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Synergistic effects of pranlukast and a leukotriene B_4 receptor antagonist on antigen-induced pulmonary reaction

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Abstract

We now attempted to differentiate effects of cysteinyl–leukotrienes and leukotriene B_4 on antigen-induced pulmonary reaction by using a selective leukotriene D_4/E_4 ($CysLT_1$) receptor antagonist and a selective LTB_4 (BLT) receptor antagonist in rats. An intratracheal challenge with ovalbumin to Brown–Norway rats actively sensitized with ovalbumin produced two phases of airway responses which were estimated based on airway resistance, the immediate-type airway response within 30 min, and the delayed-type airway response beginning from 4 to 6 h after the challenge. Pretreatment of the rats with a $CysLT_1$ receptor antagonist (pranlukast) failed to reduce the elevation of airway resistance, and pretreatment with a BLT receptor antagonist (ONO-4057; 5-[2-(2-carboxyethyl)-3-{6-(4-methoxyphenyl)-5E-hexenyl}-oxyphenoxy] valeric acid) also produced no decrease. In contrast, combined pretreatment of the rats with pranlukast and ONO-4057 did not reduce the amplitude of the immediate-type airway response, but did allow the elevated airway resistance to return to its baseline level and also significantly inhibited the delayed-type airway response. Histological examination at 6 h after ovalbumin challenge showed infiltration of inflammatory cells with a predominance of neutrophils and scattered eosinophils in the bronchial submucosa. While pretreatment with neither pranlukast nor with ONO-4057 inhibited the infiltration of inflammatory cells in the bronchial submucosa, pretreatment with the two antagonists combined significantly inhibited the infiltration of granulocytes into the bronchial submucosa. On the contrary, intratracheal administration of either leukotriene D_4 or leukotriene B_4 up to 10 μg resulted in the infiltration of granulocytes into the bronchial submucosa, but no synergism for the infiltration of granulocytes was observed after combined administration. These results suggest that leukotriene B_4 appears to play a significant role in the antigen-induced pulmonary reaction in association with cysteinyl-leukotrienes. Accordingly, the combined antagonism at the $CysLT_1$ receptor and BLT receptor may be a useful intervention for the treatment of bronchial asthma.

Keywords: Cysteinyl-leukotriene; Leukotriene B_4; Antigen-induced pulmonary reaction

1. Introduction

Recent clinical and basic studies have demonstrated 5-lipoxygenase products to be major mediators in the pathophysiology of bronchial asthma (Israel et al., 1993; Smith, 1996; Vogel, 1997). Clinical trials of several selective $CysLT_1$ receptor antagonists have demonstrated beneficial results in the management of patients with bronchial asthma (Drazen and Israel, 1995; Hay et al., 1995). The antigen-antibody reaction in the lung results in the generation of leukotriene B_4 by mast cells, alveolar macrophages, and neutrophils as well as the generation of cysteinyl–leukotrienes (Wardlaw et al., 1989). However, the role of leukotriene B_4 in bronchial asthma remains to be elucidated. Leukotriene B_4 is known to have a potent chemotactic activity on neutrophils, eosinophils, monocytes and the up-regulation of CD11b/CD18 expression on leukocytes (Turner et al., 1996). Recently, potent and selective BLT receptor antagonists have been synthesized and some of have been tested for clinical benefit in patients with bronchial asthma (Richards et al., 1989; Kishikawa et al., 1992; Showell et al., 1995; Evans et al., 1996; Marder et al., 1996). However, it remains controversial whether or not blockade of the BLT receptor by these antagonists results in relief of these symptoms or in improvement in the pathophysiology of patients with bronchial asthma (Christie and Barnes, 1996).

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On the other hand, the ocular administration of leukotriene B_4 and leukotriene D_4 has been shown to have a synergistic effect on leukocyte emigration into the conjunctiva (Spada et al., 1986, 1988, 1994). In contrast, it has yet to be shown whether such a synergism exists for the recruitment of inflammatory cells into submucosal tissues of bronchi after the intratracheal administration of leukotriene B_4 and leukotriene D_4.

