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The relationship between bronchial asthma and gastroesophageal reflux disease -The effect of proton pump inhibitor on QUEST questionnaire-positive asthmatics-

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Background : The interaction between gastroesophageal reflux disease (GERD) and asthma symptoms has been widely suggested, but the diagnosis of GERD is relatively complicated for daily practice. Although QUEST is a simple questionnaire for diagnosing GERD, the utility of QUEST in GERD-complicated asthma patients has not been well established. We prospectively investigated the effect of proton pump inhibitor (PPI) on QUEST-positive asthmatics and verified the utility of QUEST in this study. In addition, as an acid aspiration has been assumed to be a factor for asthma exacerbation, we also investigated the effect of acid exposure on bronchial epithelial cells in *in vitro* studies.

Methods : Eighty-eight asthma patients were asked to fill QUEST for the analysis of the ratio of QUEST positive patients. Among those patients, 19 QUEST-positive stable asthmatics were enrolled into study and received 20 mg of omeprazole once daily for 4 weeks. We assessed the changes in QUEST score, pulmonary function, asthma symptoms, and QOL after treatment by omeprazole. We also cultured bronchial epithelial cell line BEAS-2B in low pH medium and analyzed the survival and cytokine and chemokine generation from BEAS-2B.

Results : Forty-eight of 88 asthma patients (54.5 %) were judged as QUEST positive and the positive correlation between QUEST score and the asthma severity was observed. Omeprazole significantly improved QUEST score, cough symptoms and these factors lead to the significant improvement in QOL. In *in vitro* studies, the significant cell death of BEAS-2B was induced and the levels of PDGF-BB in culture supernatant was elevated after exposure to low pH medium for 4 hour.

Conclusions : The utility of QUEST in diagnosing and treating the GERD-complicated asthmatics was confirmed. The acid exposure induced bronchial epithelial cell death and the secretion of PDGF-BB and these effects might potentially contribute to asthma exacerbation by GERD.

Key words : Asthma, gastroesophageal reflux disease, QUEST, cough, bronchial epithelial cells

Double-Stranded RNA and TGF- α Promote MUC5AC Induction in Respiratory Cells

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Viral infection is a major trigger for exacerbation of asthma and induces overproduction of mucins. We investigated whether dsRNA could amplify the induction of mucin by TGF- α in human bronchial epithelial cells, as well as the molecular mechanisms regulating MUC5AC expression. Human pulmonary mucoepidermoid carcinoma (NCI-H292) cells and normal human bronchial epithelial cells were exposed to polyinosinic-cytidyric acid (poly(I:C)) and TGF- α . Then, MUC5AC protein production, mRNA expression, and promoter activity were evaluated. Cells were pretreated with a selective inhibitor of ERK, and phosphorylation of ERK was examined by Western blotting. Furthermore, the expression of MAPK phosphatase 3 (MKP3) mRNA was evaluated and the effect of MKP3 overexpression was assessed. Poly(I:C) synergistically increased MUC5AC induction by TGF- α in both NCI-H292 and normal human bronchial epithelial cells. This increase was dependent on MUC5AC gene transcription. A MEK1/2 inhibitor (U0126) significantly inhibited MUC5AC production. Phosphorylation of ERK was enhanced by poly(I:C). TGF- α stimulation up-regulated MKP3 mRNA expression, while costimulation with poly(I:C) inhibited this up-regulation dose-dependently. Enhanced expression of MUC5AC mRNA by poly(I:C) in wild-type cells was completely suppressed in cells transfected with the MKP3 expression vector. dsRNA can synergistically amplify the induction of MUC5AC mucin by TGF- α . This synergistic effect on MUC5AC production may be due to enhanced activation of ERK through inhibition of MKP3 by poly(I:C). *The Journal of Immunology*, 2009, 182: 293–300.

In chronic airway diseases such as asthma, goblet-cell hyperplasia is an important feature (1). Excessive secretion of mucus by hyperplastic goblet cells causes airway plugging and contributes to morbidity and mortality in asthma patients (2, 3). To date, 19 different mucin genes have been identified. Among these, MUC5AC mucin is a major component of the mucus produced by airway epithelial cells (4), and its production is regulated by epidermal growth factor receptor (EGFR) signaling pathway (5, 6). EGFR and its ligands are not only expressed in patients with malignant lung tumors, but also in those with airway inflammatory diseases such as asthma (7). TGF- α is one of the ligands of EGFR, and it is known to play a critical role in phosphorylation of EGFR that leads to MUC5AC production in the airways (5).

Viral infection is a common cause of the exacerbation of asthma. Among the many viruses that infect the airways, human rhinovirus, respiratory syncytial virus, influenza virus, and parainfluenza virus are particularly common pathogens that induce the hypersecretion of mucus and exacerbation of asthma (8–10). These are RNA viruses that synthesize dsRNA during replication in infected cells. TLR3 recognizes dsRNA and was the first antiviral TLR identified (11). Because dsRNA is a universal viral mol-

ecule, TLR3 has been assumed to have a central role in the host response to infection by viruses (11). Previous studies have shown that stimulation with a synthetic analog of viral dsRNA (polyinosinic-cytidyric acid, poly(I:C))² is mediated by a pathway involving TLR3 that induces airway inflammation due to various cytokines and chemokines such as IL-8, IL-6, and RANTES (12). Despite the importance of excessive mucin production due to viral infection in triggering the exacerbation of asthma, the mechanisms causing such overproduction remain unknown.

We hypothesized that viral infection might synergistically amplify respiratory mucin gene expression and protein production induced by growth factors that are involved in the pathogenesis of asthma. Here, we demonstrate that a synthetic analog of viral dsRNA (poly(I:C)) synergistically increases the induction of respiratory mucin MUC5AC by TGF- α in human airway epithelial cells, both at the level of mRNA expression and protein production. This action depends on the activation of ERK, and the ERK pathway is enhanced through inhibition of MAPK phosphatase 3 (MKP3) by poly(I:C).

Materials and Methods

Cell culture and stimulation

A human pulmonary mucoepidermoid carcinoma cell line (NCI-H292) was maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml) at 37°C in a humidified atmosphere with 5% CO₂. NCI-H292 cells were seeded into 12-well plates for the ELISA and luciferase assay, and into 6-cm dishes for Western blotting and mRNA analysis. Cells were grown until 70% confluence was reached,

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² Abbreviations used in this paper: poly(I:C), polyinosinic-cytidyric acid; AB-PAS, Alcian blue/periodic acid-Schiff; C_T, threshold cycle; EGFR, epidermal growth factor receptor; MKP, MAPK phosphatase; NHBE, normal human bronchial epithelial; RT, room temperature.

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Table I. Primers used for quantitative real-time PCR analysis of gene expression

Target mRNA	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
MUC5AC	TCA CAG CCG GGT ACG CGT TGG CAC AAG TGG	TGC TAT TAT GCC CTG TGT AGC CAG GAC TGC
β -actin	GTG GGG CGC CCC AGG CAC CA	CTC CTT AAT GTC ACG CAC GAT TTC
MKP3	CAC CGA CAC AGT GGT GCT CT	CTG AAG CCA CCT TCC AGG TAG
EGFR	TGC GTC TCT TGC CGG AAT	GGC TCA CCC TCC AGA AGG TT

and they were maintained overnight in serum-free medium before stimulation. Cells were exposed to poly(I:C) (Sigma-Aldrich) at 25 μ g/ml or TGF- α (R&D Systems) at 4 ng/ml, or to a combination of both agents.

