RESPIRATORY MEDICINE

The effects of regular inhaled formoterol and budesonide on preformed Th-2 cytokines in mild asthmatics

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Abstract In a recent placebo-controlled study in mild atopic asthmatics, we observed a significant decrease in eosinophils in the bronchial submucosa, after 2 months of treatment with inhaled formoterol and budesonide. Biopsy material from each treatment group; formoterol (24 μg bid), budesonide (400 μg b.i.d.) and placebo has been further assessed to investigate the role of Th-2 cytokines by immunohistochemistry using Mabs to eosinophils as an index of inflammation, IL-4 and IL-5. Treatment with formoterol significantly reduced the number of eosinophils (EG2+) in the submucosa and epithelium, but this was not paralleled by changes in cytokine immunoreactivity. In contrast, treatment with budesonide significantly reduced both the number of eosinophils (EG2+) and immunoreactivity for IL-4 and IL-5 in the submucosa. Thus, while budesonide has effects on cytokines involved in eosinophil recruitment this explanation does not apply to the eosinopaenia observed with the long-acting β2 adrenoreceptor agonist formoterol.

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INTRODUCTION

In many types of asthma, the Th-2 like T-lymphocytes orchestrate the recruitment, migration and activation of eosinophils and mast cells in the airway wall by the secretion of cytokines encoded in the interleukin (IL)-4 gene cluster, specifically IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte macrophage colony stimulating factor (GM-CSF) (1–3). Both oral and inhaled corticosteroids reduce the influx, activation and survival of inflammatory cells in the airway wall by reducing the production of Th-2 cytokines (4,5). Formoterol by inhalation is a potent and highly selective β_2-adrenoreceptor agonist (6,7) with rapid onset and prolonged duration of action of at least 12 h (8,9). Asthma management guidelines recommend that the long-acting β_2 adrenoreceptor-agonists, formoterol and salmeterol, can be given twice daily to supplement inhaled corticosteroids when these drugs at moderate to high doses fail to control asthma (10). Addition of these long-acting β_2-adrenoreceptor agonists improves symptoms and lung function (11) reduces asthma exacerbations (12,13) and enhances asthma-related quality of life (14) beyond that achieved by further increasing the dose of inhaled corticosteroid. While part of this response maybe explained by the bronchodilator action of this drug class, it has also been shown that they inhibit eosinophil activation (15), IL-8 and eotaxin release (16,17), microvascular leakage (18) and influx of inflammatory cells (19,20).

In an earlier study, investigating the effects of 8 weeks treatment of asthmatic subjects with inhaled formoterol and budesonide, we reported a significant decrease in eosinophils present in the airways (20). The present study was designed to explore the potential mechanisms that could account for the eosinopaenia observed with both the drugs. Specifically, we have tested the hypothesis that "formoterol mediates its effects on eosinophils by modulating Th-2 cytokine expression".

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METHODS

Subjects

Bronchial biopsies were collected from 30 non-smoking, atopic mild asthmatic subjects with a FEV_1 >70% of predicted, airway hyperresponsiveness defined as a provo-

cative concentration of methacholine required to reduce FEV_1 by 20% of baseline (PC_{20} methacholine) >0.1 and <6 mg/ml, who required only as required β_2 agonist over the preceding 2 months (Table 1). Selection was based on the availability of tissue. Of the 64 subjects evaluated in the original study (20), a subgroup of 30, 10 in each treatment group were selected for analysis. With a sample size of 10 in each group and assuming that 80% of the patients on active treatment compared to 10% on placebo will improve, a two-sided Fisher's exact test with a 0.05 significance level will have at least 80% power to detect a difference.

Study design

This was a parallel group, double-blind placebo-controlled study comparing the effects of 8-week treatment with formoterol 24 μg, budesonide 400 μg or placebo twice daily on airway inflammation accessed by bronchial biopsies performed before and at the end of the treatment period. The Ethics Committee of the University of Umeå approved the study and each patient gave informed written consent.

Fiberoptic bronchoscopy

Bronchoscopy was conducted using standard procedures conforming to NHLBI Guidelines (21,22). Pre-medication consisted of atropine (0.5–1.0 mg) s.c., nebulized salbutamol (2.5 mg) and ipratropium bromide (500 μg). At each bronchoscopy, 4–6 endobronchial biopsies were taken as previously described (20).

