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Comparison Study between the Mechanisms of Allergic Asthma Amelioration by a Cysteinyl-Leukotriene Type 1 Receptor Antagonist Montelukast and Methylprednisolone

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ABSTRACT

We investigated the effects of cysteinyl-leukotriene (cysLT) type 1 receptor antagonist montelukast (MK) and compared them with those of methylprednisolone (MP) in an allergic asthma model. Rats sensitized to ovalbumin (OVA) received repeated intratracheal exposure to OVA for up to 3 consecutive days. Pretreatment with MK or MP before OVA exposure inhibited late airway response (LAR) and reduced cellular infiltration into the bronchial submucosa after the triple OVA. The amount of *N*-acetyl-leukotriene E_4 in the bile was significantly reduced by pretreatment with MK or MP, suggesting that both drugs reduced the production of cysLTs in the lungs. In the in vitro study, when the fragments of lungs that had been repeatedly pretreated with MK or MP and exposed to OVA were removed and incubated with OVA, the coaddition of either drug significantly reduced cysLT production. In contrast, the cysLT production following the addition of OVA to the lung fragments that had not received in vivo pretreatment with either drug was inhibited by MK but not by MP. These results indicate that MK and MP inhibit LAR by suppressing the infiltration of inflammatory cells into the bronchial submucosa, thereby inhibiting the production of cysLTs in the lungs, and that MK but not MP may inhibit cysLT production directly. The different effects on cysLT production between the two drugs may provide a rationale for the use of combination therapy with $cysLT_1$ receptor antagonists and steroids for the treatment of asthma.

Asthma, one of the most prevalent disorders among industrialized nations, is characterized by reversible bronchoconstriction, increased mucous secretion, and complex airway inflammation (Busse and Rosenwasser, 2003). Inhalation of a specific antigen in allergic subjects usually results in dual responses, an immediate airway response (IAR) and a late airway response (LAR) (Nagy et al., 1982). The mechanisms for LAR are considered to be causally related to the infiltration of eosinophils and other inflammatory cells into the bronchial submucosa following the IAR (Bousquet et al., 1990). Recent basic and clinical studies indicate that cysteinyl-leukotrienes (cysLTs) play an important role in both responses of bronchial asthma (Smith, 1996) via the following effects on the airway system: induction of profound bronchoconstriction (Dahlen et al., 1980), enhancement of vascular leakage (Dahlen et al., 1981), enhancement of mucous secretion in the bronchi (Coles et al., 1983), and induction of chemotactic activity of eosinophils (Laitinen et al., 1993; Henderson et al., 1996). The cellular origins of cysLTs in the lungs are considered to be mast cells, eosinophils, basophils, monocytes-macrophages, and cell-cell interactions, such as those between neutrophils and platelets (Samuelsson et al., 1987; Maclouf and Murphy, 1988). Several new drugs known as "leukotriene modifiers" have been developed to modulate the actions of cysLTs (Busse, 1998; Drazen et al., 1999). Namely, cysLT type1 receptor antagonists ($cysLT_1$RAs) and 5-lipoxygenase inhibitors block the effects of cysLTs on airway tissue and decrease the generation of cysLTs, respectively. On the basis of clinical studies, $cysLT_1$RAs have been shown to be as effective at reducing asthma symptoms (Reiss et al., 1997) and inflammatory cell infiltration into the bronchial submucosa as 5-lipoxygenase inhibitors (Nakamura et al., 1998). The $cysLT_1$RA has been shown to inhibit airway eosinophilia, hyper-responsiveness, and microvascular leak-

The $cysLT_1$RA (MK) was donated by Merck & Co., Inc.
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ABBREVIATIONS: IAR, immediate airway response; LAR, late airway response; cysLT, cysteinyl-leukotriene; $cysLT_1$RA, cysteinyl-leukotriene type 1 receptor antagonist; MK, montelukast; A-LTE_4, *N*-acetyl-LTE_4; MP, metylprednisolone; EIA, enzyme immunoassay; OVA, ovalbumin; HPLC, high-performance liquid chromatography; BALF, bronchoalveolar lavage fluid.

age in mice after allergen challenge (Blain and Sirois, 2000). It has been reported that montelukast (MK), a cysLT$_1$RA, additively or synergistically improves lung function and patients' symptoms when administered in conjunction with β-adrenergic receptor agonists or steroids (Reiss et al., 1997; Price et al., 2003).

Although cysLT$_1$RAs are effective in both acute and chronic bronchial asthma and are recommended for clinical use as maintenance therapy (Busse and Lemanske, 2001; Naureckas and Solway, 2001; Price et al., 2003), the precise mechanisms by which these drugs achieve their effects remain unclear (Leff, 2001). Consequently, we investigated the effects of MK in an allergic asthma model after repeated antigen exposure by estimating pulmonary resistance (R_L), pathological findings, and biliary excretion of *N*-acetyl-leukotriene E$_4$ (A-LTE$_4$) as indices of the production of cysLTs in the lungs (Powell et al., 1995; Kodani et al., 2000). In addition, to examine the direct effects of MK or methylprednisolone (MP) on cysLT production in allergic lungs, we performed in vitro experiments using chopped lung fragments. On the other hand, steroids still remain the first-line drug for the treatment of acute exacerbation of asthma (National Institutes of Health/World Health Organization, 2002). Although there have been numerous studies concerning the mechanisms of steroids on allergic reactions, the effects of steroids on the generation of leukotrienes remain controversial (Barnes, 1998; Colamorea et al., 1999; Vachier et al., 2001; Barnes and Adcock, 2003). We therefore compared the mechanisms of the effects of MK on cysLT production in the lungs with those of MP using an allergic asthma model with repeated antigen exposures.

Materials and Methods

Materials. All experimental protocols were approved by the institutional animal care and use committee of the School of Medicine, Fukuoka University. The cysLT$_1$RA (MK sodium) was donated by Merck & Co., Inc. (Rahway, NJ). MP sodium succinate and Bordetella pertussis vaccine were purchased from Pfizer Puurs (Puurs, Belgium) and Wako Pure Chemicals Industries, Ltd. (Osaka, Japan), respectively.

Sensitization of Rats. Male Brown-Norway rats (Seakku-Yoshitomi, Fukuoka, Japan) that were 6 to 8 weeks old and that weighed around 250 g were used for the study. Active sensitization against OVA was performed by subcutaneous injection of sterile normal saline (1 ml) containing 1 mg of grade II OVA (Sigma-Aldrich, St. Louis, MO) and 200 mg of aluminum hydroxide (Sigma-Aldrich). Bordetella pertussis vaccine (50 μl) containing 6×10^9 heat-killed bacilli was given intraperitoneally as an adjuvant. Three days later, sterile normal saline (1 ml) containing 1 mg of OVA and 200 mg of aluminum hydroxide was subcutaneously injected for a booster effect. All animals selected for these studies were used from 14 to 28 days after the first injection.

