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Effect of mometasone furoate (MF)/formoterol fumarate (F) combination (MF/F) on late-phase responses in allergen-challenged Brown Norway rats

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ABSTRACT

Mometasone furoate (MF)/formoterol fumarate (F) combination is a new inhaled corticosteroid/long-acting β_2-adrenergic agonist (ICS/LABA). The purpose of this study was to evaluate the effects of different dose combinations of MF/F on a variety of late-phase responses to aerosolized antigen challenge in ovalbumin sensitized Brown Norway rats. Late-phase responses were assessed by reductions in lung function, measured by forced vital capacity (FVC) and increased numbers of inflammatory cells and pro-inflammatory cytokines in the bronchoalveolar lavage (BAL) fluid of ovalbumin challenged rats. Intratracheal administration of MF/F 5 h before aerosolized ovalbumin challenge inhibited the increase in inflammatory cells, including eosinophils and levels of interleukin (IL)-4, IL-5, IL-13 and tumour necrosis factor-α (TNF-α) appearing in the bronchoalveolar lavage fluid 24 h after the antigen challenge. The combination index for inhibition of both inflammatory cells and cytokines was consistently <1 suggesting a synergistic interaction between MF and F. Intratracheal MF/F given 24 h after the aerosolized ovalbumin challenge reversed the reduction in FVC with statistically significant effects seen over a 24 h period after drug whereas MF and F alone reversed the antigen-induced reduction in FVC at selected times only. At 5 h after drug administration, when both MF and F were partially active, the combination index for MF/F was <1 suggesting a synergistic interaction between MF and F for reversal of the lung function. These results demonstrate that MF/F combination inhibits a variety of late-phase responses induced by allergen challenge and it is likely that MF/F will have a significant benefit in clinical asthma to suppress lung inflammation and improve lung function.

1. Introduction

Fixed dose combinations of inhaled corticosteroids (ICS) and long-acting β_2-adrenergic receptor agonists (LABA) are now the standard of care for patients with moderate to severe persistent asthma and chronic obstructive pulmonary disease. Several fixed dose ICS/LABA combinations have been marketed for the treatment of these disorders and include fluticasone propionate/salmeterol (Advair; GlaxoSmithKline), budesonide/formoterol fumarate (Symbicort; AstraZeneca) and beclomethasone di-propionate/formoterol fumarate (FOSTER; Chiesi). A new ICS/LABA which is currently in development (Merck and Novartis) combines the potent ICS, mometasone furoate (MF), with formoterol fumarate (F). This combination is expected to offer many advantages since MF is the most potent ICS product currently on the market [1,2] and F has the distinct advantage over other LABAs as it possesses a rapid onset of action [3].

Combination ICS/LABA products suppress many of the important features of asthma including effects on airflow obstruction, airway hyper-responsiveness, inflammatory cell influx into the lungs, expression and production of pro-inflammatory cytokines and mediators, airway microvascular leakage and mucus hypersecretion [4]. Furthermore, ICS/LABA combinations occasionally produce these effects in a complementary or synergistic manner [4,5]. In a study with MF/F in mice [6], a synergistic interaction between MF and F was found for suppression of the airway reactivity to inhaled allergen. However, changes in lung function in this MF/F study in mice [6] were assessed using the enhanced pause (Penh) method in unrestrained animals which is a technique that may not fully reflect true changes in lung mechanics [7]. Besides

Abbreviations: BAL, bronchoalveolar lavage; CXCL1, chemokine (C-X-C motif) ligand 1; FVC, forced vital capacity; F, formoterol fumarate; ICS, inhaled corticosteroid; IFN-γ, interferon-γ; IL, interleukin; LABA, long-acting β_2-adrenergic agonist; MF, mometasone furoate; OVA, ovalbumin; TNF-α, tumour necrosis factor-α; i.t., intratracheal.

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this single study in mice, the beneficial effects of MF/F combination on inflammatory processes and improvements in lung function have not been explored.

The purpose of this study was to investigate the effects of MF/F combination on a variety of clinically relevant parameters of asthma. Specifically, MF/F was evaluated for effects on the late-phase reductions in lung function, measured by forced expiratory maneuvers, inflammatory cell influx and cytokine production in the bronchoalveolar lavage (BAL) fluid of allergen-challenged Brown Norway rats. Previous studies in Brown Norway rats have identified effects with standard anti-asthma drugs including corticosteroids [8–11] and β_2-agonist bronchodilators [12,13] on a variety of inflammatory and physiological parameters induced by allergen challenge, which make this an ideal model to assess potential complementary or synergistic interactions between these two drug combinations.