We thus intended to work on the following questions: (1) whether or not pretreatment with a $CysLT_1$ receptor antagonist or a BLT receptor antagonist alone or combined pretreatment with the two antagonists would have different effects on the time course of airway resistance and on the pulmonary histological findings after antigen challenge in the three groups; (2) whether or not a synergistic effect plays a role in the recruitment of inflammatory cells into bronchial submucosal tissue after the intratracheal administration of leukotriene D_4 and leukotriene B_4.

2. Materials and methods

2.1. Sensitization of rats

Male Brown–Norway rats (Seakku-Yoshitomi, Fukuoka, Japan) aged from 6 to 8 weeks old and weighing around 250 g were used for this study. Active sensitization against ovalbumin was obtained by subcutaneous injection of sterile normal saline (1 ml) containing 1 mg ovalbumin (grade II, Sigma, MO) and 200 mg aluminum hydroxide (Sigma, MO). At the same time 50 μl of *Bordetella pertussis* vaccine containing 6×10^9 heat-killed bacilli, which was kindly provided by The Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) was injected (i.p.) as an adjuvant. Three days later, sterile normal saline (1 ml) containing 1 mg ovalbumin and 200 mg aluminum hydroxide was injected s.c. for a booster effect. The animals were used for the study from 14 to 28 days after the first injection.

2.2. Measurement of airway resistance

The rats were anesthetized intraperitoneally with urethane (1 g/kg, 25% w/v), and the tip of the tracheal tube (5 cm length of polyethylene tubing (PE-240)) 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 the pressure in the pleural cavity with a Statham differential transducer (DP-45, Validine, Engineering, CA). The intrapleural pressure was measured with a needle (18 gauge) introduced percutaneously through the right seventh intercostal space into the pleural cavity. A Fleisch pneumotachograph and a differential transducer were used to monitor respiratory flow rate (Pulmos-II system, MIPS, Osaka). Ten ml of saline was given subcutaneously to prevent dehydration, immediately after the operation. Airway resistance was calculated according to the method of Giles et al. (1971).

To evaluate the effects of the drugs on airway resistance and on the histological findings, the drugs were administered intragastrically to the rats 2 h before the start of the intratracheal challenge with ovalbumin. The selective $CysLT_1$ receptor antagonist, pranlukast and the selective BLT receptor antagonist (ONO-4057; 5-[2-(2-carboxyethyl)-3-{6-(4-methoxyphenyl)-5E-hexenyl}-oxyphenoxy]-valeric acid) were kindly donated by the Ono Pharmaceutical (Osaka, Japan) and were suspended in saline with 0.5% carboxymethylcellulose sodium (Kishikawa et al., 1992).

2.3. Histological examination

At 6 h after the intratracheal challenge with either ovalbumin or saline, the rats were exsanguinated by cut-