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza. NHBE cells were seeded at density of $1.3 \times 10^5/cm^2$ into 12-well plates containing bronchial epithelial growth medium (Lonza) supplemented with defined growth factors and retinoic acid from the SingleQuot kit (Lonza), and were incubated at 37°C in a humidified atmosphere with 5% CO₂. Cells were exposed to poly(I:C) (25 μ g/ml) or TGF- α (4 ng/ml), or a combination of both agents, for 24 h.

Analysis of mucin

NCI-H292 cells were stained with Alcian blue and periodic acid-Schiff stains (AB-PAS). MUC5AC protein was measured as described previously (5). In brief, supernatants were collected at 24 h after stimulation and cell lysates were prepared with PBS, and 50 μ l of each sample was incubated with bicarbonate-carbonate buffer (50 μ l) at 40°C in a 96-well plate (Nunc) overnight. Plates were washed three times with PBS and blocked with 2% BSA for 1 h at 37°C. Plates were again washed three times with PBS and then incubated with 50 μ l of mouse monoclonal anti-MUC5AC Ab (1/100) (Lab Vision/NeoMarkers), which was diluted with PBS containing 0.05% Tween 20 and dispensed into each well. After 1 h, the plates were washed three times with PBS, and 100 μ l of HRP-sheep anti-mouse IgG conjugate (1/10,000) (Amersham Biosciences) was added to each well. After 1 h, the plates were washed three times with PBS. Color was developed with 3,3',5,5'-tetramethylbenzidine peroxidase solution (Kirkegaard & Perry Laboratories) and the reaction was stopped with 1 M H₂SO₄. The data were expressed as a fold induction on the same experimental day due to various mucin production with cell passage in NCI-H292 cells.

Real-time quantitative PCR analysis

Expression of MUC5AC, MKP3, and EGFR mRNA by NCI-H292 cells was determined by reverse transcription (RT), followed by the real-time quantitative PCR. Total RNA was extracted from lysates of differentiated NCI-H292 cells using Isogen (Nippon Gene) at 12 h after stimulation. RT was performed with 1 μ g of total RNA and oligo(dT) primers in a 25- μ l reaction mixture according to the manufacturer's protocol (Applied Biosystems). The sequences of the specific primer sets that were used for PCR are listed in Table I (13, 14).

Real-time PCR was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems) using SYBR Green (Applied Biosystems) as a dsDNA-specific binding dye. For MUC5AC and β -actin, initial denaturation was done at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The threshold cycle (C_T) was recorded for each sample to reflect the level of mRNA expression. A validation experiment confirmed linear dependence of the C_T value on the concentrations of MUC5AC and β -actin and the consistency of ΔC_T (mean C_T for MUC5AC - mean C_T for β -actin) in a given sample at different RNA concentrations. ΔC_T was therefore used as an indicator of relative mRNA expression. To determine the effects of different stimuli on MUC5AC gene expression compared with unstimulated cells, $\Delta\Delta C_T$ was calculated ($\Delta\Delta C_T = \Delta C_T$ for stimulated cells - ΔC_T for unstimulated cells). MUC5AC mRNA expression was indexed to β -actin mRNA expression by using the formula $1/(2^{\Delta\Delta C_T}) \times 100\%$. $2\Delta\Delta C_T$ was calculated to demonstrate the fold change of MUC5AC gene expression in stimulated cells compared with unstimulated cells.

Expression of MKP3 and EGFR mRNA by NCI-H292 cells was determined in the same manner.

FIGURE 1. Synergistic effect of poly(I:C) and TGF- α on MUC5AC production. A, AB-PAS staining of NCI-H292 cells for identification of mucus glycoconjugates. Incubation with poly(I:C) (25 μ g/ml) and TGF- α (4 ng/ml) for 24 h increased positive staining. Effect of poly(I:C) (25 μ g/ml) on TGF- α (4 ng/ml)-induced MUC5AC mucin production in NCI-H292 cell supernatant ($n = 9$) (B) and cell lysate ($n = 9$) (C). Cells were incubated with poly(I:C) and TGF- α for 24 h. Data are shown as means \pm SD. ++, $p < 0.01$ compared with non-stimulated control cells. *, $p < 0.05$ and **, $p < 0.01$.

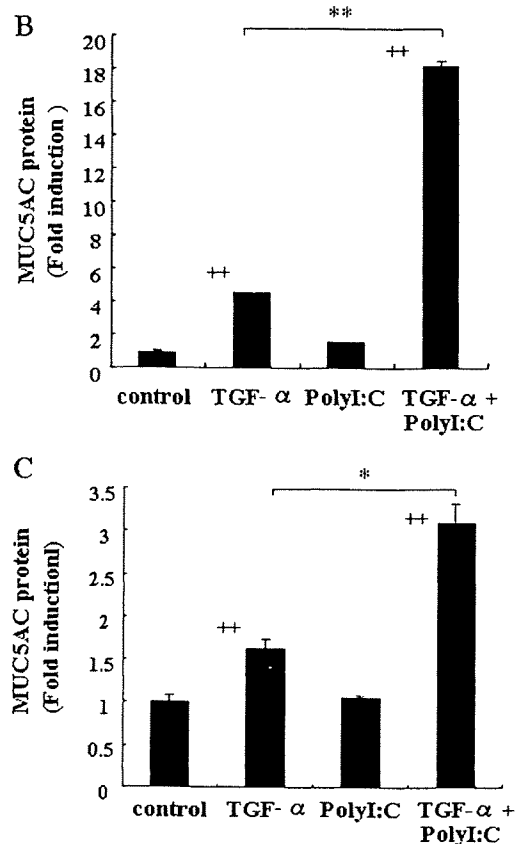
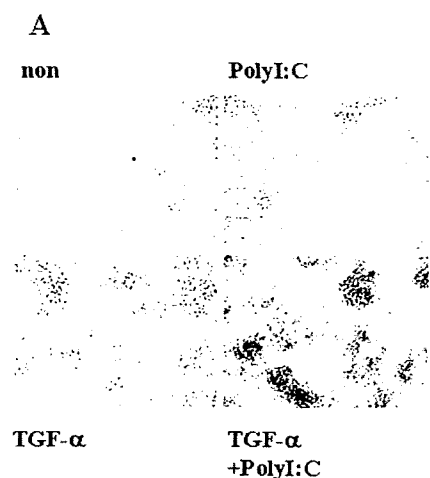
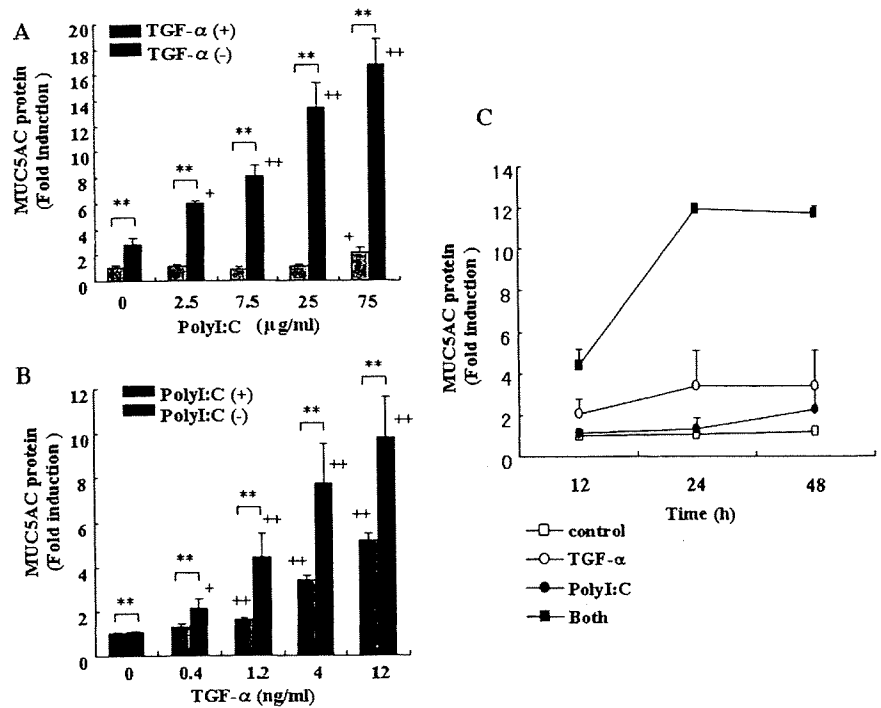