2. Material and methods

2.1. Animals

Male Brown Norway rats, weighing 150–200 g (Charles River Laboratories, Kingston, NY, USA), were used in this study. The rats were housed, three per cage, in a climate-controlled animal facility with a 12:12 light:dark cycle and were provided with food and water *ad libitum*. All procedures were approved by the Animal Care and Use Committee of the Merck Research Laboratories (Kenilworth, NJ, USA) which is a facility accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

2.2. Sensitization and allergen challenge

Animals were sensitized by an intraperitoneal injection of 1 mL allergen containing 20 μg ovalbumin (OVA, grade III) and 8 mg aluminium hydroxide (alum) suspended in 0.9% saline vehicle. A booster injection of this alum-OVA mixture was given 7 days later. Non-sensitized control animals received vehicle (alum/saline) only. Seven days later, the sensitized rats were exposed to aerosolized OVA challenge, which was performed by placing the rats into a closed Plexiglas chamber (21 L) and exposing the rats to aerosolized OVA (1%) in saline for 30 min. Non-sensitized rats received aerosolized saline (0.9% NaCl) for 30 min. The aerosols were produced by an ultrasonic nebulizer (Model Ultra-Neb 99; DeVilbiss, Somerset, PA, USA) at a flow rate of approximately 8 L min^{-1}.

2.3. Lung function measurements

A commercially available (Buxco Electronics, NC, USA) forced expiratory maneuvers system was used to assess the effects of MF/F combination on lung function [9]. This technique measures a number of pulmonary functions associated with a forced expiratory maneuver, but we focused on the measurement of forced vital capacity (FVC) to evaluate the effect of MF/F. Our experience with this measurement find a reduction in FVC by approximately 40% after OVA challenge that persists for several days [9]. Furthermore, the reductions in FVC are inhibited or reversed by both corticosteroids and β_2-adrenergic agonists [8,13] which makes this an appropriate technique to assess the effects of MF/F.

The rats were anesthetized with sodium pentobarbital (50 mg kg^{-1} i.p.), the trachea was surgically isolated and a catheter (internal diameter 1.73 mm) was inserted through a small incision in the trachea and secured in place with surgical thread. The rats were placed inside a whole body plethysmograph and the tracheal catheter was connected to an outlet port in the wall of the plethysmograph. A specially designed one-way breathing valve was connected to the tracheal port and a pressure and vacuum source were connected to the one-way breathing valve to facilitate inflation and deflation of the lungs. Pulmonary airflow was derived from the differential pressure (±2 cm H_2O) measured across a wire mesh screen built into the wall of the plethysmograph. Lung volumes were calculated by integrating the airflow signal over time. Calibrations were performed before each experiment using a 3-mL syringe. Airway opening pressure (PaO) was measured at the tracheal port with a differential pressure transducer (±70 cm H_2O).

The rats were entrained to artificial ventilation ($V_T = 10$ mL kg^{-1} and $f = 60$ breaths·min^{-1}) and after a 5-min period of equilibration on mechanical ventilation, the lungs were inflated until PaO reached (+) 20 cm H_2O. The lungs were then rapidly deflated over 0.2 s to a negative pressure of (−) 40 cm H_2O. This procedure was performed in triplicate to derive the average FVC for each animal.

2.4. Bronchoalveolar lavage collection, cell enumeration and cytokine measurements

For the collection of BAL fluid, the rats were euthanized with sodium pentobarbital (125 mg kg^{-1} i.p.) and a tracheal catheter was inserted. BAL was collected by lavaging the lungs with two aliquots of 3 mL 0.9% NaCl solution. Total recovery volume per rat was approximately 4 mL. A 2.5 mL sample of BAL fluid was centrifuged (3000 rpm for 10 min), the supernatant discarded and the cell pellet reconstituted with 1 mL of cold distilled water to osmotically lyse only the red blood cells present. One mL of 1.8% NaCl was then added and the sample centrifuged for 10 min at 3000 rpm. The supernatant was once again discarded and the cell pellet re-suspended in 2.5 mL of 0.9% NaCl. The total cells in this BAL sample were counted using a hemocytometer and the number of cells per mL of BAL fluid calculated. For the differential enumeration of the BAL cells, a 200 μL sample of the BAL fluid was centrifuged (600 rpm for 6 min) onto a microscope slide (Cytospin 3; Shandon Scientific Ltd., Runcorn, UK) and stained with methylene blue, azure A and eosin (Leukostat; Fischer Scientific, Pittsburgh, PA, USA). The number of eosinophils and neutrophils per mL of BAL fluid were calculated.