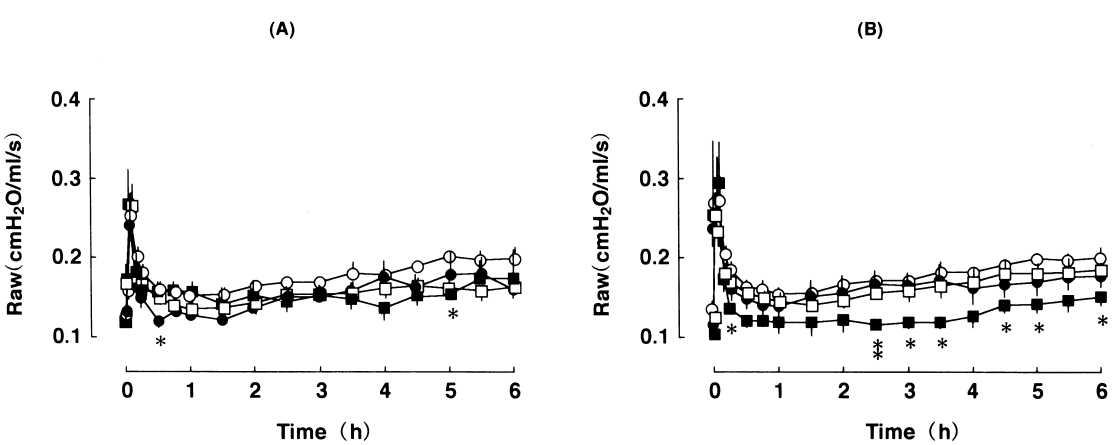

Fig. 1. Time course of airway resistance (R_{aw}) after the intratracheal administration of ovalbumin and the effects of pretreatment with pranlukast and ONO-4057, 3 mg/kg (A) and at 10 mg/kg (B), respectively. The rats were pretreated intragastrically with vehicle (○), pranlukast (●), ONO-4057 (□), or both drugs (■) 2 h before the ovalbumin challenge. Each value for R_{aw} resistance was expressed as the means ± S.E.M. ($n = 7$). $^*P < 0.05$ and $^{**}P < 0.01$ compared with vehicle alone at each time point. The details appear in Section 2.