FIGURE 2. Dose responsiveness and time course of MUC5AC protein production. *A*, Effect of the poly(I:C) concentration on TGF- α (4 ng/ml)-induced MUC5AC mucin production in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF- α for 24 h. *B*, Effect of the TGF- α concentration on poly(I:C) (25 μ g/ml)-induced MUC5AC mucin production in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF- α for 24 h. *C*, Effect of poly(I:C) (25 μ g/ml) on the time course of induction of MUC5AC mucin production by TGF- α (4 ng/ml) in NCI-H292 cells ($n = 6$). Data are shown as means \pm SD. +, $p < 0.05$ and ++, $p < 0.01$ compared with nonstimulated control cells. **, $p < 0.01$.



Reporter assay for the MUC5AC promoter

To investigate the regions of the MUC5AC promoter that were activated by poly(I:C) and TGF- α , the full-length human MUC5AC promoter was cloned into pGL3basic (a promoterless luciferase vector). This was then serially truncated using a combination of restriction enzyme digestion and PCR amplification to successively isolate regions of the promoter containing a large variety of potential transcription factor-binding sites (-1330 to -63).

NCI-H292 (0.8×10^5) cells were seeded into 12-well plates and grown overnight in complete medium. At 60% confluence, cells were rinsed with 1 ml of serum-free medium and incubated for 1 h. Then the cells were transfected using 1.3 μ l of FuGENE 6 (Roche Applied Science) in 50 μ l of RPMI 1640 medium per well plus 4 μ l of MUC5AC promoter-luciferase plasmid DNA. At 1 h after transfection, cells were stimulated with poly(I:C) (25 μ g/ml) and then incubated for 12 h before stimulation with

TGF- α (4 ng/ml). Cell lysates were prepared, and reporter gene activity was determined by using a luciferase assay kit (Promega). The total protein concentration of samples was measured by spectrophotometry (NanoDrop from Thermo Scientific) to adjust for variations in harvesting of cells.

Western blot analysis

Cells (3.0×10^5) were washed with PBS and lysed in 300 μ l of lysis buffer (0.5% Nonidet P-40, 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 3 mM *p*-aminidinophenylmethanesulfonyl fluoride (Sigma-Aldrich), 5 mg/ml aprotinin (Sigma-Aldrich), 2 mM sodium orthovanadate (Sigma-Aldrich), 5 mM EDTA). Whole-cell extracts were subjected to electrophoresis on 7.5–12% Tris-glycine gel (XV Pantera gel; DRC) and then transferred to Sequi-Blot polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T (pH 7.5)) for 30 min at room temperature (RT) and

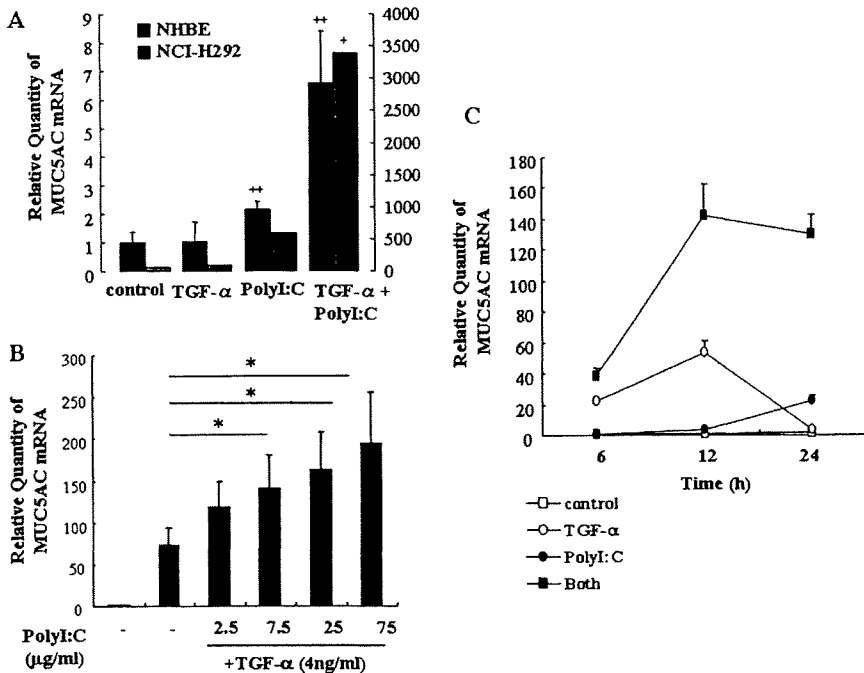


FIGURE 3. Dose responsiveness and time course of MUC5AC mRNA expression. *A*, Effect of poly(I:C) (25 μ g/ml) on TGF- α (4 ng/ml)-induced MUC5AC gene expression in NCI-H292 cells and NHBE cells. Cells were incubated with poly(I:C) and TGF- α for 24 h. Data are presented as the fold induction over the level in control NHBE cells. The right side of the y-axis is for NCI-H292 cells and the left side is for NHBE cells ($n = 6$). *B*, Effect of the poly(I:C) concentration on TGF- α (4 ng/ml)-induced MUC5AC mRNA expression in NCI-H292 cells ($n = 6$). Cells were incubated with poly(I:C) and TGF- α for 12 h. *C*, Effect of poly(I:C) (25 μ g/ml) on the time course of induction of MUC5AC mRNA expression by TGF- α (4 ng/ml) 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. *, $p < 0.05$.