Biopsy processing and immunohistochemical analysis

Bronchial mucosal biopsies were fixed overnight at $-20^{\circ}C$ in acetone-containing protease inhibitors and then processed into glycol methacrylate (GMA) (23). Two micrometer sections were cut and stained immunohistochemically using the streptavidin–biotin peroxidase detection system. Monoclonal antibodies (Mabs) directed to eosinophils (EG2, PharmaciaUp John, Milton Keynes, U.K.) as an index of inflammation, and the Th-2 cytokines IL-4 (3H4 and 4D9, AMS Biotechnology, Abingdon, U.K.) and IL-5 (MAB 7, Glaxo Smith Kline, Middlesex, U.K. (gift)) were employed. Negative control sections were incubated with isotype-matched immunoglobulins. Following staining, the samples were blinded for treatment group, and the number of EG2 and cytokine positive cells per mm^2 submucosa were enumerated with the assistance of computerised image analysis (Improvision, Birmingham, U.K.).

Data analysis

Non-parametric tests were used, the Wilcoxoń Rank-sum test for within and the Mann–Whitney *U*-test for between treatment analyses. Data are given as median and the 25–75% interquartile range, unless otherwise stated. A P value of <0.05 was considered significant.

RESULTS

Bronchial biopsies

Table 2 summarises information on immunohistochemical staining in the epithelium and submucosa before and after the placebo, formoterol and budesonide treatments. Formoterol treatment significantly reduced the number of eosinophils (EG2+) in the submucosa compared to pre-treatment values ($P=0.005$). This was significant compared to placebo ($P=0.04$), but not budesonide. Eight weeks formoterol treatment had no within group or between group effects on the number of cells immunoreactive for IL-5 or stored (4D9) and secreted (3H4) IL-4. Budesonide treatment significantly reduced the number of eosinophils ($P=0.02$) in the submucosa. This was paralleled by a significant decrease in cells staining for stored IL-4 (4D9 $P=0.008$) and IL-5 ($P=0.03$). The change in eosinophils was significant compared to placebo ($P=0.02$), but not formoterol.

DISCUSSION

Our prior observation that treatment of mild asthmatics with an inhaled long-acting β2 adrenoreceptor agonist

Table 1. Subject characteristics

	Formoterol $n=10$	Budesonide $n=10$	Placebo $n=10$
Age (years)	27 ± 9	32 ± 8	29 ± 9
Gender (male:female)	7:3	6:4	7:3
FEV_1, % predicted	94 ± 9	92 ± 10	91 ± 14
PC_{20} methacholine (mg/ml)	1.05 ± 2.34	1.79 ± 1.94	1.37 ± 2.11

The mean ± 1SD is shown for age and FEV_1 % of predicted, and geometric mean ± 1SD for PC_{20} methacholine.

Table 2. Summary of immunohistochemical data

	Treatment					
	Formoterol		Placebo		Budesonide	
Marker	Pre	Post	Pre	Post	Pre	Post
Eosinophils	21.1 (17.1–28.7)	3.9[a,b] (1.2–9.7)	6.1 (0.6–10.5)	2.8 (0.4–5.2)	2.4 (0.4–5.2)	0[a] (0–0.6)
IL-4 (3H4)	13.4 (9.8–18.0)	8.7 (6.7–10.9)	17.7 (7.5–28.6)	20.4 (12.7–23.8)	8.8 (5.7–24.2)	7.3 (4.6–11.1)
IL-4 (4D9)	2.8 (0.4–4.8)	0.7 (0–1.8)	2.5 (1.2–4.7)	2.8 (0.9–5.7)	5.1 (3.4–9.9)	1.3[a] (0.1–2.4)
IL-5	1.4 (0.6–1.8)	0.9 (0.2–1.2)	2.2 (0–3.0)	1.0 (0.6–1.4)	3.1 (0.4–4.4)	0[a] (0–0.5)

All data are median number cells (25–75 percentiles) per mm^2 of submucosa.
[a]significant within group changes pre-and post-treatment
[b]significant between group differences: formoterol *vs.* placebo.