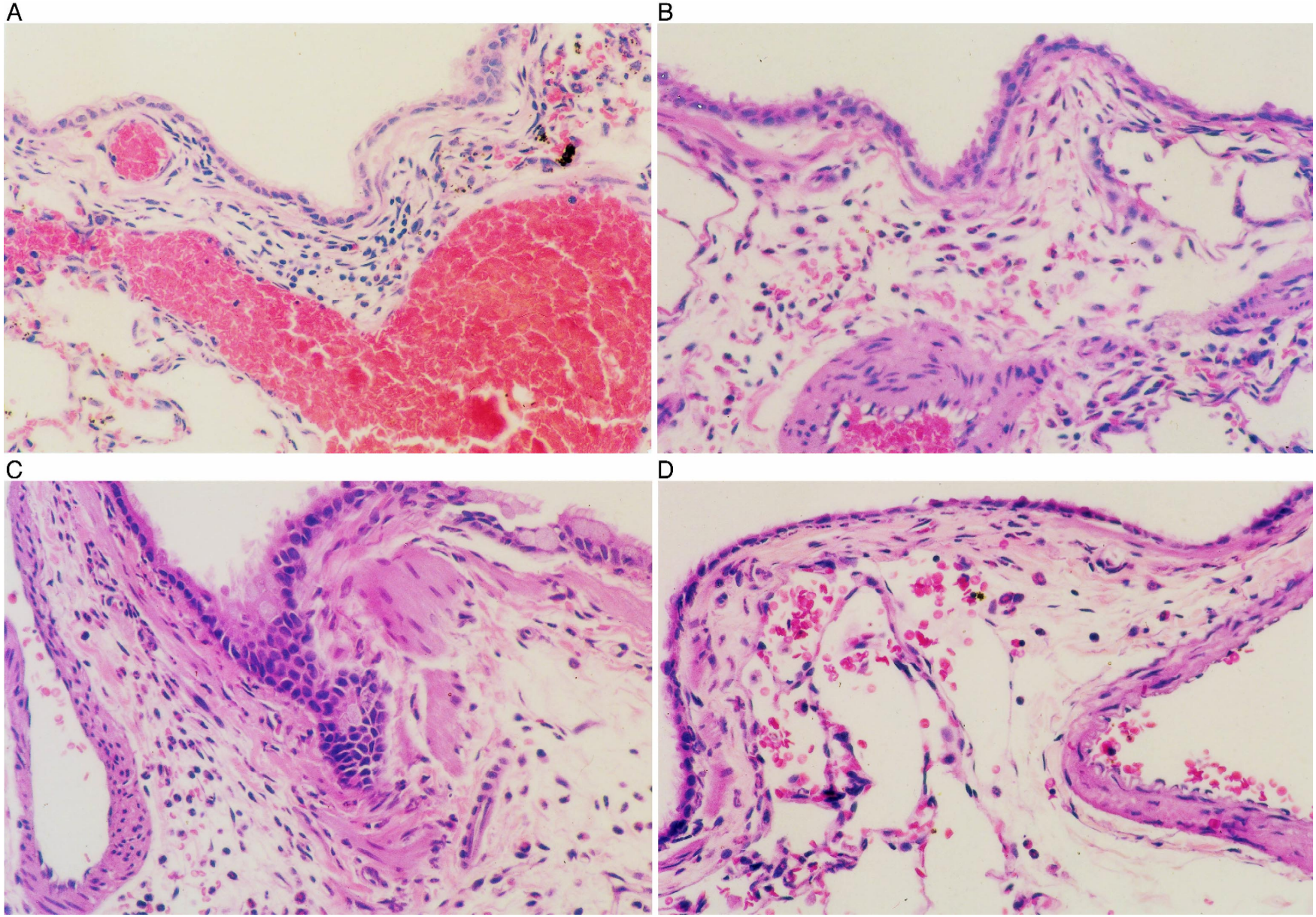

Fig. 2. Representative histology of the lungs from rats pretreated with vehicle (A), pranlukast, 10 mg/kg (B), ONO-4057, 10 mg/kg (C), or both drugs, 10 mg/kg (D) 2 h before the ovalbumin challenge. The lungs were fixed in situ at 6 h after the intratracheal challenge with ovalbumin. (Original magnification ×400, hematoxylin–eosin stained).

ting 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 intratracheal administration of 10% formaldehyde solution given at a pressure of 15 cm H_2O. The lungs were stained with hematoxylin-eosin. The inflammatory cells infiltrated into the bronchial submucosal tissues was counted under a microscope at $\times 400$ magnification, using the NIH-Image.

2.4. Statistical analysis

The data are reported as the means $\pm$ S.E.M. Statistical analysis was performed using the analysis of variance (ANOVA) with Dunnett's *t*-test as a multiple comparison method. A P value of < 0.05 was considered to be significant.

3. Results

3.1. The effects of pretreatment with pranlukast and a BLT receptor antagonist (ONO-4057) on airway resistance

After the sensitized rats were challenged with the intratracheal administration of 1.7% ovalbumin in 0.1 ml saline, the airway resistance (Raw) was measured 1, 5, 10, 15, 20, 30, 45, 60 min after the challenge. Thereafter, airway resistance was examined every 30 min for 6 h. As a control, the same volume of saline (0.1 ml) was administered intratracheally, after which airway resistance had not changed significantly after 6 h. On the other hand, the 1.7% ovalbumin challenge in the sensitized rats resulted in a rapid elevation of airway resistance to nearly twice the baseline value within 15 min after the challenge (Fig. 1). Thereafter, airway resistance returned to near its baseline value from 30 to 60 min after the challenge. Airway resistance began to rise to about 50% of its baseline value from 4–6 h. As shown in Fig. 1A, the pretreatment with pranlukast, 3 mg/kg, inhibited the rise only at 30 min over the time course of airway resistance after the challenge. Pretreatment with ONO-4057, 3 mg/kg, did not inhibit the elevation of airway resistance at all. Combined administration of the two antagonists at a dose of 3 mg/kg significantly inhibited the elevation of airway resistance only at 5 h after the ovalbumin challenge, in comparison with the effect of the vehicle (Fig. 1A). At a dose of 10 mg/kg, while pretreatment with neither pranlukast nor ONO-4057 inhibited the increase in airway resistance after ovalbumin challenge, the combined pretreatment with the two antagonists at the same dose significantly inhibited the rise in airway resistance at 20 min, 2.5, 3, 3.5, 4.5, 5, and 6 h after the ovalbumin challenge (Fig. 1B). Although the maximum amplitude of the immediate-type airway response was not inhibited by the combined pretreatment, this regimen seemed to facilitate a quicker return of the elevated airway resistance to the baseline value than with either pretreatment or no treatment at all.