Evaluation of the Effects of MK and MP. Sensitized rats were divided into groups by the number of OVA exposures and the different schedules of drug administration, as shown in Fig. 1. With respect to the number of OVA exposures, the sensitized rats were challenged daily by inhalation of OVA aerosol for two successive days (days 1 and 2) in the triple OVA exposure experiment. For this purpose, the inhalation of 0.25% OVA aerosol was accomplished by placing the rats for 20 min on each occasion in a 10-L Plexiglas chamber connected to an ultrasonic nebulizer known as the "Comfort-mini" (model-10; Sin-Ei Industries, Inc., Ageo, Japan). The next day (day 3), the final OVA challenge was performed by i.t. administration of 0.1 ml of a 1.7% OVA solution, as shown in Fig. 1, A and B. Ovalbumin grade V (Sigma-Aldrich) was used for OVA exposure (Abe et al., 2001). In the double OVA exposure, the sensitized rats were challenged by inhalation of OVA aerosol only for 1 day, and the final OVA challenge was performed by i.t. on the next day (OVA day 2). In the single OVA exposure, the rats were challenged by i.t. administration without any previous inhalation of OVA aerosol (Fig. 1C). The control indicates the OVA-sensitized rats received triple administration of saline. Administration of MK or MP was performed according to two different regimens on OVA Day 3; in one schedule, the drug was administered before every OVA exposure (triple pretreatment), whereas in the other schedule, the drug was administered only before the third OVA exposure (single pretreatment), as shown in Fig. 1, A and B, respectively. MK was dissolved in sterile saline, and the rats received the drugs gastrically at a rate of 10 mg/kg 1 h before the start of the i.t. OVA challenge. MP was dissolved in the dissolving solution supplied by the manufacturer (Pfizer Puurs) and injected into the rats intramuscularly at a rate of 10 mg/kg 1 h before the start of the i.t. challenge.

Measurement of Pulmonary Resistance (R_L). The rats were anesthetized by i.p. injection with urethane [1 g/kg, 25% (w/v)]. The tip of the tracheal tube (a 5-cm length of PE-240 polyethylene tubing) was inserted into the trachea through an open tracheostomy. The transpulmonary pressure was determined by monitoring the difference between the pressure in the external end of the tracheal cannula and esophageal cannula using a Statham DP-45 differential transducer (Validyne Engineering Corp., Northridge, CA). The intrapleural pressure was measured through a water-filled cannula (PE-240) that was placed in the lower third of the esophagus and

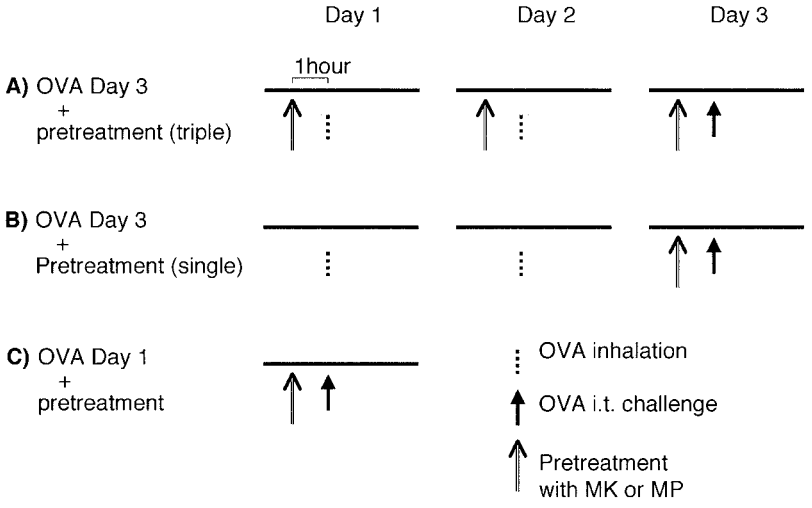

Fig. 1. Experimental schedules for the exposure of sensitized rats to OVA and pretreatment with MK, MP, or saline. Certain rats were exposed to the antigen by the inhalation of OVA aerosol, and the final challenge was conducted by an i.t. administration of OVA solution. A, triple OVA exposure with triple pretreatment before every exposure; B, triple OVA exposure with a single pretreatment only before the third exposure; and C, single OVA exposure with pretreatment.

connected to one port of a DP-45 differential pressure transducer (Validyne Engineering Corp.). A Fleisch pneumotachograph and a differential transducer were used to monitor the respiratory flow rate (PULMOS-II system; MIPS, Osaka, Japan). R_L was estimated under artificial ventilation with a Harvard Apparatus Rodent Respirator (Millis, Bedford, MA) at a respiration rate of 70 breaths/min and a tidal volume of 3.5 ml (Abe et al., 2001). The R_L was measured before the challenge (baseline value). After challenge with OVA, the R_L was measured at 1, 5, 10, 15, 30, 45, and 60 min, and thereafter, R_L was examined every 30 min for 6 h.

Histological and Cytological Examination. Six hours after the i.t. administration of OVA, the rats were exsanguinated by cutting the abdominal aorta. The trachea was joined to a tube with a three-way stopcock connected to a reservoir containing the fixative. The lungs were fixed in situ by the i.t. administration of 8% formaldehyde solution given at a pressure of 15 cm of H_2O. The lungs were then stained with hematoxylin-eosin to assess the degree of inflammation.

Bronchoalveolar lavage was performed via the tracheal cannula using 2 × 10 ml of saline containing 1 mM EDTA. The bronchoalveolar lavage fluid (BALF) was centrifuged at 300*g* for 5 min at 4°C, and the cell pellet was resuspended in 1.0 ml of sterile saline with 0.2% rat serum. The total cell count was determined by adding 50 μl of the cell suspension to 50 μl trypan blue stain and counting cells under a light microscope. The differential cell count was carried out from the smear preparation stained with Diff-Quik (International Reagents Corp., Kobe, Japan) and counting 200 cells at random under 200× magnification. The cells were identified by standard morphology.

