

**FIGURE 6.** Effect of U0126 on poly(I:C)- and TGF- $\alpha$ -induced MUC5AC mucin production in NCI-H292 cells. Cells were preincubated with U0126 (a specific inhibitor of MEK1/2) at 20  $\mu$ M (15, 16) for 1 h before adding poly(I:C) (75  $\mu$ g/ml) and TGF- $\alpha$  (4 ng/ml), and cells were analyzed 24 h after stimulation ( $n = 6$ ). Data are shown as means  $\pm$  SD. +,  $p < 0.05$  and ++,  $p < 0.01$  compared with nonstimulated control cells. \*\*,  $p < 0.01$ .

significantly induce MUC5AC mucin production in every dose, costimulation with TGF- $\alpha$  caused an increase in MUC5AC mucin production with regard to poly(I:C) in a dose-dependent manner (Fig. 2A). TGF- $\alpha$  alone induced a dose-dependent increase in MUC5AC mucin production, and poly(I:C) enhanced the effect of TGF- $\alpha$  (Fig. 2B). Subsequent studies were focused on the time course of MUC5AC mucin production. Costimulation with poly(I:C) (25  $\mu$ g/ml) and TGF- $\alpha$  (4 ng/ml) caused a small increase in MUC5AC mucin production 12 h after stimulation, with maximal levels of MUC5AC at 24 h (Fig. 2C). These results may imply that poly(I:C) synergistically up-regulates MUC5AC mucin production induced by TGF- $\alpha$ .

*Poly(I:C) synergistically enhances MUC5AC mRNA expression induced by TGF- $\alpha$*

To determine whether induction of MUC5AC mucin protein induced by poly(I:C) and TGF- $\alpha$  was a result of increased MUC5AC

gene transcription, we investigated levels of MUC5AC mRNA, determined by real-time quantitative RT-PCR in NCI-H292 and NHBE cells. TGF- $\alpha$  (4 ng/ml) alone caused little increase in MUC5AC mRNA expression in NCI-H292 cells and NHBE cells upon 24 h of stimulation (Fig. 3A). Poly(I:C) (25  $\mu$ g/ml) alone induced a small but significant increase in MUC5AC mRNA expression in both NCI-H292 cells and NHBE cells, and poly(I:C) strongly potentiated the effect of TGF- $\alpha$  (Fig. 3A). A clear dose response was observed at 12 h following stimulation with both poly(I:C) (2.5–75  $\mu$ g/ml) (Fig. 3B) and TGF- $\alpha$  (0.4–12 ng/ml) (data not shown). Costimulation with poly(I:C) and TGF- $\alpha$  caused a small increase in MUC5AC mRNA expression 6 h after stimulation, which continued to a peak at 12 h after stimulation (Fig. 3C).

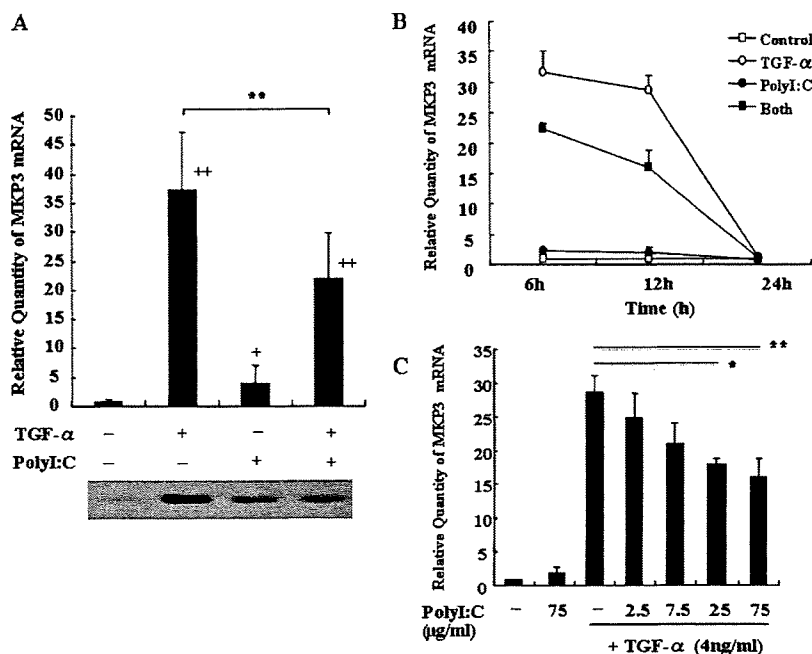
*Poly(I:C) and TGF- $\alpha$  cause synergistic trans-activation of the MUC5AC promoter*

We next investigated whether the MUC5AC promoter was activated by poly(I:C) and TGF- $\alpha$ . After 8 h, TGF- $\alpha$  alone induced a small but significant activation of the full-length MUC5AC promoter construct (–1330) (Fig. 4). Poly(I:C) did not activate the full-length MUC5AC promoter construct (–1330), but poly(I:C) strongly enhanced the activation induced by TGF- $\alpha$ , with 6-fold induction over that in unstimulated transfected control cells ( $p < 0.05$ ) (Fig. 4). This was observed when poly(I:C) was added 12 h before TGF- $\alpha$  stimulation (Fig. 4), but not when the two agents were added at the same time (data not shown). There was no difference in the level of activation of the short-length MUC5AC promoter construct (–63) among TGF- $\alpha$ , poly(I:C), and both stimulations (Fig. 4). These results indicate that the –1330 to –63 region contains the elements regulating induction of the MUC5AC promoter by poly(I:C) and TGF- $\alpha$ .

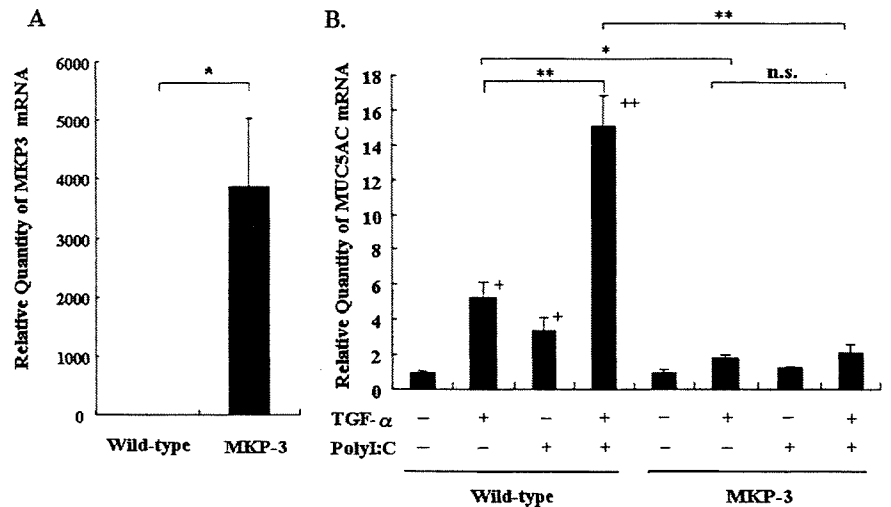
*Transactivation of the MUC5AC promoter by poly(I:C) and TGF- $\alpha$  is mediated via an ERK signaling pathway*

Since it was shown that induction of MUC5AC-specific mucin protein by poly(I:C) and TGF- $\alpha$  was a result of increased MUC5AC gene transcription, we next investigated the upstream signaling leading to activation of the promoter.

**FIGURE 7.** A, Upper, Effect of poly(I:C) (25  $\mu$ g/ml) on TGF- $\alpha$  (4 ng/ml)-induced MKP3 mRNA expression in NCI-H292 cells ( $n = 8$ ). Lower, To verify MKP3 expression in NCI-H292 cells, expression of MKP3 after stimulation with poly(I:C) (25  $\mu$ g/ml) and TGF- $\alpha$  (4 ng/ml) was examined by standard RT-PCR. In brief, 25  $\mu$ l of reaction mixture consisted of 1  $\mu$ l cDNA, 1 pmol MKP3 primer sets, and 12.5  $\mu$ l AmpliTaq Gold PCR Master mix (Applied Biosystems). PCR was performed by an initial denaturation step at 95°C for 5 min followed by 30 cycles with a denaturation step at 95°C for 30 s, an annealing step at 60°C for 30 s, and an extension step at 72°C for 30 s. B, Time course of induction of MKP3 mRNA expression by poly(I:C) (25  $\mu$ g/ml) and TGF- $\alpha$  (4 ng/ml) in NCI-H292 cells ( $n = 4$ ). C, Effect of the poly(I:C) concentration on TGF- $\alpha$  (4 ng/ml)-induced expression of MKP3 mRNA in NCI-H292 cells ( $n = 4$ ). Data are shown as means  $\pm$  SD. +,  $p < 0.05$  and ++,  $p < 0.01$  compared with nonstimulated control cells. \*,  $p < 0.05$  and \*\*,  $p < 0.01$ .



**FIGURE 8.** A, Effect of a MKP3 expression plasmid cloned into the pAcGFP1-C1 vector on MUC5AC mRNA expression in NCI-H292 cells. The level of MKP3 mRNA was enhanced significantly in cells transfected with the MKP3 expression plasmid. B, Enhanced expression of MUC5AC mRNA was noted in wild-type cells after 12 h of coincubation with TGF- $\alpha$  (4 ng/ml) and poly(I:C) (25  $\mu$ g/ml), but it was completely abolished in cells transfected with the MKP3 expression vector ( $n = 6$ ). Data are shown as means  $\pm$  SD. +,  $p < 0.05$  and ++,  $p < 0.01$  compared with nonstimulated control cells. \*,  $p < 0.05$  and \*\*,  $p < 0.01$ .



First, since TGF- $\alpha$  induces MUC5AC mucin production through the ligand-dependent *trans*-activation of EGFR in NCI-H292 cells (5), we examined the importance of EGFR activation for synergistic induction of MUC5AC mucin production by poly(I:C). We evaluated EGFR mRNA expression and phosphorylation of EGFR by RT-PCR and the Bio-Plex phosphoprotein assay, respectively. As a result, we found that poly(I:C) did not up-regulate EGFR mRNA expression upon 12 h of stimulation (data not shown) or increase the phosphorylation of EGFR (Fig. 5A).

Second, since previous studies have demonstrated that increased production of MUC5AC mucin protein after activation of the EGFR signaling pathway was exclusively MEK/ERK-dependent (17), we investigated the requirement of ERK. Western blot analysis revealed that poly(I:C) synergistically enhanced the phosphorylation of ERK by TGF- $\alpha$  stimulation (Fig. 5, B and C). This finding was compatible with the result of chemical inhibition by MEK1/2 inhibitor (U0126). U0126 inhibited the induction of MUC5AC protein production by poly(I:C) (75  $\mu$ g/ml) and TGF- $\alpha$  (4 ng/ml) compared with absence of the inhibitor at 24 h after stimulation (Fig. 6). These data suggest that *trans*-activation of the MUC5AC promoter by poly(I:C) and TGF- $\alpha$  is mediated via an ERK signaling pathway.

#### Poly(I:C) inhibits TGF- $\alpha$ -induced MKP3 mRNA expression

Having demonstrated that the ERK-dependent signaling was required in MUC5AC induction, still unclear is the mechanism interacting between TLR3-dependent signaling stimulated by poly(I:C) and EGFR-dependent signaling stimulated by TGF- $\alpha$ . Since MKP3 is known to be a member of the phosphatase family that inactivates ERK1/2, we examined the effect of poly(I:C) on MKP3 mRNA expression. A real-time quantitative RT-PCR showed that expression of MKP3 mRNA was up-regulated upon 12 h of stimulation with TGF- $\alpha$ , and MKP3 mRNA up-regulation by TGF- $\alpha$  was inhibited by stimulation with poly(I:C) (Fig. 7A). Stimulation with TGF- $\alpha$  led to a moderate increase in MKP3 mRNA expression at 6 h, followed by a decrease at 24 h (Fig. 7B). Costimulation with poly(I:C) dose-dependently inhibited this up-regulation, and inhibition was seen from 6 h after stimulation (Fig. 7, B and C).