Cytokine levels in the BAL supernatants were measured after dilution and mixing with phosphate-buffered saline containing 1% bovine serum albumin (protease free) and Complete™ Protease Inhibitor Cocktail tablet, containing EDTA and Pefabloc as directed by the manufacturer (Roche Applied Science). Supernatant aliquots were frozen at −20 °C until analysis. Twenty-five μL of thawed supernatants (in duplicate) were assessed for the presence of seven cytokines (interleukin [IL]-1β, chemokine (C-X-C motif) ligand 1 [CXCL1], IL-4, IL-5, tumour necrosis factor-α (TNF-α), interferon-γ [IFN-γ] and IL-13 using a Rat Demonstration 7-Plex Ultra-sensitive Kit (Meso Scale Discovery, Gaithersburg, MD, USA) according to the manufacturer's directions. Levels of the cytokines in the BAL fluid are expressed in electrochemiluminescence (ECL) units.

2.5. Drug administration

MF was synthesized at Merck Research Laboratories (Kenilworth, NJ, USA) and F was purchased from Bosche Scientific (New Brunswick, NJ, USA, lot #BS0706291315). The compounds were micronized, admixed with micronized lactose and given by the intratracheal route of administration [13]. Rats were lightly anesthetized with isoflurane (3% isoflurane supplemented with 100% oxygen) and a fine-tipped hand-operated DP-4 insufflator delivery device (PennCentury, Philadelphia, PA, USA) was inserted directly into the trachea with the aid of a laryngoscope. Three milligrams of the drug/lactose mixture was injected into the trachea and lungs using an air-filled 3-mL syringe. Vehicle-treated rats received 3 mg

of micronized lactose. The rats recovered from the anaesthesia within minutes of the intratracheal administrations.

Two different experimental paradigms were used in these studies. Compounds were administered either 5 h before antigen challenge with inflammatory cells or cytokines in the BAL fluid measured 24 h after antigen challenge. In another paradigm involving the measurement of FVC, the rats were first challenged with antigen and drugs were given 24 h after the antigen challenge. Measurement of drug effects were measured at selected times (1, 5 and 24 h) after drug administration. Different combinations of MF and F were used in these experiments based upon individual dose-response data with MF and F alone and on the combinations of MF and F that will likely be used in clinical studies [14].

2.6. Statistics

Statistical analysis was performed using a two-way analysis of variance using Fisher's PLSD to determine significant differences between the treatment groups. A P-value <0.05 was considered statistically significant. The results are expressed as the mean $\pm$ SEM. For dose-response experiments, the Hill equation was fitted by non-linear regression to the dose-response data. ED_{50}, Hill slope and correlation coefficients (r) were calculated by the Levenberg–Marquardt method [15]. The possible synergistic interaction between MF and F was analysed by an algebraic method that calculates results in terms of a combination index. A combination index of <1 indicates synergy [16].

3. Results

3.1. Effects on lung function

Experiments were first performed with MF and F alone to select appropriate doses for the combination MF/F study. In these experiments the rats were first challenged with aerosolized ovalbumin to induce a reduction in FVC and different doses of F (1, 10 and 100 $\mu g \cdot kg^{-1}$ i.t) and MF (100, 1000, and 3000 $\mu g \cdot kg^{-1}$ i.t) were given intratracheally to assess their ability to reverse this ovalbumin-induced FVC reduction. In these dose ranging experiments, FVC was measured 1 h after F and 5 h after MF based upon our previously published results with β_2-adrenergic receptor agonists and corticosteroids showing efficacy with these different drug classes at these post-treatment times [13]. Intratracheal administration of MF alone (100, 1000, and 3000 $\mu g \cdot kg^{-1}$ i.t.) dose-dependently reversed the deficit in FVC that was induced by the ovalbumin challenge (21 $\pm$ 9, 29 $\pm$ 6 and 51 $\pm$ 13% reversal, respectively) (Fig. 1A) and based upon these results, sub-maximal doses of 300 and 1000 $\mu g \cdot kg^{-1}$ i.t. were used in the combination studies. Much lower doses of F (1, 10 and 100 $\mu g \cdot kg^{-1}$ i.t.) were needed to reverse the ovalbumin-induced reduction in FVC (42 ± 14, 55 ± 21, and 72 ± 13% reversal, respectively), and from this dose-response experiment (Fig. 1B), sub-maximal doses of 1 and 10 $\mu g \cdot kg^{-1}$ F were selected for use in combination with MF.

In one combination study with MF alone (1000 $\mu g \cdot kg^{-1}$), F alone (1 $\mu g \cdot kg^{-1}$) and MF/F (1000 $\mu g \cdot kg^{-1}$ + 1 $\mu g \cdot kg^{-1}$), the reversal of ovalbumin-induced reduction in FVC by MF/F (54 $\pm$ 12%) was significantly ($P < 0.05$) greater than that of MF alone (23 $\pm$ 6%) but not of F alone (32 $\pm$ 7%) at 1 h (Fig. 2A) and significantly greater (91 $\pm$ 8%) than both MF (46 $\pm$ 6%) and F (33 $\pm$ 7%) alone at 5 h (Fig. 2B). By 24 h, the reversal of FVC by MF/F was 71 $\pm$ 14% (Fig. 2C) which was significantly greater than that produced by F alone (23 $\pm$ 15%) but not different from that produced by MF alone (72 $\pm$ 12%). The combination index for MF/F at the 5 h time was <1, suggesting that MF/F acted in a synergistic manner for reversal of the FVC reduction induced by antigen challenge.

