(A-to-I) conversions, are predominantly mediated by base deaminations [10]. Here we show that the RNA editing of the IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion on exon 13 is associated with the reduction in the extent of IL-12 signalling, leading to insufficient IFN-γ production and atopy. This is the first report indicating that atopy is associated with RNA editing.

Methods

Atopic and control subjects

One hundred and two atopic patients (9.3 ± 8.2 years old) with major allergic diseases such as bronchial asthma and/or atopic dermatitis having elevated levels of serum IgE and/or specific IgE antibodies, were studied. The diagnosis of bronchial asthma was made according to the criteria of the American Thoracic Society, and that of atopic dermatitis was made according to the criteria of Hanifin. The levels of specific IgE antibodies against house dust, mite, hen's egg, and cow's milk were measured by fluoroenzyme immunoassay. Scores of 3+ to 6+ were considered positive. None of the patients had been receiving systemic steroids. One hundred and four healthy controls (11.5 ± 13.7 years old) had no history of atopic diseases and their serum IgE levels were within normal limits for their age. The ethics committee of Gifu University School of Medicine approved the research project, and informed consent was obtained from all the subjects or their parents.

Cell preparation and culture

PBMCs were isolated from the heparinized blood of the controls and atopic subjects by Ficoll-Paque (Pharmacia, Sweden) gradient centrifugation. The cells were suspended to give a density of 10^6 cells/mL in the culture medium which consisted of RPMI1640 supplemented with 10% heat-inactivated fetal calf serum. PBMCs were cultured at 2 mL per tube in culture test tubes in the presence or absence of 5 IU/mL IL-12 (R&D systems, Germany), 400 ng/mL IL-18, or 10 μg/mL PHA for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 [9].

Assays for cytokines

The culture supernatants incubated for 24 h in test tubes were spun to remove the cells after the cultures. The IFN-γ concentrations of the supernatants were measured with a human enzyme-linked immunosorbent assay (ELISA) kit (Ohtsuka, Japan). The detection limit was 20 pg/mL [9].

Sequence analysis of cDNA and genomic DNA of IL-12R β2 chain

Total cellular RNA was extracted from PBMCs cultured with PHA for 24 h using an Isogen kit (Nippon Gene, Japan). Fragments of IL-12R β2 cDNA were amplified by reverse transcription-polymerase chain reaction (RT-PCR), ligated to a T-vector (Novagen) and sequenced using an autosequencer [5]. The conditions for RT-PCR were 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, for 40 cycles. For amplification of exon 13 of IL-12R β2 cDNA, the sense primer 5′-GATGACAGCTCTGACAGCTG-3′ and the anti-sense primer 5′-GGCCTGATGACCTTGGAIT-3′ were used. Genomic DNA was extracted from leukocytes. Exon 13 and the flanking region of IL-12R β2 genomic DNA were amplified by PCR with the sense primer 5′-GATGA CAGCTCTGACAGCTG-3′ and the anti-sense primer 5′-CATTGTCTCCAGGAAGATAG-3′ [11]. The conditions used for PCR were 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, for 30 cycles.

Expression constructs encoding IL-12R β2 and transfected Ba/F3 cell clones

The expression constructs encoding human wild-type IL-12R β2 or variant-type (2451 C-to-U conversion) IL-12R β2 were prepared in the PEF-BOS expression vector, as described elsewhere [5]. Ba/F3 cells were transfected by electroporation with the expression constructs encoding either the wild-type IL-12R β2 or the variant-type IL-12R β2. Then, the transfected Ba/F3 cells were cloned.

Flow cytofluorometric analysis

IL-12R β2 expressing Ba/F3 cell clones were detected by indirect immunofluoresence analysis using flow cytometry. Briefly, 10^6 cells in 100 μL of staining buffer were incubated with 1 μg/mL rat anti-hu IL-12R β2(2B6) mAb or isotype control Ab for 30 min, followed by incubation with biotinylated anti-rat-Ig $F(ab)^2$ fragments for 30 min, and finally incubated with streptavidin conjugated to PE (PharMingen) for 30 min. All incubations were performed at 4 °C in a staining buffer, and the cells were washed twice between incubations. The stained cells were analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Proliferative responses

The Ba/F3 cell clones were cultured with IL-12 (0.5, 5 or 50 IU/mL) for 24 h. DNA synthesis was measured by adding 0.5 μCi [^{3}H] thymidine per well 4 h before harvesting onto glass-fiber filters. [^{3}H] thymidine incorporation (c.p.m.) was measured by liquid scintillation counting, and the results were expressed as the means of triplicate.

