Original article

Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma

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Background: Combining inhaled corticosteroids with long-acting β_2-agonists results in improved asthma symptom control and fewer asthma exacerbations compared with those seen after inhaled corticosteroids alone. However, there are limited data as to whether these beneficial effects are due to enhanced anti-inflammatory actions or whether such combination therapies affect airway remodeling in patients with asthma.
Objective: We sought to determine the effects of inhaled budesonide/formoterol combination therapy versus inhaled budesonide alone or inhaled placebo on allergen-induced airway responses, airway inflammation, and airway remodeling.
Methods: Fourteen asthmatic subjects with dual responses after allergen inhalation were included in this prospective, randomized, double-blind, 3-period crossover study. Outcomes included early and late asthmatic responses, changes in airway responsiveness, sputum eosinophilia measured before and after allergen challenge, numbers of airway submucosal myofibroblasts, and smooth muscle area measured before and after study treatment.
Results: Allergen-induced sputum eosinophilia was significantly reduced by combination treatment to a greater extent than by budesonide alone. Allergen inhalation resulted in a significant increase in submucosal tissue myofibroblast numbers and produced a significant decrease in percentage smooth muscle area. Combination therapy, but not budesonide monotherapy, significantly attenuated these changes in myofibroblast numbers and smooth muscle area.
Conclusions: The effects on allergen-induced changes in sputum eosinophils, airway myofibroblast numbers, and smooth muscle seen with combination therapy suggest that the benefits associated with this treatment might relate to effects on airway inflammation and remodeling. The attenuation of early asthmatic responses and airway hyperresponsiveness by combination treatment was likely due to the known functional antagonistic effect of formoterol. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Bronchial provocation tests, bronchoscopy, biopsy, inhaled glucocorticoids, long-acting inhaled* β*-agonists*

Although inhaled corticosteroids (ICSs) remain the cornerstone of effective anti-inflammatory therapy for the treatment of mild asthma,[1,2] a number of large randomized controlled trials[3-8] have demonstrated that combining ICSs with long-acting β_2-agonists (LABAs) results in fewer asthma exacerbations,[5,6,8] improved asthma symptom control,[3-5,7] improved lung function,[3,5-8] and improved health-related quality of life[8,9] when compared with ICS therapy alone. Furthermore, the addition of LABAs to ICSs as a combination therapy allows reduction of the dose of ICS, thereby minimizing potential steroid-induced side effects.[10,11] Consequently, current international asthma guidelines recommend treatment with low-dose ICS and LABA combination therapy in those patients who continue to experience asthma symptoms despite being adherent to low-dose ICS monotherapy.[1,2]

Airway hyperresponsiveness (AHR) is a characteristic feature of asthma and describes the episodic bronchoconstriction that occurs in response to a variety of nonspecific stimuli.[12] Although the pathophysiologic mechanisms underlying AHR are not fully understood, it is increasingly apparent that both immune-mediated airway inflammation and airway remodeling play important roles in this process. Allergen-induced increases in eosinophilic airway inflammation are associated with increased AHR,[13,14] and a growing literature of modeling animal and human airway biopsy studies supports a functional role for airway remodeling in the pathogenesis of AHR.[15-18]

Based on published data to date, it is not clear whether the clinically beneficial effects of the ICS-LABA combination are due to a synergistic effect or to the bronchodilator effect of

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Abbreviations used
AHR: Airway hyperresponsiveness
α-SMA: α-Smooth muscle actin
ICS: Inhaled corticosteroid
IQR: Interquartile range
LABA: Long-acting β_2-agonist
MCh: Methacholine

LABAs alone. Allergen-induced increases in airway eosinophilia and AHR are well-recognized features of allergen-challenge models in asthmatic subjects.[19] However, less well appreciated is the allergen-induced increase in the number of myofibroblasts present in the airway walls of asthmatic subjects 24 hours after allergen inhalation.[20] Myofibroblasts are believed to play a key role in the pathogenesis of airway remodeling because of their contractile properties and synthesis of extracellular matrix proteins.[21,22] No clinical studies have yet examined whether currently available asthma therapies, including combinations of ICSs and LABAs, can attenuate allergen-induced airway remodeling, which might partly account for the clinical benefits observed in clinical trials.

The purpose of this study was to determine the effects of inhaled budesonide/formoterol combination therapy (Symbicort Turbuhaler; AstraZeneca, Lund, Sweden) versus inhaled budesonide alone or inhaled placebo on allergen-induced airway responses, airway inflammation, and aspects of airway remodeling, primarily myofibroblast numbers, in subjects with mild stable asthma in an allergen-challenge clinical model. Some of the results of this study have been reported previously in abstract form.[23-25]

METHODS

Subjects

Fourteen nonsmoking subjects (mean age, 26 years; 8 female subjects; see Table E1 in this article's Online Repository at www.jacionline.org) with clinically stable, mild atopic asthma were included in the study. Additional characteristics are provided in the Methods section of this article's Online Repository at www.jacionline.org. The study was approved by the Ethics Committee at McMaster University Health Sciences Centre, and all subjects provided written informed consent to participate in the study.

Study design and protocol

The study was a prospective, double-blind, placebo-controlled, randomized, 3-period crossover design. Subjects underwent an initial diluent bronchial challenge with bronchoscopy performed 24 hours later, at which time endobronchial biopsy specimens were obtained. These biopsy specimens served as the baseline for all subsequent treatment periods. After a 3-week washout period, subjects were randomized to receive one of 3 treatment regimens of 11 days each separated by washout periods of at least 21 days (see Fig E1 in this article's Online Repository at www.jacionline.org). The 3 treatment regimens were (1) inhaled budesonide (Pulmicort Turbuhaler), 200 μg, 2 inhalations taken twice daily; (2) a combination of inhaled budesonide, 200 μg, and formoterol, 6 μg, in a single inhaler (Symbicort Turbuhaler), 2 inhalations taken twice daily; and (3) placebo Turbuhaler.

On the first day of each treatment period, methacholine (MCh) inhalation challenge and sputum induction were performed to determine baseline pretreatment airway responsiveness and airway inflammatory status, respectively (day 1). Subjects began taking their study medications on the evening of day 1 and continued morning and evening thereafter through day 11. On day 8, MCh challenge and sputum induction were repeated to establish posttreatment preallergen baseline levels. On day 9, subjects underwent an allergen inhalation challenge; 7 hours after allergen inhalation, sputum induction was repeated to assess airway inflammation. Bronchoscopy was performed on day 10, 24 hours after allergen inhalation, at which time endobronchial biopsy specimens were obtained. The final dose of study medication was given on the morning of day 11, after which MCh challenge and sputum induction were performed 24 hours after bronchial biopsy. Additional details are provided in the Methods section of this article's Online Repository.

Outcome measurements

The primary outcome was the effect of treatment on allergen-induced increases in airway myofibroblast numbers. Secondary outcomes were the effects of treatment on (1) allergen-induced sputum eosinophilia, (2) allergen-induced early and late asthmatic airway responses, and (3) allergen-induced AHR. Additional details are provided in the Methods section of this article's Online Repository. The analysis of airway smooth muscle area was a *post hoc* analysis not originally specified in the study protocol.

Laboratory procedures

MCh inhalation challenge, allergen inhalation challenge, sputum induction and analysis, bronchoscopy, and endobronchial biopsy. Details of these procedures are provided in the Methods section of this article's Online Repository.

Tissue processing, imaging, and analysis. Formalin-fixed tissues were processed for histologic and immunohistochemical analysis and morphometry, as described in the Methods section of this article's Online Repository.

Electron microscopy. Cells with the typical features of myofibroblasts, including peripheral myofilament bundles and fibronexi, were identified on ultrastructural examination, as described by us previously.[26] Details are provided in the Methods section of this article's Online Repository.

Statistical analysis

Descriptive statistics were used to summarize the baseline clinical characteristics of study participants. MCh PC_{20} measurements were $\log_2$-transformed to normalize the data and are reported as geometric means.[27] Comparisons between baseline diluent challenge and allergen-induced changes in asthmatic responses, AHR, and sputum eosinophil numbers were made by using 2-factor, repeated-measures ANOVA to analyze the effect of the 2 independent variables of treatment and time on these dependent variables. Comparisons between tissue inflammatory cell numbers, myofibroblast numbers, and percentage areas (including smooth muscle) in the diluent- and allergen-challenged groups were analyzed by using univariate analyses weighted for area. Additional details are provided in the Methods section of this article's Online Repository.

RESULTS

Early and late asthmatic responses

There were no significant differences in the early asthmatic airway responses between the placebo and inhaled budesonide treatment groups. However, inhaled budesonide/formoterol significantly attenuated the early asthmatic response (48.0% reduction) when compared with placebo ($P < .001$) and inhaled budesonide ($P < .001$). In addition, there was a modest but not significant difference in the late asthmatic airway response between the placebo and inhaled budesonide groups ($P = .06$). However, inhaled budesonide/formoterol significantly attenuated the late asthmatic response (42.8% reduction) when compared with placebo ($P = .03$, Fig 1).