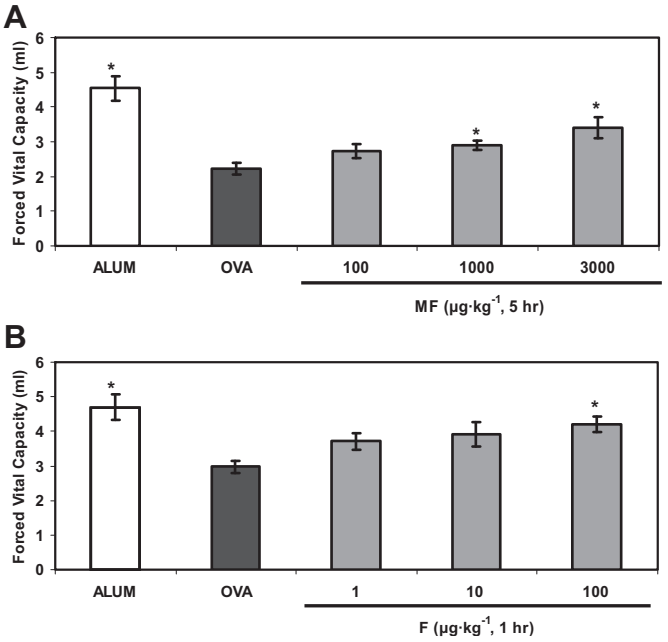

Fig. 1. Effect of MF (A) and F (B) alone on forced vital capacity (FVC) in allergen-challenged rats. Drugs or vehicle were given 24 h after allergen ovalbumin (OVA) challenge and FVC was measured 5 h after MF and 1 h after F. $^*P < 0.05$ versus OVA group. Values are mean $\pm$ SEM ($n = 7$–8 per group).

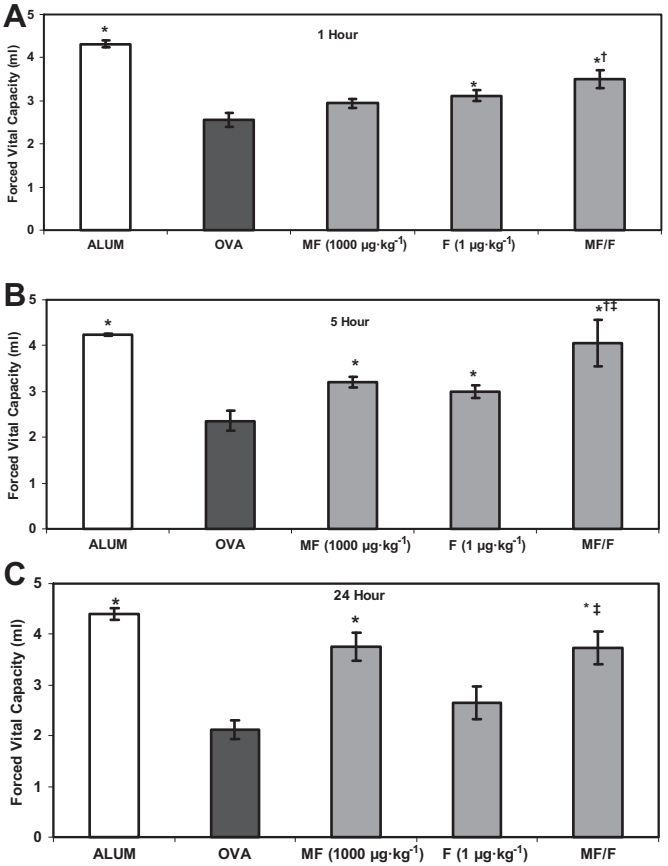

Fig. 2. Effect of MF, F, and MF/F on the reduction in forced vital capacity (FVC) induced by aerosolized ovalbumin (OVA) challenge. Drugs or vehicle were given 24 h after OVA challenge and FVC was measured 1 (A), 5 (B) and 24 (C) h after MF, F and MF/F. $^*P < 0.05$ versus OVA group. †$P < 0.05$ versus MF. ‡$P < 0.05$ versus F. Values are mean $\pm$ SEM ($n = 7$–8 per group).