Immunoprecipitation assay for phosphorylated Stat4

PBMCs from the patients and controls were stimulated with PHA and IL-12 (5 IU/mL) or the control culture medium for 15 min. The cells were lysed in 1% Triton X-100, 150 mM NaCl, 20 mM Na_2PO_4, 1% aprotinin, 5 mM PMSF, 100 mM NaF and 2 mM Na_3VO_4, and were immunoprecipitated with rabbit antisera for Stat4 (Santa Cruz Biotechnology). Precipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to a nylon membrane, the blots were probed with antibody for phosphotyrosine. Equal loading of Stat4 was confirmed by stripping the same membranes and reprobing them with the antiserum for Stat4.

Suppression of in vitro *IgE production*

PBMCs (10^6 cells/mL) from the atopic patients with or without the 2451 C-to-U conversion were cultured at 2 mL per tube in culture test tubes with Derf 1 (5 μg/mL, Asahi, Tokyo, Japan) and IL-4 (500 U/mL, Genzyme/Techne, USA) for 14 days at 37 °C in a humidified atmosphere containing 5% CO_2. For suppression of IgE production, IFN-γ (1000 U/mL, Genzyme/Techne, USA) or IL-12 (5 IU/mL) was added to the culture. The IgE concentrations of the culture supernatants were measured by ELISA.

Statistics

The significance of difference between groups was analysed by the Mann–Whitney's *U* test or χ^2-test.

Results

RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion associated with atopy

In this study, we found that IL-12R β2 mRNA editing modifies cytidine in an alanine codon (GCU) at nucleotide 2451 in the extracellular domain to a uridine (GUU), converting to a valine codon (Ala 604 Val) in atopic patients. Fragments of IL-12R β2 cDNA were amplified by RT-PCR, ligated to a T-vector and sequenced using an autosequencer. Interestingly, sequence analysis of the cDNA clones representing IL-12R β2 mRNA transcripts revealed the C-to-U conversion at nucleotide 2451 (Ala 604 Val) on exon 13 in some of the atopic patients (Fig. 1). Very recently, van Rietschoten et al. [11] reported the genomic organization of the human IL-12R β2 chain gene. Therefore, we determined the sequence of the genomic DNA of the IL-12R β2 chain. Surprisingly, sequence analysis of the genomic DNA of the IL-12R β2 chain from the atopic patients showed neither 2451 C-to-T (Ala 604 Val) mutation on exon 13 nor mutation in the flanking region of exon 13 (Fig. 1). Therefore, it was suggested that the C-to-U mismatch observed upon comparison of IL-12R β2 genomic DNA with cDNA clones had arisen at the RNA level. RNA editing is formally defined as any RNA-processing event (excluding RNA splicing) that generates an RNA transcript with a primary nucleotide sequence different from that of its gene. Therefore, we concluded that the observed C-to-U mismatch in the cDNA clone of the IL-12R β2 chain is due to the RNA editing of this transcript.

To determine whether the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA is associated with atopy, we conducted a genetic association study on atopy. The C-to-U conversion was observed in 21 (20.6%) of the 102 atopic patients, whereas this conversion was observed in only 4 (3.8%) of the 104 non-atopic subjects. There was a significant ($P<0.001$, by χ^2-test) difference in the C-to-U conversion frequency between the non-atopic subjects and the atopic subjects (Table 1). The subjects exhibited neither this conversion nor any mutations in the flanking region of exon 13 in the genomic DNA of the IL-12R β2 chain.

IFN-γ production by PBMCs stimulated with IL-12, IL-18 or PHA

To determine whether the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA affects the IL-12 signal

Table 1. An association study of the C-to-U conversion at nucleotidc 2451 (Ala 604 Val) in IL-12R β2 chain cDNA responsible for atopy

	C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA			
	n	–	+	*P*-value
Non-atopic subjects	104	100	4 (3.8%)	<0.001
Atopic patients	102	81	21 (20.6%)	

P-value was calculated by χ^2-test.
Sequence analysis of genomic DNA of the IL-12R β2 chain showed no 2451 C-to-T (Ala 604 Val) mutation in any of the subjects tested.