Airway responsiveness

Baseline MCh PC_{20} values were similar across the 3 treatment groups (Fig 2). There were no significant differences in airway

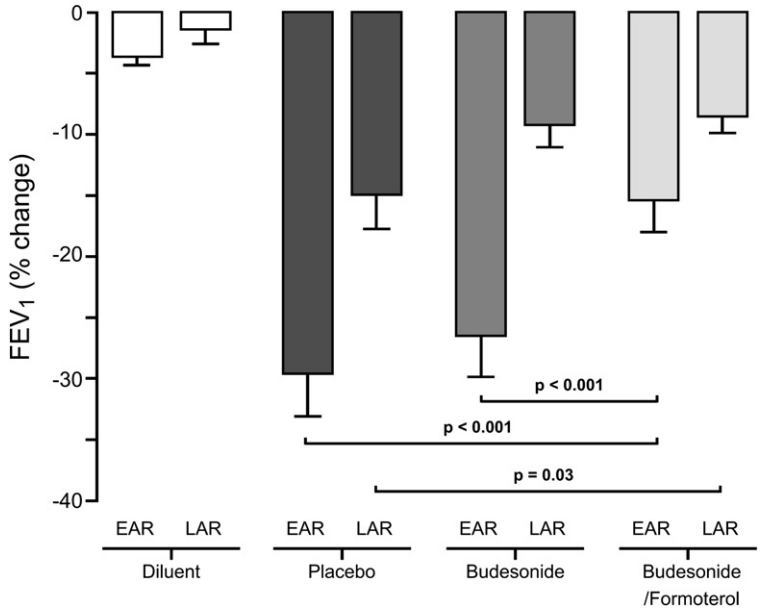


FIG 1. Effect of study treatment on allergen-induced early *(EAR)* and late *(LAR)* asthmatic responses.

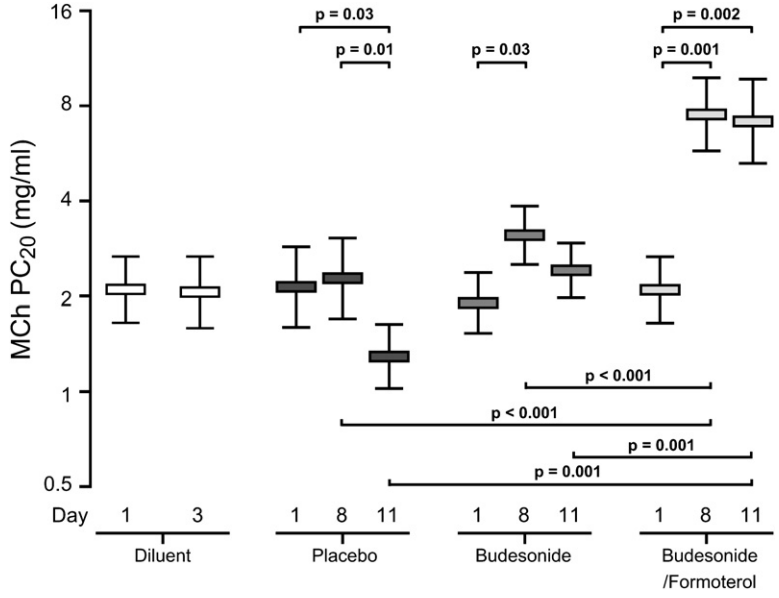


FIG 2. Effect of study treatment on AHR (MCh PC_{20}) before (day 8) and after (day 11) allergen challenge.

responsiveness after diluent challenge and bronchoscopy. After treatment with inhaled placebo, there was no significant difference in the MCh PC_{20} value between pretreatment baseline values and the posttreatment preallergen values. However, AHR increased significantly 48 hours after allergen challenge when compared with pretreatment baseline ($P = .03$) and posttreatment preallergen ($P = .01$) values (Fig 2). After treatment with inhaled budesonide, MCh PC_{20} values increased significantly between pretreatment baseline and posttreatment preallergen ($P = .03$) values, and budesonide prevented an allergen-induced increase in AHR. After treatment with inhaled budesonide/formoterol, MCh PC_{20} values increased significantly between pretreatment baseline and posttreatment preallergen values ($P < .001$, Fig 2). This increase was significantly greater than that seen after treatment with inhaled budesonide alone ($P < .001$). Furthermore, inhaled budesonide/formoterol prevented an allergen-induced increase in AHR, resulting in a significantly higher postallergen MCh PC_{20} value than seen in the group treated with inhaled budesonide alone ($P = .001$, Fig 2).

Sputum eosinophilia

A significant increase in sputum eosinophilia was measured 7 hours after allergen inhalation (day 9) after treatment with either inhaled placebo ($P = .04$) or inhaled budesonide ($P = .009$) when compared with posttreatment preallergen measurements (day 8,

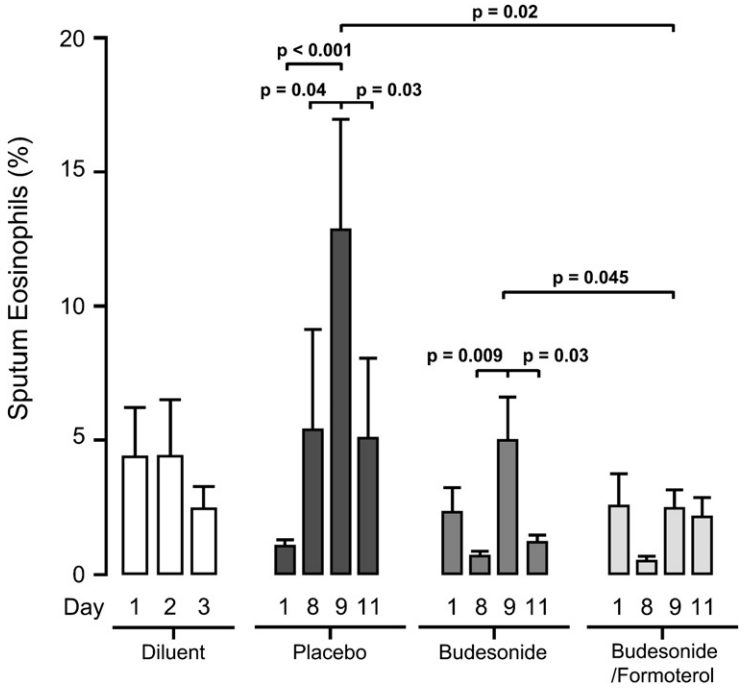


FIG 3. Effect of study treatment on sputum eosinophil numbers before (day 8) and 7 hours (day 9) and 48 hours (day 11) after allergen challenge.

Fig 3). Although the allergen-induced sputum eosinophilia was attenuated by treatment with budesonide, this reduction was not significantly different when compared with the increase seen after placebo treatment. In contrast, treatment with inhaled budesonide/formoterol resulted in a significant reduction in the percentage of sputum eosinophils 7 hours after allergen challenge compared with that seen after placebo ($P = .02$), as well as compared with that seen after budesonide treatment alone ($P = .045$, Fig 3). The allergen-induced sputum eosinophilia was attenuated at 48 hours (day 11) in all study treatment groups, and there were no differences in sputum eosinophil numbers when comparing the posttreatment preallergen (day 8) and 48-hour postallergen (day 11) time points in any of the treatment groups. Similar differences were observed when sputum eosinophils were expressed as absolute cell numbers (data not shown).

Inflammatory cells in intraepithelial and subepithelial tissue

There were no significant differences in the numbers of intraepithelial and subepithelial inflammatory cells when diluent and allergen challenge were compared after placebo treatment (see Tables E2 and Table E3 in this article's Online Repository at www.jacionline.org). Intraepithelial eosinophil numbers were decreased after both active treatments compared with those after placebo, but only the budesonide group showed a statistically significant reduction ($P = .034$, see Table E2 and Fig E2, *A*, in this article's Online Repository at www.jacionline.org). Although eosinophil numbers in the subepithelial tissue were reduced after budesonide or budesonide/formoterol treatment, these reductions were not significant when compared with those seen in the placebo-treated group (see Table E3 and Fig E2, *B*). There were no significant differences in other inflammatory cell types between the different study treatment groups within either the intraepithelial or subepithelial compartments (see Tables E2 and E3).

Inflammatory cells in airway smooth muscle

Although relatively few cells infiltrated the airway smooth muscle, the majority of these were mast cells and occasionally $CD3^+$ lymphocytes. There were no significant differences in the numbers of mast cells in airway smooth muscle after diluent challenge (median, 34 cells/mm^2; interquartile range [IQR], 52 cells/mm^2) compared with those seen in biopsy specimens after allergen challenge in the placebo-treated (median, 22 cells/mm^2; IQR, 60 cells/mm^2), budesonide-treated (median, 13 cells/mm^2; IQR, 45 cells/mm^2), or budesonide/formoterol-treated (median, 21 cells/mm^2; IQR, 41 cells/mm^2) groups.

