Combined budesonide/formoterol therapy in conjunction with allergen avoidance ameliorates house dust mite-induced airway remodeling and dysfunction

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Allergic asthma is a chronic disease of the airways characterized by immune-driven inflammation, remodeling changes in the airway wall, and bronchial hyperreactivity (2, 3, 10, 19, 39). Although there has been remarkable progress toward understanding these processes, the impact of currently available treatment strategies on the full pathophysiology of asthma remains unclear. Indeed, although the anti-inflammatory and bronchodilatory capabilities of inhaled corticosteroids and long-acting β2-agonists, respectively, have been well documented, the consequences of these interventions on airway structure and function remain either controversial or poorly understood (reviewed in Ref. 41).

In this study, we investigated the impact of corticosteroid and β2-agonist combination therapy, which is now emerging as the treatment of choice for many asthmatics, on the immunoinflammatory, structural, and physiological processes associated with chronic allergen exposure. In BALB/c mice, chronic intranasal exposure to house dust mite extract (HDM) results in sustained, severe eosinophilic airway inflammation, peribronchial remodeling, and severe bronchial hyperreactivity similar to the phenotype of human asthma. Interestingly, discontinuation of allergen exposure results in rapid resolution of the immunoinflammatory process; however, bronchial hyperreactivity is resolved only partially, whereas remodeling changes do not resolve at all (18). With this background, we elected to examine the therapeutic impact of combination therapy with a corticosteroid, budesonide (BUD), and a long-acting β2 agonist, formoterol (FORM), under a variety of conditions designed to evaluate the efficacy of this intervention in the presence or absence of continued allergen exposure (Fig. 1). In all experiments, combination treatment with BUD and FORM (BUD/FORM) was initiated once airway inflammation was established. The first aim of this study was to evaluate the impact of this treatment on airway inflammation, structure, and function in the context of continuous allergen exposure. Treatment was either initiated early, when the airway inflammatory response to HDM had plateaued but remodeling changes to the airway wall were incipient (Protocol 1), or later in the disease process, when both inflammation and remodeling were fully established (Protocol 2). Second, we investigated whether the effectiveness of BUD/FORM therapy could be improved by treatment following withdrawal of the inciting allergen; as before, treatment was initiated early (Protocol 3) or later, 2 wk after lung pathology was fully established (Protocol 4). Collectively, our data show that BUD/FORM therapy, given to mice with severely inflamed airways, led to a remarkable reduction in airway inflammation despite continued allergen exposure. However, better control of the spectrum of immunoinflammatory, structural, and functional abnormalities associated with allergic asthma was achieved when BUD/FORM therapy was initiated early with the concomitant cessation of allergen exposure.

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METHODS

Animals. Female BALB/c mice were purchased from Charles River Laboratories (Montréal, Québec, Canada) and initiated into experiments at 7–9 wk of age (~20 g wt). Mice were housed under specific pathogen-free conditions following a 12-h light-dark cycle. Cages, food, and bedding were autoclaved, and all mice were handled in a laminar flow hood by gloved, gowned, and masked personnel. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University and followed the guidelines put forward by the Canadian Council on Animal Care.

Allergen administration. Mice were exposed to purified HDM whole body extract (Greer Laboratories, Lenoir, NC) intranasally (25 μg of protein in 10 μl of saline) under inhaled anesthesia (Isoflurane; Pharmaceutical Partners of Canada, Richmond Hill, Ontario, Canada) for 5 consecutive days, followed by 2 days rest, for 5 or 7 consecutive weeks. No exogenous adjuvant was given at any time. Negative control animals were age-matched allergen-naïve mice.

Drug delivery. Drug delivery was performed 5 days per week for 2 consecutive weeks. In some experiments, animals were exposed to a dry powder aerosol drug formulation [as described previously by Wiley et al. (38)] of either lactose vehicle or BUD/FORM combination therapy for 10 min immediately before daily allergen administration given for the last 2 wk of a 5- or 7-wk allergen exposure protocol (concurrent treatment). In other studies, mice were given allergen for either 5 or 7 consecutive weeks, at which point allergen exposure was stopped and mice were treated with either lactose or BUD/FORM daily for the next 2 wk (postexposure treatment). Allergen and drug delivery protocols are shown in Fig. 1. The dry powder formulations of BUD/FORM/lactose (90:5:90 wt/wt/wt) or lactose alone were delivered as aerosols at a total powder concentration of ~160 μg/l. The actual aerosol concentrations (analyzed from filter in 10 l of saline) for 5 days a week for 5 (Protocol 1) or 7 (Protocol 2) consecutive weeks. Dry powder inhalation of either lactose vehicle or BUD/FORM was provided for 10 min immediately before daily HDM challenge during the last 2 wk of allergen exposure. In the postexposure treatment groups, mice were exposed to HDM (25 μg of protein in 10 μl of saline) for 5 days a week for 5 (Protocol 3) or 7 (Protocol 4) consecutive weeks. Dry powder inhalation of either lactose vehicle or BUD/FORM was initiated following cessation of allergen exposure and continued for 2 wk. Treatment was provided for 10 min daily. Mice were killed 24 h after the last dose of BUD/FORM.