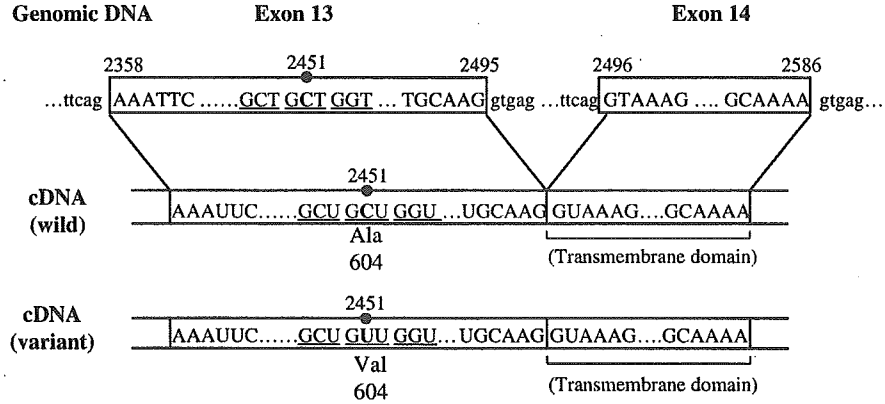

Fig. 1. Sequence analysis of genomic DNA and the cDNA of IL-12R β2 exons 13 and 14 and their flanking regions in non-atopic healthy controls and atopic patients. Sequence analysis of genomic DNA of the IL-12R β2 chain from any of the non-atopic healthy controls and any of the atopic patients showed no 2451 C-to-T (Ala 604 Val) mutation in exon 13. Sequence analysis of cDNA (variant) clones representing IL-12R β2 mRNA transcripts indicates the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in some of the atopic patients. Therefore, it is indicated that the observed C-to-U mismatch in the cDNA clone of 1L-12R β2 chain is due to RNA editing of this transcript. The number above each sequence indicates the number of the nucleotide according to the Genbank database U 64198, and the number under each sequence indicates the number of amino acid.

Table 2. IFN-γ production by PBMCs stimulated with IL-12, IL-18 or PHA in the subjects (atopic patients and non-atopic subjects) with or without the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in IL-12R β2 chain cDNA

	IFN-γ concentration (pg/mL)*		
	Without C-to-U conversion† ($n = 169$)	With C-to-U conversion† ($n = 25$)	*P*-value‡
Stimulated			
with IL-12	154.7 (34.9 ~ 685.9)	69.3 (16.0 ~ 266.5)	0.013
with IL-18	68.6 (11.8 ~ 398.7)	57.3 (9.0 ~ 364.5)	0.962
with PHA	1568.2 (582.1 ~ 4224.5)	1578.1 (711.8 ~ 3498.6)	0.970

*Genometric means are shown, and the ranges of SD are shown in parentheses. †at nucleotide 2451 (Ala 604 Val) in IL-12R β2 chain cDNA. ‡*P*-values were calculated by Mann–Whitney's *U* test.

cascade, we, next, conducted an association study on IFN-γ production by PBMCs following stimulation with IL-12, IL-18 or PHA. After PBMCs were cultured with IL-12, IL-18 or PHA for 24 h, the IFN-γ concentration in the culture supernatants was measured (Table 2). The results revealed that IFN-γ production by PBMCs stimulated with IL-12 in the subjects with the C-to-U conversion was significantly ($P<0.013$) lower than that in the subjects without the C-to-U conversion. In contrast, there was no significant difference in IFN-γ production by PBMCs stimulated with IL-18 or PHA between the subjects with the C-to-U conversion and those without the conversion.

Expression of IL-12R β2 chain

To investigate the expression of the wild-type or variant-type IL-12R β2 chain, Ba/F3 cells were transfected by electroporation with wild-type IL-12R β2 cDNA or variant-type IL-12R β2 cDNA containing the C-to-U conversion at nucleotide 2451 in the PEF-BOS expression vector and then cloned [5]. The IL-12R β2-chain-expressing cells were detected by flow cytometry using the anti-IL-12R β2 antibody. The results revealed that the staining intensity and the percentage of cells expressing the IL-12R β2 chain in the clone with the C-to-U conversion were lower than those in the clone without the conversion (Fig. 2a). Furthermore, the degree of proliferative responses of the cells was measured. As a result, the proliferative response of the Ba/F3 cell clones transfected with variant-type IL-12R β2 cDNA containing the C-to-U conversion at nucleotide 2451 to IL-12 (0.5, 5 IU/mL) was lower than that of the Ba/F3 cells transfected with wild-type IL-12R β2 chain cDNA (Fig. 2b).