Measurement of A-LTE$_4$ in Bile. The rats were anesthetized with urethane and then the common bile duct was exposed and cannulated by a PE-20 polyethylene tube (15 cm in length) after the ligation of the duodenal end. The rats were allowed to stabilize for a period of 2 h prior to i.t. challenge with OVA. The bile was collected every hour on ice in 1.5-ml Eppendorf tubes under a stream of argon before and after the i.t. challenge and then was stored at −80°C until analyzed. A-LTE$_4$ was measured in bile using precolumn extraction/reverse-phase high-performance liquid chromatography (RP-HPLC) according to the previously reported method (Powell et al., 1995; Kodani et al., 2000). Briefly, ethanol was added to the aliquots (including [^{3}H]LTE$_4$ as an internal standard) to give a final concentration of 15%. After adjusting to pH 3.0 to 3.5, the samples were loaded onto a Sep-Pak cartridge (Waters Corporation, Milford, MA). Methanol-eluted fractions passed through the minicolumn were concentrated under reduced pressure by a Speed Vac Concentrator (Savant Instruments, Holbrook, NY). After resuspension with 150 μl of HPLC solvent A, 75 μl of the concentrated fraction was injected onto a Novapak C18 5-μm column (0.39 × 15 cm) (Waters Corporation). The A-LTE$_4$ and LTE$_4$ fractions were collected using a model 201 fraction collector (Gilson, Villiers le Bel, France), and then evaporated under reduced pressure. The residue was analyzed by enzyme immunoassay (EIA) using a Leukotriene C$_4$/D$_4$/E$_4$ EIA kit (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. The values of LTC$_4$, LTD$_4$, and LTE$_4$ were normalized based on the recovery rates of [^{3}H]LTE$_4$ (31.6 ± 1.0%, n = 27).

In Vitro Experiments for Estimation of cysLT Production in Lung Fragments. The lungs were removed from the actively sensitized rats with or without previous OVA exposure, and then the large bronchi or blood vessels were dissected from the lung tissue. The tissues were chopped into small pieces (approximately 2 × 2 × 2 mm) by fine scissors. The chopped lung tissue (300 mg) was preincubated in Tyrode's buffer with or without the coaddition of MK or MP for 5 min at 37°C and further incubated for 30 min at 37°C after the addition of OVA solution (100 μg/ml). For incubation in the controls, saline was added instead of OVA. After terminating the reaction by the addition of cold ethanol, LTs were partially purified through a Sep-Pak cartridge (Waters Corporation). After evaporation of methanol eluates under reduced pressure and resuspension with HPLC, solvent A, LTC$_4$, LTD$_4$, and LTE$_4$ were separated with the Novapak C18 column and each fraction was collected. LTC$_4$ and LTD$_4$ fractions were assayed using a cysLT-EIA kit (Cayman Chemical, Ann Arbor, MI), and the LTE$_4$ fraction was assayed using an LTE$_4$-EIA kit (Cayman Chemical). The sum of the amounts of LTC$_4$, LTD$_4$, and LTE$_4$ was considered the cysLT amount.

HPLC. The HPLC system consisted of a model 600 controller, a 717 autosampler (Waters Corporation), and the Novapak C18 column. We used solvent A [acetonitrile/methanol/water/acetic acid, 30:12:58:0.03 (v/v)] containing 0.03% EDTA-free acid (Dojindo, Kumamoto, Japan) and solvent B, which consisted of acetonitrile/methanol/water/acetic acid [68:12:20:0.01 (v/v)] containing 0.001% EDTA. All solvents were adjusted to pH 5.6 with ammonia solution (Nacalai Tesque, Kyoto, Japan). The mobile phase began with solvent A and then was changed to solvent B at 20 min. The flow rate was 1 ml/min. The retention times for LTC$_4$, A-LTE$_4$, LTD$_4$, and LTE$_4$ were approximately 4.2, 9.1, 13.1, and 15.1 min, respectively.

Statistical Analysis. Data are reported as the means ± S.E.M. The statistical analysis was performed using the General Linear Models Procedure in Statistical Analysis System. A p value of less than 0.05 was considered to be statistically significant.

Results

Time Course for Changes of R_L. Figure 2 shows the time course for changes of R_L after the third OVA challenge. Although control rats given 0.1 ml of saline i.t. did not show any significant changes in R_L up to 6 h after the challenge, rats that received the triple OVA exposure showed prominent LAR. As shown in Fig. 2A, triple pretreatment with MK or MP significantly suppressed LAR, but the intervention with MP seemed to be more potent than that with MK. The control indicates the OVA-sensitized rats that received triple i.t. administration of saline. Single pretreatment with MK or MP only before the third OVA exposure also significantly inhibited LAR, but the inhibition by either drug was less than that by the triple pretreatment (Fig. 2B).

Table 1 shows the peak height of IAR and LAR after the third OVA exposure with or without pretreatment with MK or MP. Although triple pretreatment with MP significantly suppressed IAR, both single and triple pretreatment with MK tended to suppress IAR, but not significantly. On the other hand, both drugs with either administration schedule significantly suppressed LAR.

Cytological Studies in BALF. Cytological studies were performed to examine the changes of total leukocyte number and the recovery of cellular differentiation in BALF and to evaluate the effects of MK and MP on the infiltration of inflammatory cells into airway space. The results are shown in Fig. 3. Alveolar macrophages made up more than 90% of recovered cells in BALF after the i.t. saline challenge (control). The triple OVA exposure resulted in significantly more leukocytes in BALF than in the controls and showed a diathesis toward increase in leukocyte number compared with the double exposure (OVA day 2). Concerning cellular differentiation, eosinophils and neutrophils were the predominant cells, and the lymphocyte number also increased significantly. As shown in Fig. 3, A and B, repeated pretreatment before every OVA exposure with either drug suppressed the accumulation of all types of leukocytes in BALF, but the effect of the single pretreatment only before the third exposure was weaker than that of the triple pretreatment. The single pretreatment with MK or MP did not significantly