In another combination study, MF alone (300 μg·kg^{-1}), F alone (10 μg·kg^{-1}) and MF/F (300 μg·kg^{-1} + 10 μg·kg^{-1}) were given intratracheally to ovalbumin challenged rats and FVC measured 1, 5 and 24 h after their administration. Similar results were obtained with this MF/F combination showing evidence of a synergistic interaction (combination index <1) between MF and F at the 5 h time (Table 1).

3.2. Effects on inflammatory cells

The number of total inflammatory cells, eosinophils and neutrophils in the BAL fluid of non-sensitized rats was 102 ± 15, 29 ± 12 and 11 ± 3 × 10^3 cells/ml, respectively. Nebulized ovalbumin challenge to sensitized rats significantly increased the number of BAL total inflammatory cells, eosinophils and neutrophils to 371 ± 34, 156 ± 22 and 123 ± 15 × 10^3 cells/ml, respectively. Treatment of rats with MF alone 5 h before the ovalbumin challenge inhibited the increase in total cells, eosinophils and neutrophils that were induced by the antigen challenge (Fig. 3). The Hill slope for inhibition of total cells and eosinophils by MF were 0.4 and 0.6, respectively. Furthermore, the ED_{50} values for inhibition of total cells and eosinophils by MF were 82 (r = 0.68) and 35 μg·kg^{-1} (r = 0.65) i.t., respectively. Treatment of rats with F alone 5 h before the ovalbumin challenge reduced the numbers of total cells, eosinophils and neutrophils (Fig. 3). The Hill slope for inhibition of total cells and eosinophils by F were 0.2 and 0.5, respectively. Furthermore, the ED_{50} values for inhibition of total cells and eosinophils by F were 5.5 (r = 0.96) and 0.8 μg·kg^{-1} (r = 0.50), i.t., respectively.

In combination studies with MF and F, most of the MF/F combinations had a combination index <1 for inhibition of total cells and eosinophils (Table 2) which is indicative of synergy [16,17]. There was no linearity in the dose-responses against neutrophils (Fig. 3C) and a combination index for neutrophils could not therefore be calculated.

3.3. Effects on cytokines

Allergen challenge to sensitized rats significantly increased the levels of IL-4 (non-sensitized, 4.6 ± 0.2, sensitized 15.0 ± 4.0 × 10^2 ECL units) IL-5 (non-sensitized 2.5 ± 0.3, sensitized 27.0 ± 8.2 × 10^2 ECL units), IL-13 (non-sensitized 16 ± 2, sensitized 84 ± 17 × 10^2 ECL units) and TNF-α (non-sensitized 4.5 ± 0.2, sensitized 15.3 ± 2.0 × 10^2 ECL units) in the BAL fluid. Levels of three other cytokines, IL-1β, CXCL1 and IFN-γ, however, did not change. Treatment of sensitized rats with intratracheal MF (30, 100 and 300 μg·kg^{-1}) and F (1, 3 and 10 μg·kg^{-1}) alone dose-dependently inhibited the increase in levels of IL-4, IL-5, IL-13 and TNF-α (Fig. 4).

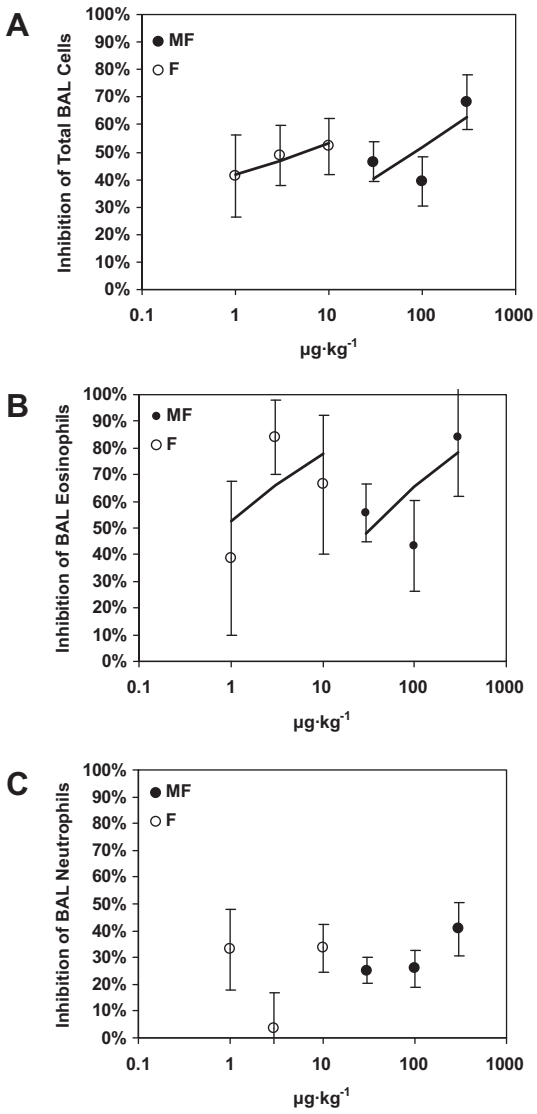

Fig. 3. Inhibition of the numbers of BAL total cells (A), eosinophils (B) and neutrophils (C) by MF and F alone. Drugs or vehicle were given 5 h before OVA challenge and inflammatory cells were measured 24 h after OVA challenge. Values are mean ± SEM (n = 8–15 per group).