Tyrosine phosphorylation of Stat4

Furthermore, to investigate the functional aspects of the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA, we examined the tyrosine phosphorylation of Stat4. Although PBMCs from the patient without the C-to-U conversion (patient 2) and the control (control 1) cultured with IL-12 and PHA showed the tyrosine phosphorylation of Stat4, PBMCs from the patient with the C-to-U conversion (patient 1) cultured with IL-12 and PHA faintly showed the tyrosine phosphorylation of Stat4 (Fig. 3 a). These results suggest that the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA is associated with reduced signal transduction of IL-12 for IFN- γ production by PBMCs.

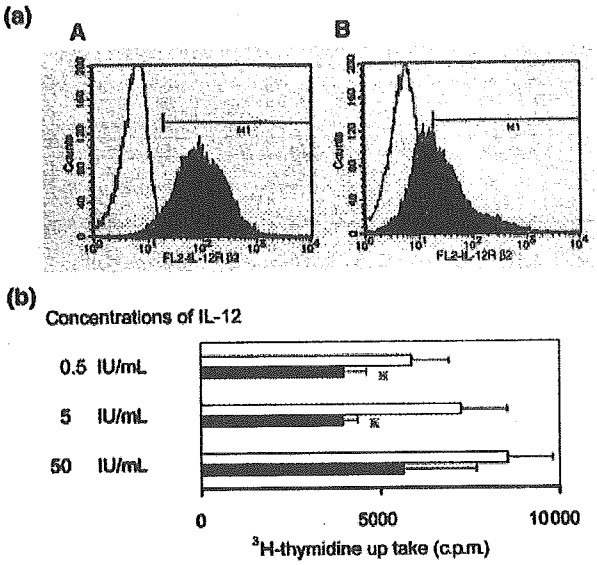

Fig. 2. (a) Expression of the IL-12R β2 chain without or with the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in Ba/F3 cell clones transfected with wild-type (A) or variant-type (B) IL-12R β2 cDNA. 10^6 cells in 100 μL of staining buffer were incubated with 1 μg/mL rat anti-hu IL-12R β2(2B6) mAb (black) or isotype control Ab (white) for 30 min, followed by incubation with biotinylated anti-rat-Ig $F(ab)^2$ fragments for 30 min, and finally incubated with streptavidin conjugated to PE (PharMingen) for 30 min. IL-12R β2 expressing cells were detected by flow cytometry. The staining intensity and the percentage (50.1%) of cells expressing the IL-12R β2 chain in the clone with the C-to-U conversion were lower than those (the percentage: 94.4%) in the clone without the conversion. (b) Proliferative responses of the Ba/F3 cell clones transfected with wild-type or variant-type IL-12R β2 cDNA containing the C-to-U conversion at nucleotide 2451. Ba/F3 were stimulated with IL-12 (0.5, 5 or 50 IU/mL) for 24 h. The proliferative responses of the Ba/F3 cell clones transfected with variant-type IL-12R β2 cDNA (closed column) to IL-12 (0.5 IU/mL, 5 IU/mL) were significantly (*$P<0.05$ for each) lower than those of the Ba/F3 cell clones transfected with wild-type IL-12R β2 chain cDNA (open column). Means+SD (c.p.m.) of triplicate are shown.

In vitro *IgE production suppressed by IL-12*

Next, we examined the effects of IL-12 on *in vitro* IgE production by PBMCs from the atopic patients with the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA (Fig. 3 b). IgE production by PBMCs cultured with IL-4 and Derf 1 for 14 days was suppressed by IL-12 as well as by IFN-γ in the atopic patients without the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA. In contrast, in the