Area of epithelium, submucosal glands, and smooth muscle

There was no significant difference in the percentage area composed of epithelium between any of the study groups. Submucosal glands were identified in 47.2% of biopsy specimens and composed of areas ranging from 1% to 88.3% (expressed as a percentage of total biopsy area: mean, 14.6%; SD, 17.2%). There was no change in the area of submucosal glands between any of the groups.

Smooth muscle was identified in 83% of biopsy specimens, with the area comprising smooth muscle in these biopsy specimens ranging from 1.1% to 55.4% (expressed as a percentage of total biopsy area: mean, 18.1%; SD, 16.5%). There was a significant decrease in the proportion of airway smooth muscle after allergen challenge and placebo ($P = .03$) or budesonide ($P = .004$) treatment but not after combination

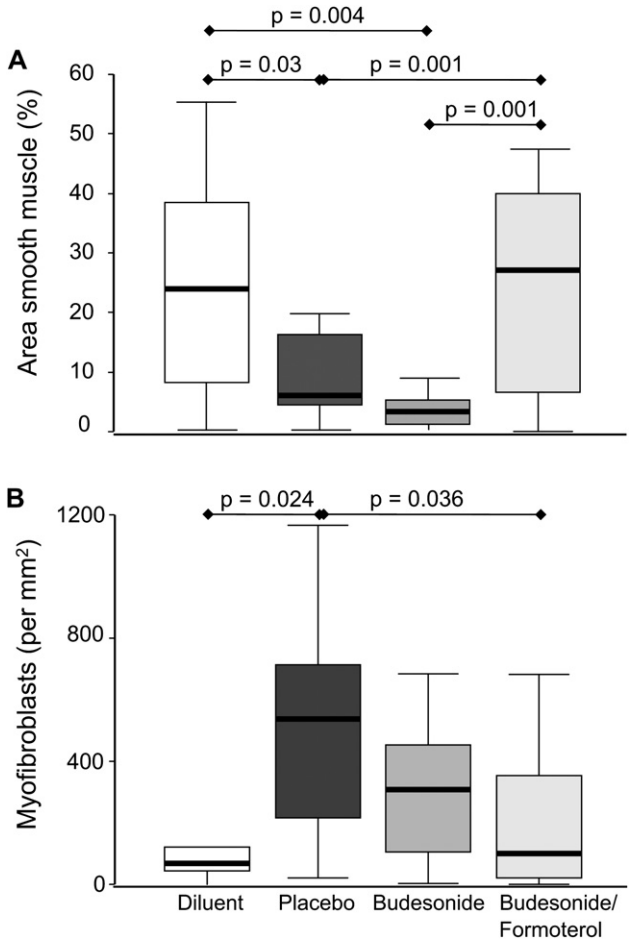


FIG 4. Smooth muscle (percentage of area) and numbers of myofibroblasts in the subepithelial compartment in bronchial biopsy specimens. *Box plots* represent the medians and IQRs.

therapy when compared with that after diluent challenge (Fig 4, *A*). The effects of treatment with combination therapy were significantly different from those of budesonide alone ($P = .001$) or placebo ($P = .001$).

Allergen-induced increases in airway myofibroblast numbers

There was a significant allergen-induced increase in the number of airway myofibroblasts identified on light microscopy and transmission electron microscopy (see Fig E3 in this article's Online Repository at www.jacionline.org) in the placebo-treated group (median, 53.7 cells/mm^2; IQR, 114.5 cells/mm^2) compared with that seen in the diluent-challenged control group (median, 6.9 cells/mm^2; IQR, 11.9 cells/mm^2; $P = .024$; Fig 4, *B*). Treatment with budesonide/formoterol combination therapy significantly attenuated this allergen-induced increase in myofibroblast numbers to near baseline levels (median, 10 cells/mm^2; IQR, 37.9 cells/mm^2; $P = .036$), but the reduction in myofibroblast numbers with budesonide treatment alone (median, 30.8 cells/mm^2; IQR, 37.8 cells/mm^2) was not statistically significant and remained substantially greater than diluent-induced baseline levels (Fig 4, *B*). Although myofibroblasts were distributed widely throughout all levels of the submucosa, there were focal areas evident where they were closely applied to smooth muscle bundles (see Fig E4, *A* and *B*, in this article's Online Repository at www.jacionline.org). In some areas myofibroblast-like cells were more closely apposed than usual and arranged in a parallel pattern to each other (see Fig E4, *C*, in this article's Online Repository at www.jacionline.org).

DISCUSSION

This study demonstrated that 11 days of treatment with the combination of budesonide/formoterol significantly attenuated allergen-induced early and late asthmatic airway responses compared with placebo and significantly attenuated early asthmatic airway responses compared with budesonide alone. In addition, the combination therapy significantly attenuated allergen-induced increases in AHR and sputum eosinophilia 7 hours after challenge when compared with the placebo- and budesonide-treated groups. Importantly, inhaled budesonide/formoterol treatment significantly attenuated the marked allergen-induced increase in airway myofibroblast numbers. The decrease in airway myofibroblast numbers after budesonide therapy alone did not reach statistical significance, although it was not significantly different from that seen after combination therapy. We also found that the proportion of smooth muscle area significantly decreased after allergen challenge in the placebo- and budesonide-treated groups compared with the diluent-challenged group, whereas treatment with combination therapy prevented this allergen-induced reduction in percentage smooth muscle area.

It is likely that the effects of combination therapy on allergen-induced early and late asthmatic airway responses and AHR were largely due to the known functional antagonistic effects of formoterol. This is the ability of inhaled β_2-agonists to protect against bronchoconstriction. Nonetheless, the results of our study provide novel and substantive evidence that budesonide/formoterol combination therapy has greater anti-inflammatory and antiremodeling effects than inhaled budesonide monotherapy. Our findings support the paradigm that, as evidenced by previous clinical trials,[3-8] ICS and LABA combination therapies exert their clinically beneficial effects not only by providing bronchodilation and functional antagonism but also by increased anti-inflammatory activity and by attenuating aspects of airway remodeling when compared with ICS therapy alone.

The mechanistic basis for the therapeutic benefits seen with budesonide/formoterol compared with budesonide alone in our study is not yet fully understood. Traditional dogma has held that ICSs and LABAs likely act independently on distinct pathophysiologic processes in asthma, with ICSs attenuating the inflammatory component and LABAs targeting the bronchoconstrictive component of the disease. Consequently, there are at least 2 plausible theories, not necessarily mutually exclusive, which might explain the increased anti-inflammatory and antiremodeling activity of budesonide/formoterol combination therapy over budesonide monotherapy. The first of these is that budesonide and formoterol activate mechanistically distinct anti-inflammatory pathways that combine to produce an additive clinical response. The second holds that formoterol augments the activity of budesonide through a common mechanism or mechanisms to produce a degree of synergistic anti-inflammatory response. The weight of evidence as to which of these paradigms is likely to be more relevant in explaining our findings can be deduced from a number of earlier clinical studies.

Despite some *in vitro* evidence that LABAs are able to suppress several indices of inflammation,[28] they do not evoke clinically relevant anti-inflammatory effects *in vivo* when given as monotherapy.[29,30] This argues against separate anti-inflammatory mechanisms that combine to elicit an additive effect as being the major mode of action. In contrast, the Formoterol and Corticosteroids Establishing Therapy and Oxis and Pulmicort Turbuhaler In the Management of Asthma (OPTIMA) studies[5,6] found that the addition of formoterol to low-dose inhaled budesonide reduced exacerbation rates to a significantly greater degree than did treatment with low-dose budesonide alone. This suggests that formoterol was able to enhance the clinical efficacy of budesonide to a level that could not be achieved with budesonide monotherapy. The results of the present study are consistent with these and other previous observations[31] but also offer advances in understanding how these 2 classes of drugs, when administered together, result in superior clinical efficacy. Although not the first bronchial biopsy study to demonstrate that combination therapy results in additional anti-inflammatory effects when compared with ICS treatment alone,[32] this is the first study to demonstrate that the combination of budesonide and formoterol in a single inhaler device has additive/synergistic anti-inflammatory effects when compared with those of inhaled budesonide alone. In recent *in vitro* studies synergistic augmentation of anti-inflammatory effects of ICSs by LABAs appeared to be due to enhanced glucocorticoid response element–dependent transactivation of anti-inflammatory genes.[28,33,34]

Our study is also the first to demonstrate that inhaled budesonide/formoterol combination therapy significantly attenuates allergen-induced airway myofibroblasts compared with placebo, and this effect was not seen in those subjects receiving inhaled budesonide alone. The increase in myofibroblast numbers after allergen challenge is in keeping with a previous study in which myofibroblast numbers were also significantly increased after allergen challenge.[22] In that study these cells persisted along with allergen-induced AHR, despite resolution of airway inflammation, suggesting that AHR might be dependent on the presence of myofibroblasts and not inflammatory cells.