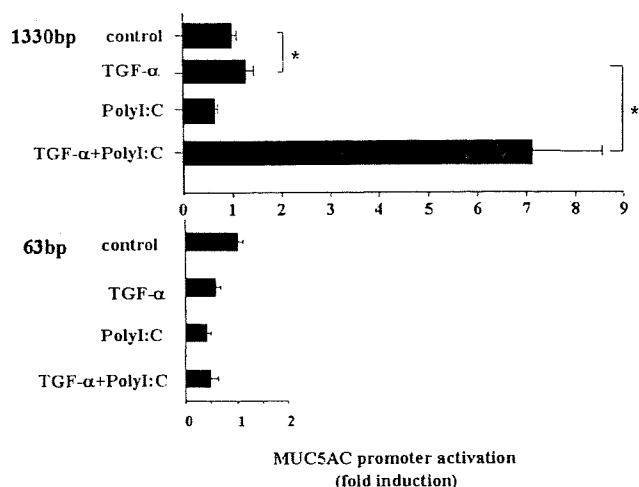


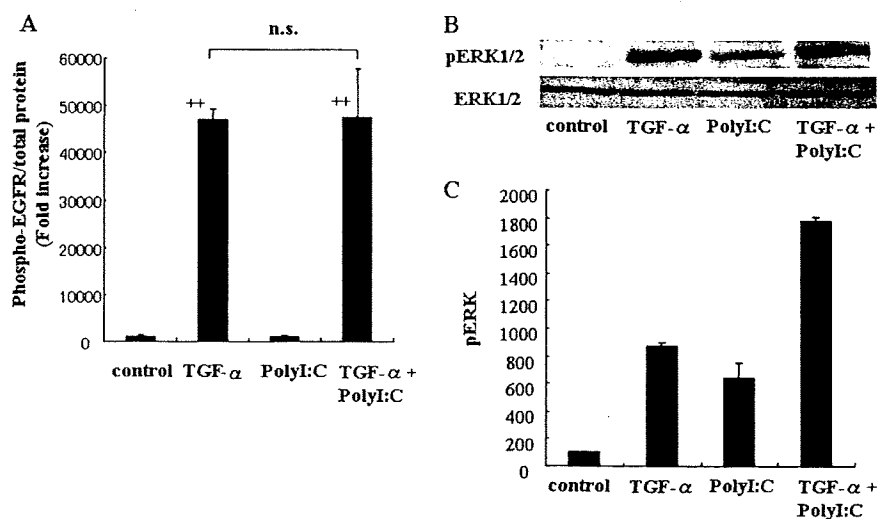
FIGURE 4. Effect of poly(I:C) (25 μ g/ml) on TGF- α (4 ng/ml)-induced *trans*-activation of the full-length (-1330) and the short-length (-63) MUC5AC promoter in NCI-H292 cells ($n = 6$). Data are shown as means \pm SD. *, $p < 0.05$.

probed with primary anti-human phospho-p44/42 MAPK (Thr²⁰²/Tyr²⁰⁴) Ab and p44/42 MAPK Ab (Cell Signaling Technology) for 1 h at RT. The membranes were then washed with TBS-T and incubated with secondary donkey anti-rabbit Ig Ab conjugated to HRP (Amersham Biosciences) for 1 h at RT. Finally, Ab-Ag complexes were detected using an ECL chemiluminescent detection system according to the manufacturer's instructions (ECL plus Western blot detection system; Amersham Biosciences).

Cloning of MKP3 expression vector and transfection into NCI-H292 cells

A DNA fragment of the coding sequence of MKP3 was amplified by PCR using cDNA from poly(I:C)-treated NCI-H292 cells. The purified PCR product was digested with *Bam*HI and *Sal*I and cloned into the pAcGFP1-C1 vector (Clontech Laboratories). The plasmid was analyzed by digestion with restriction enzymes and DNA sequencing. Plasmids for transfection were purified with HiSpeed Plasmid Maxi kit (Qiagen). H292 cells were seeded into 6-well plates and grown to 50% confluence. Cells were transfected with 4 μ g of expression vector with 10 μ l of Lipofectamine 2000 (Promega) and grown in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml). After 24 h, the medium was changed to RPMI 1640 supplemented with 10% FBS without antibiotics. Then, the cells were exposed to poly(I:C) (25 μ g/ml), TGF- α (4 ng/ml), or a combination of both agents. After 12 h, the expression of MUC5AC and MKP3 mRNA was evaluated.

FIGURE 5. A, Relative phosphorylation of EGFR by poly(I:C) and TGF- α in NCI-H292 cells. Proteins extracted from samples collected were tested for the presence of phosphorylated EGFR by a Bio-Plex phosphoprotein assay kit using the Bio-Rad Luminex machine. The values plotted show the ratios of phosphorylated EGFR to total EGFR expressed as fold increase over control ($n = 6$). B, Phosphorylation of ERK1/2 by poly(I:C) (25 μ g/ml) and TGF- α (4 ng/ml) in NCI-H292 cells assessed by performing Western blot analysis. C, Phospho-ERK was expressed as the fold increase in relative intensity ($n = 3$). Data are shown as means \pm SD. ++, $p < 0.01$ compared with nonstimulated control cells.



Other reagents

U0126 (a MEK1/2 inhibitor) was purchased from Sigma-Aldrich and monoclonal anti-human CXCL8/IL-8 Ab was purchased from R&D Systems. U0126 was dissolved in DMSO, while the monoclonal anti-human CXCL8/IL-8 Ab was dissolved in PBS. In all studies, the concentration of DMSO was 0.02–0.06%. U0126 (20 μ M) (15, 16) and the anti-IL-8 Ab (2 μ g/ml) were preincubated with cells for 1 h before adding poly(I:C) and TGF- α .

Phosphoprotein assay

Cells (3.0×10^5 /ml) were seeded into 6-cm dishes and were treated with poly(I:C) for 1 h and then with TGF- α for 15 min. Protein lysates were prepared by using a cell lysis kit (Bio-Rad), and phosphorylated EGFR was detected with an EGFR (Tyr) assay kit (Bio-Rad) and a phosphoprotein testing reagent kit (Bio-Rad) according to the manufacturer's protocol. Briefly, 50 μ l of cell lysate (adjusted to a protein concentration of 200–400 μ g/ml) was plated into a 96-well filter plate coated with EGFR Ab-coupled beads and incubated overnight on a platform shaker at 300 rpm at RT. Total protein was measured with a Bio-Rad DC protein assay kit.

Statistical analysis

All data are expressed as the means \pm SD. Results were analyzed by using the paired Student's *t* test or ANOVA as appropriate. Analyses were done with SPSS II software (SPSS Japan), and *p* values of <0.05 were considered significant.

Results

Poly(I:C) synergistically enhances MUC5AC protein production induced by TGF- α

First, we examined the ability of TGF- α and poly(I:C) to induce mucous glycoconjugate production assessed by AB-PAS staining in NCI-H292 cells (Fig. 1A). Twenty-four hours of incubation with TGF- α (4 ng/ml) increased PAS-positive staining, while poly(I:C) (25 μ g/ml) alone did not affect staining. However, poly(I:C) enhanced the stimulatory effect of TGF- α on mucous glycoconjugate production (Fig. 1A). To quantify the MUC5AC mucin production, an ELISA was performed. TGF- α alone caused a 5-fold increase in MUC5AC mucin protein in cell supernatant (Fig. 1B) and a 1.5-fold increase in cell lysate (Fig. 1C) from NCI-H292 cells 24 h after stimulation. Poly(I:C) alone caused little increase in MUC5AC mucin protein; however, poly(I:C) strongly potentiated the effect of TGF- α . Thereafter, we evaluated MUC5AC mucin protein only in cell supernatant, because it was more prominent than cell lysate.

Next, we determined effects of dose responses of poly(I:C) (2.5–75 μ g/ml) and TGF- α (0.4–12 ng/ml) on MUC5AC mucin production (Fig. 2, A and B). Although poly(I:C) alone did not

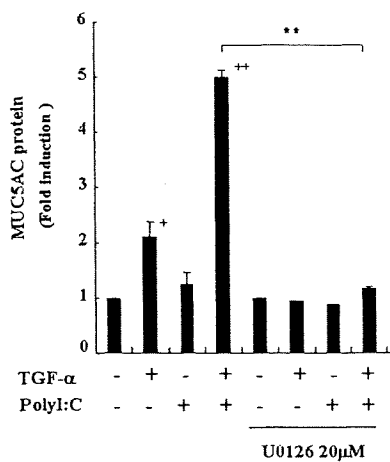


FIGURE 6. Effect of U0126 on poly(I:C)- and TGF- α -induced MUC5AC mucin production in NCI-H292 cells. Cells were preincubated with U0126 (a specific inhibitor of MEK1/2) at 20 μ M (15, 16) for 1 h before adding poly(I:C) (75 μ g/ml) and TGF- α (4 ng/ml), and cells were analyzed 24 h after stimulation ($n = 6$). 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- α caused an increase in MUC5AC mucin production with regard to poly(I:C) in a dose-dependent manner (Fig. 2A). TGF- α alone induced a dose-dependent increase in MUC5AC mucin production, and poly(I:C) enhanced the effect of TGF- α (Fig. 2B). Subsequent studies were focused on the time course of MUC5AC mucin production. Costimulation with poly(I:C) (25 μ g/ml) and TGF- α (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- α .