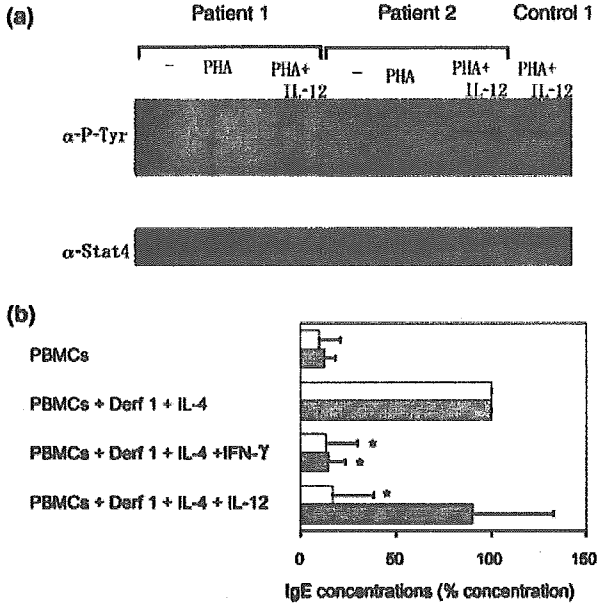

Fig. 3. (a) Tyrosine phosphorylation of Stat4 in PBMCs. PBMCs from the patients with or without the C-to-U conversion at nucleotide 2451 (Ala 604 Val) on the IL-12R β2 chain and the control subject were cultured with PHA and IL-12. Cell lysates were immunoprecipitated with anti-Stat4, resolved by SDS-PAGE, transferred to a nylon membrane and blotted sequentially with anti-phosphotyrosine (α-P-Tyr, upper panel) or anti-Stat4 (a Stat4, lower panel). No band was shown on blotting with α-P-Tyr in PBMCs from the patients cultured with or without PHA. Although PBMCs from the patient without the C-to-U conversion (patient 2) and the control (control 1) cultured with both IL-12 and PHA showed a band on blotting with α-P-Tyr, indicating phosphorylation of Stat4, PBMCs from the patient with the C-to-U conversion (patient 1) cultured with both IL-12 and PHA showed a very faint band. (b) Suppression of *in vitro* IgE production. IgE production by PBMCs was induced by Derf 1 and IL-4. Suppression by IFN-γ (1000 U/mL) and IL-12 (5 IU/mL) was represented by % concentration. % concentration = 100 X IgE concentration in the culture with IFN-γ or IL-12/IgE concentration in the culture without both IFN-γ and IL-12. In the atopic patients (open column, mean +SD, $n = 3$) without the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA, IgE production was significantly ($^*P < 0.05$ for each) suppressed by IL-12 as well as by IFN-γ. In contrast, in the atopic patients (shadow column, mean+SD, $n = 3$) with the C-to-U conversion at nucleotide 2451 in IL-12R β2 chain cDNA, IgE production was not suppressed by IL-12, whereas it was significantly ($^*P < 0.05$) suppressed by IFN-γ.

atopic patients with the C-to-U conversion, IgE production by PBMCs was not suppressed by IL-12 whereas it was suppressed by IFN-γ.

Discussion

Our results showed that RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion in atopic subjects caused impairment of the IL-12 signal cascade, and then reduced IFN-γ production by PBMCs following IL-12 stimulation, resulting in impaired down-regulation of IgE production.

Recently, it has been reported that a homozygous nonsense mutation of the IL-12R β1 chain gene causes impairment of salmonella and mycobacterial immunity [12, 13]. The development of Th1 lymphocytes is disturbed in IL-12 or IL-12R β1 knockout mice [8, 14]. In this study, atopic subjects with RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion, did not exhibit any impairment of salmonella and mycobacterial immunity. The IL-12R β2 subunit, similar to the IL-12R β1 subunit, is a member of the gp130-type subgroup of the cytokine receptor superfamily. However, each of the two IL-12R subunits itself is more closely related to gp130 than to each other. In contrast to IL-12R β1, which does not contain any tyrosine residues, the cytoplasmic region of IL-12R β2 contains three tyrosine residues, suggesting an important role of the β2 subunit in IL-12 signal transduction [5]. Presky et al. [5] reported that Ba/F3 cells transfected with IL-12R β2 alone proliferates in response to human IL-12 although the role of endogenous mouse IL-12R β1 in IL-12 signal transduction in these transfectants cannot be ruled out. Thus, IL-12R β2 is different from IL-12 β1 in both structure and function.

It has been noted that RNA editing, a post-transcriptional modification, plays an important role in achieving molecular diversity [10]. The forms of RNA editing are classified into two categories, namely, C-to-U and A-to-I conversions that occur by nucleotide deamination. The best example of C-to-U editing occurs within RNA transcripts encoding apolipoprotein B RNA [15, 16] and is mediated by the activity of cytidine deaminase. Recently, A-to-I conversions have been observed within a growing number of RNAs, including those encoding several glutamate receptor subunits [17–19] and the G-protein-coupled serotonin 2C receptor [20]. Moreover, RNA editing of WT1, that is thought to be a susceptibility gene for Wilm's tumour, converts U-to-C at nucleotide 839, transforming genomically encoded leucine into proline [21]. The leucine, non-edited form of the protein, is a more potent transcriptional repressor than the proline-containing isoform, suggesting that this editing might be associated with the development of Wilm's tumour. The neurofibromatosis type-1 gene product neurofibromin, associated with an increased risk of neurofibromatosis type 1 (NF1), is thought to serve as a tumour suppressor [10]. Although editing of C 2914 of this gene occurs at low levels (<2%) in control subjects, patients with NF1 show almost eight times the level of editing at this position. The editing at this site converts a CGA (Arg) codon into a UGA (stop) codon, suggesting that NF1 patients lack sufficient quantities of neurofibromin [22]. Furthermore, it is suggested that a reduction in the amount of this potential tumour suppressor may prevent appropriate regulation of the Ras signalling pathway, leading to unchecked cellular proliferation and cancer [22].