3.2. Effects of pretreatment with pranlukast and ONO-4057 on pulmonary histology

Fig. 2 shows the representative histological findings for the lungs at 6 h after ovalbumin challenge; (A) pretreated with vehicle, (B) pretreated with pranlukast 10 mg/kg, (C) pretreated with the BLT receptor antagonist (ONO-4057) 10 mg/kg, (D) pretreated with pranlukast and ONO-4057 at a dose of 10 mg/kg each. Ovalbumin challenge induced infiltration of the granulocytes, predominantly neutrophils with scattered eosinophils, into the bronchial submucosa. Pretreatment with either antagonist alone did not significantly inhibit the infiltration of granulocytes into bronchial submucosal tissue, as shown in Fig. 3. In contrast, only combined pretreatment with the two antagonists at a dose of 10 mg/kg resulted in a statistically significant reduction in the infiltration of granulocytes into the bronchial submucosa. When the granulocytes were counted separately as eosinophils and neutrophils, the percentages of eosinophils among the granulocytes were $9 \pm 2\%$, $8 \pm 1\%$, $10 \pm 1\%$, and $10 \pm 2\%$ for pretreatment with vehicle, pranlukast 10 mg/kg, ONO-4057 10 mg/kg, and both, respectively ($n = 5$). Consequently, the relative numbers of eosinophils to neutrophils infiltrating the bronchial submucosa were not influenced by pretreatment with either drug alone or with the combination.

3.3. Effects of intratracheal administration of leukotriene D_4 and leukotriene B_4 on the submucosal infiltration of granulocytes

Under anesthesia with urethane, the actively sensitized rats received intratracheally leukotriene D_4, leukotriene

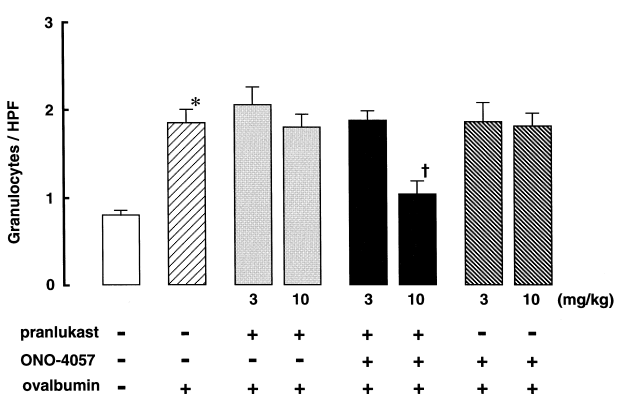

Fig. 3. Comparison of the effects of the pretreatment with pranlukast and ONO-4057 on infiltrating granulocytes in bronchial submucosal tissue at 6 h after the challenge with ovalbumin. The infiltrated granulocytes were counted using the NIH-Image under microscopic guidance at a magnification of $\times 400$. The results are expressed as the granulocyte number (mean $\pm$ S.E.M., $n = 7$) per high-power field (HPF, $= 6800\ \mu m^2$). $^*P < 0.05$ for comparison between ovalbumin alone and saline or $^\dagger P < 0.05$ for comparison between the pretreatment levels with the drugs and vehicle (ovalbumin alone).