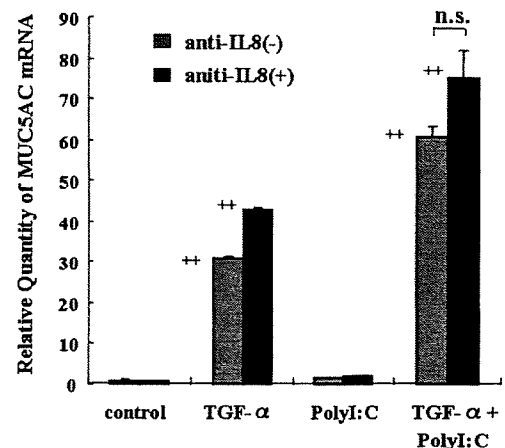
#### Effect of the MKP3 expression vector

To further demonstrate the role of MKP3 on MUC5AC mucin induction, we investigated the effect of MKP3 expression vector. MKP3 mRNA levels were significantly enhanced in cells trans-

ected with the MKP3 expression plasmid cloned into the pAcGFP1-C1 vector when compared with wild-type cells (Fig. 8A). Enhanced expression of MUC5AC mRNA was noted in wild-type cells 12 h after coincubation with TGF- $\alpha$  (4 ng/ml) and poly(I:C) (25  $\mu$ g/ml), but was completely abolished in cells transfected with the MKP3 expression vector (Fig. 8B). These data suggest that the inhibition of MKP3 mRNA expression by poly(I:C) leads to synergistic MUC5AC mucin induction.

#### Anti-IL-8 Ab does not inhibit poly(I:C)- and TGF- $\alpha$ -induced MUC5AC mRNA expression

Poly(I:C) is known to increase the expression of mRNA for various chemokines and cytokines (18, 19). In our preliminary study, we measured the cytokine and chemokine levels in the supernatant after stimulation with costimulation of TGF- $\alpha$  and poly(I:C) by using a Bio-Plex cytokine assay. In that study, only the IL-8 level was synergistically high due to costimulation of TGF- $\alpha$  and poly(I:C). Therefore, we chose IL-8, and to investigate the role of IL-8, NCI-H292 cells were preincubated with anti-IL-8 Ab 1 h before stimulation with poly(I:C) (75  $\mu$ g/ml) and TGF- $\alpha$  (4 ng/ml). Anti-IL-8 Ab did not inhibit the increase in the expression of MUC5AC mRNA induced by 12 h of stimulation with poly(I:C) and TGF- $\alpha$  (Fig. 9).



**FIGURE 9.** Effect of the anti-IL-8 Ab on poly(I:C) (75  $\mu$ g/ml) and TGF- $\alpha$  (4 ng/ml)-induced MUC5AC mRNA expression in NCI-H292 cells ( $n = 4$ ). Data are shown as means  $\pm$  SD. ++,  $p < 0.01$  compared with nonstimulated control cells.

## Discussion

In this study, we found that poly(I:C) synergistically increased the production of MUC5AC induced by TGF- $\alpha$  in both NCI-H292 and NHBE cells. This increase was dependent on activation of the MUC5AC promoter, and the upstream signaling pathway was ERK-dependent. The most interesting finding of this study was that expression of MKP3, which is one of the negative regulators of MAPK, was up-regulated by TGF- $\alpha$  and this up-regulation was inhibited by poly(I:C), indicating that MKP3 has a central role in the synergistic induction of MUC5AC production by poly(I:C) and TGF- $\alpha$ .

Mucin hypersecretion and goblet cell hyperplasia are characteristic features of airway inflammatory diseases such as asthma (1, 2). Since hypersecretory diseases are associated with abnormal epithelial cell growth and differentiation, and epithelial damage leads to repair and remodeling (19, 20), both inflammatory mediators and growth factors may be involved in stimulating mucin production from goblet cells. It has been postulated that activation of the EGFR pathway is a common denominator in the induction of MUC5AC mucin, a major component of mucus in the airways. Takeyama et al. have shown that stimulation of EGFR by its ligands, EGF and TGF- $\alpha$ , causes MUC5AC production by airway epithelial cells both *in vitro* and *in vivo* (5), and this effect is potentiated by TNF- $\alpha$  (5). In the present study, we found that using AB-PAS staining, ELISA, and RT-PCR, poly(I:C) synergistically amplified the induction of MUC5AC mucin induced by TGF- $\alpha$  at both the mRNA and protein levels in NCI-H292 cells.

In NHBE cells, MUC5AC mRNA expression was much lower than that in NCI-H292 cells, but poly(I:C) still synergistically amplified the expression of MUC5AC mRNA induced by TGF- $\alpha$ , indicating that synergic induction of MUC5AC by poly(I:C) and TGF- $\alpha$  may be generalizable to normal human epithelial cells. The lower expression of MUC5AC mRNA may be explained by not using an air-liquid interface in culturing NHBE cells. Indeed, studies done in air-liquid interface or monolayers would provide us important results. However, previous studies have demonstrated that both NCI-H292 and NHBE cells share key components of the signaling pathways upstream and downstream of EGFR responsible for mucin production (21), suggesting that NCI-H292 cells are a valid model of mucin production in normal cells. Therefore, our further studies investigating the mechanisms of the signaling pathway were done in NCI-H292 cells.

In the present study, we found that synergistic induction of MUC5AC mucin production by poly(I:C) and TGF- $\alpha$  was dependent on activation of the MUC5AC promoter within the proximal -1330/-63 region. Additionally, we investigated upstream signaling by using an inhibitor and Western blot analysis, and we found that the process was ERK-dependent. Our data are in agreement with findings reported by Hewson and coworkers, showing that increased production of MUC5AC mucin protein after activation of the EGFR signaling pathway was exclusively MEK/ERK-dependent (17). Furthermore, we found that poly(I:C) synergistically enhanced the phosphorylation of ERK induced by TGF- $\alpha$ . Therefore, we concluded that *trans*-activation of the MUC5AC promoter by poly(I:C) and TGF- $\alpha$  occurs exclusively via an ERK signaling pathway.

Receptor regulation has an important role in controlling the actions of several mediators. Yamamoto et al. demonstrated that IL-4-induced production of eotaxin-3 in airway epithelium was enhanced due to up-regulation of the IL-4 receptor by IFN- $\gamma$  (22). In the present study, to determine whether the synergistic effect of poly(I:C) was due to up-regulation of the EGFR, we evaluated EGFR mRNA expression and EGFR phosphorylation. However,

up-regulation of EGFR mRNA expression and the phosphorylation of this receptor by stimulation with poly(I:C) were not observed.

Since we had found that ERK was required for the synergistic effect of poly(I:C) on MUC5AC production induced by TGF- $\alpha$ , we proceeded to investigate this further by evaluating the role of MKP3, which is a member of the phosphatase family that inactivates ERK1/2. MKP3 is predominantly localized in the cytoplasm and has a highly specific role in the dephosphorylation and inactivation of ERK1/2 (23-26). MKP3 is an immediate early gene and is transcriptionally up-regulated after ERK2 activation (27, 28). Our present finding that MKP3 mRNA expression was 37-fold higher following stimulation with TGF- $\alpha$  is in agreement with previous reports that MKP3 is up-regulated after activation of the ERK2 pathway (27-29). Additionally, we found that this up-regulation was inhibited by stimulation with poly(I:C), and that overexpression of MKP3 completely abolished the increase in expression of MUC5AC mRNA. These data indicate that when NCI-H292 cells are stimulated by TGF- $\alpha$  alone, MUC5AC protein production remains under autoregulation to a certain extent by negative feedback via MKP3. However, when additional stimulation with poly(I:C) is added, MKP3 mRNA expression is partially down-regulated. This leads to synergistic activation of ERK, synergistic *trans*-activation of the MUC5AC promoter, and finally to synergistic production of MUC5AC protein.

Posttranscriptional events are also important in regulation of gene expression. A detailed examination of the time course of MUC5AC mRNA expression revealed that it was maximal at 12 h and decreased at 24 h after treatment with TGF- $\alpha$  alone. In contrast, costimulation with poly(I:C) and TGF- $\alpha$  caused a significant time-dependent increase in MUC5AC mRNA expression for up to 24 h. Furthermore, analysis of mRNA stability by real-time quantitative RT-PCR demonstrated that poly(I:C) did not alter the stability of MUC5AC mRNA (data not shown). Accordingly, the additional stimulation with poly(I:C) significantly increased and prolonged the induction of MUC5AC mRNA expression induced by TGF- $\alpha$  without affecting the rate of MUC5AC mRNA degradation.

Poly(I:C) is known to increase the expression of mRNA for various chemokines (IP-10, RANTES, LARC, MIP1 $\alpha$ , IL-8, GRO- $\alpha$ , and ENA-78) and cytokines (IL-1 $\beta$ , GM-CSF, and IL-6), as well as the cell adhesion molecule ICAM-1 (18, 19). To determine whether IL-8 has an important role in the synergistic effect of poly(I:C) and TGF- $\alpha$  on MUC5AC production, we investigated the potential role of IL-8 by preincubation with an anti-IL-8 Ab in the cells. The anti-IL-8 Ab did not inhibit MUC5AC mRNA expression, indicating that IL-8 has no role in the process. This finding was consistent with a previous report showing that IL-8 alone had no effect on MUC5AC protein production in NCI-H292 cells (30).

Also, the role of IFN may be an important point particularly in the context of poly(I:C) and asthma. We have not done studies directly on IFN- $\alpha$  and IFN- $\beta$ . However, to further investigate whether extracellular factors (such as chemokines and cytokines) released by poly(I:C) stimulation up-regulated TGF- $\alpha$ -induced MUC5AC production, we changed the culture medium at 12 h after poly(I:C) stimulation and then stimulated the cells with TGF- $\alpha$ . Although the extracellular factors had been removed, it did not alter the synergic expression of MUC5AC mRNA (data not shown), suggesting that extracellular factors including IFN- $\alpha$  and IFN- $\beta$  released by poly(I:C) may not contribute to the enhanced MUC5AC expression.

In conclusion, poly(I:C) synergistically increases the production of MUC5AC induced by TGF- $\alpha$  in airway epithelial cells, due to inhibition of MKP3 expression. Studies completed with viruses,

especially rhinovirus and also inactivated viruses, would provide us with important perspectives. Further studies will be needed to analyze the interaction between viruses and TGF- $\alpha$ . Viral respiratory tract infections are the most common triggers for the exacerbation of asthma (31, 32), and mucin overproduction is one of the mechanisms involved. The present findings may help to explain the excessive production of mucus in asthmatic patients with viral infection. Mucus plugging of the airways is a feature of fatal asthma in both adults and children (33, 34). At present, there are no effective therapies to relieve the symptoms induced by hypersecretion of mucus due to viral infection in asthmatic patients. Our findings may provide a mechanism to explain mucin overproduction and a potential strategy for therapy.

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### Disclosures

The authors have no financial conflicts of interest.