resulted in a reduction in the airway eosinophilia deserved explanation since this effect may contribute to its therapeutic efficacy. We have previously found no support for the hypothesis that NF-κB regulated expression of the cytokines IL-8, GM-CSF and tumour necrosis factor-α (TNFα) or endothelial adhesion molecules were involved (24). To address the possible involvement of Th-2, cytokines, we assessed the level of expression of IL-4 and IL-5. Budesonide and placebo were included as positive and negative controls against which to compare the reponse to formoterol. Despite showing a 81% decrease in submucosal eosinophils, formoterol had no discernable effect on Il-4 or IL-5 which contrasted with the inhibitory effects that budesonide, but not placebo had on these markers. Thus, while confirming the potent anti-inflammatory action of an inhaled steroid this study has failed to incriminate inhibition of IL-4 or IL-5 production as being responsible for the tissue eosinopaenia.

In the present study, 8 weeks of treatment with budesonide had a powerful effect in reducing airway eosinophils which is in agreement with our earlier observation (20) and that of others (4,25). The reduction in eosinophils is probably in part the result of a loss of cells that store IL-4 and IL-5 or a selective loss of their Th-2 cytokine content within the submucosa together with the adhesion molecule vascular adhesion molecule-1 (VCAM-1), as we have previously reported (24). IL-4 and IL-5 are key cytokines in eosinophil-mediated disease (reviewed in 26). In combination with TNFα, IL-4 specifically enhances and stabilises the expression of VCAM-1 on endothelial cells, an adhesion molecule that plays an important role in eosinophil recruitment by interacting with the integrin CD49d expressed on circulating eosinophils. IL-4 is also a chemoattractant for primed eosinophils. IL-5 is a key eosinophilopoietic cytokine, which stimulates their development, enhances their maturation, and primes them for degranulation and chemotaxis, whilst having a chemotactic activity itself. In addition, IL-5 inhibits eosinophil apoptosis thereby enhancing survival. The importance of IL-4 and IL-5 is highlighted in two recent studies in symptomatic asthmatics (27,28). Soluble IL-4 receptor which binds to free IL-4 has been shown to be therapeutically effective (27) and a blocking humanised antibody against IL-5 has a profound effect in reducing both peripheral blood and airways eosinophils (28). A decrease in stored IL-4 and IL-5 after inhaled corticosteroid treatment has been reported by other workers (4).

Although in this present study, we have confirmed the effect of 8 weeks inhaled formoterol on eosinophils as shown in our previous study (20), we could not find any evidence for the mechanism of action. Several alternative mechanisms could be involved. Important for the chemotaxis/recruitment of eosinophils are the chemokines eotaxin and RANTES together with leukotrienes. Eotaxin is a specific chemokine for eosinophils that together with IL-5 is important for their recruitment, survival and activation (26). RANTES and eotaxin interact with eosinophils through the chemokine receptor (CCR-3) whose expression is high on eosinophils (26). Eotaxin is regulated by NF-κB, whose activation in the epithelium we have recently demonstrated to be reduced by formoterol compared to placebo (24). *In vitro* studies have shown that β_2-adrenoreceptor agonists can inhibit cytokine-induced eotaxin release from cultured human smooth muscle cells (17). Another NF-κB regulated pathway that maybe involved is the cysteinyl leukotrienes and nitric oxide (NO), mediators which can influence eosinophil recruitment (29–31).

An alternative pathway is an effect of formoterol on eosinophil apoptosis. The survival of eosinophils is affected by IL-3, IL-5 and GM-CSF (26), involving both FAS antigen and p38 MAP kinase (32,33). FAS has been found

to induce eosinophil apoptosis, and FAS antibodies to inhibit *in vitro* and *in vivo* survival of the eosinophils (34).

Formoterol may also have a direct effect on eosinophil recruitment. In a guinea pig cutaneous inflammatory model, salmeterol has been shown to directly inhibit the accumulation of eosinophils. The exact mechanism for this is not known, but one suggestion is that decreased CDI8 expression on the surface of the eosinophils, an integrin, that is necessary for its migration into tissue (35).

In conclusion; 8 weeks treatment with inhaled budesonide and formoterol in mild allergic asthmatic subjects reduces eosinophil cell numbers. For budesonide, but not formoterol treatment, this was paralleled with a decrease in Th-2 cytokines. However, the mechanism of formoterol action on eosinopaenia still needs elucidation.

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