The expression levels of IL-12R β2 and the proliferative responses to IL-12 in variant-type IL-12R β2 transfected Ba/F3 were lower than those of wild-type. The RNA editing of IL-12R β2, 2451 C-to-U (Ala 604 Val) conversion, associated with atopy in this study may disturb conformational binding of IL-12 to IL-12R, although the possibility that the antibody is affected by the conformational change cannot be excluded.

In the immunological system, RNA editing has not been reported. An immunological system as well as a neurological system require molecular diversity. From this viewpoint, it is natural that lympocytes utilize RNA editing for the regulation of the function on the cytokine and cytokine receptor. Activation induced cytidine deaminase (AID), which is the causative gene for the hyper-IgM syndrome, is homologous to that of mammalian RNA editing deaminase, APOBEC1.

AID had deaminase activity when tested for deoxycytidine deamination. Therefore, AID may be another RNA editing

deaminase although its substrate has not yet been identified. AID or other enzymes may be the candidate for RNA editing in an immunological system. It is indicated that the regulatory spacer and mooring sequences (such as UGAUAC, AAUU, UGAUCAGUAUA, respectively in human apolipoprotein B) may provide binding sites for distinct components of the cellular editing machinery: once bound, the factor(s) would be correctly positioned to edit nucleotides within a certain distance upstream from the binding site [10, 23]. Therefore, we investigated these sequences in the IL-12R β2 chain gene. However, we were not able to find any motifs.

Atopic disorders develop by a combination of genetic risk factors and environmental factors. Very recently, Karcher et al. found the temperature sensitivity of RNA editing reaction in the plastid ndhB transcript [24]. Therefore RNA editing, one of post-transcriptional modifications, in atopic patients may be induced by a combination of genetic factors and environmental factors. Experiments along these lines are now under way. Our results indicate that several candidate genes that have failed to show association should be investigated at the mRNA level. Although several polymorphisms or mutations of the genes associated with atopy have been reported [9, 25–27], this study is the first report indicating that atopy is associated with RNA editing, a post-transcriptional modification.

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Original Article

Urinary leukotriene E_4 and 11-dehydro-thromboxane B_2 excretion in children with bronchial asthma

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Abstract

Background: Cysteinyl leukotrienes (CysLTs) and thromboxane (TX) A_2 have been implicated in the pathogenesis of bronchial asthma. Urinary leukotriene E_4 (LTE_4) and 11-dehydro-TXB_2 ($11DTXB_2$) levels are often used to assess the production of CysLTs and TXA_2. However, few studies have examined the products of these two mediators in the same asthmatic patients. To define the potential roles of CysLTs and TXA_2 in the pathogenesis of bronchial asthma in children, their urinary levels were measured in the present study.

Methods: Urinary LTE_4 and $11DTXB_2$ levels were measured by enzyme immunoassay (EIA) and radioimmunoassay (RIA), respectively. Urine samples from asthmatic children were measured during the stable condition and during an acute attack.

Results: Urinary LTE_4 levels during an acute attack (median 476 pg/mg creatinine; range 191–1100 pg/mg creatinine) and during the stable condition (median 332 pg/mg creatinine; range 128–965 pg/mg creatinine) were significantly higher ($P < 0.05$) than those of controls (median 233 pg/mg creatinine; range 103–389 pg/mg creatinine). Urinary $11DTXB_2$ levels during an acute attack and during the stable condition (median 1666 (range 110–5105) and 1009 (range 46–6070) pg/mg creatinine, respectively) were significantly higher ($P < 0.05$) than those of controls (median 252 pg/mg creatinine; range 41–716 pg/mg creatinine). Comparing different stages of asthma, LTE_4 levels during an acute attack were significantly higher ($P < 0.05$) than during the stable condition; however, there was no difference in urinary TXB_2 levels.

Conclusions: The present findings suggest that high levels of CysLTs and TXA_2 are associated with the pathogenesis of bronchial asthma. The measurement of urinary LTE_4 and $11DTXB_2$ would be useful in understanding the individual pathogenesis of asthmatic children.

Key words: bronchial asthma, cysteinyl leukotrienes, 11-dehydro-thromboxane B_2, leukotriene E_4, thromboxane A_2.