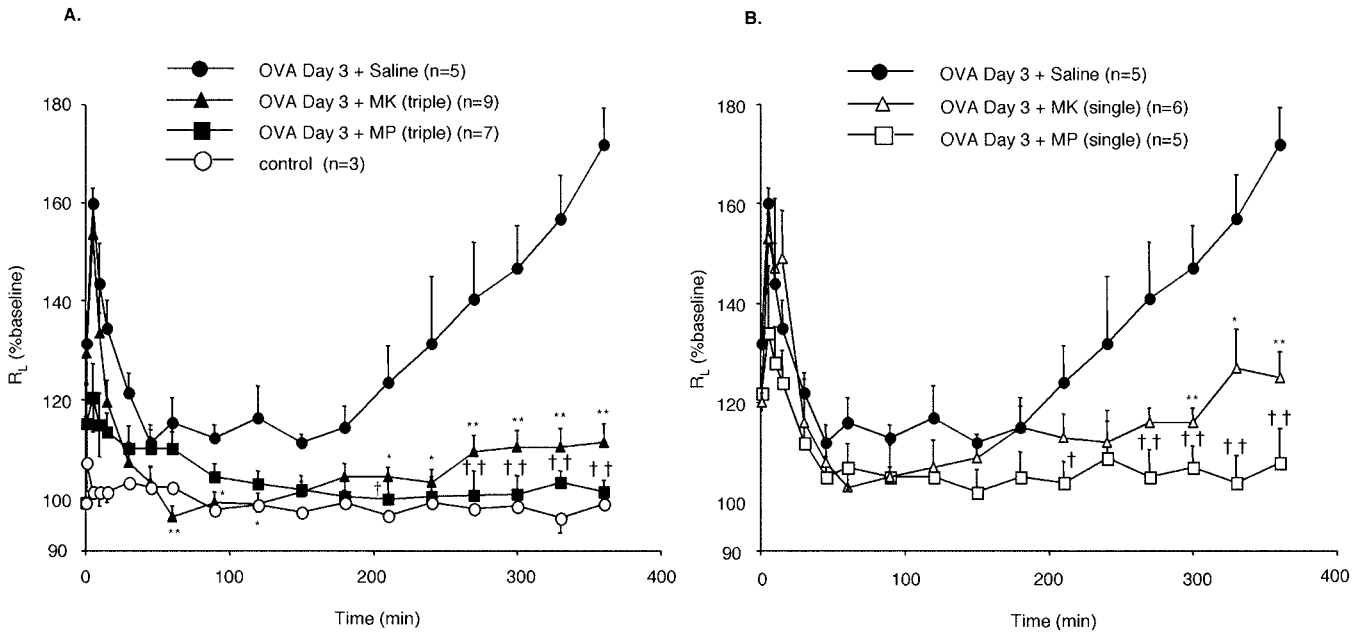

Fig. 2. A, time course of changes in pulmonary resistance (R_L) after the third OVA exposure to actively sensitized rats with or without pretreatment with MK (10 mg/kg gastrically) or MP (10 mg/kg intramuscularly) 1 h before every OVA exposure. Control rats were given 0.1 ml of saline i.t. instead of OVA solution, namely triple administration of saline. B, time course of R_L changes after the third OVA exposure in rats pretreated with 10 mg/kg MK or 10 mg/kg MP only before the final OVA exposure. The rats inhaled OVA aerosol for the last 2 consecutive days. The baseline value of saline was 0.217 ± 0.003 cm of H_2O/(milliliters/second). The baseline value of each group differed from that of the saline group by less than 10%. Data are expressed as a percentage of the baseline value (R_L value before challenge = 100%) and presented as the means ± S.E.M. *, $p < 0.05$ and **, $p < 0.01$ between the positive control group (OVA day 3 + saline) and the OVA day 3 + MK group; +, $p < 0.05$ and ++, $p < 0.01$ between the positive control group and OVA day 3 + MP group.

TABLE 1

Effects of pretreatment with MK or MP on IAR and LAR following the third OVA exposure

The peak heights of IAR and LAR after the third OVA exposure with or without pretreatment with MK (10 mg/kg gastrically) or MP (10 mg/kg intramuscularly) are given. The peak height of IAR was the highest value over the first 30 min after the challenge, and that of LAR was the highest value at 240 to 360 min. OVA + MK or MP (triple) indicates that the rats were pretreated with MK 1 hour before every OVA exposure, and OVA + MK or MP (single) indicates that the rats were pretreated with MK only before the last OVA exposure. Data are expressed as percent baseline (pulmonary resistance value before challenge = 100%), and values are presented as the mean ± S.E.M. The number of rats used is given in parentheses.

	Peak Height of IAR	Peak Height of LAR
	% baseline	
Control	1.099 ± 0.026 (n = 3)	0.960 ± 0.038 (n = 3)
OVA + saline	1.722 ± 0.130 (n = 7)	1.733 ± 0.069 (n = 5)
OVA + MK (triple)	1.467 ± 0.076 (n = 10)	1.239 ± 0.080 (n = 9)**
OVA + MP (triple)	1.315 ± 0.104 (n = 7)*	1.190 ± 0.090 (n = 7)**
OVA + MK (single)	1.555 ± 0.074 (n = 7)	1.303 ± 0.122 (n = 6)*
OVA + MP (single)	1.501 ± 0.087 (n = 5)	1.204 ± 0.071 (n = 5)**

* $P < 0.05$ and ** $P < 0.01$ compared with the positive controls (OVA + saline).

inhibit infiltration of eosinophils in the airway space after the third exposure.

Histological Studies. When the rats received double or triple OVA exposures, histological findings in bronchial tissue were examined at 6 h after the last exposure. As shown in Fig. 4, A and B, an extremely high infiltration of inflammatory cells including eosinophils and neutrophils was recognized in the bronchial submucosa after the third exposure compared with the double exposure. Although triple pretreatment with MK suppressed the infiltration of inflammatory cells into the bronchial submucosa, as shown in Fig. 4C, that with MP almost completely inhibited the cellular infiltration (Fig. 4D). On the other hand, when the other administration schedule, single pretreatment only before the third OVA exposure, was used to evaluate the effects of both drugs, pretreatment with either MK or MP also moderately suppressed the infiltration of inflammatory cells into the bronchial submucosa, and these suppressions were less potent than those by triple pretreatment (Fig. 4, E and F).

***N*-Acetyl-LTE$_4$ Level in Bile.** To examine the time course of the generation of cysLTs in the lungs, we measured A-LTE$_4$ excretion in biliary fluid after the third exposure to OVA. Saline administration did not change the level of A-LTE$_4$ (control). The third OVA challenge resulted in significantly greater biliary excretion of A-LTE$_4$ up to 6 h after challenge. As shown in Fig. 5A, triple pretreatment with MK or MP significantly reduced A-LTE$_4$ excretion in biliary fluid, but pretreatment with MP seemed to be more potent than that with MK. On the other hand, the single pretreatment with either drug only before the third challenge also significantly reduced A-LTE$_4$ excretion in bile (Fig. 5B), suggesting that these two drugs suppressed cysLT production in the lungs after antigen challenge.