Table 1
Reversal of allergen-induced reduction in forced vital capacity produced by mometasone furoate (MF), formoterol fumarate (F) and MF/F combination.

MF (μg·kg^{-1}, i.t.)	F (μg·kg^{-1}, i.t.)	Time after treatment (h)		
		1	5	24
0	1	32 ± 7	33 ± 7	23 ± 15
1000	0	23 ± 6	46 ± 6	72 ± 12
1000	1	54 ± 12[a]	91 ± 8[a, b]	71 ± 14[b]
0	10	81 ± 19	49 ± 15	24 ± 11
300	0	38 ± 6	20 ± 20	49 ± 13
300	10	73 ± 13	86 ± 19[a, b]	60 ± 7[b]

Drugs or vehicle were given 24 h after ovalbumin challenge and FVC was measured 1, 5, and 24 h after treatment with drugs or vehicle. Values are mean ± SEM (n = 7–8 per group) values for percent reversal of allergen-induced reduction in FVC.
[a] $P < 0.05$ versus MF alone
[b] $P < 0.05$ versus with F alone.

Table 2
Combination index for mometasone furoate (MF), formoterol fumarate (F), and the MF/F combination to inhibit total inflammatory cells and eosinophils in the bronchoalveolar lavage fluid of ovalbumin challenged rats.

Dose μg·kg^{-1} i.t.		Total inflammatory cells		Eosinophils	
MF	F	Inhibition (%)	CI	Inhibition (%)	CI
100	0	41	–	49	–
100	1	76	0.06	83	0.21
100	3	73	0.09	94	0.03
30	0	35	–	42	–
30	1	61	0.13	79	0.15
30	3	52	0.66	56	2.77

Drugs or vehicle were given 5 h before aerosolized ovalbumin challenge and numbers of inflammatory cells and eosinophils in the BAL fluid were measured 24 h after ovalbumin challenge. Values represent the average % inhibition and combination index (CI) for each MF/F dose. A combination index <1 indicates drug synergy.