Introduction

Cysteinyl leukotrienes (CysLTs), namely leukotrienes C_4, D_4 and E_4, and thromboxane (TX) A_2 are considered to play important roles in bronchial asthma.[1–10] Cysteinyl leukotrienes are derived from arachidonic acid by the action of 5-lipoxygenase and increase vascular permeability, stimulate mucus secretion and induce bronchial hyperreactiveness and bronchoconstriction. Moreover, increased production of CysLTs in asthmatic patients *in vivo* has been observed in several studies.[1–5,11,12] A potent bronchoconstrictor, TXA_2 is generated from arachidonic acid by cyclooxygenase. Enhanced TXA_2 release has also been reported in asthmatic patients after allergen challenge.[7] Owing to the significant roles of CysLTs and TXA_2, their inhibitors or receptor antagonists have been developed extensively and recently some drugs have become available.[13]

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Although these mediators of asthma have been discussed previously, few studies have examined the products of these two mediators in the same asthmatic patients. In addition, few studies have compared the TXA_2 products of asthmatic patients with those of healthy control subjects. Leukotriene E_4 is a stable product of CysLTs and is considered an index of the systemic production of CysLTs production in humans.[14] 11-Dehydro-thromboxane B_2 ($11DTXB_2$) is the most abundant degradation product of TXB_2 and is also considered an index of systemic TXA_2 production.[15–17] The measurement of urinary LTE_4 and $11DTXB_2$ is a non-invasive method for assessing the production of CysLTs and TXA_2 *in vivo*, respectively. Oosaki *et al.*[18] previously established sensitive and selective methods of determination of urinary LTE_4 by enzyme immunoassay (EIA) and Ruitta *et al.*[16] established the method of determining urinary $11DTXB_2$ by radioimmunoassay (RIA). In the present study, using these methods, the products of CysLTs and TXA_2 were assessed *in vivo* in control subjects and in children with bronchial asthma during the stable condition and during an acute attack.

METHODS

Subjects

Twenty-nine children with bronchial asthma (19 males, 10 females) and nine control subjects (six males, three females) were enrolled in the study (Table 1). The mean age of asthmatic children and control subjects was 7 years (range 1–15 years) and 8 years (range 1–15 years), respectively. None of the patients had a history of aspirin sensitivity. Twenty-six of the asthmatic patients were receiving theophylline, all were using inhaled disodium cromoglycate (DSCG) and β_2-adrenergic receptor agonists, 10 were receiving inhaled corticosteroids and 17 were using a leukotriene receptor antagonist (LTRA). All patients were classified into one of four categories (intermittent, mild persistent, moderate persistent and severe persistent) according to Glolabal Initiative for Asthma (GINA) guidelines (http://www/ginasthma.com/ Table 1). None of the patients had been treated previously with oral prednisolone (PSL) prior to their enrollment in the study. In the present study, the urinary excretion of LTE_4 or $11DTXB_2$ was not influenced by the usage of LTRA or corticosteroids, because the patients kept the treatments unmodified throughout the duration of the study. Informed consent to participate in the study was obtained from all subjects or their parents.

Urine samples from children with bronchial asthma were measured during the stable condition and during an acute attack. Urine samples from nine asthmatic children selected at random (Table 1, patients 1–9) were also measured 2 days after treatment. The 'stable condition' refers to the condition in which the patients did not complain of any symptoms with or without receiving their usual medications. The 'acute attack' refers to a condition in which the patients complained of some active symptoms, cough and/or wheezing and/or chest tightness, which were occurring repeatedly on waking and/or disturbing sleep at night; therefore, they needed additional treatment to their usual treatments.[19] Patients were treated with steroid and/or theophylline by injection and/or inhaled β_2-adrenergic receptor agonists and, 2 days after treatment, they felt better but were still complaining slightly of some asthmatic symptoms, such as cough and/or wheezing and/or chest tightness.

Urine samples were collected when the asthmatic children visited our hospitals during the stable condition maintaining their usual treatments. Urine samples from asthmatic children were also collected on arrival at hospital when they had acute asthma attacks. Patients were treated with theophylline and/or β_2-adrenergic receptor agonists and/or corticosteroid drip infusion. Urine samples from nine asthmatic children were collected 2 days after treatment.

Measurement of LTE_4

Urine samples were stored at –80°C and analyzed within 1 month of collection. An aliquot of urine was removed to determine creatinine concentration. The urinary creatinine level was determined using a Creatinine test kit (Pure Auto S CRE-L; Daiichi-kagaku, Tokyo, Japan).