The additional findings relating to the maintenance of smooth muscle area by budesonide/formoterol combination therapy, but not by budesonide alone, are novel and initially seem to be counterintuitive given that increased smooth muscle is an important component of airway remodeling.[35-37] However, if one considers that 24 hours after allergen challenge is an early time point in the airway-remodeling process, it is plausible that the reduction in smooth muscle is due to myocyte dedifferentiation into myofibroblasts and subsequent migration into the submucosa. This would account for the significant allergen-induced increase in myofibroblast numbers seen at this same time point. Because myofibroblasts are large cells with numerous contractile elements (see Fig E3), it would seem that the myocyte is the most likely cell capable of differentiating into a myofibroblast and migrating through the submucosa within 24 hours, which represents a relatively short period. We postulate that myofibroblasts originate from smooth muscle and migrate into the submucosa along a chemotactic gradient toward the epithelium, as has been previously suggested.[35] Evidence that increased smooth muscle in patients with asthma is due to hyperplasia rather than hypertrophy[38] is supportive of this hypothesis. Although the myofibroblasts appeared to be randomly distributed throughout all levels of the submucosa, we observed focal areas where myofibroblasts and smooth muscle were in close apposition (see Fig E4). It is possible that myofibroblasts would have been concentrated around the smooth muscle if the bronchial biopsies had been performed at earlier time points rather than at 24 hours.

In several sections of tissue (see Fig E3, *A*), myofibroblasts completely fill the submucosal area, and it can be postulated that these conglomerates effect AHR by altering the extracellular matrix, by contracting and stiffening the airway wall, or both because they are capable of isometric tension.[39,40] Eventually these myofibroblasts might differentiate back into smooth muscle, with myofibroblasts and myocytes existing in a state of dynamic equilibrium, as suggested by Ward and Walters.[41] This would result in the remodeled smooth muscle being closer to the epithelial surface, as has been described in asthmatic subjects.[42,43] Fig E4, *C*, shows a localized collection of myofibroblasts arranged loosely in a pattern reminiscent of smooth muscle, and it is tempting to speculate that these represent cells transitioning from smooth muscle to myofibroblasts or *vice versa*. The lack of a significant effect of budesonide monotherapy on the allergen-induced increase in myofibroblast numbers and decrease in smooth muscle is in agreement with *in vivo* studies that have shown that airway smooth muscle mitogenesis and growth might be resistant to glucocorticoid inhibition.[44,45] The importance of our findings are emphasized by the fact that (myo)fibroblast accumulation and increased airway smooth muscle in proximal airways are the only aspects of remodeling that discriminate severe persistent asthma from milder forms.[42,46] Several studies have also demonstrated that increased contractile tissue mass in the airway wall accounts for most of the functional contribution of airway remodeling to the pathophysiology of AHR.[15,47-51]

Our study has several limitations that might influence interpretation of the results. We did not include a study treatment period of inhaled formoterol alone, primarily because this is not clinically relevant given that none of the current guidelines advocate LABAs as a monotherapy for the treatment of asthma.[1,2] It is unlikely that formoterol alone would have demonstrated any anti-inflammatory effects in our study, given previous *in vivo* evidence.[29,30]

Second, most of the differences in inflammatory cell counts likely did not reach significance because of the wide variation in cell count numbers after allergen challenge in the placebo group. It should be emphasized that these were secondary outcomes, and that our study was not powered *a priori* to detect significant differences in inflammatory cell numbers in the various tissue compartments examined between the diluent- and various allergen-challenged study treatment groups. It is salient to note that many previous allergen-challenge airway biopsy studies did not include a diluent challenge as a control,[22] and our results suggest that diluent challenge might have some inflammatory effects. Sputum eosinophil numbers were maximally and significantly increased 7 hours after allergen challenge and after placebo treatment before returning to normal by 48 hours after allergen challenge. Eosinophil numbers were not significantly increased in the bronchial tissues 24 hours after allergen challenge, suggesting that eosinophils traffic rapidly through the airway wall into the lumen.

In summary, we have confirmed that treatment with the single-inhaler combination of budesonide/formoterol provided greater anti-inflammatory effect with regard to allergen-induced sputum eosinophilia compared with that seen after inhaled budesonide alone. Importantly, this combination therapy also significantly attenuated the allergen-induced increase in airway myofibroblast

numbers and prevented the concomitant decrease in airway smooth muscle. Budesonide alone did not significantly modulate myofibroblast numbers or area of airway smooth muscle. It seems plausible that our findings represent an inhibitory effect on the proliferation, differentiation, and migration of smooth muscle cells by budesonide/formoterol combination therapy, which is not seen with ICS monotherapy. The allergen-induced decrease in smooth muscle and increase in myofibroblast numbers provides strong, albeit circumstantial, evidence of a smooth muscle origin of myofibroblasts, with important implications for future studies. Together these effects likely explain, at least in part, the beneficial effects of ICS and LABA combination therapies compared with ICS monotherapies that have been consistently observed in a number of randomized, controlled clinical trials.

We thank all the subjects who participated in the clinical study. We also thank the McMaster Electron Microscopy unit, especially Ernie Spitzer, for technical assistance.

Clinical implications: The mechanism by which budesonide/formoterol combination therapy exerts its clinically beneficial effects appears to relate to increased anti-inflammatory activity and attenuation of aspects of airway remodeling in asthma.

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METHODS

Subjects

Fourteen nonsmoking subjects (mean age, 26 years; 8 female subjects; Table E1) with clinically stable, mild atopic asthma were included in the study. All subjects had symptoms of asthma for more than a year, a PC_{20} value of less than 16 mg/mL, and allergen-induced early and late bronchoconstrictor responses of at least 15% reduction in FEV_1 during a screening challenge. Subjects who had been treated with any asthma medication other than inhaled short-acting β_2-agonists or who used inhaled short-acting β_2-agonists more frequently than once daily during the 4-week period before screening were not eligible to participate in the study. The study protocol was approved by the Ethics Committee at McMaster University Health Sciences Centre, and all subjects provided written informed consent to participate in the study.

Study design and protocol

The study was performed by using a prospective, double-blind, placebo-controlled, randomized, 3-period crossover design. Subjects demonstrating dual asthmatic responses during an initial screening allergen challenge underwent a separate diluent challenge followed by bronchoscopy 24 hours later during which endobronchial biopsy specimens were obtained. This procedure was carried out at least 3 weeks after the screening allergen challenge and served as a baseline for all subsequent treatment periods. After a further 3-week washout period, subjects were randomized to receive one of 3 different treatment regimens of 11 days each separated by washout periods of at least 21 days (Fig E1). The 3 treatment regimens were (1) inhaled budesonide (Pulmicort Turbuhaler), 200 μg, 2 inhalations taken twice daily (total daily dose, 800 μg); (2) a combination of inhaled budesonide, 200 μg, and formoterol, 6 μg, in a single inhaler (Symbicort Turbuhaler), 2 inhalations taken twice daily (total daily dose, 800/24 μg); and (3) placebo Turbuhaler.

On the first day of each treatment period, MCh inhalation challenge and sputum induction were performed to determine baseline pretreatment airway responsiveness and airway inflammatory status, respectively (day 1). Subjects began receiving their study medications on the evening of day 1 and continued thereafter, and on day 8, MCh challenge and sputum induction were repeated to establish posttreatment preallergen baseline levels. On day 9, after receiving the morning dose of study medication, subjects underwent an allergen inhalation challenge; spirometric results was monitored throughout the day to check for early and late asthmatic responses. Seven hours after allergen inhalation, sputum induction was repeated to assess airway inflammation. After receiving the morning dose of study medication on day 10, bronchoscopy was performed, and endobronchial biopsy specimens were obtained. The final dose of study medication was given on the morning of day 11, after which MCh challenge and sputum induction were performed 24 hours after bronchial biopsy. Bronchial biopsies were performed 24 hours after allergen or diluent challenge because it has been demonstrated that airway myofibroblast numbers are significantly increased at this time.[E1] All measurements for each subject were made at the same time of day (±2 hours) at baseline and after each treatment period. Study treatments were consistently administered in the morning before MCh challenge (days 8 and 11), allergen challenge (day 9), and bronchial biopsy (day 10). Study subjects were allowed to use inhaled β_2-agonists on an as-needed basis throughout the treatment and washout periods but were asked to refrain from doing so 8 hours before any study visit.

Randomization and allocation concealment

Randomization was performed by using computer-generated randomization codes, which were maintained by a research pharmacist at McMaster University, who was independent of the study. Treatment allocation was concealed from the investigators and participants for the duration of the study. All study medications were independently packaged and labeled by the hospital pharmacy. Placebo Turbuhalers were identical in appearance and labeling to budesonide Turbuhalers (Pulmicort Turbuhaler, 200 μg per dose) and to budesonide/formoterol combination Turbuhalers (Symbicort Turbuhaler, 200/6 μg per dose), all of which were supplied by AstraZeneca (AstraZeneca Canada, Inc, Mississauga, Ontario, Canada). At the start of a treatment period, each subject was given a new coded Turbuhaler. Inhaler technique was checked at each visit and corrected if necessary. At the end of each treatment period, study medication was returned, and compliance was monitored by counting the number of Turbuhaler doses remaining.