Assessment of airway hyperresponsiveness. Airway responsiveness was measured 24 h after the last dose of BUD/FORM by assessing total respiratory system resistance (Rrs) to increasing intravenous (internal jugular vein) doses of methacholine (MCh) as previously described (8). Briefly, mice were anesthetized with triphysane and bronchoalveolar lavage (BAL) fluid, and lungs were collected 24 h after the last dose of BUD/FORM. BAL fluid was collected by dissection of the lungs and cannulation of the trachea with polyethylene tubing (Becton Dickinson, Sparks, MD) as previously described (32). Briefly, the lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Approximately 0.3 ml of the instilled fluid was consistently recovered. Total cell counts in BAL fluid were performed using hemocytometer. After centrifugation, cell pellets were resuspended in PBS, and smears were prepared by cytospin centrifugation (Shandon, Pittsburgh, PA) at 300 rpm for 2 min. The Hema 3 stain set (Biochemical Sciences, Swedeshor, NJ) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hematoxylogical criteria to classify them as mononuclear cells, neutrophils, or eosinophils. Additionally, peripheral blood was collected by retroorbital bleeding using heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA). Serum was prepared by incubating whole blood for 30 min at 37°C.

Collection and measurement of specimens. Isoflurane-anesthetized mice were killed by exsanguination and bronchoalveolar lavage (BAL) fluid, and lungs were collected 24 h after the last dose of BUD/FORM. BAL fluid was collected by dissection of the lungs and cannulation of the trachea with polyethylene tubing (Becton Dickinson, Sparks, MD) as previously described (32). Briefly, the lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml). Approximately 0.3 ml of the instilled fluid was consistently recovered. Total cell counts in BAL fluid were performed using hemocytometer. After centrifugation, cell pellets were resuspended in PBS, and smears were prepared by cytospin centrifugation (Shandon, Pittsburgh, PA) at 300 rpm for 2 min. The Hema 3 stain set (Biochemical Sciences, Swedeshor, NJ) was used to stain all smears. Differential counts of BAL cells were determined from at least 500 leukocytes using standard hematoxylogical criteria to classify them as mononuclear cells, neutrophils, or eosinophils. Additionally, peripheral blood was collected by retroorbital bleeding using heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA). Serum was prepared by incubating whole blood for 30 min at 37°C.

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in Tris-buffered saline for 15 min at room temperature. Sections were incubated with α-SMA primary antibody (diluted 1:150 in 1% NSS; clone 1A4, Dako) for 1 h at room temperature and then with biotinylated rabbit anti-mouse secondary antibody (diluted 1:300 in 1% NSS; Dako) for 1 h at room temperature. Subsequently, sections were incubated in streptavidin-peroxidase conjugate (diluted 1:600 in 1% NSS; Dako) for 45 min at room temperature, placed in acetate buffer (pH 5.0) for 5 min, and then incubated in freshly prepared chromogen substrate solution for 15 min. Tissues were counterstained in Mayer’s hematoxylin for 1 min, washed, and mounted in glycerin gelatin.

Morphometry. Images for morphometric analysis were captured using OpenLab software version 3.0.3 (Improvision, Guelph, Ontario, Canada) via a Leica camera and microscope attached to a Macintosh computer (Mac OS 9 operating system). Analysis (6–10 animals per group) was performed on a custom computerized image analysis system [Northern Eclipse software version 6 (Empix Imaging, Mississauga, Ontario, Canada) on a Pentium IV computer (2.4 GHz processor, Windows XP operating system)]. Morphometric quantification involved calculating the percent of tissue area that was positively stained within regions of interest (1 slide per mouse per stain). For analysis of epithelial mucus production, images were subjected to digital color inversion, and the airway epithelium was digitally isolated. Positive mucus stain (bright green) was selected using RGB color modulation, 70–98 red, 119–254 green, and 109–219 blue. For analysis of subepithelial remodeling, sections of the airway that were associated with connective tissue attachments to associated vessels were excluded from the analysis. Analysis of sections stained for PSR was performed on a 30-μm wide section extending from the basement membrane into the parenchyma, and positive stain was selected using RGB color modulation, 0–359 red, 116–212 green, and 40–255 blue. Analysis of sections stained for α-SMA was performed on a 20-μm wide section extending from the basement membrane into the parenchyma, and positive stain was selected using RGB color modulation, 0–255 red, 20–195 green, and 0–192 blue.