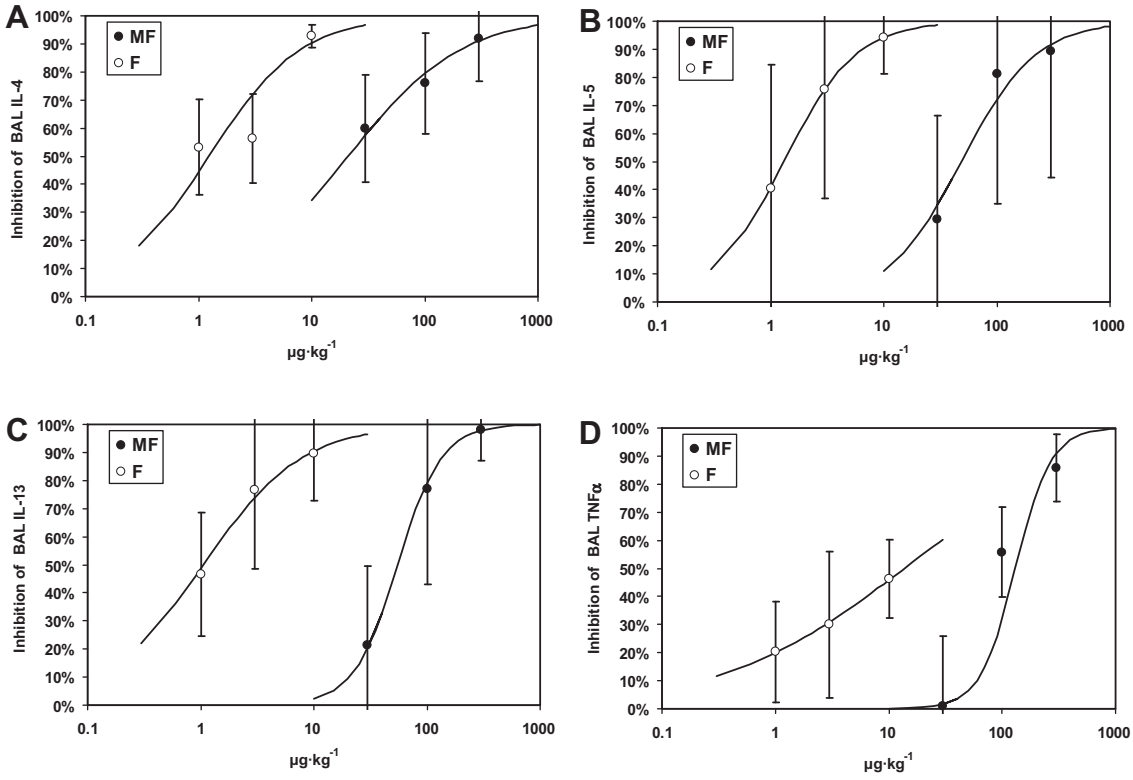

Fig. 4. Inhibition of the cytokine levels in the BAL fluid by MF and F alone. Drugs or vehicle were given 5 h before OVA challenge and BAL cytokines were measured 24 h after OVA challenge. Values represent the mean % inhibition of interleukin (IL)-4 (A), IL-5 (B), IL-13 (C) and TNF-α (D) by MF and F (n = 8–15 per treatment).

The rank order potency of MF for inhibition of these cytokines was IL-4 (ED_{50} = 21 μg·kg^{-1}, r = 0.99), IL-5 (ED_{50} = 49 μg·kg^{-1}, r = 0.96), IL-13 (ED_{50} = 55 μg·kg^{-1}, r = 0.96) and TNF-α (ED_{50} = 131 μg·kg^{-1}, r = 0.97). F was equipotent for its effects to inhibit IL-4 (ED_{50} = 1.2 μg·kg^{-1}, r = 0.90), IL-5 (ED_{50} = 1.3 μg·kg^{-1}, r = 0.99) and IL-13 (ED_{50} = 1.1 μg·kg^{-1}, r = 0.99) but was less potent for inhibition of TNF-α (ED_{50} = 13.7 μg·kg^{-1}, r = 0.99). In the MF/F studies, the combination index was <1 at most dose combinations (Table 3) suggesting that MF and F may act in a synergistic manner to inhibit the production of IL-4, IL-5, IL-13 and TNF-α in the lungs following allergen challenge.

4. Discussion

The results of this study demonstrate synergistic and long-acting effects of the MF/F combination for the improvement of lung function, the inhibition of inflammatory cell influx and increase in pro-inflammatory cytokines in the BAL fluid of allergen-challenged Brown Norway rats. For lung function improvement, intratracheal MF/F, given 24 h after ovalbumin challenge, reversed the reduction in FVC and was efficacious at all time points studied. Evidence of synergy between MF and F was demonstrated at 5 h after drug administration. Intratracheal MF/F given 5 h before the ovalbumin challenge also inhibited the increase in total inflammatory cells, eosinophils and neutrophils and levels of IL-4, IL-5, IL-13 and TNF-α in the BAL fluid of allergen-challenged rats. The combination index of MF/F for inhibition inflammatory cells, cytokines and improvement in lung function was generally <1 suggesting that drug synergy exists for the MF/F combination.

The allergen-challenged Brown Norway rat displays many of the important features of human asthma such as acute and late-phase airflow obstruction [9,18,19], bronchial hyper-responsiveness [20,21], inflammatory cell influx into the lungs [8,9,19–21], production of pro-inflammatory mediators and cytokines [19,21] and mucus hypersecretion [9]. In this study, evaluations were performed on the late-phase reduction in lung function, measured

Table 3
Combination index for mometasone furoate (MF), formoterol fumarate (F), and the MF/F combination to inhibit interleukin (IL)-4, IL-5, IL-13 and tumour necrosis factor-α (TNF-α) levels in the bronchoalveolar lavage fluid of ovalbumin challenged rats.

Dose μg·kg^{-1} i.t.		IL-4		IL-5		IL-13		TNF-α	
MF	F	Inhibition (%)	CI	Inhibition (%)	CI	Inhibition (%)	CI	Inhibition (%)	CI
100	0	76	–	81	–	77	–	56	–
100	1	85	0.81	85	0.79	86	0.97	63	0.66
100	3	99	0.01	99	0.07	99	0.09	84	0.43
30	0	60	–	29	–	21	–	1	–
30	1	83	0.43	89	0.29	81	0.50	58	0.25
30	3	78	1.10	82	0.95	57	2.59	47	0.51

Drugs or vehicle were given 5 h before aerosolized ovalbumin challenge and levels of cytokines in the BAL fluid were measured 24 h after ovalbumin challenge. Values represent the average % inhibition and combination index (CI) for each MF/F dose. A combination index <1 indicates drug synergy.

by the decrease in FVC, the numbers of inflammatory cells, including eosinophils and neutrophils, and levels of pro-inflammatory cytokines, including IL-4, IL-5, IL-13 and TNF-α in the BAL fluid of sensitized rats following aerosolized ovalbumin challenge. Our experience with pharmacological agents in this model, including evaluations with corticosteroids and β_2-agonists in both a prophylactic and therapeutic dosing paradigm, have demonstrated a broad range of effects including effects on forced expiratory lung functions and inflammatory cell influx into the lungs [8,9,13]. However, these experiments are the first studies investigating the combined effects of these drug classes on lung functions and inflammatory end points in this model.