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## Forum News & Views

# Does Leukotriene Affect Intracellular Glutathione Redox State in Cultured Human Airway Epithelial Cells?

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### ABSTRACT

Leukotrienes (LTs) are one of the most important mediators in the pathophysiology of asthma. We measured the intracellular amounts of reduced glutathione (GSH) and oxidized glutathione (GSSG) in cultured human airway epithelial cells. LTC<sub>4</sub> affects the GSH/GSSG ratio by activating signals to increase interleukin-8 (IL-8) production. Pretreatment with a reducing agent, glutathione monochrome ester (GSH-OEt), and with a leukotriene receptor antagonist, montelukast, significantly suppressed LTC<sub>4</sub>-induced time-dependent changes in the intracellular redox state, and also suppressed upregulation of IL-8 production by suppressing NF- $\kappa$ B activation. Our observations led to the hypothesis that LTC<sub>4</sub>-induced oxidative stress is likely to contribute to amplification of airway inflammation. *Antioxid. Redox Signal.* 10, 821–828.

### AIRWAY INFLAMMATION AND REDOX

**A**STHMA IS A CHRONIC INFLAMMATORY AIRWAY DISEASE that affects children and adults of all ages (4). Although the pathogenesis of asthma remains incompletely defined, there is ample evidence that asthma is mediated by oxidative stress. The imbalance between reactive oxygen species (ROS) and antioxidants is termed oxidative stress. Most environmental factors, including oxidants, ultraviolet light, radioactivity, infections, and allergic responses to allergens, act as oxidative stress upon cells (6, 12). The cells obtained from the peripheral blood and lungs of patients with asthma generate increased amounts of ROS, such as superoxide radicals and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the increase correlates with disease severity (16). Cell functions are activated and inactivated by the balance between intracellular oxidation and reduction (redox state), which in turn closely correspond to the surrounding environment. Epithelial cells are the first cells to encounter inhaled allergens, and asthma is a disorder involving the airway epithelium that is more vulnerable to environmental injury and responds to this by impaired healing, in addition to inflammation (9). As the

first barrier of the airway, to maintain a steady state, airway epithelial cells possess mechanisms that eliminate oxygen radicals, tending to counteract intracellular shifts toward the oxidized state (20). Understanding how allergic respiratory diseases are exacerbated requires consideration of the effect of inflammation as an oxidative stress on airway epithelial cells, the initial site of injury from inflammatory cells, and/or pro-inflammatory mediators.

Glutathione, the most abundant nonprotein tripeptide containing a sulfhydryl group, plays a prominent role in antioxidant protection of the lung. In humans, glutathione is 100-fold more concentrated in the airway epithelial lining fluid than in plasma (29). Oxidants or oxidative stress have been reported to activate transcription factors, including NF- $\kappa$ B and phosphorylation of MAP kinase (19, 22). Inflammation-related changes in intracellular redox state in lung macrophages and monocytes have a potent effect on cytokine production (11, 28). Furthermore, the intracellular redox system is affected by oxidative stress induced by inflammatory cells during allergic reactions in the airway mucosa. However, changes of intracellular redox state and redox-related reactions in airway epithelial cells are largely uncharacterized.

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## LEUKOTRIENE AND INFLAMMATION

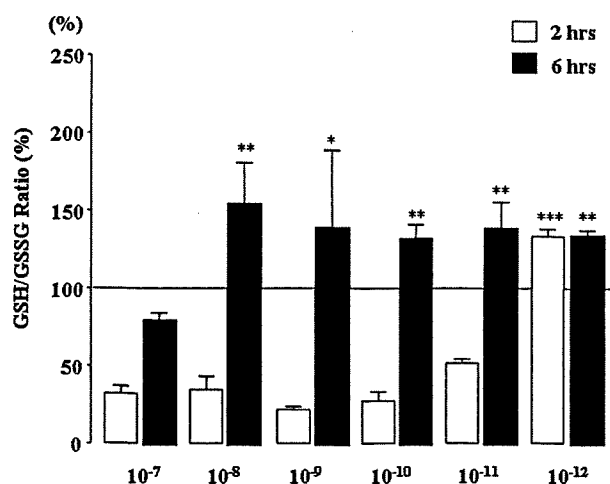
Leukotrienes (LTs), one of the most important mediators of the pathophysiology of asthma, are known to induce bronchoconstriction, airway inflammation, edema, and mucus hypersecretion (18). There are two families of leukotrienes: LTB<sub>4</sub> and a second group (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>), called cysteinyl-leukotrienes (Cys-LTs), bind to highly selective receptors to induce bronchoconstriction and inflammation associated with asthma (14). Analysis by enzyme immunoassay of exhaled breath condensate from patients with asthma has proved that asthma patients have higher levels of Cys-LTs and 8-isoprostane (a marker of oxidative stress) than normal subjects (1). In a B cell line (BL41-E95-A), oxidative stress (H<sub>2</sub>O<sub>2</sub>, diamide) enhanced activity of the cellular 5-lipoxygenase (5-LO), which catalyzes the first two steps in leukotriene biosynthesis (30). Depletion of thiol antioxidants induced the production of Cys-LTs and selective phosphorylation of MAP kinase in lung fibroblasts (2). Previous studies have demonstrated an association between production of Cys-LTs and oxidative stress. In this study, we investigated whether Cys-LTs can induce oxidative stress in cultured human airway epithelial cells, and evaluated the direct effect of Cys-LTs as an oxidative stressor through measurement of reduced glutathione (GSH) and oxidized glutathione (GSSG). We also studied cytokine regulation induced by Cys-LTs with a leukotriene receptor antagonist, montelukast, which is widely used as an anti-asthmatic drug.

## LEUKOTRIENE AND INTRACELLULAR REDOX STATE

### *LTC<sub>4</sub> exposure and intracellular redox state*

To determine an informative concentration and duration of Cys-LTs exposure, we compared six various stimulated concentration of LTC<sub>4</sub>: from 10<sup>-7</sup> to 10<sup>-12</sup> M at 2 and 6 h. LTC<sub>4</sub> exposure dose-dependently induced an oxidized state within Calu-3 cells at 2 h after stimulation (Fig. 1). We chose a final LTC<sub>4</sub> concentration of 10<sup>-8</sup> M as suitable for subsequent study because it induced a reproducible oxidized state at 2 h and a rebound reaction at 6 h.

We measured the intracellular redox state in Calu-3 cells immediately and at 2, 4, 6, 12, 24, and 48 h after LTC<sub>4</sub> exposure. In the 10<sup>-8</sup> M group after exposure for 2 h, the GSH/GSSG ratio decreased immediately to a mildly oxidized state, whereas the GSH/GSSG ratio fell below 100% compared with the control state. At 4 h after LTC<sub>4</sub> exposure, the ratio gradually declined to a severely oxidized state where the GSH/GSSG ratio fell to the minimal value, then the GSH/GSSG ratio increased dramatically to reach a maximum reduction at 6 h after LTC<sub>4</sub> exposure (Fig. 2A). Figure 2B shows the time course of the absolute values of GSH and GSSG. At 4 h after LTC<sub>4</sub> exposure, the GSH slightly decreased, and thereafter the GSH increased significantly to reach a maximum reduction at 6 h after LTC<sub>4</sub> exposure (Fig. 2B). The GSSG increased at 24 h after LTC<sub>4</sub> exposure. The control group, exposed to PBS as vehicle (10 μl/well), did not show any significant changes in the intracellular redox state at any time point (data not shown).



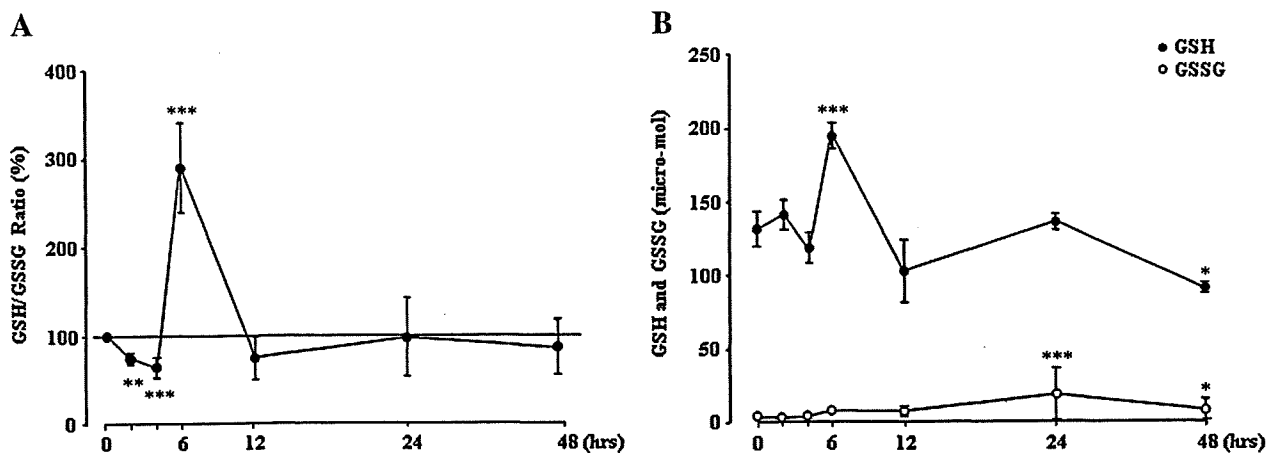
**FIG. 1.** Dose-dependent effect of LTC<sub>4</sub> on intracellular redox. Results following LTC<sub>4</sub> (10<sup>-7</sup>–10<sup>-12</sup> M) exposure for 2 h show the intracellular redox state declining to indicate an oxidized state, with the GSH/GSSG ratio showing <100% of control-state values (*n* = 6). At 6 h after exposure, the GSH/GSSG ratio increased. In 10<sup>-8</sup> M concentration, LTC<sub>4</sub> induced a clear series of oxidation and reduction. \**p* < 0.05, \*\**p* < 0.02, \*\*\**p* < 0.001, compared with 10<sup>-7</sup> M LTC<sub>4</sub>.

### *LTC<sub>4</sub> exposure and pretreatment with glutathione modulators*

Calu-3 cells were pretreated with the reducing reagent, glutathione monochrome ester (GSH-OEt), at concentrations of 10<sup>-4</sup>, 10<sup>-5</sup>, and 10<sup>-6</sup> M for 4 h, and then exposed to LTC<sub>4</sub> at a concentration of 10<sup>-8</sup> M. In the LTC<sub>4</sub> group (exposed to LTC<sub>4</sub> without pretreatment), the GSH/GSSG ratio at 2 h after LTC<sub>4</sub> exposure showed a decreased of >25%, indicating that cells were in an oxidized state, peaking at 6 h after LTC<sub>4</sub> exposure (Figs. 3A and B). On the other hand, in the OEt+LTC<sub>4</sub> group (GSH-OEt pretreatment and exposed to LTC<sub>4</sub>), the GSH/GSSG ratio exceeded 100%, and a reduced intracellular state was maintained for 6 h after LTC<sub>4</sub> exposure. LTC<sub>4</sub>-induced intracellular oxidative state at 2 h was inhibited by pretreatment with GSH-OEt, thus resulting in the continuous reductive state in human epithelial cells.

### *LTC<sub>4</sub> exposure and pretreatment with Cys-LTs receptor antagonists*

Calu-3 cells were pretreated with Cys-LTs receptor antagonist, montelukast, at concentrations of 10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>, and 10<sup>-9</sup> M for 2 h before and during exposure to LTC<sub>4</sub> at a concentration of 10<sup>-8</sup> M. In the LTC<sub>4</sub> group (exposed to LTC<sub>4</sub> without pretreatment), the GSH/GSSG ratio at 4 h after LTC<sub>4</sub> exposure decreased to nearly 50%, indicating that cells were in a severely oxidized state (Fig. 4), while addition of montelukast at concentrations of 10<sup>-6</sup> and 10<sup>-7</sup> M attenuated the significant decreases (Fig. 4). There was no significant difference in the GSH/GSSG ratio between montelukast pretreatment (10<sup>-6</sup>, 10<sup>-7</sup> M) group and control group at 4 h after LTC<sub>4</sub> exposure.