Poly(I:C) synergistically enhances MUC5AC mRNA expression induced by TGF- α

To determine whether induction of MUC5AC mucin protein induced by poly(I:C) and TGF- α 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- α (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 μ 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- α (Fig. 3A). A clear dose response was observed at 12 h following stimulation with both poly(I:C) (2.5–75 μ g/ml) (Fig. 3B) and TGF- α (0.4–12 ng/ml) (data not shown). Costimulation with poly(I:C) and TGF- α 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- α cause synergistic trans-activation of the MUC5AC promoter

We next investigated whether the MUC5AC promoter was activated by poly(I:C) and TGF- α . After 8 h, TGF- α 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- α , 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- α 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- α , 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- α .

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

Since it was shown that induction of MUC5AC-specific mucin protein by poly(I:C) and TGF- α 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 μ g/ml) on TGF- α (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 μ g/ml) and TGF- α (4 ng/ml) was examined by standard RT-PCR. In brief, 25 μ l of reaction mixture consisted of 1 μ l cDNA, 1 pmol MKP3 primer sets, and 12.5 μ 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 μ g/ml) and TGF- α (4 ng/ml) in NCI-H292 cells ($n = 4$). C, Effect of the poly(I:C) concentration on TGF- α (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 **, $p < 0.01$.

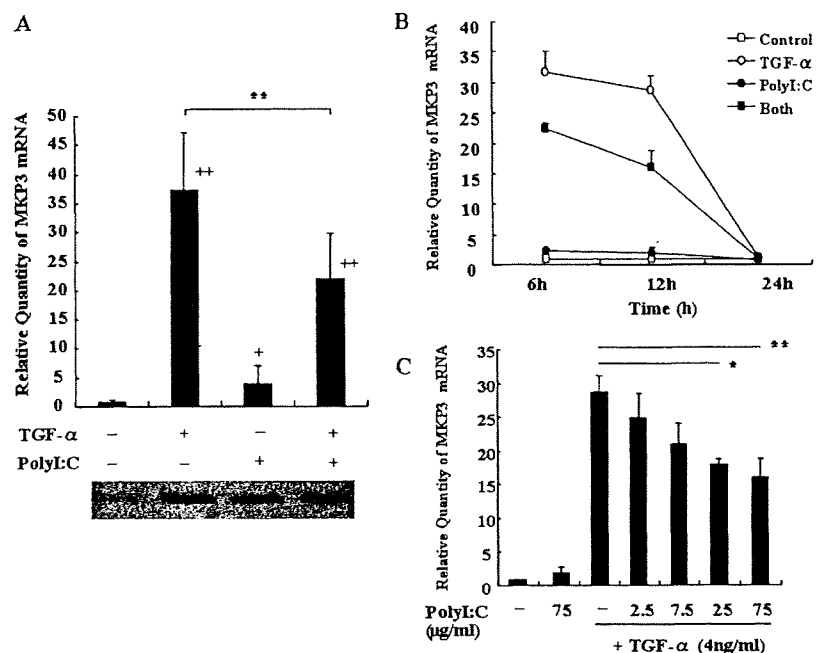
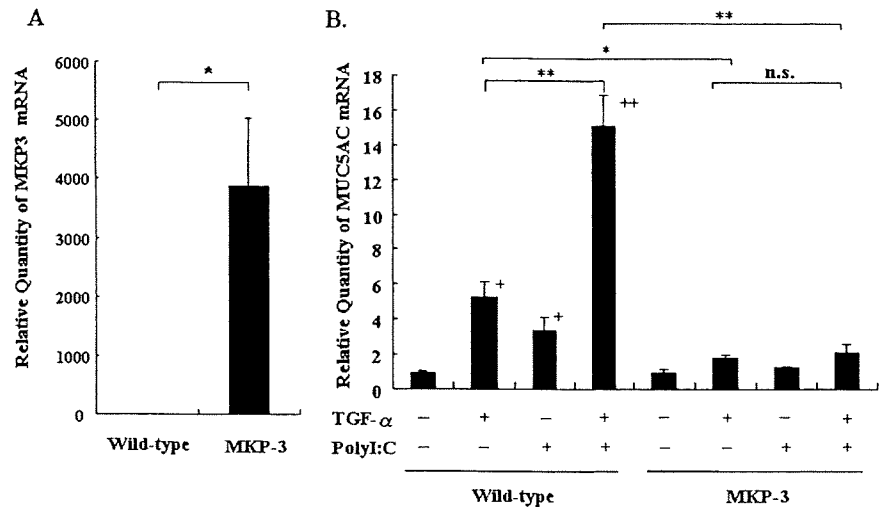


FIGURE 8. *A*, Effect of a MKP3 expression plasmid cloned into the pAcGFP1-C1 vector on MUC5AC mRNA expression in NCI-H292 cells. The level of MKP3 mRNA was enhanced significantly in cells transfected with the MKP3 expression plasmid. *B*, Enhanced expression of MUC5AC mRNA was noted in wild-type cells after 12 h of coincubation with TGF- α (4 ng/ml) and poly(I:C) (25 μ 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- α 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- α 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 μ g/ml) and TGF- α (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- α is mediated via an ERK signaling pathway.

Poly(I:C) inhibits TGF- α -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- α . 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- α , and MKP3 mRNA up-regulation by TGF- α was inhibited by stimulation with poly(I:C) (Fig. 7A). Stimulation with TGF- α 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-

fected 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- α (4 ng/ml) and poly(I:C) (25 μ 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- α -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- α 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- α 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 μ g/ml) and TGF- α (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- α (Fig. 9).

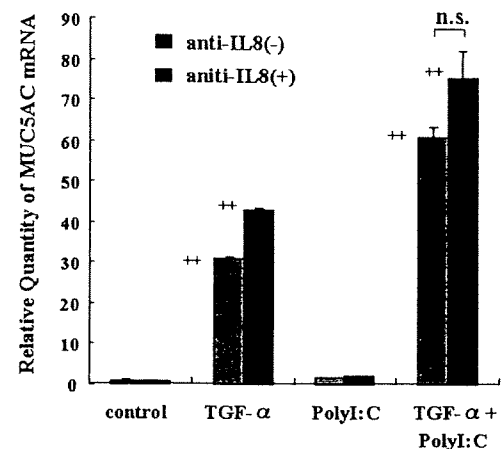


FIGURE 9. Effect of the anti-IL-8 Ab on poly(I:C) (75 μ g/ml) and TGF- α (4 ng/ml)-induced MUC5AC mRNA expression in NCI-H292 cells ($n = 4$). Data are shown as means \pm SD. ++, $p < 0.01$ compared with nonstimulated control cells.

Discussion

In this study, we found that poly(I:C) synergistically increased the production of MUC5AC induced by TGF- α 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- α 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- α .