Data analysis. Data are expressed as means ± SE unless otherwise indicated. Results were interpreted using Student’s t-test except for analysis of airway hyperreactivity data, which was performed using one-way ANOVA for repeated measures. Differences were considered to be statistically significant when P < 0.05.

RESULTS

Airway inflammation in mice exposed to HDM. Mice were chronically exposed to HDM extract for 5 or 7 consecutive weeks. We observed robust airway inflammation, as evidenced by elevated cellular infiltration into the BAL (Fig. 2A). The nature of the inflammation following either 5 or 7 wk of allergen exposure was characterized by significant numbers of eosinophils in the BAL (Fig. 2B). BUD/FORM treatment delivered during the last 2 wk of allergen exposure led to a significant decrease in both total cellular airway inflammation and BAL eosinophilia (Protocols 1 and 2). Total inflammation and eosinophilia returned to baseline levels (indicated by the dotted line, Fig. 2A) after 2 wk of allergen avoidance (Protocols 3 and 4).

Histopathological evaluation of lung tissue stained for hematoxylin and eosin (Fig. 2C) demonstrated extensive peribronchial and perivascular inflammation in HDM-exposed animals given lactose vehicle during the last 2 wk of allergen exposure. In contrast, there was a marked decrease in inflammatory infiltrates in tissues from BUD/FORM-treated mice (Protocols 1 and 2). Lung inflammation was drastically reduced 2 wk after the final HDM exposure, in both vehicle control and BUD/FORM treatment groups (Protocols 3 and 4).

Epithelial mucus production and epithelial thickness. Epithelial mucus production was quantified morphometrically (Fig. 3A) by evaluating lung tissues stained with PAS (Fig. 3B). Mucus production increased compared with baseline levels (indicated by the dotted line, Fig. 3A) with allergen exposure and was significantly reduced in mice given BUD/FORM early with continued allergen exposure (Protocol 1), whereas this effect was not seen when BUD/FORM treatment was initiated later in the disease process (Protocol 2). Allergen withdrawal was associated with decreased epithelial mucus production, with further improvement seen in mice given BUD/FORM (Protocols 3 and 4).

Subepithelial collagen deposition. Increased collagen deposition in the airway subepithelium (Fig. 4B) was apparent in mice exposed to HDM for either 5 or 7 wk. The observed increases, compared with allergen-naive controls (indicated by the dotted line), in collagen accumulation in the airway wall were quantified morphometrically (Fig. 4A). No significant differences were observed in collagen deposition in mice given BUD/FORM compared with lactose vehicle in either of the concurrent treatment protocols (Protocols 1 and 2). However, postexposure BUD/FORM treatment was associated with a significant decrease in total collagen deposition in mice exposed to HDM for 5 wk (Protocol 3), although there was no significant drug effect observed when BUD/FORM treatment was initiated after 2 additional weeks of allergen exposure (Protocol 4).

Expression of α-SMA. Increased α-SMA expression in the airway subepithelium (Fig. 5B) was apparent in mice exposed to HDM for either 5 or 7 wk. The observed increases compared with allergen-naive controls (indicated by the dotted line) in α-SMA expression in the airway wall were quantified morphometrically (Fig. 5A). A statistically significant reduction in α-SMA expression was observed in mice given BUD/FORM in both of the concurrent treatment protocols (Protocols 1 and 2). Furthermore, early postexposure BUD/FORM treatment was associated with a significant decrease in α-SMA expression (Protocol 3), although there was no significant drug effect on this parameter when BUD/FORM treatment was initiated after 2 additional weeks of allergen exposure (Protocol 4).

Bronchial hyperresponsiveness to MCh. The two key indicators of airway responsiveness (as described in Ref. 20), namely airway reactivity (the rate of increase in respiratory resistance for a given increase of dose of MCh) and maximal inducible bronchoconstriction (maximum respiratory resistance), were evaluated in lactose vehicle- and BUD/FORM-treated mice continuously exposed to HDM for 5 or 7 wk, and in vehicle- and BUD/FORM-treated mice 2 wk after cessation of allergen exposure (Fig. 6). BUD/FORM treatment given concurrently with allergen exposure had no effect on maximal bronchoconstriction or airway reactivity, although a reduction in airway resistance was observed at 33 mg/kg MCh in mice given BUD/FORM during the last 2 wk of a 5-wk period of allergen exposure (Protocol 1). In animals given postexposure BUD/FORM treatment for 2 wk following 5 wk of HDM challenge (Protocol 3), both airway reactivity and maximal inducible bronchoconstriction returned to baseline levels. A partial improvement in airway function was also observed in animals treated with BUD/FORM for 2 wk following 7 wk of HDM challenge (Protocol 4), with a reduction in airway resistance but no improvement in airway reactivity.
DISCUSSION