Outcome measurements

The primary outcome was the effect of treatment on allergen-induced increases in airway myofibroblast numbers. Secondary outcomes were the effects of treatment on (1) allergen-induced sputum eosinophilia, (2) allergen-induced early and late bronchoconstrictor responses, and (3) allergen-induced AHR. The analysis of airway smooth muscle area was a *post hoc* analysis not originally specified in the study protocol. The sample size was considered adequate because previous studies from our group[E1] have shown that 12 or more subjects have greater than 90% power to detect a clinically important 3-fold increase in airway myofibroblast numbers 24 hours after allergen challenge when compared with numbers after diluent challenge. Our study was also sufficiently powered to demonstrate differences in allergen-induced airway eosinophilia and in allergen-induced early and late asthmatic responses.[E2,E3]

Laboratory procedures

MCh inhalation challenge. MCh inhalation challenge was performed by using the method described.[E4] Subjects inhaled through a mouthpiece attached to a Wright nebulizer (Roxon Medi-Tech; Montreal, Quebec, Canada). Normal saline followed by doubling concentration increases in MCh were nebulized for 2 minutes each. FEV_1 was measured at 30, 90, 180, and 300 seconds after each inhalation by using a Collins water-sealed spirometer (Warren E. Collins, Braintree, Mass) and kymograph. The test was terminated when the FEV_1 had decreased to a level at least 20% less than the postsaline measurement. The concentration of MCh required to achieve a decrease in FEV_1 of 20% (MCh PC_{20}) was calculated through linear interpolation of the percentage decrease in FEV_1 against the log-transformed MCh concentration.

Allergen inhalation challenge. Allergen challenge was performed as previously described.[E5] The allergen producing the largest skin wheal diameter after skin prick testing was used for subsequent airway challenges. The concentration of allergen extract for inhalation was determined by using a formula derived by Cockcroft et al[E6] using the results from skin test titrations and the MCh PC_{20}. During the screening allergen challenge, the starting concentration of allergen extract for inhalation was 2 doubling concentrations less than that predicted to cause a 20% decrease in FEV_1. Doubling increases in allergen were inhaled every 10 minutes until a 15% reduction in FEV_1 was achieved. FEV_1 was then measured at 10, 20, 30, 45, 60, 90, and 120 minutes after allergen inhalation and then each hour until 7 hours after allergen inhalation. The early bronchoconstrictor response was taken to be the largest percentage decrease in FEV_1 within 2 hours after allergen inhalation, and the late bronchoconstrictor response was taken to be the largest percentage decrease in FEV_1 in the period beginning 3 hours and ending 7 hours after allergen inhalation. Maximal decreases in FEV_1 were chosen to quantify the early and late response magnitudes based on earlier studies from our group, indicating that these measurements have utility in detecting treatment effects.[E2] Only subjects who achieved a 15% or greater early and late decrease in FEV_1 on the allergen-screening challenge were randomized, and the same allergen concentrations were used on all subsequent allergen inhalation challenges.

Sputum induction and analysis. Sputum induction was performed after MCh challenge on days 1, 8, and 11 and 7 hours after allergen challenge on day 9 of each treatment period. MCh challenge performed before sputum induction does not significantly alter the cellular and biochemical constituents of sputum.[E7] Sputum was induced as previously described.[E8,E9] Briefly, after pretreatment with inhaled albuterol (200 μg), subjects inhaled an aerosol of 3%, 4%, and 5% hypertonic saline for 7 minutes each from a Medix ultrasonic nebulizer (Clement Clarke, Harlow, Essex, United Kingdom). After each inhalation period, subjects expectorated sputum into a container. Sputum was processed within 2 hours of collection, as previously described.[E10] Total cell counts were performed with a hemocytometer and were expressed as cells per milliliter of sputum. Cells were resuspended in Dulbecco PBS at 1.0×10^6/mL, and cytospin preparations were prepared with a Shandon III

cytocentrifuge (Shandon Southern Instruments, Sewickly, Pa). Slides were stained with Diff-Quik (American Scientific Products, Mcgaw Park, Ill), and a 400 nonsquamous differential cell count was performed on all slides by a single observer blind to the clinical data; the mean count from 2 slides per subject was used for analysis.

Bronchoscopy and endobronchial biopsy. Flexible fiberoptic bronchoscopy was performed according to recommendations of the National Institutes of Health[E11] as a day-case procedure. To obtain the best possible quality biopsy samples, we adhered to published recommendations.[E12] Six mucosal biopsy specimens were taken from segmental and subsegmental carinae on either the right or left lung, with biopsy specimens during subsequent treatment periods taken from alternating opposite sides. Two biopsy specimens were collected in 10% buffered formalin, and 2 were collected in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for subsequent electron microscopy. One of the investigators, who is an anatomic pathologist (MMK), was present at all bronchoscopies and checked that biopsy specimens were of adequate size, as recommended by Jeffery et al.[E12] If a biopsy specimen appeared to consist mainly of blood or mucus, an additional specimen was taken to ensure that adequate biopsy samples were obtained.

Tissue processing for light microscopy. Formalin-fixed tissues were processed into paraffin blocks, and 3-μm-thick serial sections were cut onto positively charged slides (Fisher-Plus slides; Fisher Scientific, Nepean, Ontario, Canada). The sections were stained with hematoxylin and eosin to determine the adequacy and general morphology of the sample and with Congo Red stain to identify eosinophils. Immunohistochemistry was performed as described to identify cells as follows: total leukocytes ($CD45^+$), T lymphocytes ($CD3^+$), T-lymphocyte subsets ($CD4^+$ and $CD8^+$), B lymphocytes ($CD20^+$), neutrophils (anti-neutrophil elastase), macrophages ($CD68^+$), mast cells (anti-tryptase, AA1), plasma cells ($CD138^+$), myofibroblasts and pericytes (α-smooth muscle actin [α-SMA]), and blood vessels ($CD34^+$). All antibodies were obtained from DAKO Diagnostics (Ontario, Canada). 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. Representative photomicrographs of myofibroblasts and smooth muscle stained with α-SMA are shown in Figs E3, E4, and E5. Representative photomicrographs of biopsy specimens stained for eosinophils, $CD3^+$ T cells, plasma cells, and neutrophils are shown in Fig E6, and mast cells in the subepithelial and smooth muscle compartments are shown in Fig E7.

Electron microscopy. Biopsy specimens fixed in 2% glutaraldehyde were rinsed in sodium cacodylate buffer, postfixed in 1% osmium tetroxide for 1 hour, dehydrated in graded ethanol solutions, and embedded in Spurr resin. One-micrometer-thick sections were stained with toluidine blue, and areas of intact submucosa were selected for further electron microscopic analysis. Ultrathin sections (90 nm) were examined with a transmission electron microscope (Philips CM10, Eindhoven, The Netherlands), as described by us previously.[E13] Cells with the typical features of myofibroblasts, including peripheral myofilament bundles and fibronexi, were identified on ultrastructural examination.[E1,E13-E15] Representative photomicrographs of myofibroblasts and smooth muscle identified on transmission electron microscopy are shown in Figs E3, *C* and *D*, and E5, *C* through *E*, respectively.

Tissue imaging and area and cell quantification

Biopsy specimens were photographed at 200× magnification, and the total areas comprising epithelium, subepithelium, submucosal glands, and smooth muscle were measured with Openlab morphometry software (Improvision, Inc, Lexington, Mass) by selecting the areas manually. Areas of poor integrity and crush artifact were excluded. Detached epithelial strips were not included. All area measurements were performed by a single operator (CG). Subepithelium refers to mucosal tissue within 200 μm beneath the basement membrane, excluding submucosal glands and smooth muscle. The area of the epithelium, rather than the length, was measured to allow for variations in thickness of epithelium, as well as tangential sectioning of the biopsy specimens. The slides were coded, and area profile counts were used to count inflammatory cells and myofibroblasts within the different tissue compartments by a single observer (MMK) blinded to clinical details. The cell counts were performed by viewing the original stained slides at 200× or 400× magnification, depending on how easy it was to identify the positively stained cells, and were expressed as cells per square millimeter. Plasma cells were not counted in the epithelium because epithelium stains positively for CD138.

Myofibroblasts were identified on light microscopy based on their characteristic morphology and α-SMA staining.[E15] Because pericytes also stain with α-SMA, slides stained with this immunomarker were compared with those stained with CD34, an endothelial marker, to exclude blood vessels. To verify that cells identified on light microscopy were myofibroblasts, areas with large numbers of these cells were identified in plastic-embedded sections, and these areas were then examined by means of transmission electron microscopy to confirm they were myofibroblasts based on ultrastructural criteria.[E1,E13-E15] Photomicrographs of bronchial biopsy specimens stained for α-SMA to identify myofibroblasts on light microscopy with corresponding transmission electron microscopic photomicrographs are shown in Fig E3.