Two different dosing regimes were used to evaluate the effects of the MF/F combination. To capture the potential synergistic effects of MF and F on lung function, rats were first challenged with aerosolized ovalbumin and drug combinations were given intratracheally 24 h after the ovalbumin challenge. Drug effects were assessed by the reversal of the reductions in FVC induced by the ovalbumin challenge at selected times after administration of the drug. As previously shown by our group, both β_2-adrenergic agonists and corticosteroids are active in this therapeutic dosing paradigm [13] and assess therefore the effects of drugs to reverse airflow obstruction. Likely mechanisms of action for drugs in this therapeutic dosing regime include bronchodilation, reversal of airway edema and suppression of inflammatory mechanisms contributing to airway closure. Previous studies have found that both MF and F inhibit airway microvascular leakage induced by allergen challenge [12,22–24] and F is a potent bronchodilator with a rapid onset of action [3]. Therefore, the efficacy of MF and F to increase FVC in allergen-challenged rats is to be expected given the pharmacological properties of these two drugs. The other paradigm used to profile the effects of MF/F on inflammatory cells and cytokines in the BAL fluid used a prophylactic dosing regime in which MF/F was given before the antigen challenge. This paradigm blocks many of the upstream factors contributing to the development of lung inflammation and reductions in lung function. It will include therefore effects of drugs on mast cell activation and mechanisms leading to the recruitment and activation of inflammatory cells into the lungs.

The MF/F combination was highly efficacious for lung function improvement and demonstrated several advantages over MF and F alone. At 5 h after MF/F administration, the reversal of FVC reduction was greater than that produced by both MF and F alone with a combination index of <1 indicating a synergistic interaction between MF and F. The MF/F combination was also active at all times studied over a 24-h period whereas MF and F were active at selected times only. Corticosteroids and β_2-agonists act in a complementary, additive and synergistic manner on inflammatory processes [4–6,25] and the results from our study demonstrate rapid and long-acting effects of MF/F to improve lung function. The mechanisms responsible for the combined effects of MF/F, including the apparent synergy seen after 5 h, were not investigated in this study, but likely involve effects on airway microvascular, airway smooth muscle contraction and mucus hypersecretion as these components contribute to the reduction in FVC after antigen challenge in rats [9,12,23] It is important to note that studies with other ICS/LABA combinations find additive and occasionally synergistic effects on airway hyper-responsiveness, lung inflammation and lung edema after antigen challenge [6,12,26]. The results of our study using a well established technique to assess lung mechanics i.e. forced expiratory maneuvers, demonstrate that combined MF/F has superiority over both MF and F alone to improve lung function.

MF/F was also superior to MF and F alone in reducing the increase in BAL inflammatory cells and levels of the pro-inflammatory cytokines IL-4, IL-5, IL-13 and TNF-α in the BAL fluid induced by the allergen challenge. In these studies, which were designed to assess the potential synergistic interactions between MF and F, the calculated combination index was consistently <1 in most dose combinations which is indicative of synergy [17]. Although the anti-inflammatory activity of corticosteroids is well established, the anti-inflammatory effect of β_2-agonists is less clear. Potential sites of action of F in our study could be the inhibition of mast cell mediator release [4], reduced adhesion of eosinophils and neutrophils to vascular endothelial cells [27] or possibly to an effect on airway epithelial cells and airway smooth muscle inhibiting the release of pro-inflammatory cytokines from these tissues [5,28]. Previous studies have identified an important role for CD4+ and CD8+ T cells in the airway eosinophilia and late-phase airway response to allergen challenge in Brown Norway rats [18,19,29] which raises the possibility that F had effects on T cell functions. Indeed, *in vitro* studies have demonstrated a synergistic effect of salmeterol and fluticasone on T cell activation and apoptosis in asthma [25].

In summary, the results of this study in allergen-challenged Brown Norway rats demonstrate that combined MF/F produces rapid and sustained improvement in lung function. Furthermore, the anti-inflammatory activity of MF/F shows evidence of a synergistic interaction between these two drugs for suppression of both inflammatory cell influx and release of pro-inflammatory cytokines into the lungs following antigen challenge On the basis of these results, it is likely that MF/F will have significant clinical benefit for the treatment of asthma.

Conflicts of interest

All authors are full-time employees of the Merck Research Laboratories.

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