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- α , causes MUC5AC production by airway epithelial cells both in vitro and in vivo (5), and this effect is potentiated by TNF- α (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- α 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- α , indicating that synergistic induction of MUC5AC by poly(I:C) and TGF- α 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- α 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- α . Therefore, we concluded that *trans*-activation of the MUC5AC promoter by poly(I:C) and TGF- α 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- γ (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- α , 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- α 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- α 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- α alone. In contrast, costimulation with poly(I:C) and TGF- α 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- α 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 α , IL-8, GRO- α , and ENA-78) and cytokines (IL-1 β , 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- α 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- α and IFN- β . However, to further investigate whether extracellular factors (such as chemokines and cytokines) released by poly(I:C) stimulation up-regulated TGF- α -induced MUC5AC production, we changed the culture medium at 12 h after poly(I:C) stimulation and then stimulated the cells with TGF- α . Although the extracellular factors had been removed, it did not alter the synergistic expression of MUC5AC mRNA (data not shown), suggesting that extracellular factors including IFN- α and IFN- β 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- α 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- α . 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. LTC₄ 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 LTC₄-induced time-dependent changes in the intracellular redox state, and also suppressed upregulation of IL-8 production by suppressing NF- κ B activation. Our observations led to the hypothesis that LTC₄-induced oxidative stress is likely to contribute to amplification of airway inflammation. *Antioxid. Redox Signal.* 10, 821–828.

AIRWAY INFLAMMATION AND REDOX

ASTHMA 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 (H₂O₂), 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- κ 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: LTB₄ and a second group (LTC₄, LTD₄, LTE₄), 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-isoprotane (a marker of oxidative stress) than normal subjects (1). In a B cell line (BL41-E95-A), oxidative stress (H₂O₂, 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 oxidative 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

LTC₄ exposure and intracellular redox state

To determine an informative concentration and duration of Cys-LTs exposure, we compared six various stimulated concentration of LTC₄: from 10⁻⁷ to 10⁻¹² M at 2 and 6 h. LTC₄ exposure dose-dependently induced an oxidized state within Calu-3 cells at 2 h after stimulation (Fig. 1). We chose a final LTC₄ concentration of 10⁻⁸ 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 LTC₄ exposure. In the 10⁻⁸ 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 LTC₄ 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 LTC₄ exposure (Fig. 2A). Figure 2B shows the time course of the absolute values of GSH and GSSG. At 4 h after LTC₄ exposure, the GSH slightly decreased, and thereafter the GSH increased significantly to reach a maximum reduction at 6 h after LTC₄ exposure (Fig. 2B). The GSSG increased at 24 h after LTC₄ 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).

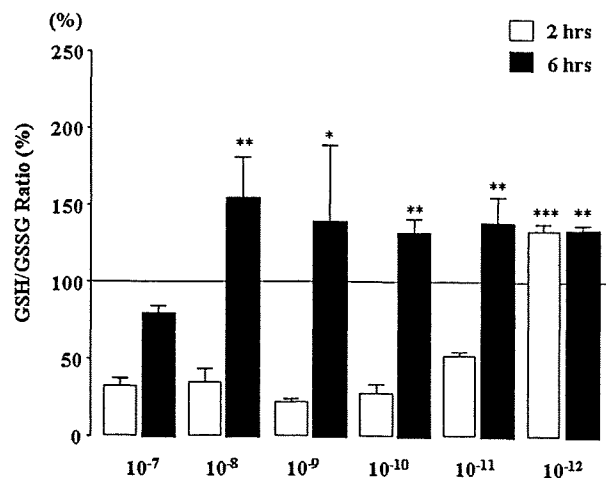


FIG. 1. Dose-dependent effect of LTC₄ on intracellular redox. Results following LTC₄ (10⁻⁷–10⁻¹² M) exposure for 2 h show the intracellular redox state declining to indicate an oxidized state, with the GSH/GSSG ratio showing <100% of control-state values (*n* = 6). At 6 h after exposure, the GSH/GSSG ratio increased. In 10⁻⁸ M concentration, LTC₄ induced a clear series of oxidation and reduction. **p* < 0.05, ***p* < 0.02, ****p* < 0.001, compared with 10⁻⁷ M LTC₄.

LTC₄ exposure and pretreatment with glutathione modulators

Calu-3 cells were pretreated with the reducing reagent, glutathione monochrome ester (GSH-OEt), at concentrations of 10⁻⁴, 10⁻⁵, and 10⁻⁶ M for 4 h, and then exposed to LTC₄ at a concentration of 10⁻⁸ M. In the LTC₄ group (exposed to LTC₄ without pretreatment), the GSH/GSSG ratio at 2 h after LTC₄ exposure showed a decreased of >25%, indicating that cells were in an oxidized state, peaking at 6 h after LTC₄ exposure (Figs. 3A and B). On the other hand, in the OEt+LTC₄ group (GSH-OEt pretreatment and exposed to LTC₄), the GSH/GSSG ratio exceeded 100%, and a reduced intracellular state was maintained for 6 h after LTC₄ exposure. LTC₄-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.

LTC₄ exposure and pretreatment with Cys-LTs receptor antagonists

Calu-3 cells were pretreated with 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 LTC₄ at a concentration of 10⁻⁸ M. In the LTC₄ group (exposed to LTC₄ without pretreatment), the GSH/GSSG ratio at 4 h after LTC₄ exposure decreased to nearly 50%, indicating that cells were in a severely oxidized state (Fig. 4), while addition of montelukast at concentrations of 10⁻⁶ and 10⁻⁷ M attenuated the significant decreases (Fig. 4). There was no significant difference in the GSH/GSSG ratio between montelukast pretreatment (10⁻⁶, 10⁻⁷ M) group and control group at 4 h after LTC₄ exposure.

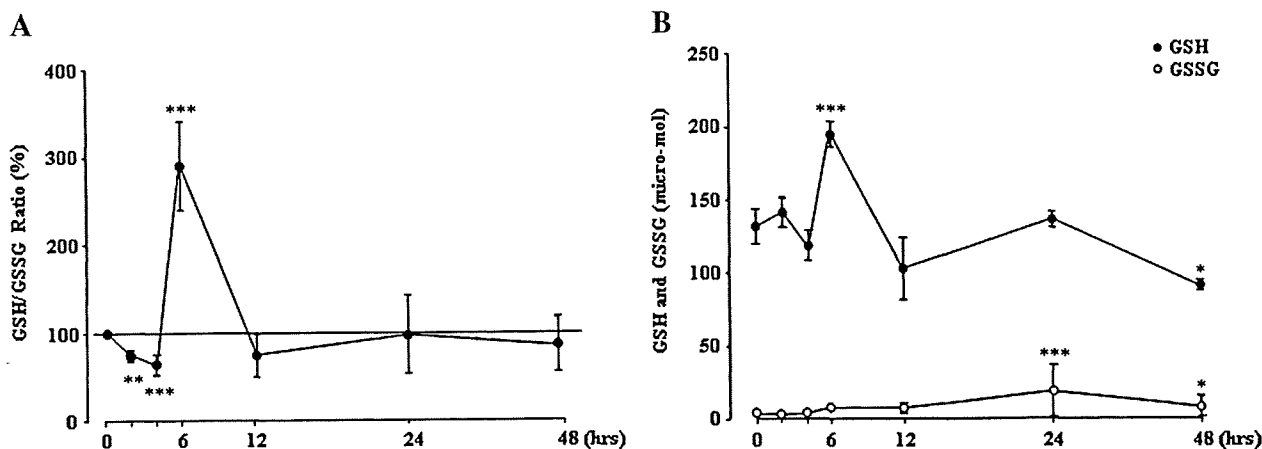


FIG. 2. (A) Change of GSH/GSSG ratio after LTC₄ exposure. Results following LTC₄ (10⁻⁸ M) exposure for 2 h show the intracellular redox state declining to indicate a mildly oxidized state, with the GSH/GSSG ratio showing <100% of control-state values (n = 6). At 4 h after exposure, the ratio gradually 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 LTC₄ exposure. **p < 0.02, ***p < 0.001, compared with 0 h. **(B) Change of GSH and GSSG after LTC₄ exposure.** At 4 h after LTC₄ exposure, the GSH slightly decreased, thereafter the GSH increased significantly to reach a maximum reduction at 6 h after LTC₄ exposure. The GSSG increased at 24 h after LTC₄ 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 LTC₄ was 185.75 ± 23.92 ng/ml. After exposing Calu-3 cells to LTC₄, we measured the changes in production

of IL-8. At 24 h after exposure, IL-8 values (282.49 ± 16.14 ng/ml) were significantly higher than in the control group (p < 0.001, Fig. 5). We also investigated the effects of the LTC₄-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 LTC₄-induced upregulation of epithelial cell-derived IL-8 production (Fig. 5). Also, pretreatment with 10⁻⁶ or 10⁻⁷ M montelukast