The methods used to quantify inflammatory cells and myofibroblast density in this study expressed the number of cells counted within a defined 2-dimensional area measured in square millimeters and are similar to those used in several previous studies.[E12,E16-E18] To minimize bias, we sampled tissue and processed blocks and sections in a systematic and random fashion. Endobronchial biopsy specimens are inherently elastic and do not lie flat; this favors random orientation of tissue structures during embedding and sectioning. Cell counts might be biased unless all cells of interest have an equal probability of being counted, and this depends on the cell size, orientation, and thickness of the tissue section. Most inflammatory cells are relatively uniform and small compared with the reference area in which they were measured, and we only examined nucleated cells in single 5-μm tissue sections. Myofibroblasts are relatively large cells, but we opted to count only nucleated cells, which reduced bias related to large cell size.[E12] We are thus confident that comparisons of relative cell densities between our various study groups are valid and make our data directly comparable with the many other published studies that have counted cells in this way.[E12] Although there are complex issues related to the assessment of airway smooth muscle,[E19] measurement of the area of smooth muscle is a standard technique to assess smooth muscle bulk.[E20,E21]

Statistical analysis

Data were analyzed with SPSS for Windows, release 11.0 (SPSS, Inc, Chicago, Ill). Descriptive statistics were used to summarize the baseline clinical characteristics of study participants. Measurement variability was expressed by using SDs for baseline subject characteristics and SEMs for outcome variables. MCh PC_{20} measurements were log_2-transformed to normalize the data and are reported as geometric means.[E22] The choice of base 2 for the logarithmic transformation allows differences between PC_{20} values to be expressed as doubling concentrations. Data for eosinophils were normalized by using logarithmic transformation. Comparisons between baseline diluent challenge– and allergen-induced changes in asthmatic responses, AHR, and sputum eosinophil counts were made by using 2-factor, repeated-measures ANOVA to analyze the effect of the 2 independent variables of treatment and time on these dependent variables. Comparisons between tissue inflammatory, myofibroblast cell counts and percentage areas (including smooth muscle) in the diluent- and allergen-challenged groups were analyzed by using univariate analyses weighted for area. Tukey least significant *post hoc* testing was performed to assess for significant effects while controlling for multiple comparisons. All comparisons were 2-tailed, and P values of less than .05 were considered significant.

Assessment of quality of sputum cytospin preparations and endobronchial biopsy specimens

By using published criteria,[E23] 93.7% of sputum cytospin preparations were suitable for evaluation. Endobronchial biopsy specimens were assessed for overall quality and suitability for analysis based on the presence of intact epithelium and a subepithelial area of at least 0.3 mm^2, as recommended by Jeffery et al.[E12] Based on these criteria, 96.4% of biopsy specimens were considered suitable for evaluation. The repeatability of measurement for cell counts (expressed as the intraclass correlation coefficient) was greater than 0.97 for all counts.

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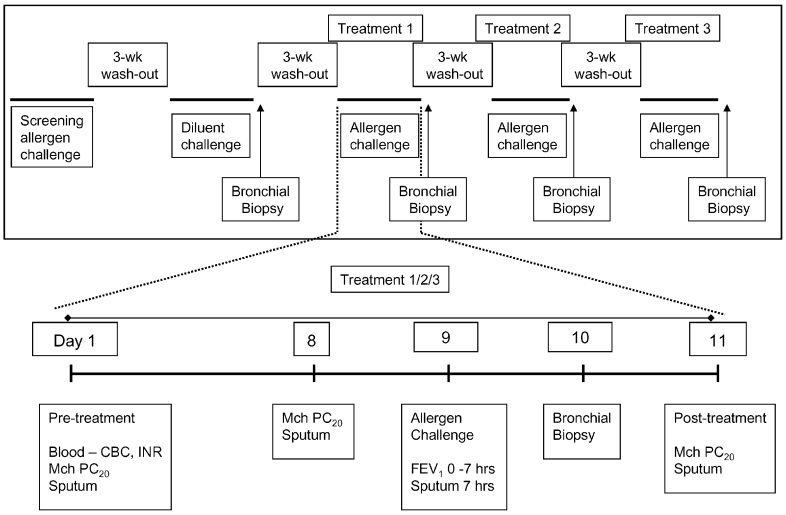


FIG E1. Study protocol. *CBC*, Complete blood count; *INR*, international normalized ratio.

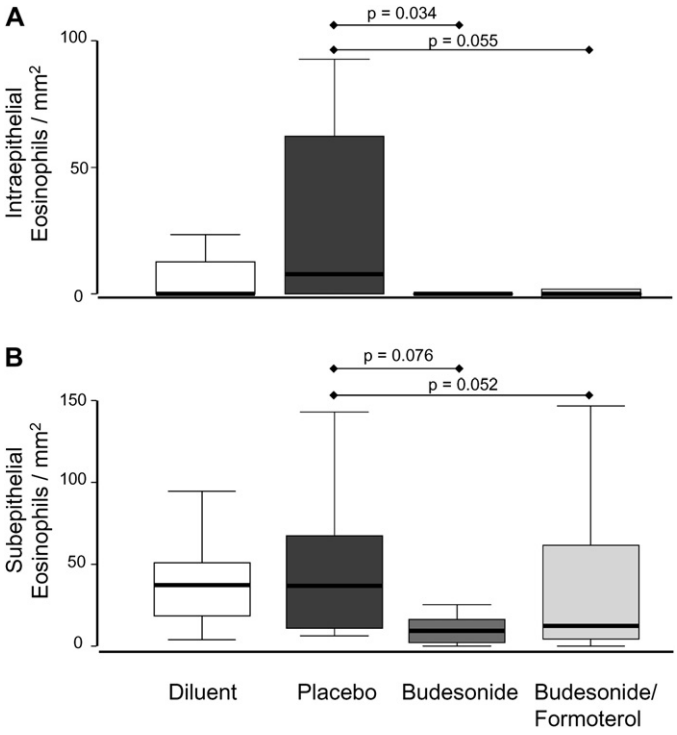


FIG E2. Numbers of intraepithelial **(A)** and subepithelial **(B)** eosinophils in bronchial biopsy samples. *Box plots* represent medians and IQRs.

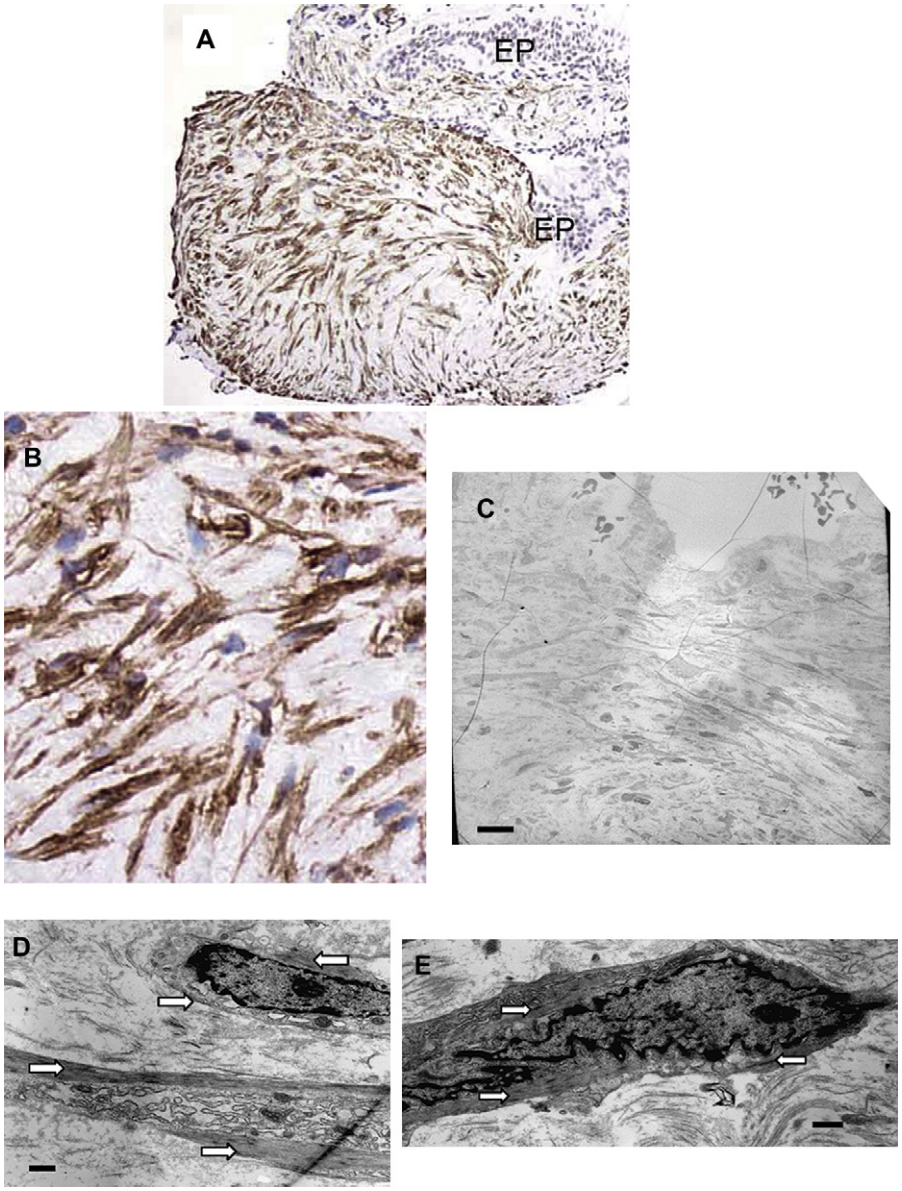


FIG E3. Immunohistochemistry to detect α-SMA in myofibroblasts (**A** and **B**, brown stain). Transmission electron microscopy of area rich in myofibroblasts (**C** and **D**). The similar morphology of myofibroblasts when viewed under light microscopy and low-power transmission electron microscopy can be appreciated by comparing Fig E3, *B*, and Fig E3, *C*. Fig E3, *D* and *E*, represent high-power photomicrographs of the area seen in Fig E3, *C*, which confirms the ultrastructural features of myofibroblasts. *White arrows* indicate microfilament bundles within myofibroblast cytoplasm. Original magnification: Fig E3, *A*, × 200; Fig E3, *B*, × 400. The *bar* in Fig E3, *C*, represents 20 μm, and the *bar* in Fig E3, *D* and *E*, represents 1 μm. *EP*, Epithelium.