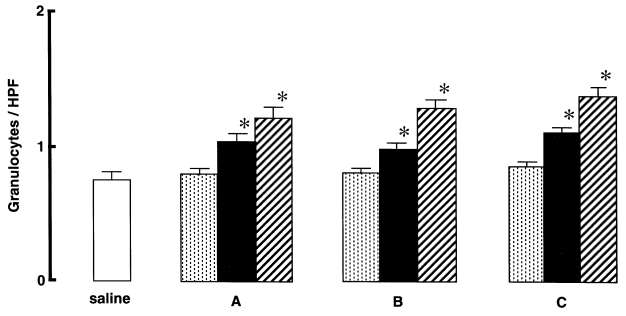

Fig. 4. Comparison of the infiltrating granulocytes in bronchial submucosal tissue at 6 h after the intratracheal administration of leukotriene D_4 (A), leukotriene B_4 (B), or leukotriene D_4 plus leukotriene B_4 (C) at doses of 100 ng (dotted column), 1 μg (black column), and 10 μg (hatched column). The infiltrated granulocytes were counted using the NIH-Image under microscopic guidance at ×400 magnification. The results are expressed as the means ± S.E.M. ($n = 5$) of the granulocyte number per high-power field (HPF). $^*P < 0.05$ for comparison between leukotriene administration and saline.

B_4, and the combination (leukotriene D_4 + leukotriene B_4) at various doses consisting of 100 ng, 1, 10 μg per 0.1 ml saline through the tracheal cannula. Six hours after the administration, the rats were exsanguinated by cutting the abdominal aorta and the lungs were fixed by flooding with 10% formalin. The granulocytes infiltrating the bronchial submucosa were counted and the counts were compared among the three groups at each dose under a microscope at a magnification of ×400. As shown in Fig. 4, a significantly ($p < 0.05$) greater number of granulocytes infiltrated into the bronchial submucosa after either leukotriene at 1 and 10 μg than after saline. However, the number of granulocytes infiltrating after the combination (leukotriene D_4 + leukotriene B_4) was not significantly different from that after either leukotriene D_4 or leukotriene B_4 alone at all three doses. When the granulocytes infiltrated into bronchial submucosa were counted separately as eosinophils and neutrophils, the percentages of eosinophils were 7 ± 1%, 9 ± 2%, and 8 ± 1% after the challenges with 10 μg leukotriene D_4, 10 μg leukotriene B_4, and 10 μg leukotriene D_4 plus leukotriene B_4, respectively ($n = 5$). Therefore, no synergy was found between leukotriene D_4 and leukotriene B_4 for the effect on the numbers of total granulocytes or on the relative numbers between eosinophils and neutrophils infiltrated in the bronchial submucosa.