Relationship between *N*-Acetyl-LTE$_4$ Level and Leukocyte Number in BALF. When the A-LTE$_4$ level in bile and leukocyte number in BALF from the individual rats were plotted, a significant correlation was observed between the two parameters, as shown in Fig. 6, A and B. The correlation coefficient for this relationship (OVA day 3 + saline) was 0.849 (p = 0.0051). When rats were pretreated with MK or MP, the relationship between the A-LTE$_4$ level in bile and the leukocyte number in BALF was well correlated under the single pretreatment regimen (Fig. 6B) but not under the triple pretreatment regimen (Fig. 6A). The correlation coefficients in the former treatment were 0.719 (p = 0.0266) for the single pretreatment with MK and 0.862 (p = 0.0092) for that with MP, respectively.

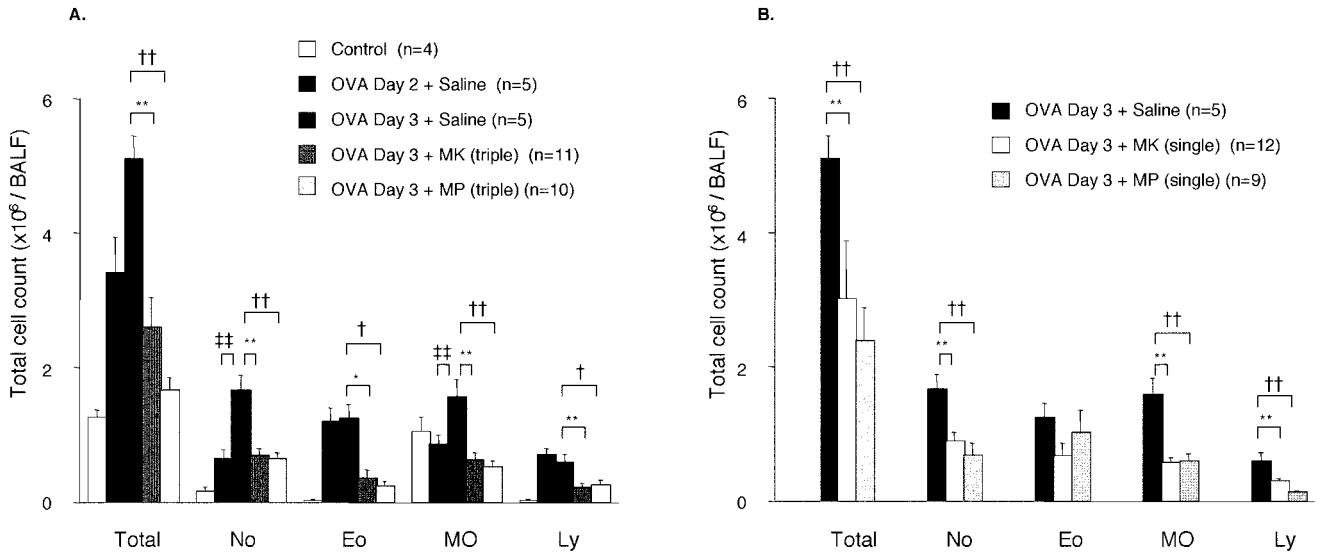

Fig. 3. Cytological studies for changes in leukocyte number and cellular differentiation in BALF and effects of MK and MP. A, pretreatment with 10 mg/kg MK or 10 mg/kg MP performed 1 h before every OVA exposure. The number of total inflammatory cells in BALF was counted 6 h after the second or third OVA challenge. Cellular differentiation of the infiltrating inflammatory cells showed that the levels of neutrophils (No), eosinophils (Eo), lymphocytes (Ly), and macrophages (Mo) were recovered in BALF 6 h after the second or third OVA challenge. Control rats were given 0.1 ml of saline i.t. instead of OVA solution. B, pretreatment with 10 mg/kg MK or 10 mg/kg MP performed only before the third exposure. Values are presented as the means ± S.E.M. *, $p < 0.05$ and **, $p < 0.01$ between the positive control group (OVA day 3 + saline) and the OVA day 3 + MK group; +, $p < 0.05$ and ++, $p < 0.01$ between the positive control group and OVA day 3 + MP group.

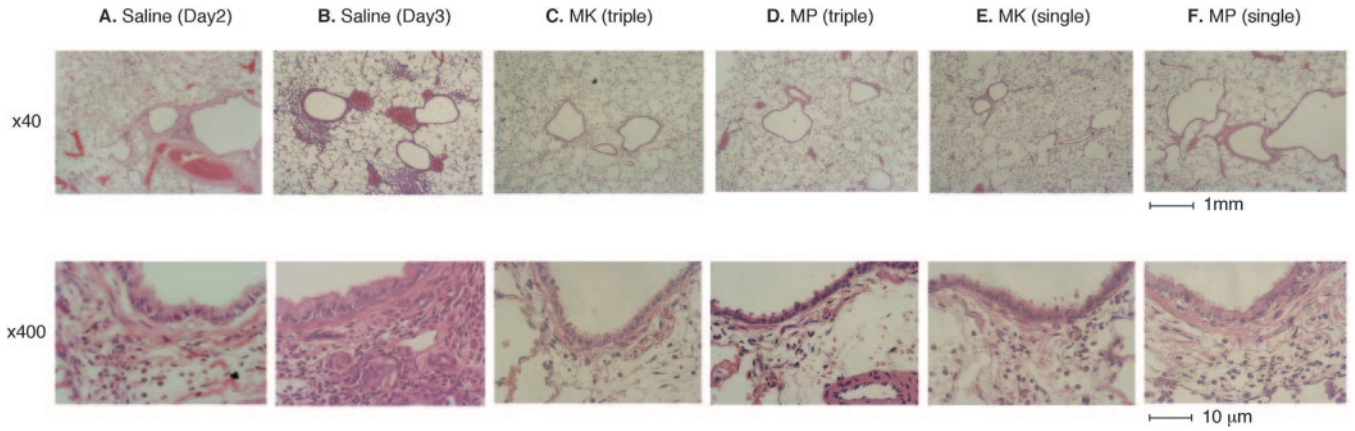

Fig. 4. Histological findings in bronchial tissues after the third OVA exposure of actively sensitized rats, and the effects of pretreatment with MK or MP. A, double OVA exposure (OVA day 2 + saline); B, triple OVA exposure (OVA day 3 + saline); C, triple OVA exposure with triple pretreatment with 10 mg/kg MK before every exposure; D, triple OVA exposure with triple pretreatment with 10 mg/kg MP before every exposure; E, triple OVA exposure with single pretreatment with 10 mg/kg MK only before the third exposure; F, triple OVA exposure with single pretreatment with 10 mg/kg MP only before the third exposure. The top and bottom panels show 40× and 400× magnification by light microscopy following hematoxylin-eosin staining, respectively.