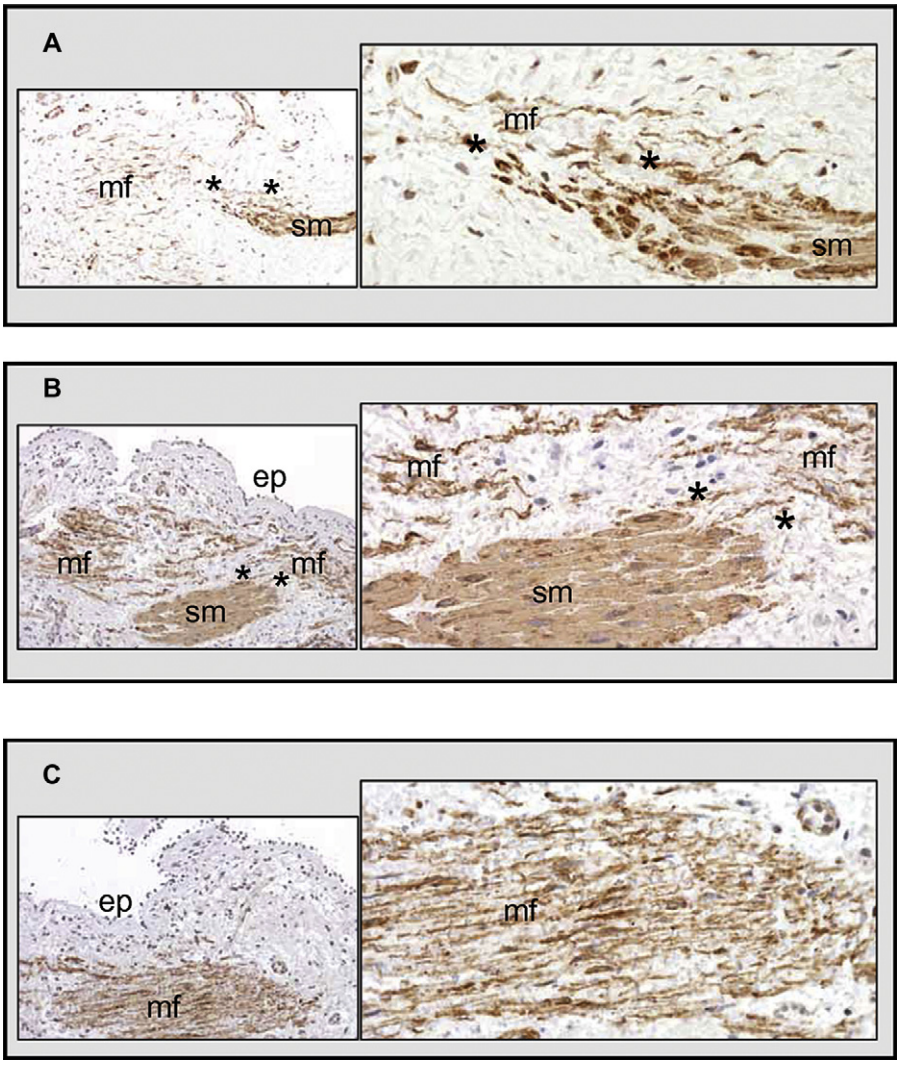


FIG E4. Immunohistochemistry directed against α-SMA in myofibroblasts and smooth muscle (brown stain). **A** and **B**, Areas of possible transition between smooth muscle *(sm)* cells and myofibroblasts *(mf; *)*. The epithelial surface *(ep)* has been mostly stripped away, with only the basal layer intact. **C**, Myofibroblasts within the submucosa that have a similar orientation and appear to form a syncytium. Original magnification: × 200 for left-sided panels and × 400 for right-sided panels.

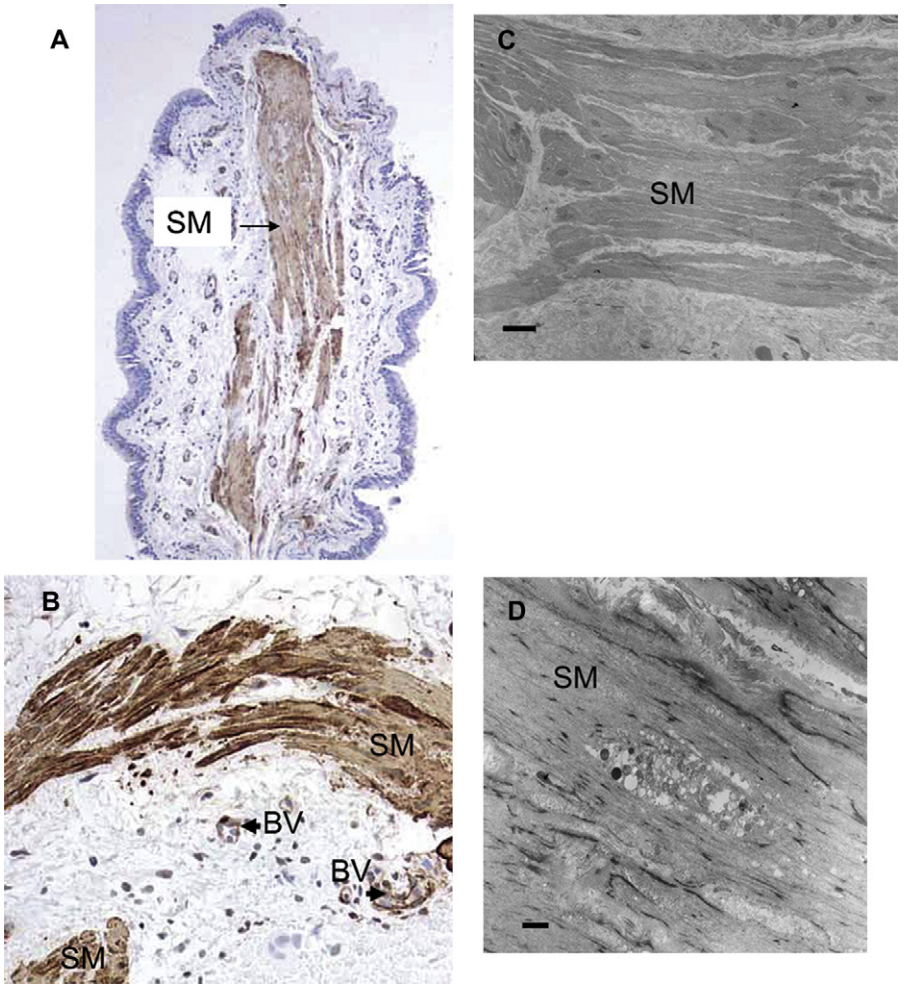


FIG E5. Immunohistochemistry to detect α-SMA in smooth muscle (**A** and **B**, brown stain). Transmission electron microscopy of smooth muscle (**C** and **D**). The similar morphology of smooth muscle bundles when viewed under light microscopy and low-power transmission electron microscopy can be appreciated by comparing Fig E5, *B*, and Fig E5, *C*. Fig E5, *D*, represents a high-power photomicrograph of Fig E5, *C*, which confirms the ultrastructural features of smooth muscle. Original magnification: Fig E5, *A*, × 60; Fig E5, *B*, × 400. The *bar* in Fig E5, *C*, represents 20 μm, and the *bar* in Fig E5, *D*, represents 1 μm. *SM*, Smooth muscle; *BV*, blood vessel.