4. Discussion

Recent clinical trials of $CysLT_1$ receptor antagonists have shown them to be effective for the treatment of patients with bronchial asthma (Drazen and Israel, 1995; Hay et al., 1995). While cysteinyl-leukotrienes, leukotriene C_4, D_4, and E_4, show various effects on the airway system, profound bronchoconstriction (Dahlen et al., 1980), enhanced mucous secretion in bronchi (Coles et al., 1983), and chemotactic activity for eosinophils (Chan et al., 1990; Foster and Chan, 1991; Laitinen et al., 1993), leukotriene B_4 is also known to be a potent chemoattractant for neutrophils and eosinophils (Ford-Hutchinson et al., 1980; Martin et al., 1989; Sampson et al., 1997). On the other hand, leukotriene B_4 is produced in the lungs along with cysteinyl–leukotrienes following an antigen-antibody reaction (Wardlaw et al., 1989). We therefore attempted to differentiate the effects of leukotriene B_4 from those of cysteinyl–leukotrienes on the airway system in the present study. The intratracheal administration of leukotriene B_4 induced a granulocyte infiltration in bronchial submucosal tissue which was consistent with the findings in a similar experiment using guinea pigs (Silbaugh et al., 1987). Since a BLT receptor antagonist, CP-105696 ((+)-1-(3*S*,4*R*)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]cyclopentane carboxylic acid), inhibited leukotriene B_4-mediated monkey neutrophil chemotaxis and CD11b up-regulation, leukotriene B_4 was suggested to partially mediate the acute and chronic airway responses following antigen challenge in an experimental primate asthma model (Turner et al., 1996). In addition, CP-105696 was reported to inhibit leukotriene B_4-induced eosinophil and neutrophil infiltration into the dermis of guinea pigs, to inhibit leukotriene B_4-induced neutrophil chemotaxis and CD11b up-regulation of neutrophils in in vitro experiments, and leukotriene B_4-induced Ca^{2+} mobilization in monocytes (Showell et al., 1995). Regarding two other BLT receptor antagonists, LY293111(2-[2-propyl-3-(3-{2-ethyl-4-(4-fluorophenyl)-5-hydroxyphenoxy} propoxy) phenoxy] benzoic acid) inhibits the leukotriene B_4-induced Macrophage-1 antigen (Mac-1) up-regulation of neutrophils (Evans et al., 1996; Marder et al., 1996) while U-75302 (6-[6-(3-hydroxy-1*E*,5*Z*-undecadien-1-yl)-2-pyridinyl]-1,5-hexanediol) inhibits eosinophil infiltration but not neutrophil infiltration after antigen challenge in guinea pigs (Richards et al., 1989). In humans, the airway administration of leukotriene B_4 has also been reported to result in polymorphonuclear leukocyte recruitment into the bronchial submucosa (Martin et al., 1989; Sampson et al., 1997). Although we expected an inhibitory effect of the selective BLT receptor antagonist (ONO-4057) on the time course of airway resistance and cellular infiltration in the bronchial submucosa after antigen challenge, neither BLT receptor antagonist nor $CysLT_1$ receptor antagonist alone inhibited either the time course of the airway resistance or granulocyte infiltration into the bronchial submucosa. Only the combined pretreatment with the two antagonists significantly inhibited both the elevation of the airway resistance and the infiltration of inflammatory cells after the antigen challenges. The early clinical trial of a BLT receptor antagonist (LY293111) with mildly asthmatic patients suggested no measurable improvement in lung function, despite inhibition of the neutrophil influx in the broncho-alveolar lavage fluid (Evans et al., 1996; Marder et al., 1996).

It should be noted that the present model is different from human asthma in two points, the predominance of neutrophils among granulocytes infiltrated into the bronchial submucosa and the lack of effects of the $CysLT_1$ receptor antagonist alone on the immediate-type airway response following antigen challenge. In contrast, eosinophils are considered to be major cells in airway inflammation of patients with bronchial asthma, and additionally, the administration of a $CysLT_1$ receptor antagonist is reported to inhibit the antigen-induced bronchoconstriction (Laitinen et al., 1993; Smith, 1996).

As for any possible interaction of two kinds of leukotrienes, cysteinyl-leukotrienes and leukotriene B_4, the topical application of leukotriene B_4 and leukotriene D_4 in ocular tissues was found to exert a synergistic effect on ocular infiltration with inflammatory cells (Spada et al., 1988). Although we examined the possibility of a synergistic effect of the intratracheal administration of leukotriene B_4 and leukotriene D_4 on cellular infiltration in the lungs, there was no significant synergism, based on the histological findings for the lungs. The anatomical complexity of the lung in comparison to the eye may be one of the reasons for these negative results.

In conclusion, the combined antagonism of leukotriene receptors by both $CysLT_1$ receptor and BLT receptor antagonists indicated a synergistic inhibition of the airway response and cellular infiltration into the bronchial submucosa after the antigen challenge. It is thus speculated that these pharmacological effects obtained from the combined administration of the two antagonists mimic the effects of a 5-lipoxygenase inhibitor. However, it has been reported that the inhibition of 5-lipoxygenase shunts arachidonic acid to another lipoxygenase pathway, such as either 12- or 15-lipoxygenase (Vanderhoek and Bailey, 1984; Fogh et al., 1988; Tanaka et al., 1992). Therefore, the combined antagonism at $CysLT_1$ and BLT receptors may be more useful than 5-lipoxygenase inhibition in the treatment of bronchial asthma.

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