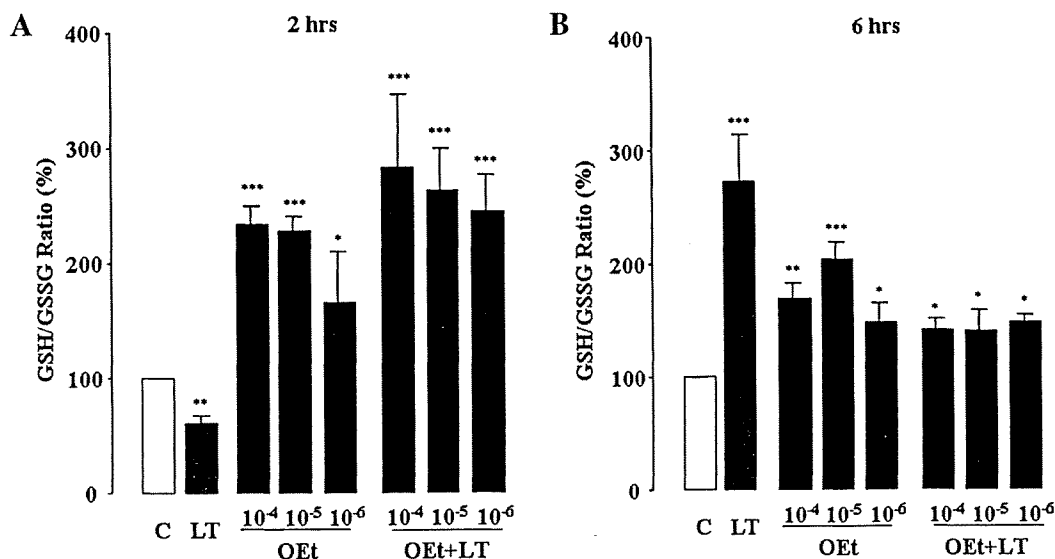


FIG. 3. (A) Effect of antioxidant on LTC₄-induced redox change at 2 h. GSH-OEt increased GSH/GSSG ratio after LTC₄ exposure in Calu-3 cells at 2 h (n = 6). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC₄ was 2 h. *p < 0.05, ***p < 0.001, compared with control. **(B) Effect of antioxidant on LTC₄-induced redox change at 6 h.** GSH-OEt suppressed after LTC₄-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 LTC₄ was 2 h. *p < 0.05, **p < 0.01, ***p < 0.001, compared with control.

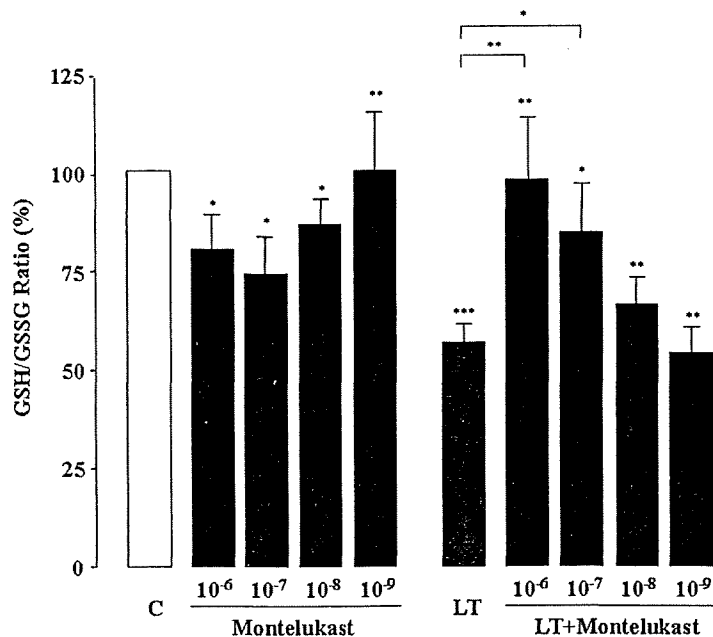


FIG. 4. Effect of LT antagonist on LTC₄-induced redox change. Montelukast had a protective effect on GSH/GSSG ratio after LTC₄ exposure in Calu-3 cells ($n = 6$). Pretreatment of montelukast was for 2 h, and incubation time of LTC₄ was 2 h. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.001$, compared with control.

significantly inhibited LTC₄-induced upregulation of epithelial cell-derived IL-8 production (Fig. 6).

Effect of Cys-LTs exposure upon NF- κ B p65 binding activity

Compared with the control sample, the concentration of NF- κ B p65 showed a significant increase after LTC₄ exposure ($p < 0.001$, Fig. 7). Pretreated with 10⁻⁴ or 10⁻⁵ M, GSH-OEt suppressed LTC₄-induced activation of NF- κ B p65 in Calu-3 cells, and the

inhibitory effect was dose dependent. This was also the case for montelukast at concentrations of 10⁻⁶ and 10⁻⁷ 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 LTC₄-induced oxidative stress. At 4 h after Calu-3 cells were exposed to 10⁻⁸ M LTC₄, 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,

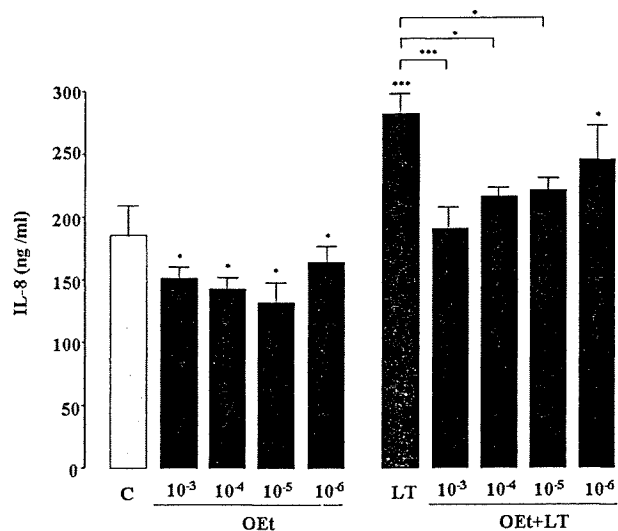
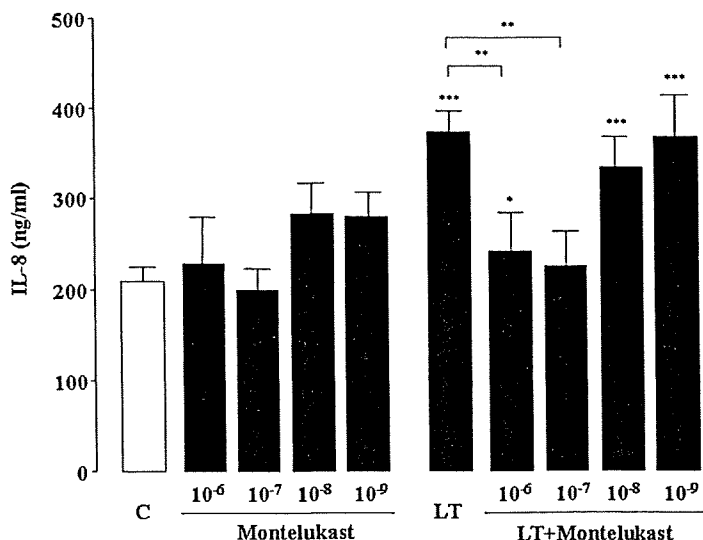