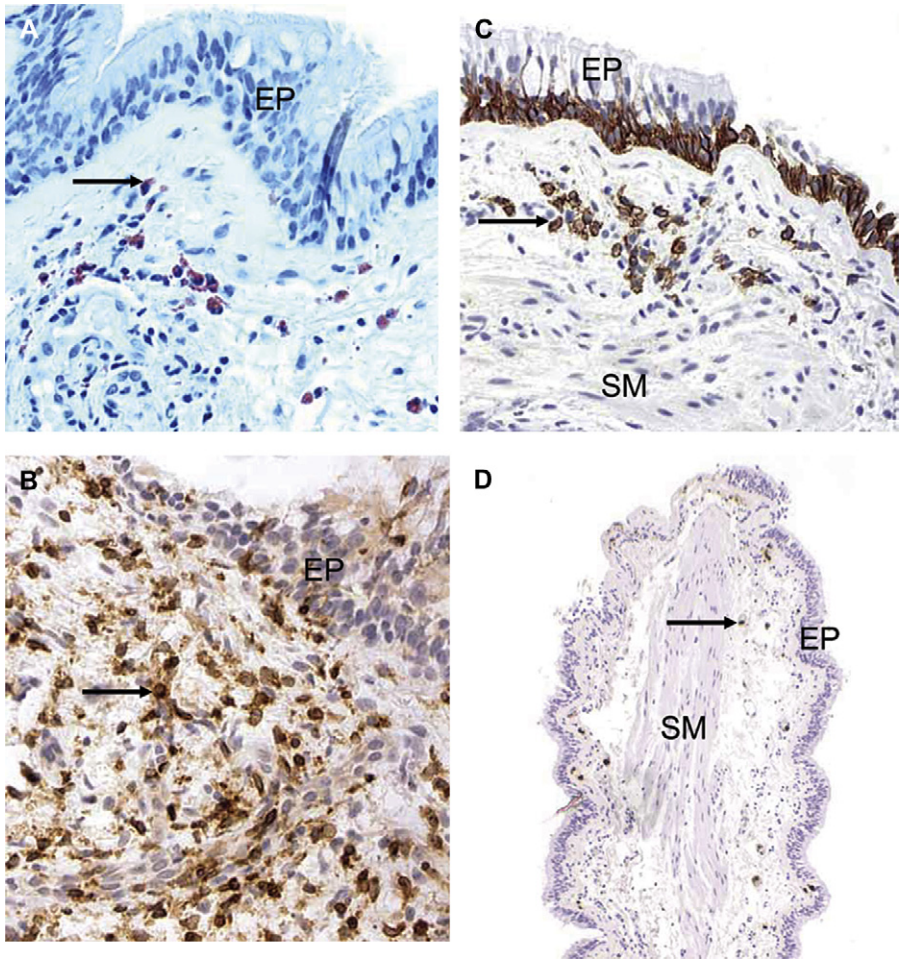


FIG E6. Special stains for eosinophils (**A**; Congo Red, red stain), $CD3^+$ lymphocytes (**B**; anti-CD3, brown stain), plasma cells (**C**; anti-CD138, brown stain), and neutrophils (**D**; anti-neutrophil elastase, brown stain). Original magnification: Fig E6, *A*, × 400; Fig E6, *B*, × 400; Fig E6, *C*, × 400; and Fig E6, *D*, × 60. Anti-CD138 stains the basal bronchial epithelial cells, as well as the plasma cells. *Arrows* indicate cells of interest. *SM*, Smooth muscle; *EP*, bronchial epithelium.

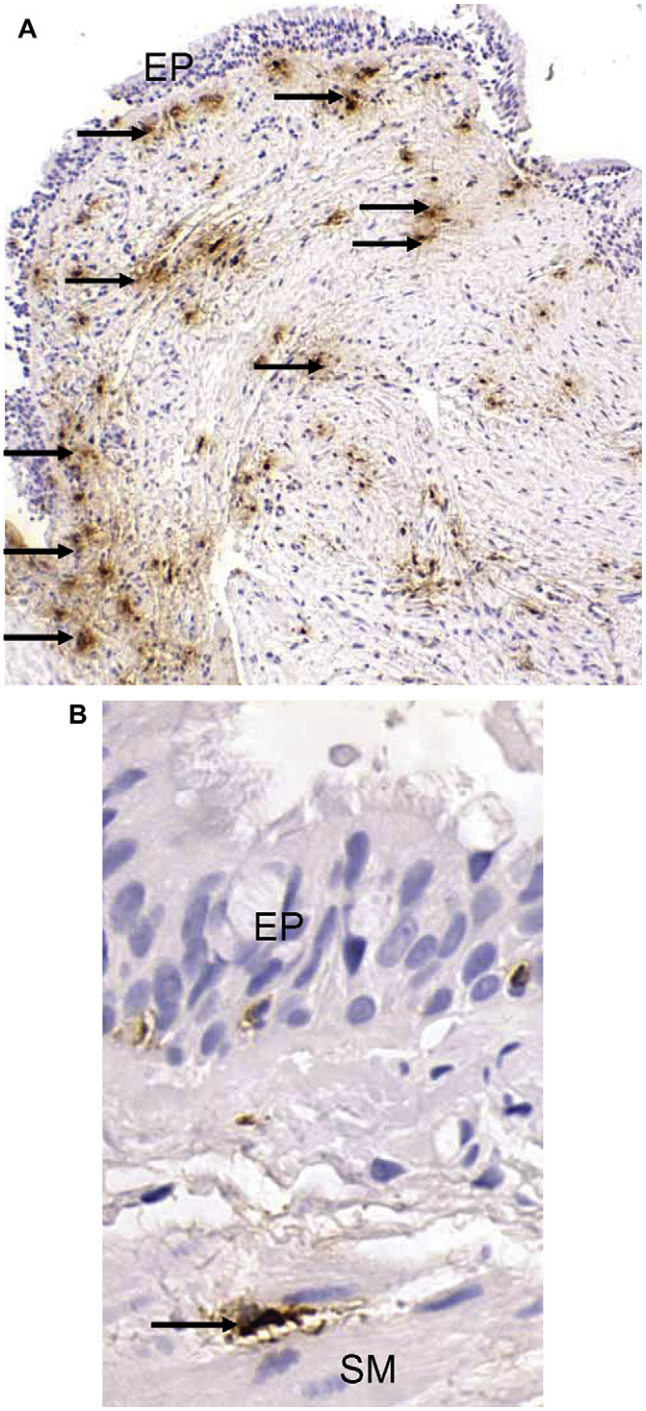


FIG E7. Special stains for mast cells (anti-tryptase, brown stain). Original magnification: **A**, × 60; **B**, × 400. *Arrows* indicate cells of interest. *SM*, Smooth muscle; *EP*, bronchial epithelium.

TABLE E1. Subjects' characteristics

Age (y), mean (SD)	29.0 (11.7)
Sex, no. (%)	
Male	6 (43)
Female	8 (57)
FEV_1 (% predicted)	
Mean (SD)	82.2 (10.2)
Range	69.3-103.2
MCh PC_{20}	
Mean* (SD)	3.9 (4.0)
Range	0.5-15.1
Antigen used in allergen challenge	
House dust mite *(Dermatophagoides pteronyssinus)*	9 (64.3)
House dust mite *(Dermatophagoides farinae)*	2 (14.3)
Grass	2 (14.3)
Cat	1 (7.1)
Allergen responses during screening	
EAR, mean (SD) decrease	−32.5 (5.8)
LAR, mean (SD) decrease	−22.2 (7.8)

EAR, Early airway response; *LAR*, late airway response.
*Geometric mean.

TABLE E2. Inflammatory cells within epithelium

Cell type	Diluent	Allergen challenge		
		Placebo	Budesonide	Budesonide/formoterol
CD45 cells	482 (447)	523 (561)	350 (283)	380 (341)
$CD3^+$ cells	388 (337)	298 (610)	237 (285)	270 (369)
$CD4^+$ T cells	21 (108)	30 (61)	3 (26)	16 (29)
$CD8^+$ T cells	359 (465)	288 (441)	134 (276)	134 (230)
CD20 B cells	0 (2)	0 (53)	0 (0)	0 (0)
Eosinophils	0 (15)	0.8 (6.3)	0 (0.0)*	0 (4)
Mast cells ($AA1^+$)	35 (86)	38 (83)	38 (65)	30 (71)
$CD68^+$ cells	9 (29)	8 (25)	0 (14)	0 (12)
Neutrophils	45 (81)	67 (138)	58 (380)	22 (66)

Numbers of cells are expressed as medians (IQRs) per square millimeter.
*$P = .034$, budesonide treatment compared with placebo treatment.

TABLE E3. Inflammatory cells in bronchial subepithelium

		Allergen challenge		
Cell type	**Diluent**	**Placebo**	**Budesonide**	**Budesonide/formoterol**
CD45 cells	686 (839)	780 (973)	376 (1360)	625 (883)
$CD3^+$ cells	513 (315)	364 (404)	274 (865)	372 (489)
$CD4^+$ T cells	211 (268)	145 (223)	69 (435)	175 (234)
$CD8^+$ T cells	251 (172)	210 (168)	16.2 (56.8)	276 (312)
CD20 B cells	20 (73)	33 (55)	4.0 (7.1)	32 (47)
Eosinophils	37 (37)	37 (63)	9 (15)	12 (78)
Mast cells ($AA1^+$)	52 (96)	48 (66)	99 (75)	78 (98)
Plasma cells	103 (121)	90 (209)	11 (74)	34 (66)
$CD68^+$ cells	14 (16)	3.0 (2.1)	2.7 (2.9)	7 (35)
Neutrophils	140 (167)	150 (282)	174 (710)	147 (162)

Numbers of cells are expressed as medians (IQRs) per square millimeter.