Inhaled corticosteroids and β2-adrenoceptor agonists are the two most effective and commonly used pharmacotherapies for allergic asthma. These treatments have been shown to effectively control airway inflammation and bronchoconstriction, respectively (2, 4, 7, 9, 13, 14). Recently, combination therapy, i.e., the addition of a long-acting β2-agonist to low-to-medium doses of inhaled corticosteroids, has become the preferred treatment for moderate persistent and severe asthma in adults and children. Indeed, clinical studies have demonstrated that combination therapy is superior to corticosteroid monotherapy at improving asthma symptoms and reducing the frequency of exacerbations (1, 11, 17, 24, 27, 29, 40).

Very little is known about the impact of corticosteroids and, particularly, combination therapy, on the airway structural changes, commonly referred to as remodeling, associated with chronic asthma. Remodeling includes a number of changes in the structure of the airway wall such as goblet cell hyperplasia, peribronchial collagen deposition, and increased contractile elements (3). A variety of factors have been associated with the development of airway remodeling, including the persistence and duration of asthma symptoms, the number of mast cells, and eosinophils in the airway and the degree of T lymphocyte infiltration (3, 15, 19). Importantly, whether remodeling can be prevented or reversed is highly relevant because these changes to the airway wall are thought to contribute to airway dysfunction, even in cases of mild asthma (5, 28, 36).

The relationship between chronic inflammation, airway remodeling, and airway dysfunction is complex because these are interacting events each with distinct kinetics. For this reason, an experimental model mimicking the phenotype of chronic asthma is useful as it may allow for addressing questions that for technical and/or ethical reasons cannot be addressed in humans. In this study, we set out to investigate the impact of combination therapy in an experimental model in
mice driven by continuous intranasal exposure to a HDM extract for up to 7 wk. This protocol leads to maintained inflammation, robust remodeling, and bronchial hyperreactivity (18). That this protocol is associated with sustained eosinophilic inflammation is relevant because in ovalbumin (OVA)-based models, chronic OVA exposure, whether continuous or intermittent, leads to a progressive diminution, in fact a complete abrogation, of eosinophilic inflammation (22, 33, 34).

Our data show that combination BUD/FORM therapy had a profound effect on reducing established airway inflammation. The reduction in BAL inflammation was confirmed in the lung tissue by histopathology, thus demonstrating that BUD/FORM treatment reduced inflammation in the entire target organ. Although the anti-inflammatory effect of combination therapy was expected, it is notable that it occurred in the context of continuous allergen exposure. On cessation of HDM exposure, both BAL and lung inflammation decreased remarkably in both lactose vehicle- and BUD/FORM-treated mice, demonstrating that airway inflammation is entirely dependent on the presence of allergen.

Our studies on the effect of combination therapy on airway structural changes yielded divergent results. First, we examined changes occurring in the airway epithelium. As expected, epithelial mucus production was significantly increased in mice chronically exposed to HDM. Combination therapy significantly decreased epithelial mucus production during concurrent allergen exposure but only when it was administered early in the disease process, i.e., after 3 wk of exposure. It also decreased when allergen exposure was discontinued, indicating that epithelial mucus production is primarily dependent on the presence of inflammation in the airway. In the airway subepithelium, combination therapy was able to reduce expression of α-SMA when given concurrently with allergen. In contrast, combination therapy had no effect on subepithelial collagen deposition as long as mice were exposed to allergen. Unlike airway inflammation and epithelial mucus production, collagen deposition and α-SMA expression did not resolve at all with allergen withdrawal. However, a significant decrease in these subepithelial structural changes was observed when combination therapy was administered after 5 wk, but not after 7 wk, of allergen exposure along with discontinuation of allergen. It should be noted that the degree of airway inflammation is similar at these two time points. Thus these findings suggest that a reversal of several aspects of structural changes, i.e., subepithelial collagen deposition and α-SMA expression, is dependent on both the discontinuation of allergen exposure and the duration of the remodeling process. As the data show, the observed amelioration was partial; whether further reversal of airway remodeling could have been achieved with combination therapy for longer than 2 wk remains to be investigated.