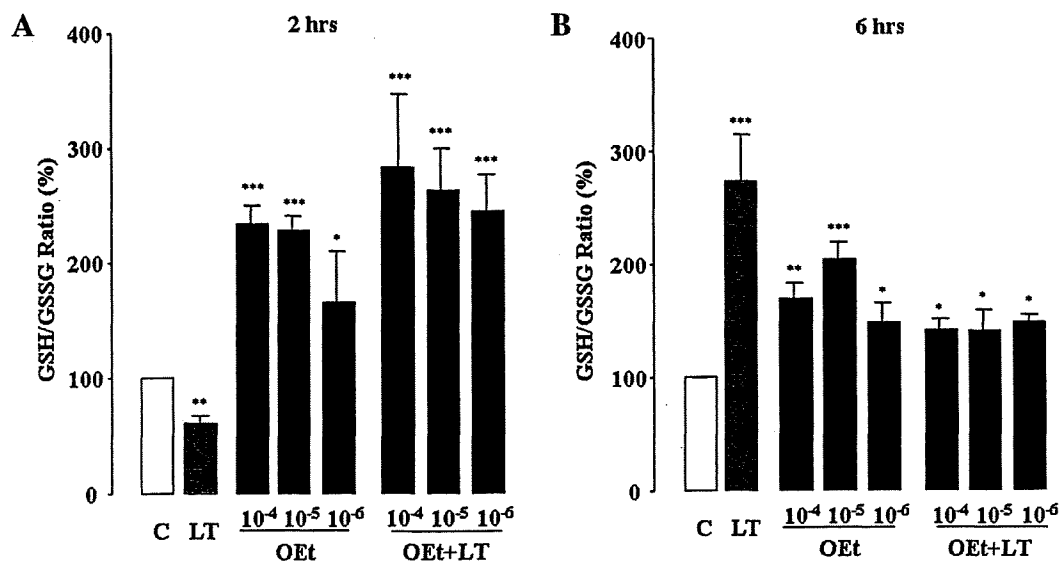
**FIG. 2. (A) Change of GSH/GSSG ratio after  $\text{LTC}_4$  exposure.** Results following  $\text{LTC}_4$  ( $10^{-8}$  M) exposure for 2 h show the intracellular redox state declining to indicate a mildly oxidized state, with the GSH/GSSG ratio showing <100% of control-state values ( $n = 6$ ). At 4 h after exposure, the ratio gradually declined to severely oxidized state where the GSH/GSSG ratio fell to the minimal value. Then the GSH/GSSG ratio increased dramatically, and the maximum reduction was reached at 6 h after  $\text{LTC}_4$  exposure. \*\* $p < 0.02$ , \*\*\* $p < 0.001$ , compared with 0 h. **(B) Change of GSH and GSSG after  $\text{LTC}_4$  exposure.** At 4 h after  $\text{LTC}_4$  exposure, the GSH slightly decreased, thereafter the GSH increased significantly to reach a maximum reduction at 6 h after  $\text{LTC}_4$  exposure. The GSSG increased at 24 h after  $\text{LTC}_4$  exposure ( $n = 6$ ). \* $p < 0.05$ , \*\*\* $p < 0.001$ , compared with 0 h.

## LEUKOTRIENE AND CYTOKINE/CHEMOKINE UPREGULATION

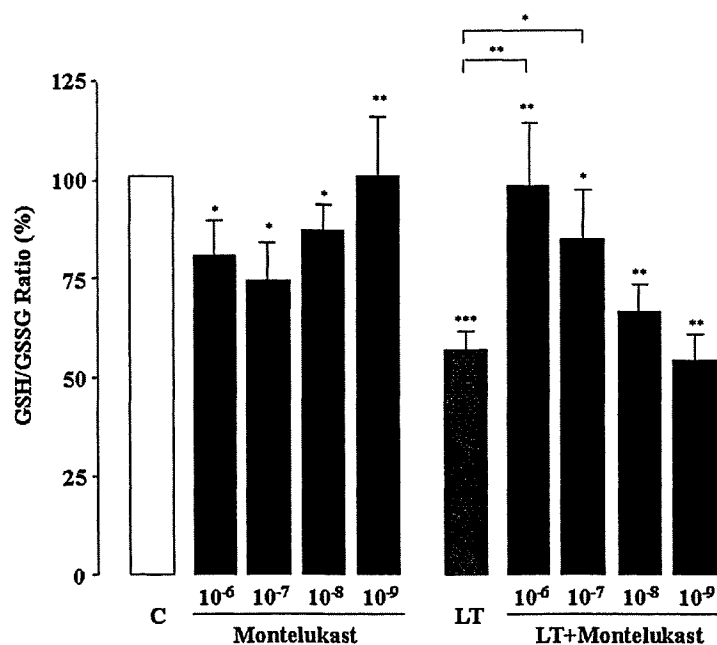
### *Cys-LTs* -induced upregulation of cytokine production

The concentration of interleukin-8 (IL-8) in control cells unexposed to  $\text{LTC}_4$  was  $185.75 \pm 23.92$  ng/ml. After exposing Calu-3 cells to  $\text{LTC}_4$ , we measured the changes in production

of IL-8. At 24 h after exposure, IL-8 values ( $282.49 \pm 16.14$  ng/ml) were significantly higher than in the control group ( $p < 0.001$ , Fig. 5). We also investigated the effects of the  $\text{LTC}_4$ -induced changes in IL-8 production following pretreatment with GSH-OEt ( $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$  M) or montelukast ( $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ , and  $10^{-9}$  M). Pretreatment with GSH-OEt ( $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  M) dose-dependently suppressed  $\text{LTC}_4$ -induced upregulation of epithelial cell-derived IL-8 production (Fig. 5). Also, pretreatment with  $10^{-6}$  or  $10^{-7}$  M montelukast



**FIG. 3. (A) Effect of antioxidant on  $\text{LTC}_4$ -induced redox change at 2 h.** GSH-OEt increased GSH/GSSG ratio after  $\text{LTC}_4$  exposure in Calu-3 cells at 2 h ( $n = 6$ ). Pretreatment of GSH-OEt was for 4 h, and incubation time of  $\text{LTC}_4$  was 2 h. \* $p < 0.05$ , \*\*\* $p < 0.001$ , compared with control. **(B) Effect of antioxidant on  $\text{LTC}_4$ -induced redox change at 6 h.** GSH-OEt suppressed after  $\text{LTC}_4$ -induced increase of GSH/GSSG ratio in Calu-3 cells at 6 h ( $n = 6$ ). Pretreatment of GSH-OEt was for 4 h, and incubation time of  $\text{LTC}_4$  was 2 h. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared with control.



**FIG. 4.** Effect of LT antagonist on LTC<sub>4</sub>-induced redox change. Montelukast had a protective effect on GSH/GSSG ratio after LTC<sub>4</sub> exposure in Calu-3 cells ( $n = 6$ ). Pretreatment of montelukast was for 2 h, and incubation time of LTC<sub>4</sub> was 2 h. \* $p < 0.05$ , \*\* $p < 0.02$ , \*\*\* $p < 0.001$ , compared with control.

significantly inhibited LTC<sub>4</sub>-induced upregulation of epithelial cell-derived IL-8 production (Fig. 6).

#### Effect of Cys-LTs exposure upon NF- $\kappa$ B p65 binding activity

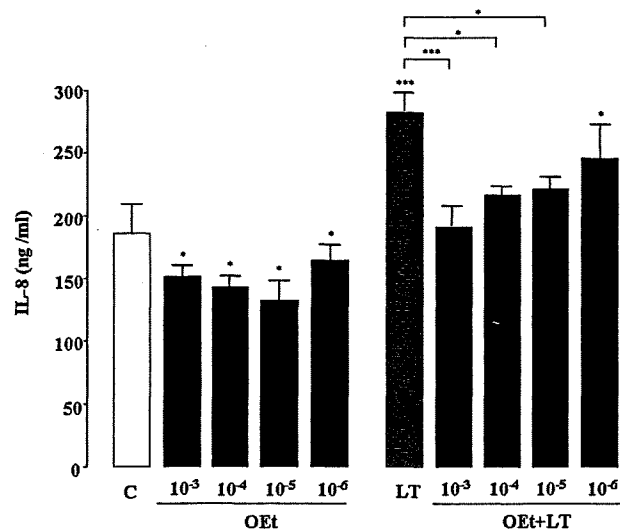
Compared with the control sample, the concentration of NF- $\kappa$ B p65 showed a significant increase after LTC<sub>4</sub> exposure ( $p < 0.001$ , Fig. 7). Pretreated with 10<sup>-4</sup> or 10<sup>-5</sup> M, GSH-OEt suppressed LTC<sub>4</sub>-induced activation of NF- $\kappa$ B p65 in Calu-3 cells, and the

inhibitory effect was dose dependent. This was also the case for montelukast at concentrations of 10<sup>-6</sup> and 10<sup>-7</sup> M (Fig. 7).

## CONCLUSION AND OPEN QUESTIONS

A vast body of evidence suggests that the intracellular redox state regulates various aspects of the cellular function (12), while that in various cell types of glutathione constitutes the first line of cellular defense against oxidative injury, acting as the major intracellular redox buffer (20). However, in the studies published so far, little has been reported regarding whether some important inflammatory molecules, such as Cys-LTs, can induce oxidative stress on the glutathione redox system in human airway epithelial cells or how changes in the intracellular redox state affect cytokine regulation. In this study, we examined whether Cys-LTs can induce oxidative stress in cultured human airway epithelial cells (Calu-3) and evaluated the direct effect of Cys-LTs as an oxidative stressor through measurement the intracellular redox state in Calu-3 cell. We monitored the intracellular glutathione redox balance, as indicated by the GSH/GSSG ratio. We also studied cytokine regulation induced by this oxidative stress.

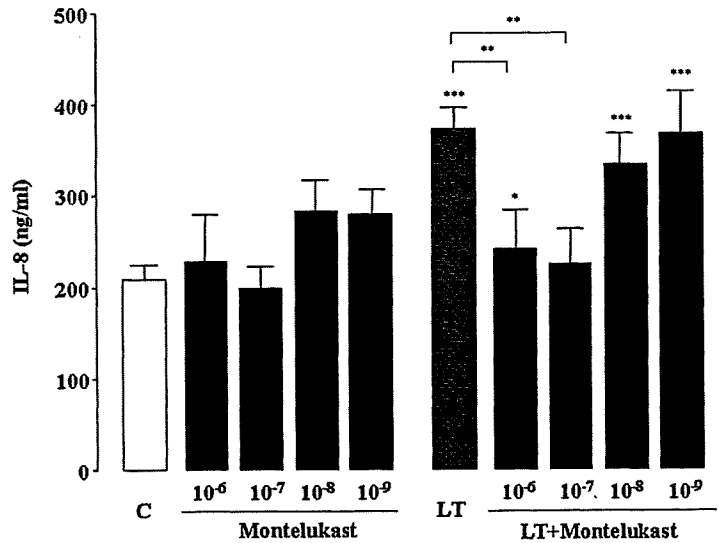
One key finding of the present study is that the glutathione redox balance in airway epithelial cells was affected by LTC<sub>4</sub>-induced oxidative stress. At 4 h after Calu-3 cells were exposed to 10<sup>-8</sup> M LTC<sub>4</sub>, the intracellular redox state gradually changed from a reduced to a severely oxidized state, followed by a prolonged reduced state. Airway epithelial cells have effective mechanisms to prevent a prolonged oxidized state, particularly the glutathione redox system, which can rapidly return cells from an oxidized to a reduced state (27). Although previous studies have demonstrated an association between the production of Cys-LTs and oxidative stress (2, 30), this is the first study of the effect of Cys-LTs on the intracellular redox state,



**FIG. 5.** Effect of LTC<sub>4</sub> and antioxidant on IL-8 production in Calu-3 cells. GSH-OEt suppressed LTC<sub>4</sub>-induced IL-8 production in Calu-3 cells ( $n = 6$ ). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC<sub>4</sub> was 2 h. \* $p < 0.05$ , \*\*\* $p < 0.001$ , compared with control.