In Vitro Production of cysLTs from Chopped Lung Fragments. To examine the influence of MK or MP on the production of cysLTs in lung tissue after stimulation with the antigen, the chopped-sensitized lung fragments without previous OVA exposure were incubated with OVA in Tyrode's buffer in the presence or absence of either drug at various concentrations for 30 min at 37°C. An approximately 3-fold higher amount of cysLT was produced in the chopped lung fragments supplemented with OVA compared with those supplemented with saline (control), as shown in Fig. 7. When MK was added at various concentrations (1–100 μg/ml), the production of cysLTs was significantly suppressed at the highest concentration (100 μg/ml). In contrast, the addition of MP at 1 to 100 μg/ml seemed to increase the production of cysLTs but not to a significant extent compared with that by OVA alone. The cysLT amount in the presence of MK (100 μg/ml) was significantly lower than that in the presence of MP (100 μg/ml). Next, the influence of MK or MP on cysLT production was evaluated in the lungs that were removed after repeated exposures to the antigen (Fig. 8). The lungs were removed from rats that were sequentially exposed to OVA for the last 2 days without pretreatment of MK or MP (Fig. 8A). The lungs were chopped into small pieces and then incubated with OVA in the buffer with or without the coaddition of either 100 μg/ml MK or 100 μg/ml MP for 30 min at 37°C. Whereas the amount of cysLT from the chopped lung fragments was significantly greater by the addition of OVA than by the addition of saline (control), the coaddition of MK but not MP significantly inhibited

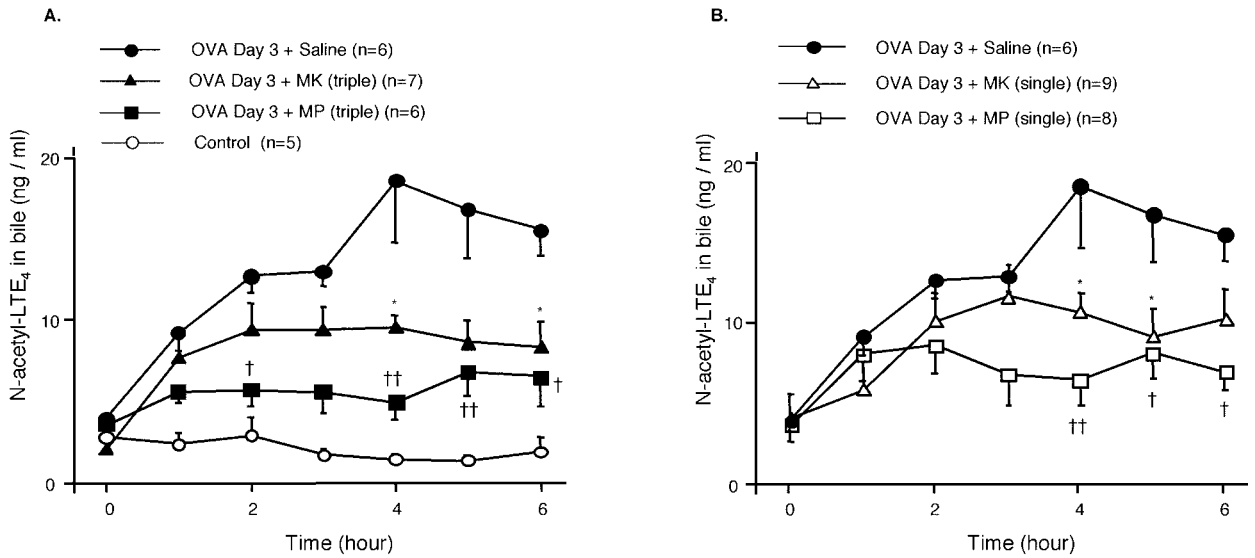

Fig. 5. Estimation of *N*-acetyl-leukotriene E_4 excreted in the biliary fluid after the third OVA exposure, and effects of pretreatment with 10 mg/kg MK and 10 mg/kg MP. A-LTE_4 was measured as described under *Materials and Methods* and expressed as nanograms per milliliter of bile per hour for consecutive periods commencing 1 h before challenge and up to 6 h after OVA challenge. A, pretreatment with 10 mg/kg MK or 10 mg/kg MP performed 1 h before every OVA exposure. B, 10 mg/kg MK or 10 mg/kg MP pretreated only before the third exposure. Values are presented as the means ± S.E.M. ∗, $p < 0.05$ between the positive control group (OVA day 3 + saline) and OVA day 3 + MK group; +, $p < 0.05$ and ++, $p < 0.01$ between the positive control group and OVA day 3 + MP group.

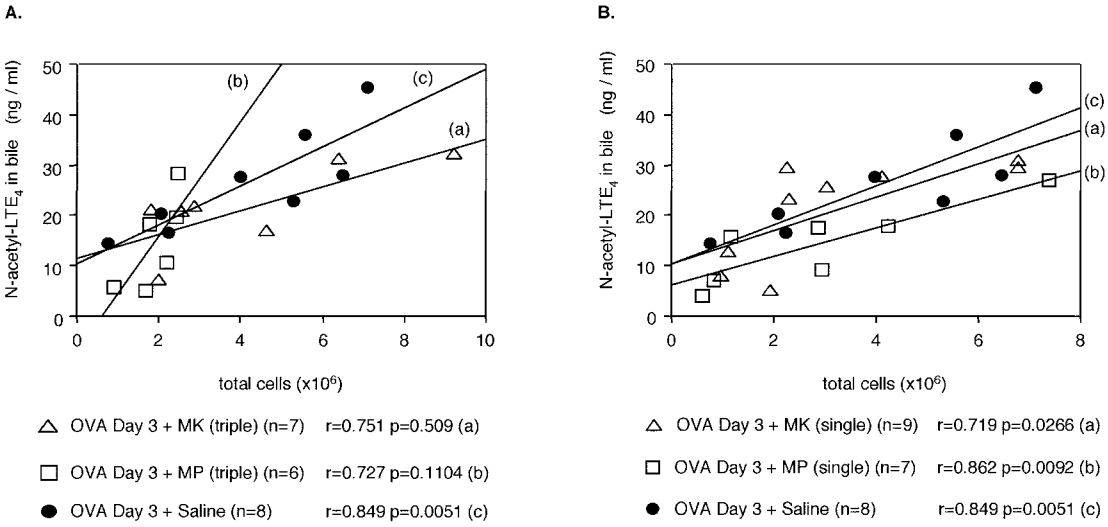

Fig. 6. Relationship between the amount of *N*-acetyl-LTE_4 in bile and total number of leukocytes in BALF at 6 h after the third OVA exposure with or without pretreatment of MK or MP. A, pretreatment with 10 mg/kg MK or 10 mg/kg MP was performed 1 h before every OVA exposure. B, pretreatment with 10 mg/kg MK or 10 mg/kg MP performed only before the third exposure.

cysLT production. The cysLT amount in the presence of MK was significantly lower than that in the presence of MP (Fig. 8A). In another trial, rats were daily challenged by the inhalation of OVA aerosol for 2 successive days with or without repeated pretreatment of 10 mg/kg MK or 10 mg/kg MP before every challenge. On the next day, the lungs were removed and chopped into small pieces. When the chopped lung fragments were incubated with the OVA solution for 30 min at 37°C, the coaddition of either 100 μg/ml MK or 100 μg/ml MP significantly inhibited cysLT production (Fig. 8B).

