects of 4 weeks of treatment with salmeterol (100 µg) with placebo in 12 mild asthmatics. Salmeterol improved the appearance of the inflamed airway mucosa as seen during bronchoscopy and lowered the bronchoalveolar fluid ECP levels. In contrast, GARDINER *et al*. [31] found that 8 weeks of treatment with salmeterol in nine asthmatics receiving maintenance inhaled steroid improved the peak expiratory flows, but had no effect on bronchoalveolar lavage cell profile or on proportion of activated lymphocytes. BOULET *et al*. [32] found no effect of 9 weeks of treatment with salmeterol (100 µg) on baseline bronchoalveolar lavage and bronchial biopsy measurements (total and differential cell counts and markers of eosinophil and lymphocytes activation studied by immunohistochemistry) or on the effects of allergen inhalation.

We conclude that the results of the present study confirm that salmeterol is a highly effective bronchodilator and protector against bronchoconstriction. The effect of a single dose 15 min before allergen inhalation did not produce measurable inhibition of allergen-induced airway inflammatory cell infiltration as indicated by sputum eosinophils, blood eosinophils and serum ECP. However, neither did a single dose of beclomethasone demonstrate these anti-inflammatory effects. These results indicate that the physiological effects of salmeterol can mask inflammatory cell infiltration but they do not exclude an anti-inflammatory effect of this drug. In future, the anti-inflammatory effects of drugs should be examined after the drug has been given regularly rather than in a single dose or when other inflammatory indices are measured.

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