**FIG. 6. Effect of LTC<sub>4</sub> and montelukast on IL-8 production in Calu-3 cells.** Montelukast had a significant effect on LTC<sub>4</sub>-induced IL-8 production in Calu-3 cells (*n* = 6). Pretreatment of monterukast was for 2 h, and incubation time of LTC<sub>4</sub> was 2 h. \**p* < 0.05, \*\**p* < 0.02; \*\*\**p* < 0.001, compared with control.



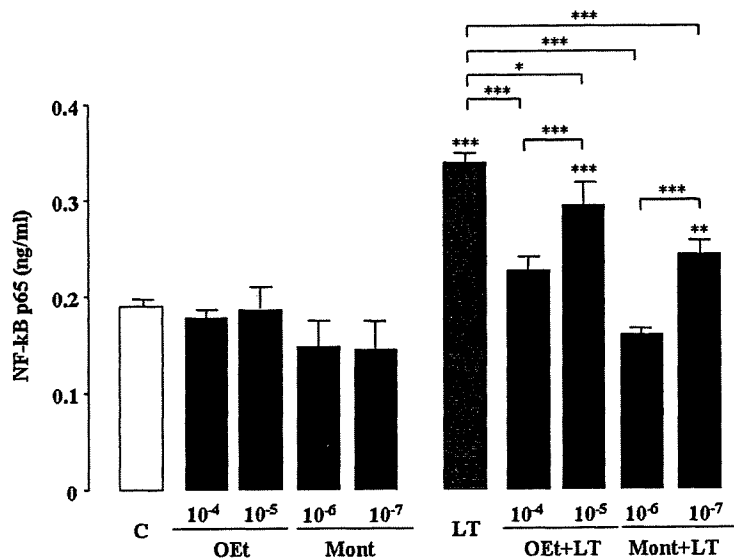
and it proved that Cys-LTs can induce oxidative stress in cultured human airway epithelial cells. The decreased intracellular GSG/GSSG ratio in Calu-3 cells may be induced by the increased ROS formation after exposure to LTC<sub>4</sub>. Electron spin resonance is the only method that can be used to measure free radicals directly, but the evanescent nature of many ROS makes them difficult to measure. Therefore, the most common technique is to quantify ROS indirectly by measuring the products damage caused by ROS, including the shift in the balance of reduced and oxidized glutathione (6). GSH is capable of reducing a wide variety of disulfides by transhydrogenation and acts as a major reductant of cellular protein disulfides (8).

This study clearly demonstrated that LTC<sub>4</sub> stimulated airway epithelial cells to produce IL-8. Cys-LTs are one of the most important mediators of the pathophysiology of asthma (18). They are produced mainly by eosinophils and mast cells. Studies have proved that Cys-LTs not only elicit bronchoconstriction as potent constrictors of smooth muscle, but also play an

important role in airway remodeling. Recently, Perng *et al.* reported that LTC<sub>4</sub> induced transforming growth factor β<sub>1</sub> (TGF-β<sub>1</sub>) in the airway epithelium through a P38 mitogen-activated protein (MAP) kinase activation mechanism (21). In our experiment, LTC<sub>4</sub> upregulated epithelial cell-derived IL-8 production. IL-8, a member of the α-chemokine family and one of the most abundant cytokines produced by airway epithelial cells, exhibits a variety of biological activities including neutrophils and T-lymphocyte chemotactic activity (3, 9). Recently, IL-8 has been implicated in the pathogenesis of the allergic inflammation of asthma, and neutrophils have been found to predominate over eosinophils as the major inflammatory cell type in bronchoalveolar lavage fluid and sputum samples of patients with acute exacerbated asthma (5).

Studies of inflammation-related changes in the intracellular redox state in pulmonary macrophages and monocytes show that these changes have a potent effect on cytokine production (11, 28). The IL-8 gene has been reported to be activated by

**FIG. 7. Effect of LTC<sub>4</sub> on NF-κB p65 binding activity in Calu-3 cells.** LTC<sub>4</sub> had an effect on NF-κB p65 binding activity in Calu-3 cells (*n* = 5). Pretreatment of GSH-OEt and montelukast was for 4 h and for 2 h. Incubation time of LTC<sub>4</sub> was 2 h. \**p* < 0.05, \*\**p* < 0.02, \*\*\**p* < 0.001, compared with control.



oxidative stress (10, 17). In our previous study, oxidative stress affected the signal transfer system in airway epithelial cells, resulting in upregulation of cytokine and chemokine production, especially of IL-8 (27). It may be surmised that LTC<sub>4</sub> upregulated IL-8 production by means of oxidative stress in Calu-3 cells.

Oxidants or oxidative stress have been reported to activate transcription factors, including NF- $\kappa$ B, and to promote phosphorylation of MAP kinase (19, 22). We also confirmed that oxidative stress influenced the NF- $\kappa$ B pathway. Like most inflammatory mediators, IL-8 expression can be induced by activation of specific transcription factors such as NF- $\kappa$ B, which then bind to DNA sites located in the promoter region of the relevant gene (17). NF- $\kappa$ B has been shown to be redox state responsive (19), with binding of NF- $\kappa$ B to IL-8 promoters being highly sensitive to changes in the intracellular redox state (29).

On the other hand, Cys-LTs are recognized by at least two receptor types: Cys-LT<sub>1</sub> and Cys-LT<sub>2</sub>. The Cys-LT<sub>1</sub> receptor mRNA is found in the spleen, lung tissue, and smooth muscle cells (1), and Cys-LT<sub>2</sub> receptor mRNA is confirmed in human and rat airway epithelial cells (25). It remains unclear which of these is involved in the mechanism of LTC<sub>4</sub>-induced upregulation of IL-8 production. Also, we cannot ascertain whether LTC<sub>4</sub> is able to directly induce NF- $\kappa$ B activation via the Cys-LTs receptor in human airway epithelial cells. A previous study reported that LTC<sub>4</sub> did not directly induce NF- $\kappa$ B activation in U-937 cells (human monocytic leukemia cell line) (15). Further investigations are necessary to clarify the relationship between the LTC<sub>4</sub>-induced intracellular redox state and IL-8 related signal transfer in airway epithelial cells.

Our results also suggested that pretreatment with montelukast, a Cys-LT<sub>1</sub> receptor antagonist, suppressed LTC<sub>4</sub>-induced oxidation and significantly reduced the upregulation of IL-8 production in cultured human airway epithelial cells. At present, montelukast is widely used in the treatment of asthma. The anti-asthmatic effect may be rendered not only by the antileukotriene activity, but also by other types of pharmacological activity. Simeonova *et al.* demonstrated that montelukast inhibited TNF- $\alpha$ -induced NF- $\kappa$ B activation in THP-1 cells (human monocytic leukemia cell line) (24). Pranlukast, another Cys-LT<sub>1</sub> receptor antagonist, has been reported to inhibit TNF- $\alpha$ -induced NF- $\kappa$ B activation in U-937 cells, which have Cys-LT<sub>1</sub> receptors on their membranes, and T cells (Jurkat) which do not have Cys-LT<sub>1</sub> receptors on their membranes (15). The underlying mechanism of the inhibitory effect on NF- $\kappa$ B activation is unclear. Taking a previous study into consideration (15, 24), antagonism of Cys-LT<sub>1</sub> receptor may be partially related to the inhibition of NF- $\kappa$ B activation, and it is likely that Cys-LT<sub>1</sub> receptor antagonists have another antagonism that inhibits NF- $\kappa$ B activation. Cys-LT<sub>1</sub> receptor antagonists compose a new class of drugs, currently being investigated, and further research will expand our knowledge of their anti-inflammatory potential.

Our results demonstrated that, in cultured human airway epithelial cells, pretreatment with a reducing agent significantly suppressed LTC<sub>4</sub>-induced time-dependent changes in the intracellular redox state and LTC<sub>4</sub>-induced upregulation of epithelial cell-derived IL-8 production by suppressing NF- $\kappa$ B activation, which in turn confirmed that the LTC<sub>4</sub>-induced upregulation of IL-8 production was mainly a consequence of

oxidative stress. This finding suggests that pretreatment with antioxidative agents could protect against LTC<sub>4</sub>-induced oxidative stress. Accordingly, pretreatment with reducing agents may protect against ozone-induced upregulation of IL-8 production in cultured human airway epithelial cells (27), so antioxidative drugs may have a prophylactic effect against inflammation-induced exacerbation of respiratory symptoms. The modulation of IL-8 production, in relation to oxidative stress, has an important role in inflammatory cell recruitment and activation (24), and treatment with reducing agents might benefit patients with airway diseases associated with inflammation, such as asthma. The protective effects of reducing reagents on Cys-LTs exposure *in vivo* require further preclinical investigation.

In conclusion, LTC<sub>4</sub> can affect the intracellular glutathione redox state in human airway epithelial cells, thereby activating signals and thus causing them to increase the production of cytokine. These findings may provide a basis for understanding the interrelationships between oxidative stress and airway inflammation. Therapeutic interventions that either augment endogenous antioxidant defenses or result in a decreased exposure to environmental oxidative stress might therefore be beneficial as adjunctive therapies for asthma and allergic respiratory disorders.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

GSH, reduced glutathione; GSH-OEt, glutathione monochrome ester; GSSG, oxidized GSH; redox, reduction-oxidation; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

## APPENDIX

### *Culture of human airway epithelial cells*

The Calu-3 cell line obtained frozen from the American Type Culture Collection (Rockville, MD), was grown in T75 tissue-culture flasks (Coster, CA), containing a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium with 10% fetal calf serum (FCS) at 37°C in an atmosphere including 5% CO<sub>2</sub> and 95% air. When the cells were 80–90% confluent, they were detached with 0.05% trypsin and 0.02% ethylenediamine tetraacetate (EDTA), and seeded into wells in six-well plastic tissue culture dishes at 10<sup>5</sup> cells/cm<sup>2</sup>. They then were grown to confluence, which typically required 10–12 days (23).

### *LTC<sub>4</sub> stimulation*

In experiments involving Cys-LTs stimulation, confluent Calu-3 cells were serum-starved for 24 h before the addition of LTC<sub>4</sub>. Cells were stimulated with LTC<sub>4</sub> at various concentrations or exposed to ve-

hicle (phosphate buffer solution, PBS). Based on previous experiments of cultured airway epithelial cell, we finally exposed confluent Calu-3 cells to  $10^{-8}$  M LTC<sub>4</sub>.

#### Cell exposure to glutathione modulators or pretreated with Cys-LTs receptor antagonists

To increase the concentration of cellular GSH, some Calu-3 cells were either incubated with GSH-OEt at concentrations of  $10^{-4}$ ,  $10^{-5}$ , or  $10^{-6}$  M for 4 h before and during exposure to LTC<sub>4</sub> (7). Glutathione modulators were added in the presence of FCS-free medium to minimize the influence of FCS on cellular GSH. At the same time, FCS-free medium was added to the control samples. In selected experiments, Calu-3 cells were incubated with the Cys-LTs receptor antagonist, montelukast, at concentrations of  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ , and  $10^{-9}$  M for 2 h before and during exposure to LTC<sub>4</sub>. Cell viability, determined by trypan blue dye exclusion, always exceeded 90%.