Table 2 summarizes the percent ratios of LTC_4, LTD_4, and LTE_4 in cysLTs produced from each incubation mixture containing the chopped lung fragments. LTC_4 was a major metabolite and occupied 55 to 70% of cysLTs produced by incubation for up to 30 min.

Discussion

This study indicates that either cysLT$_1$R antagonist or steroid suppresses LAR and infiltration of inflammatory cells into the bronchial submucosa following repeated antigen challenge. In a previous study, Henderson et al. (1996) reported that a 5-lipoxygenase inhibitor inhibited the infiltration of eosinophils into the bronchial wall following antigen challenge in a murine asthma model. Equivalent effects have been observed using cysLT$_1$R antagonists in similar models (Muñoz et al., 1997). It has already been reported that LTE_4 shows chemotactic activity toward eosinophils (Laitinen et al., 1993). In the present study, a cysLT$_1$R antagonist and a steroid each inhibited the accumulation of inflammatory cells in the bronchial submucosa and airway space in parallel with a decrease of A-LTE_4 excretion into bile. We speculate that

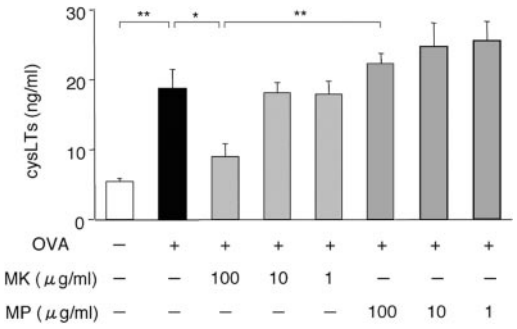

Fig. 7. Generation of cysLTs from the lung fragments without previous OVA exposure by stimulation with OVA and effects of coaddition with MK or MP. Various doses of MK or MP (1–100 μg/ml) were added to the incubation mixture, which contained 300 mg of chopped-sensitized lung fragments. Each mixture containing either drug was preincubated for 5 min at 37°C and then further incubated for 30 min after the addition of OVA (final concentration = 100 μg/ml). Values are presented as the means ± S.E.M. (n = 3–10). *, $p < 0.05$ and **, $p < 0.01$ compared with the controls.

the decrease in the number of cells accumulated in the lung, especially in the bronchial submucosal tissues, contributed to the decreased excretion of A-LTE_4 into the bile, suggesting a reduction in the generation of cysLTs in the lungs (Powell et al., 1995). In support of this idea, we observed a linear relationship between the number of leukocytes in BALF and the A-LTE_4 levels in bile. Pretreatment with either drug suppressed bronchoconstriction while maintaining the linear relationship between these two parameters. The single pretreatment with MP or MK significantly suppressed A-LTE_4 in the bile but did not inhibit the number of eosinophils in BALF. These results suggest that the cellular origin of cysLTs may come from macrophages rather than from eosinophils during LAR, as previously reported (Yu et al., 1995). These results may suggest that the suppression in the infiltration of leukocytes into the airway tissues by MP or MK contributes to the reduced production of cysLTs in the lungs. However, whether or not MK and MP directly reduce cysLT production from the sensitized lungs after antigen challenge remained unclear in these in vivo experiments.

To further analyze the mechanisms by which the two drugs reduce the generation of cysLTs, the effects of either drug on cysLT production in the sensitized chopped lungs were evaluated in vitro. The two drugs had different effects on the production of cysLTs induced by incubation with the antigen (see Fig. 7). Although MK reduced cysLT production at the high dose, MP showed a diathesis to increase cysLT production from the chopped lungs after antigen challenge. This relationship between the two drugs was also similar in the experiment using chopped lung fragments after repeated antigen exposure (see Fig. 8A). Namely, the coaddition of MK at the high dose reduced cysLT production from the lungs with or without previous OVA exposure following the addition of OVA, but this effect was not observed with MP. This result suggests that MK is able to directly suppress the generation of cysLTs in the lung tissue, but MP is not. On the other hand, when we performed a similar experiment using the lung fragments from rats subjected to repeated OVA exposure and repeated in vivo pretreatment with either drug (see Fig. 8B), both drugs suppressed the in vitro generation of cysLTs after the third OVA exposure, and the suppression of cysLTs by MP was similar to that by MK. Consequently, it is concluded that MP does not directly inhibit cysLT generation from the lung tissue following antigen challenge, but MP is able to suppress cysLT production in the case of repeated treatment through broad anti-inflammatory effects, including inhibition of cellular infiltration into the bronchial submucosa after repeated antigen exposure. We could not propose an explanation of how cysLT production from the chopped lungs was inhibited by MK. On the other hand, Ramires et al. (2003) reported that montelukast directly inhibited 5-lipoxygenase activity in mast cells at the lower micromolar ranges when stimulated by calcium ionophore A23187. However, this inhibition required cellular integrity, because MK did not inhibit 5-lipoxygenase activity in the homogenates from the cells. The dose of MK (100 μg/ml) used in the in vitro study is much higher than the concentrations required to block $CysLT_1R$ in human lung preparations (Fregonese et al., 2002). However, in our in vivo experiments, MK was administered to rats at 10 mg/kg, which is approximately 50 times more than the usual clinical dosage. Concerning the in vivo dose of MK used in the present animal studies, other groups have used similar doses (10–25 mg/kg) (Wu et al., 2003; Leick-Maldonado et al., 2004). A blood concentration of 100 μg/ml may hardly be achievable after the administration of the in vivo dose at 10 mg/kg. However, this concentration may be achievable if used at high doses of 25 mg/kg or more, because the dose at 25 mg/kg raised the blood concentration nearly up to 80 μg/ml as shown by Wu et al. (2003). The dose of MK needed to block LTC_4 is much higher than that needed to block LTD_4 (Jones et al., 1995). The ratios of production of LTC_4 to LTD_4 in the rat lungs in the present study suggests that conversion of LTC_4 to LTD_4 was slower in this species than in humans (see Table 2). Concerning human chopped lung, Kumlin and Dahlen (1990) reported that LTC_4 was rapidly converted to LTD_4 and LTE_4, and only 10% of LTC_4 remained intact after 30 min of incubation at 37°C. Consequently, the discrepancy in γ-glutamyl transpeptidase activity between the two species may be one reason that the dose of $cysLT_1RA$ required to ameliorate asthma in rats is higher than that in humans (Shi et al., 2001).