FIG. 5. Effect of LTC₄ and antioxidant on IL-8 production in Calu-3 cells. GSH-OEt suppressed LTC₄-induced IL-8 production in Calu-3 cells ($n = 6$). Pretreatment of GSH-OEt was for 4 h, and incubation time of LTC₄ was 2 h. * $p < 0.05$, *** $p < 0.001$, compared with control.

FIG. 6. Effect of LTC₄ and montelukast on IL-8 production in Calu-3 cells. Montelukast had a significant effect on LTC₄-induced IL-8 production in Calu-3 cells ($n = 6$). Pretreatment of monterukast was for 2 h, and incubation time of LTC₄ was 2 h. * $p < 0.05$, ** $p < 0.02$; *** $p < 0.001$, compared with control.



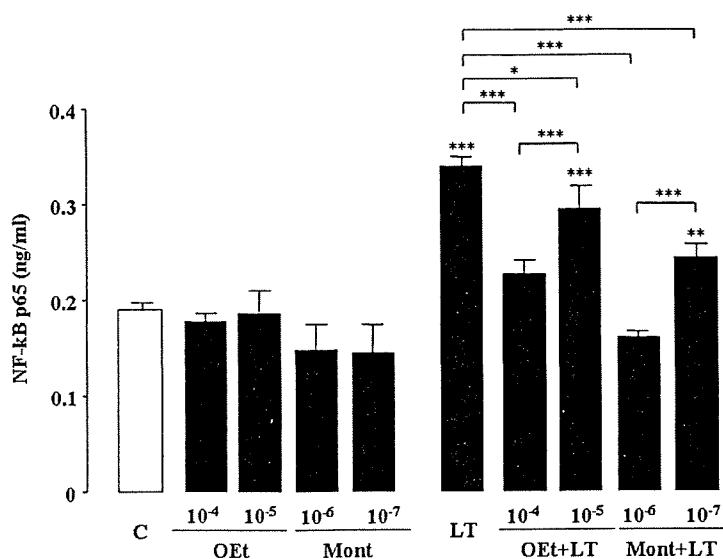
and it proved that Cys-LTs can induce oxidative stress in cultured human airway epithelial cells. The decreased intracellular GSG/GSSG ratio in Calu-3 cells may be induced by the increased ROS formation after exposure to LTC₄. Electron spin resonance is the only method that can be used to measure free 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 LTC₄ 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 LTC₄ induced transforming growth factor β_1 (TGF- β_1) in the airway epithelium through a P38 mitogen-activated protein (MAP) kinase activation mechanism (21). In our experiment, LTC₄ 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 LTC₄ on NF- κ B p65 binding activity in Calu-3 cells. LTC₄ had an effect on NF- κ B 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 LTC₄ 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 LTC₄ 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- κ B, and to promote phosphorylation of MAP kinase (19, 22). We also confirmed that oxidative stress influenced the NF- κ B pathway. Like most inflammatory mediators, IL-8 expression can be induced by activation of specific transcription factors such as NF- κ B, which then bind to DNA sites located in the promoter region of the relevant gene (17). NF- κ B has been shown to be redox state responsive (19), with binding of NF- κ 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-LT₁ and Cys-LT₂. The Cys-LT₁ receptor mRNA is found in the spleen, lung tissue, and smooth muscle cells (1), and Cys-LT₂ receptor mRNA is confirmed in human and rat airway epithelial cells (25). It remains unclear which of these is involved in the mechanism of LTC₄-induced upregulation of IL-8 production. Also, we cannot ascertain whether LTC₄ is able to directly induce NF- κ B activation via the Cys-LTs receptor in human airway epithelial cells. A previous study reported that LTC₄ did not directly induce NF- κ B activation in U-937 cells (human monocytic leukemia cell line) (15). Further investigations are necessary to clarify the relationship between the LTC₄-induced intracellular redox state and IL-8 related signal transfer in airway epithelial cells.

Our results also suggested that pretreatment with montelukast, a Cys-LT₁ receptor antagonist, suppressed LTC₄-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- α -induced NF- κ B activation in THP-1 cells (human monocytic leukemia cell line) (24). Pranlukast, another Cys-LT₁ receptor antagonist, has been reported to inhibit TNF- α -induced NF- κ B activation in U-937 cells, which have Cys-LT₁ receptors on their membranes, and T cells (Jurkat) which do not have Cys-LT₁ receptors on their membranes (15). The underlying mechanism of the inhibitory effect on NF- κ B activation is unclear. Taking a previous study into consideration (15, 24), antagonism of Cys-LT₁ receptor may be partially related to the inhibition of NF- κ B activation, and it is likely that Cys-LT₁ receptor antagonists have another antagonism that inhibits NF- κ B activation. Cys-LT₁ 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 LTC₄-induced time-dependent changes in the intracellular redox state and LTC₄-induced upregulation of epithelial cell-derived IL-8 production by suppressing NF- κ B activation, which in turn confirmed that the LTC₄-induced upregulation of IL-8 production was mainly a consequence of

oxidative stress. This finding suggests that pretreatment with antioxidative agents could protect against LTC₄-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, LTC₄ 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.

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ABBREVIATIONS

GSH, reduced glutathione; GSH-OEt, glutathione monochrome ester; GSSG, oxidized GSH; redox, reduction-oxidation; NF- κ B, nuclear factor- κ 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% CO₂ 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 10⁵ cells/cm². They then were grown to confluence, which typically required 10–12 days (23).

LTC₄ stimulation

In experiments involving Cys-LTs stimulation, confluent Calu-3 cells were serum-starved for 24 h before the addition of LTC₄. Cells were stimulated with LTC₄ 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 LTC₄.

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 LTC₄ (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 LTC₄. 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 μ 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 μ l of 0.1 N HCl, and protein was precipitated with 15 μ 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 β -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 μ l of 2-vinylpyridine per 100 μ 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 LTC₄ or PBS exposure. The assays could detect IL-8 concentrations exceeding 3 pg/ml.

Nuclear protein extraction and quantitation of NF- κ 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 LTC₄ or PBS, respectively. At 6 h following the LTC₄ 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 MgCl₂, 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 MgCl₂, 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- κ B p65 expression was measured using the TransA NF- κ B p65 kit (Active Motif).

Reagents

GSH-OEt, LTC₄, IL-4, and TNF- α were purchased from Sigma Chemical. (St. Louis, MO). montelukast sodium was purchased from Merck Co. (Whitehouse Station, NJ). Nicotinamide adenine dinucleotide phosphate (β -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)₃) 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 NaHCO₃. 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 H₂SO₄, 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°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 cytopsin 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 H_2O . The trachea was clamped until fixation was completed. Tissue specimens were sectioned in the midsagittal plane to a thickness of 6 μm , 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-

dicade 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- α , IFN- γ 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-