#### Measurements of intracellular GSH and GSSG concentration

Calu-3 cells were washed three times with cold wash buffer (0.1 M sodium phosphate and 5 mM EDTA; pH 7.5) and immediately thawed in 100  $\mu$ l of lysis buffer (0.1% Triton-X, 0.1 M sodium phosphate, and 5 mM EDTA; pH 7.5) for 5 min. Lysates then were acidified with 15  $\mu$ l of 0.1 N HCl, and protein was precipitated with 15  $\mu$ l of 50% sulfosalicylic acid. After centrifugation, the supernatant was collected for GSH and GSSG assays. The total cellular glutathione concentration was assayed by a GSSG-reductase-DTNB recycling procedure by the method of Tietze (26), as modified by Buchmüller-Rouiller and co-workers (7). GSH was oxidized by DTNB and then reduced by  $\beta$ -NADPH in the presence of glutathione reductase.

Formation of 2-nitro-5-thiobenzoic acid was monitored by comparing absorbance at 405 nm with that of standard samples of GSH in lysis buffer. GSSG was assayed by Griffith's method (13). Briefly, standard solutions of GSSG or aliquots of samples were mixed with 2  $\mu$ l of 2-vinylpyridine per 100  $\mu$ l of sample volume. All solutions were adjusted to pH 7.5 with triethanolamine. After incubation for 60 min at room temperature, the assay was performed as described for total glutathione.

#### Quantitation of IL-8

Concentrations of IL-8 in culture supernatants were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems, Minneapolis, MN), in accordance with the manufacturer's instructions. Samples of each supernatant were collected 24 h after LTC<sub>4</sub> or PBS exposure. The assays could detect IL-8 concentrations exceeding 3 pg/ml.

#### Nuclear protein extraction and quantitation of NF- $\kappa$ B p65 binding activity

Nuclear protein extraction was carried out using a nuclear extract kit (Active Motif, Carlsbad, CA) as follows. Calu-3 Cells ( $10^5/cm^2$ ) in six-well plastic tissue culture dishes were cultured for 10–12 days. After incubation with varied concentrations of GSH-OEt for a pretreatment time of 4 h or with montelukast for a pretreatment time of 2 h, the cells were exposed to LTC<sub>4</sub> or PBS, respectively. At 6 h following the LTC<sub>4</sub> exposure, nuclear proteins were extracted. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were harvested after washing twice with 2 ml of ice-cold PBS, centrifuging at 500 g for 5 min after each wash. Cells were resuspended in lysis buffer (10 mM HEPES at pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail). A syringe with a 27-

gauge needle was used to disrupt the cells by 10 repetitions of drawing the cell suspension and then ejecting it. The disrupted cell suspension then was centrifuged at 11,000 g for 20 min; the supernatant was removed, and the nuclear pellet was resuspended in extraction buffer [20 mM HEPES at pH 7.9, 1.5 mM MgCl<sub>2</sub>, 0.42 M NaCl, 0.2 mM EDTA, 25% (vol/vol) glycerol, 1 mM DTT, and protease inhibitor cocktail]. Nuclei were disrupted with a syringe using the method described above, and centrifuged for 5 min at 21,000 g. Protein concentration of the nuclear extract was measured by the Lowry method.

NF- $\kappa$ B p65 expression was measured using the TransA NF- $\kappa$ B p65 kit (Active Motif).

#### Reagents

GSH-OEt, LTC<sub>4</sub>, IL-4, and TNF- $\alpha$  were purchased from Sigma Chemical. (St. Louis, MO). montelukast sodium was purchased from Merck Co. (Whitehouse Station, NJ). Nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADPH), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), and glutathione reductase were obtained from Wako Pure Chemical Industries (Osaka, Japan).

#### Statistical analysis

All values are expressed as mean  $\pm$  SE. Nonparametric analysis of variance (Kruskal–Wallis method) was used to determine significant overall differences between groups. We used the Mann–Whitney *U* test to determine significant differences between individual groups. A value of  $p < 0.05$  was considered to indicate significance.

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# Age-Related Difference in the Persistency of Allergic Airway Inflammation and Bronchial Hyperresponsiveness in a Murine Model of Asthma

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## Key Words

Cytokines · Development · Infant · Maturation · Mouse

## Abstract

**Aim:** Asthmatic children are more likely to outgrow their symptoms than adult patients. Thus, we wanted to know whether there were any age-related differences in the time course of the allergic airway inflammation. **Methods:** BALB/C mice at different ages (young: 3 days after birth, and mature: 8 weeks of age) were sensitized with ovalbumin (OVA). Subsequently, animals were challenged with aerosolized OVA during 1, 2, 4 or 8 consecutive weeks. Bronchial hyperresponsiveness (BHR), serum IgE levels, the degrees of inflammatory cell infiltration (ICI) and goblet cell metaplasia (GCM) in the airways, and the number of eosinophils and cytokine levels in bronchoalveolar lavage fluid (BALF) were examined. **Results:** At 1 week, airway inflammation and BHR occurred similarly between young and mature mice. However, BHR disappeared at 4 weeks in young, whereas it persisted even at 8 weeks in mature mice. GCM, ICI and eosinophilia in BALF attenuated with time, with more remarkable reduction in young mice. The BALF IL-4 level was high during the first 2 weeks in both groups, while the IL-2 level was significantly increased at 2 weeks solely in young mice. **Conclu-**

**sion:** Different time courses in airway inflammation and in BHR may relate to the different prognoses between childhood and adult asthma. The understanding of the mechanisms underlying this age-related differences may be helpful to induce remission in asthmatic patients.

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## Introduction

Recently, asthma has been believed to be a chronic inflammatory and partly irreversible airway disease, mainly based on findings in adult patients [1]. In children, however, details on airway inflammation and remodeling are inconclusive compared with those in adult patients. A difference in the natural history of asthma has been shown in patients regarding the onset of illness in childhood and after adolescence. Longitudinal studies indicate that about 50% of all asthmatic children are virtually free of symptoms within 10–20 years [2], in contrast to only about 10% of asthmatic adults [3], showing that pediatric patients are more likely to outgrow their symptoms than adult patients, although the exact mechanism of this age-related difference remains to be elucidated. Most patients with childhood asthma are known

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to be atopic [4]. Therefore, an age-related difference in the development of allergic airway inflammation and remodeling may be responsible for the differences.

The pathophysiology of atopic asthma is characterized by airway eosinophilia, an elevated serum IgE level, bronchial hyperresponsiveness (BHR), inflammatory cell infiltration (ICI) and goblet cell metaplasia (GCM) in the airways. So-called T-helper (Th) 2 cytokines, e.g. interleukin (IL)-4, IL-5, and IL-13 produced by Th2 cell subsets, are thought to play a pivotal role in this process [5, 6]. The Th2 deviation has been reported to be induced by several factors, including the genetic background, the doses of antigen for sensitization [7] and the cytokines involved during early T-cell activation [6]. The details for these pathophysiological and immunological abnormalities have been based on data in humans and animal models of asthma.

It has been shown that immune responses in the perinatal and neonatal period were at variance with those seen in mature individuals [8, 9]. The different immune responses may induce altered airway inflammation and remodeling via immune modulators such as cytokines.

The purpose of the present study was to determine whether there are any differences in allergic airway inflammation and in BHR depending on the stages of maturation in animals. Thus, the time courses of airway inflammation and BHR were examined in sensitized young and mature mice. In addition, cytokine levels in the BALF were also assessed to know the role of immune responses in these changes.

## Materials and Methods

### *Animals*

BALB/C mice at different stages of maturation (young mice 3 days after birth and mature mice at 8 weeks of age) were studied. Eight-week-old adult mice and pregnant mice were obtained from Charles River Japan (Shizuoka, Japan). After delivery of newborn mice, each mother and the litter were housed separately. The protocol of the experimental study was approved by the institutional animal care and use committee.

### *Sensitization and Airway Challenge Protocol*

Young and adult mice were randomly divided into two groups. One group of mice was immunized with 10 µg OVA i.p. (grade V, Sigma, St. Louis, Mo., USA) in 20 mg of alum (Al(OH)<sub>3</sub>) on day 0 and boosted on days 7 and 14 as described previously [7, 10]. Thereafter, they were challenged with aerosolized 2.5% OVA solution using an ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan) in a 4.5-liter inhalation box 3 times a week from 1 to 8 weeks. Another group of animals (non-immunized control group) received injections of alum alone (vehicle for OVA) for 3 times and were repeatedly challenged with vehicle (saline) under the same schedule. We chose 10 µg OVA as the dose of sensitization, be-

cause we have previously established that this dose, but not higher doses (e.g. 1,000 µg OVA), induced Th2-biased responses both in juvenile and mature mice [7]. Twenty-four hours after the last challenge of repetitive inhalation for either 1, 2, 4 or 8 weeks, BHR was examined in each animal. Thereafter, serum IgE levels, eosinophil and cytokine counts in BALF, and the degrees of ICI and GCM in the airways were examined.

### *Measurement of BALF Cytokines*

The levels of eight BALF cytokines, including IL-2, IL-4, IL-5, IL-10 and IL-12, tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ), were measured using a commercially available kit (Bio-Plex Suspension Array System; Bio-Rad, Richmond, Calif., USA). The detection range was 1.0–32,000 pg/ml for all cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α and IFN-γ).

### *Determination of OVA-Specific IgE*

OVA-specific IgE levels were determined by ELISA as described previously [7]. Ninety-six-well microtiter plates were coated with 200 µg/ml of OVA (grade V, Sigma) diluted in 0.1 M NaHCO<sub>3</sub>. After 2 h of incubation at 37°C, plates were washed with washing buffer (Sigma) and blocked with PBS-bovine serum albumin for 2 h at 37°C. After washing with the buffer for 5 times, serially diluted serum samples (100 µl) were added and incubated for 2 h at 37°C. Plates were washed for 5 times with 300 µl of the washing buffer. Subsequently, 100 µl of 1:800-diluted rat anti-mouse IgE monoclonal antibodies (Biosource International, Camarillo, Calif., USA) were added. After 2 h of incubation at 37°C, plates were washed for 5 times with 300 µl of the washing buffer. After 2 h of incubation at 37°C, the reaction chromogen was generated with FAST (Sigma). After the reaction was stopped with H<sub>2</sub>SO<sub>4</sub>, plates were read in a multiplate reader at 490 and 620 nm. The serum pooled from adult mice that was sensitized and challenged with OVA was used as a positive control. The OVA-specific IgE titer was determined as the reciprocal of the highest dilution giving a positive value. The results were expressed as indices (the ratio of test serum to positive control).

### *Determination of BHR*

BHR to increasing concentrations of aerosolized methacholine (Mch) was studied on unstrained conscious mice as described previously [7]. Mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Sharon, Conn., USA), and continuous measurement of the pressure-time wave was made. The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation with airway resistance [7, 11], was calculated. Mice were challenged with Mch (3.13, 6.25, 12.50 and 25.00 mg/ml) aerosol generated by an ultrasonic nebulizer (NE-U12, Omron) for 2 min. Respiratory mechanics were measured for 3 min after each aerosol inhalation and averaged. BHR was evaluated utilizing two parameters, (1) the leftward shift of the dose-response curve and (2) the absolute value of Penh corresponding to the maximum Mch concentration (25.00 mg/ml) referred to as maximum reactivity.

### *The Sampling Procedure of Blood and BALF*

After assessment of BHR, animals were killed with an overdose of pentobarbital (50 mg/animal i.p.) to obtain serum samples. After sampling, a 24-gauge cannula was introduced into the proximal portion of the trachea, and lungs were lavaged 3 times

with PBS. The amount of lavage fluid was 0.4 ml each time for mature mice and young animals exposed to OVA or vehicle for 4 and 8 weeks. From the young mice exposed to OVA or vehicle for less than 2 weeks, 0.3 ml of lavage fluid were sampled because of their smaller body size [7]. The BALF was centrifuged at 800 rpm for 5 min. For cytokine level measurements, the supernatant was stored at  $-70^{\circ}\text{C}$ . The cell pellet was resuspended in 0.3 ml of RPMI-1640 medium (Sigma). Total cell counts were performed with a hemocytometer, and differential cell counts were performed on cytospin preparations stained with Diff-Quick (Kokusai-Siyaku, Tokyo, Japan). A blinded observer counted a minimum of 200 cells for each sample.