With respect to the two administration schedules used in this study, both were effective at inhibiting late bronchoconstriction, cellular infiltration into the bronchial submucosa, and cysLT production in the lungs, but the triple pretreatment regimen resulted in more complete suppression than the single pretreatment regimen. These results suggest that both drugs are also effective for treatment in the later advanced stages of the disease, when inflammation is already present. MP seemed to show similar but more potent effects than MK did. These results may be compatible with the previous clinical observation that severe asthma attack was not always ameliorated by $cysLT_1R$ antagonists alone but required the coadministration of steroids (Tomari et al., 2001). This result provides further evidence for the effectiveness of steroids and $cysLT_1RAs$ on allergic disorders as anti-inflammatory therapy. Concerning anti-inflammatory effects, Wu et al. (2003) reported that high doses of MK exerted anti-inflammatory effects in an animal model of acute asthma by inhibiting cytokine production. Since steroids have various anti-inflammatory and immunosuppressive effects on allergic reactions, including inhibition of cytokine gene induction, inhibition of

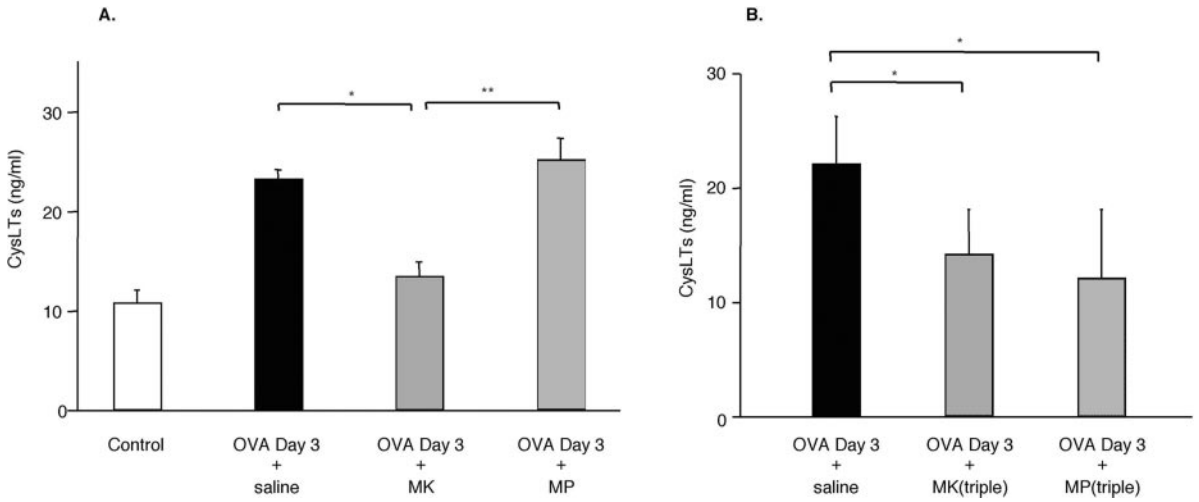

Fig. 8. Generation of cysLTs from the lung fragments with previous OVA exposure by stimulation with the third OVA challenge and effects of coaddition with MK or MP. A, sensitized rats exposed to the antigen by inhalation of OVA aerosol for the last 2 consecutive days without pretreatment. The next day, the lungs were removed and cut in small pieces (chopped lung). Each incubation mixture containing 300-mg chopped lung fragments was preincubated for 5 min at 37°C in the presence or absence of 100 μg/ml MK or 100 μg/ml MP and then in vitro-challenged with OVA (final concentration = 100 μg/ml) and incubated for 30 min at 37°C. Saline was used in place of OVA solution for the controls. B, sensitized rats exposed to the antigen by inhalation of OVA aerosol for the last 2 consecutive days with or without pretreatment of 10 mg/kg MK or 10 mg/kg MP before every exposure. The next day, the lungs were removed and cut in small pieces (chopped lung). Each incubation mixture containing 300-mg chopped lung fragments in the presence or absence of 100 μg/ml MK or 100 μg/ml MP was similarly incubated as in A. Values are presented as the means ± S.E.M. (n = 4–8). *, $p < 0.05$ and **, $p < 0.01$ compared with the controls.

TABLE 2

Percent ratios of LTC_4, LTD_4, and LTE_4 in cysLTs produced from each incubation mixture

The percent ratios of LTC_4, LTD_4, and LTE_4 in cysLTs produced from the chopped lung fragments are shown. The sensitized lung fragments (each 300 mg) without previous OVA inhalation were incubated with OVA at 100 μg/ml (OVA day 1 + saline) or without (control) for 30 min at 37°C. The sensitized rats were exposed to OVA by inhalation for the last 2 consecutive days, and on the next day the lungs were removed, cut in small pieces (chopped lung), and incubated with OVA (OVA day 3 + saline). Values are expressed as the means ± S.E.M. The number in parentheses indicates the number of experiments.

	LTC_4	LTD_4	LTE_4
		%	
Control (5)	68.0 ± 5.0	6.8 ± 3.7	25.1 ± 4.7
OVA day 1 + saline (5)	54.7 ± 9.7	5.5 ± 2.4	39.8 ± 9.9
OVA day 3 + saline (6)	70.8 ± 3.6	2.8 ± 1.3	26.4 ± 3.7

chemokine synthesis, repression of genes encoding cell surface receptors, and repression of adhesion molecules involved in leukocyte activation, migration, and recruitment (Karin, 1998), the present finding that the effects of the cysLT$_1$R antagonist were almost equal to those of the steroid suggests that cysLTs play a major role in allergic asthma through cysLT$_1$R, as supported by previous reports using receptor-disrupted mice (Maekawa et al., 2002).

In conclusion, this study revealed that MK may have the novel effect of directly inhibiting cysLT generation when administered at a high dose in addition to the previously reported ameliorative effects of cysLT$_1$R antagonists on bronchoconstriction, recruitment of inflammatory cells into loci, and inflammation (Wu et al., 2003). The finding that MK and MP had different effects on cysLT production may provide a further rationale for the use of combination therapy with cysLT$_1$RAs and steroids for treatment of asthma.

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