Regardless, that allergen avoidance in conjunction with combination therapy delivered locally was able to reverse established structural abnormalities of the airway is a finding of potential clinical significance.
The effect of corticosteroids on airway remodeling is a subject under intense study at present both in animal models of allergic airway inflammation and in human asthmatics. Two recent studies employing either prophylactic (23) or therapeutic (6) corticosteroid treatment demonstrated decreased collagen deposition associated with drug treatment, although therapeutic intervention with corticosteroids had a beneficial impact on airway remodeling only following the cessation of allergen exposure, thus supporting our findings. However, these studies were conducted in a model that used the surrogate allergen OVA and dexamethasone delivered systemically. Biopsy studies in human asthmatics show variable results (16, 25, 31, 35, 37), likely as a consequence of the fact that the impact of inhaled corticosteroid therapy is influenced by a number of variables, including disease severity at the time when treatment is initiated, extent of the disease, the duration of treatment, and whether the individual patient continues to be exposed to the inciting allergen.

Despite the fact that early intervention with combination therapy markedly reduced airway inflammation, mucus production, and α-SMA expression even with ongoing allergen exposure, bronchial hyperreactivity was not affected under these conditions. In contrast, airway reactivity and maximal inducible bronchoconstriction returned to baseline levels when allergen exposure was discontinued after 5 wk, and combination therapy implemented an effect that was associated with an improvement in all of the remodeling parameters investigated in this study (mucus, collagen, and α-SMA expression). Interestingly, delaying allergen discontinuation and pharmacological treatment by another 2 wk was associated with still a marked improvement in mucus but no changes at all in collagen and α-SMA expression; under these conditions, the improvement in lung function was only partial. Collectively, these findings indicate that different elements of the allergic airway disease phenotype are susceptible to reversal under different conditions and show that the success of treatment on both the structure and the function of the lung is significantly influenced not only by continuing allergen exposure, but also by the duration and/or severity of the disease.

It is becoming increasingly evident, from our findings and other observations, that the management strategy that furnishes the greater beneficial effect on lung structure and function is that which combines avoidance of the inciting allergen or irritant with a pharmacological treatment that includes BUD/FORM combination therapy. It is also of particular interest that the greatest beneficial effect was observed when the combination of allergen avoidance and pharmacological treatment was implemented early.

In summary, we have evaluated the impact of BUD/FORM treatment, alone or combined with allergen avoidance, in a murine model of sustained eosinophilic inflammation and airway remodeling. We show that BUD/FORM combination therapy remarkably suppresses established airway inflammation, goblet cell hyperplasia, and subepithelial...
α-SMA expression, even with ongoing allergen exposure. However, treatment under these conditions was not associated with an improvement in collagen deposition or a return to normal lung function. Our data also demonstrate that neither BUD/FORM combination therapy nor allergen avoidance alone fully resolve airway remodeling or lung dysfunction. In contrast, treatment with BUD/FORM along with allergen avoidance significantly ameliorates established airway remodeling and function. These data intimate that the optimal therapeutic impact on the asthmatic phenotype can be achieved by combining pharmacotherapy early in the disease process with allergen avoidance or, presumably, avoidance of other factors that can perpetuate the immune-inflammatory syndrome.

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Fig. 5. Impact of BUD/FORM and allergen avoidance on subepithelial α-smooth muscle actin (α-SMA) expression. Mice were exposed to HDM extract for 5 or 7 wk and treated with lactose vehicle (black bars) or BUD/FORM (gray bars) according to study protocols. A: lung sections were stained by immunohistochemistry for α-SMA and were analyzed morphometrically to quantify the accumulation of subepithelial contractile elements. Means ± SE, n = 8–14 from 2 independent experiments; *P < 0.05 compared with lactose-treated control mice. Statistical analysis was performed using Student’s t-test. Dotted line denotes basal levels in allergen-naïve mice. B: lung sections were stained by immunohistochemistry for α-SMA to demonstrate the accumulation of subepithelial contractile elements. Bar indicates 50 μm.

Fig. 6. Evaluation of airway hyperreactivity to methacholine in mice treated with either lactose vehicle (black) or BUD/FORM (gray) according to study protocols. The dose-response relationship between respiratory resistance and increasing intravenous doses of methacholine was evaluated in individual mice. Means ± SE, n = 8–10; *P < 0.05 compared with lactose-treated control mice (for each dose of methacholine); †P < 0.05 compared with lactose vehicle-controls (maximal bronchoconstriction); and ‡P < 0.05 compared with lactose vehicle-controls (airway reactivity). Statistical analysis was performed using 1-way ANOVA for repeated measures. Allergen-naïve mice were used as negative control (Neg Ctrl; white).
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