#### *Tissue Preparation*

After getting BALF, the lungs were inflated at a pressure of 25 cm  $\text{H}_2\text{O}$ . The trachea was clamped until fixation was completed. Tissue specimens were sectioned in the midsagittal plane to a thickness of 6  $\mu\text{m}$ , embedded in paraffin and stained with hematoxylin-eosin and Alcian blue/periodic acid-Schiff (AB/PAS). The slides were coded and graded in a blinded fashion, and the degrees of ICI and GCM were examined.

#### *Evaluation of ICI*

ICI in the lung was evaluated using a modification of a reproducible scoring system described previously [7, 12]. A value from 0 to 3 per criterion was ascribed to each tissue section scored. Three criteria were scored to document the pulmonary inflammation: peribronchial inflammation, perivascular inflammation and alveolar inflammation. For peribronchial and perivascular lesions, a value of 0 was assigned when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value 2 when most bronchi or vessels were surrounded by a thin layer (1–5 cells thick) of inflammatory cells and a value of 3 when most bronchi or vessels were surrounded by a thick layer ( $>5$  cells) of inflammatory cells. To assess alveolar wall inflammation, a value of 1 was defined as increased numbers of inflammatory cells in alveolar walls, a value of 2 as 1–3 foci per section showing cellular alveolar exudate and atelectasis, and a value of 3 as  $>3$  foci per section showing cellular alveolar exudate and atelectasis additionally. The total score (the cellular infiltration score) was the sum of these three subscores (ranging from 0 to 9).

#### *Evaluation of GCM*

The degree of GCM was analyzed using a semiquantitative 5-point-scale, as described previously [7, 13]. The AB/PAS-stained slides were examined with a light microscope (IX 70, Olympus, Tokyo, Japan) and graded on a 5-point scale: grade 0 = 0%, grade 1 = 0–25%, grade 2 = 25–50%, grade 3 = 50–75% and grade 4 = 75–100% of epithelial cells staining positive for AB/PAS, respectively. The mean of the grade in the main bronchus and the large membranous airways was scored separately in each animal. The average of both points was referred to as the mucus cell score.

#### *Statistical Analysis*

All data were expressed as means  $\pm$  SEM unless otherwise mentioned. Non-parametric analysis of variance (Kruskal-Wallis test for unmatched pairs) was used to determine the significance of variance between groups. If a significant difference was found, the Mann-Whitney U test was performed to assess differences between groups. A p value of less than 0.05 was considered to in-

dicating statistical significance. The statistical analysis was performed utilizing Statview version 4.5 (Abacus Concepts, Berkeley, Calif., USA).

## Results

### *Cytokine Levels in BALF*

Of the eight cytokines examined in this study, only IL-4, a Th2 cytokine, and IL-2, a Th1 cytokine, were significantly increased compared to control animals of corresponding age. Other cytokines, including IL-5 and IL-12, did not show any significant change. In both young and mature animals, the IL-4 level was increased during the first 2 weeks and then declined. In contrast, the IL-2 level was significantly increased at 2 weeks in young but not in mature animals (fig. 1). Around 2 weeks after the challenge, Th1 and anti-inflammatory cytokines, including TNF- $\alpha$ , IFN- $\gamma$  and IL-10, were detected in some of the young animals sensitized and challenged with OVA. However, these changes did not reach statistical significance (fig. 1).

### *OVA-Specific IgE Antibodies*

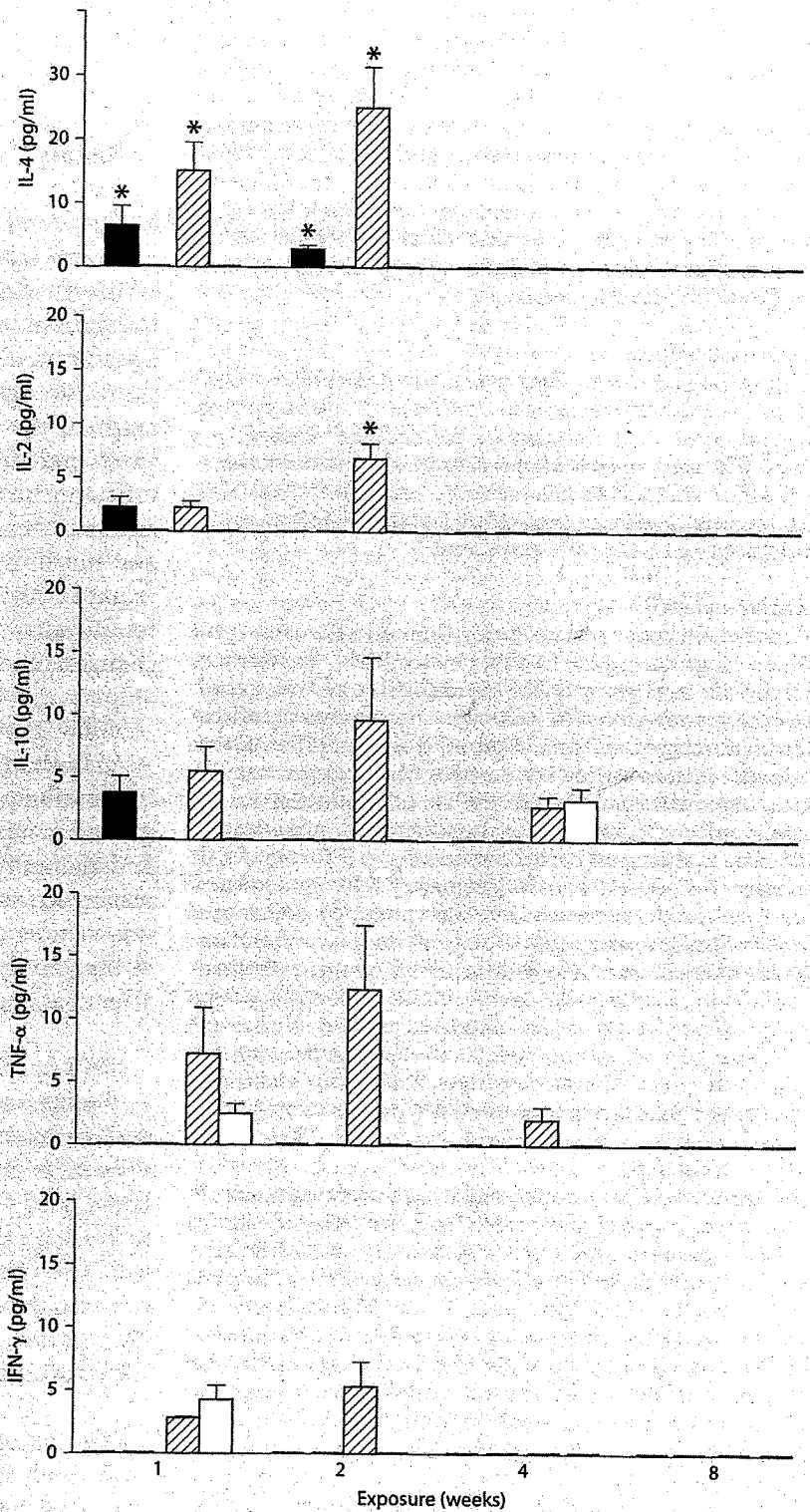
In both mature and young animals, the OVA-specific IgE antibody level was significantly higher in OVA-sensitized groups compared to the corresponding control, and did not fluctuate throughout the study period. Comparing sensitized animals of different age groups, there was a tendency to a higher level in mature mice compared to the young ones, which was significant 1 and 4 weeks after challenge ( $p < 0.05$ , fig. 2).

### *BHR to Mch Challenge*

Figure 3 shows the results of BHR to Mch in mature and young mice. One and 2 weeks after exposure, BHR showed a marked leftward shift of the dose-response curve, and a significant increase in maximum reactivity in both age groups of animals sensitized and challenged with OVA compared to controls. In mature animals, this was also the case 4 and 8 weeks after repeated exposures. By contrast, in young mice, BHR disappeared 4 and 8 weeks after exposure compared to control animals.

### *The Number of Eosinophils in BALF*

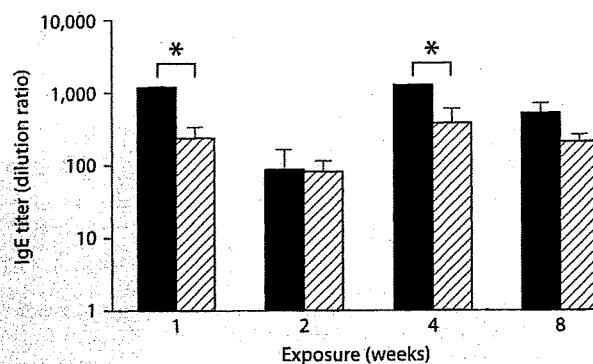
In both age groups of animals sensitized and challenged with OVA, the number of eosinophils was significantly elevated compared to controls 1 week after exposure. In both age groups, the number of eosinophils significantly declined with time, although it was significantly higher compared to control animals without sen-



**Fig. 1.** Cytokine levels in BALF. The values of mature animals with (■) and without sensitization (-) and those of young ones with (▨) and without sensitization (□) were shown. Values are means ± SEM. \*  $p < 0.05$  vs. control animals at corresponding age ( $n = 5$  for each group).

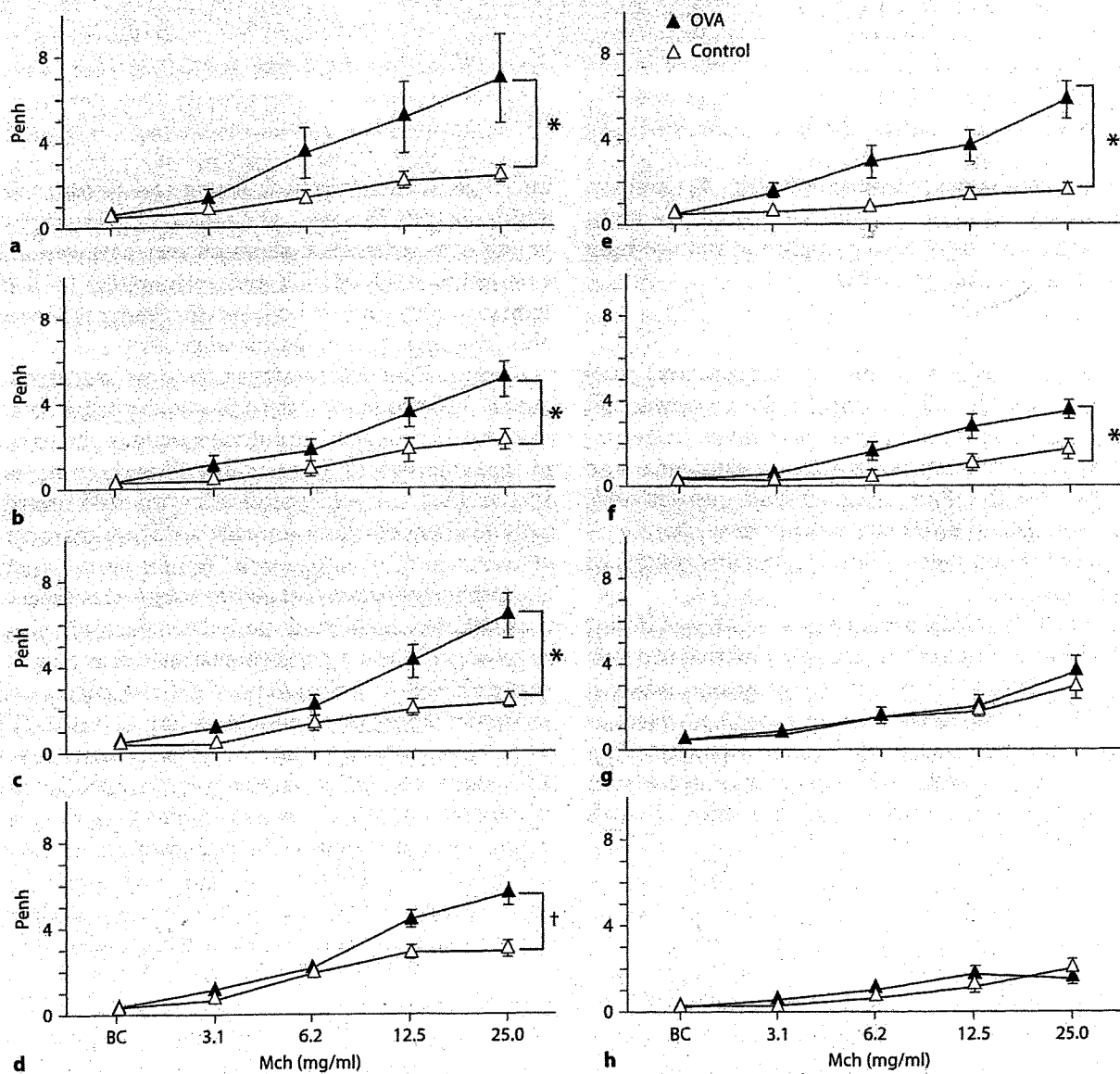


**Fig. 2.** OVA-specific IgE antibody titers determined as the reciprocal of the highest dilution giving a positive value. Mature (■) and young (▨) animals sensitized and challenged with OVA. Values are means ± SEM. \*  $p < 0.05$  between groups ( $n = 5$  for each group).

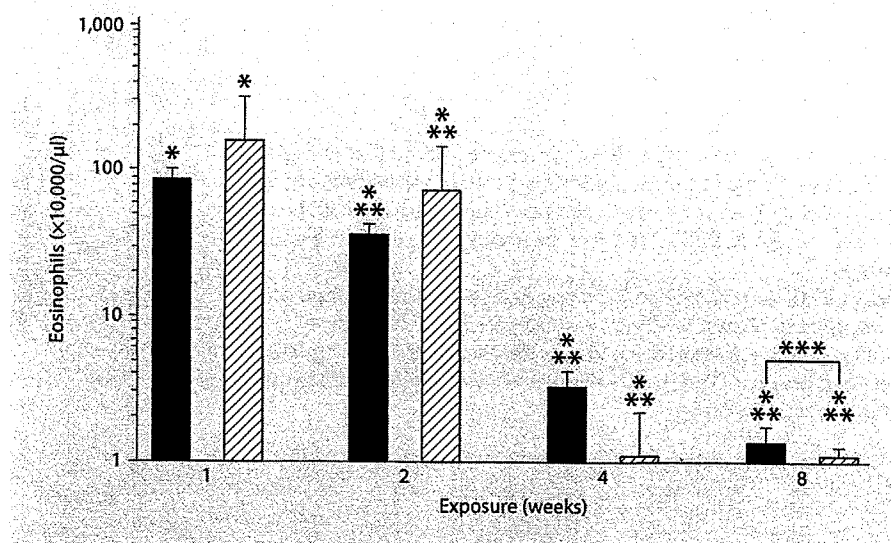


2

**Fig. 3.** BHR to aerosolized Mch in adult (a-d) and juvenile (e-h) mice after exposure to OVA or vehicle (control) for 1 (a, e), 2 (b, f), 4 (c, g) and 8 weeks (d, h). Values are means ± SEM. \*  $p < 0.05$  between groups (OVA:  $n = 7-17$ , control:  $n = 4-10$  for each group). BC = Before challenge.



3



**Fig. 4.** Eosinophil counts in BALF of mature animals (■) and young ones (▨) with and without sensitization are shown. Values are means  $\pm$  SEM. \*  $p < 0.05$  vs. control animals at corresponding age, \*\*  $p < 0.01$  vs. animals exposed to OVA for 1 week, \*\*\*  $p < 0.05$  between groups ( $n = 4-9$  for each group).

sensitization and challenge even at 8 weeks (fig. 4). Comparing sensitized animals at different age groups, the number at 8 weeks was significantly higher in mature mice than in young animals ( $p < 0.05$ ).

#### ICI and GCM

In both age groups of animals sensitized and challenged with OVA, the cellular infiltration score was significantly higher than that in control animals 1 week after exposure ( $3.8 \pm 0.7$  vs.  $0.5 \pm 0.3$  in mature mice, and  $4.6 \pm 1.2$  vs.  $0.4 \pm 0.3$  in young animals, respectively). However, a statistical difference was not seen after the 2-week exposure in animals of both age groups compared to control animals.

The mucus cell score, a parameter of GCM, was almost zero in both age groups of the controls, and that in sensitized and challenged mice of both age groups was significantly higher than in control animals even at 8 weeks. In young animals, however, the degree attenuated significantly at 4 and 8 weeks compared to that 1 week after exposure, although it did not change in mature animals (table 1).

#### Discussion

In the present study, we have demonstrated that the persistency of both airway inflammation and BHR were different between young and mature mice. One week after the aerosol challenge with OVA, airway inflammation

and BHR occurred in both young and mature mice to a similar degree. However, at 4 weeks, BHR disappeared in young mice, whereas it persisted even at 8 weeks in mature animals. GCM, ICI and eosinophilia in BALF attenuated with time in both groups, being more remarkable in young than in mature animals.

In animal models of asthma, the time course of allergic airway inflammation after chronic exposure to antigen differs depending on the animal species or the strain even in the same species. In mice, Temelkovski et al. [14] sensitized BALB/c mice systemically with OVA and chronically challenged these animals with low particle mass concentrations of aerosolized OVA for up to 8 weeks. As a result, they showed that airway inflammation and BHR persisted during the study period. On the other hand, Shinagawa and Kojima [15] demonstrated that only A/J, but neither BALB/C, C57BL/6 nor C3H/HeJ mice, developed airway inflammation and remodeling as well as BHR up to 12 weeks after exposure to antigen, when sensitizing animals by instillation via the nose. Yiamouyiannis et al. [16] recently reported that neither allergic airway inflammation nor BHR persisted in the airways of C57BL/6 after the exposure to antigen for 6 weeks, although these responses were seen in the period of acute challenge (10 days). Our present study confirmed the findings of Temelkovski et al. [14] that BHR persisted for 8 weeks after the aerosol challenge with antigen in mature BALB/C mice. However, airway inflammation gradually attenuated with time, although it persisted even at 8 weeks, but the reason for this difference remains to be elucidated.

**Table 1.** Mucus cell score at the different periods of exposure

Animals	Mucus cell score			
	1 week	2 weeks	4 weeks	8 weeks
Mature				
Control	0.00 ± 0.00	0.20 ± 0.20	0.25 ± 0.25	0.00 ± 0.00
OVA	3.00 ± 0.45*	2.17 ± 0.48*	2.10 ± 0.19*	2.00 ± 0.41*
Young				
Control	0.10 ± 0.10	0.10 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
OVA	3.20 ± 0.26*	2.92 ± 0.33*	2.14 ± 0.32*,**	1.00 ± 0.00*,**

OVA and control indicate animals given OVA and vehicle for sensitization and challenge, respectively. Values are means ± SEM. \*  $p < 0.05$  vs. control animals at corresponding age, \*\*  $p < 0.01$  vs. animals exposed to OVA for 1 week ( $n = 4-9$  for each group).

Previously, we have shown that the major area of airway remodeling after repeated exposure to antigen differed depending on the stage of maturation of guinea pigs *in vivo* [17]. In brief, the prominent areas of thickening caused by chronic exposure to antigen were different among juvenile, adult and old animals. Inner wall thickening was more remarkable in juvenile and adult animals than in old ones. By contrast, a thickened smooth muscle was only observed in old animals. In addition, it was suggested that increased renewal of epithelial cells contributed to the thickening of the inner wall in juvenile and adult animals, and that thickening of smooth muscle areas in old animals was due to factors such as hypertrophy. In the present study, BHR and airway inflammation subsided earlier in young than in mature mice, suggesting that the time course of allergic airway inflammation and BHR is also affected by the stage of maturation of animals.

The mechanism underlying the age-related differences in the persistency of airway inflammation and BHR observed in this study is particularly of concern. Recently, Schramm et al. [18] have reported that continuous inhalational exposure (6 weeks) to OVA resulted in attenuation of airway eosinophilia and BHR without reduction in OVA-specific IgE levels. The finding of our present study that elevated IgE levels persisted in the absence of allergic airway inflammation was similar to those of Schramm et al. [18]. Thus our blunted local airway responses were attributed to local mechanisms rather than systemic energy.

Th2 cytokines, e.g. IL-4, IL-5 and IL-13, are responsible for the development of various features of allergic airway inflammation, including increased production of antigen-specific IgE, infiltration of eosinophils into the airways and GCM. In the present study, we have em-

ployed a relatively low dose of OVA in order to produce Th2-biased allergic airway inflammation [7, 19]. As a result, both allergic airway inflammation and BHR occurred in sensitized mice in both age groups. In BALF, IL-4 was increased until 2 weeks after sensitization and aerosol challenge, confirming that a low dose of antigen induced Th2-biased airway inflammation in both young and mature mice [7]. However, the time course of cytokine levels in BALF was slightly different between these groups, i.e. IL-2 was significantly increased at 2 weeks in young mice but not in mature animals. The cause and the role of the increased IL-2 level in the development of allergic inflammation and BHR in the young airways was not clear in the present study. IL-2 is necessary for any clonal expansion of T cells during immune responses irrespective of the Th type (Th1 or Th2) [20]. Thus, it might be possible that an increased level of local IL-2 modified the development of allergic inflammation and BHR by altering immune responses. In addition, Th1 cytokines, including TNF- $\alpha$  and IFN- $\gamma$ , increased in young but not in mature mice around 2 weeks after challenge, although these changes did not reach statistical significance. The shift toward a Th1 response may partly contribute to the earlier disappearance of allergic airway inflammation in young animals. Further investigations assessing antibody production (IgG2a and IgG1) and subsets of T lymphocytes are required to elucidate the underlying mechanisms of these age-related differences.

In the present study, except for IL-2 and IL-4, cytokines did not significantly increase in the BALF. Of note, IL-5, a major survival and maturation factor for eosinophils, was not detected, although remarkable eosinophilia was observed in the BALF. In the present study, we collected BALF samples 24 h after the last challenge, as was report-

ed by Schramm et al. [18]. By contrast, Ohkawara et al. [21] examined a detailed time course of cytokine levels in BALF. They found that the inflammatory events were observed 3–24 h after antigen challenge. Thus, other cytokines might have been detected in earlier BALF samples.

The specific IgE antibody level in the peripheral blood was more prominent in mature than in young mice, whereas IL-4 responses in the BALF were similar or greater in juvenile animals, confirming our previous observation [7]. The results may indicate a functional immaturity of B lymphocytes or other cells that are responsible for the immune system in juvenile animals, although details remain to be elucidated.

In the present study, there was a tendency to an earlier attenuation in both BHR and airway inflammation in young compared to mature mice. The age-related differ-

ences in the persistency of airway responses may be partly responsible for the different outcome between patients with adult and childhood onset of asthma. Local cytokine levels may contribute to this difference.

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