Approximately 3000 d.p.m. [^{3}H]-LTE_4 was added to each urine sample as an internal standard and the urine was applied to a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) that had been preconditioned by the serial addition of methanol and distilled water. Then, the cartridge was washed with distilled water, followed by 40% methanol; LTE_4 was eluted with 80% methanol. This elution was dried with nitrogen gas and was dissolved in the elution buffer used in high-performance liquid chromatography (HPLC; 486 Tunable Absorbance Detector; Waters) and the solution was injected onto a C18 reverse-phase column (CAPPCELL PAC UG 120; Shiseido, Tokyo, Japan). The fractions that contained peak [^{3}H]-LTE_4 radioactivity and also corresponded to the

Table 1 Characteristics of patients and urinary levels of leukotriene E_4 and 11-dehydro-thromboxane B_2

Patient no.	Gender	Age (years)	Serum IgE (U/mL)	HD score	Mite score	Severity (GINA)	Steroid treatment during attack	Theophylline	LTRA	DSCG	β_2-Adrenergic receptor agonist	Steroid inhalant	Stable condition LTE_4	Stable condition $11DTXB_2$	Acute attack LTE_4	Acute attack $11DTXB_2$	After treatment LTE_4	After treatment $11DTXB_2$
1	M	7	1784	5	6	Moderate	+	+	−	+	+	−	183	861	671	1285	156	2130
2	M	5	200	4	5	Mild	+	+	−	+	+	−	212	2166	611	1188	295	820
3	M	14	900	6	6	Moderate	+	−	−	+	+	+	482	745	1100	563	443	785
4	M	1	21	0	0	Severe	+	+	+	+	+	−	373	282	280	3122	524	2708
5	M	9	70	0	0	Moderate	+	+	+	+	+	−	480	1059	546	2096	771	2137
6	F	10	199	2	2	Moderate	+	+	+	+	+	−	253	1515	437	2247	231	842
7	F	5	82	6	6	Mild	+	+	−	+	+	−	188	2150	409	589	340	492
8	F	9	294	4	4	Severe	+	+	−	+	+	+	484	131	797	166	473	533
9	F	7	1281	4	4	Moderate	+	+	+	+	+	−	556	1009	483	2087	872	1515
10	M	10	653	2	3	Moderate	+	+	+	+	+	+	378	904	615	1847		
11	M	4	208	4	5	Moderate	+	+	+	+	+	−	341	1842	468	3254		
12	M	5	2892	4	4	Moderate	+	+	+	+	+	+	274	163	553	2461		
13	M	3	401	5	5	Moderate	+	+	+	+	+	−	519	2462	567	2352		
14	M	9	265	5	5	Mild	+	+	+	+	+	−	254	1476	369	2499		
15	M	3	180	5	6	Moderate	+	+	+	+	+	+	332	1392	424	480		
16	M	3	1381	3	4	Moderate	+	+	−	+	+	+	606	167	191	379		
17	M	9	890	2	1	Mild	+	+	+	+	+	−	165	873	269	1259		
18	M	9	703	6	5	Severe	+	+	+	+	+	+	278	804	334	939		
19	M	2	1417	6	6	Moderate	+	+	+	+	+	−	755	3535	743	4075		
20	M	9	578	5	6	Mild	+	+	+	+	+	−	286	793	284	1700		
21	M	8	62	2	0	Intermittent	+	−	−	+	+	−	365	1992	371	258		
22	M	4	334	3	3	Moderate	+	+	+	+	+	+	328	3483	394	2548		
23	M	2	394	5	6	Mild	+	+	+	+	+	−	610	6070	980	5104		
24	F	15	301	0	0	Moderate	+	+	−	+	+	+	284	46	1100	311		
25	F	5	866	6	6	Intermittent	−	−	−	+	+	−	727	196	245	110		
26	F	7	1300	5	6	Mild	+	+	−	+	+	−	259	1837	476	2413		
27	F	7	1000	2	1	Mild	−	+	−	+	+	−	128	903	289	1318		
28	F	13	597	5	5	Mild	−	+	−	+	+	−	332	1026	504	1666		
29	F	3	600	6	6	Moderate	+	+	+	+	+	+	965	261	528	429		
Median			577.5										332	1009	476	1666	443	842
Average		7	684.5	4	4								393.0	1384.2	518.6	1680.9	456.1	1329.2

GINA, Global Initiative for Asthma (GINA) guideline (http://www.ginasthma.com/).

HD, house dust; LTRA, leukotriene receptor antagonist; DSCG, disodium cromoglycate; LTE_4, leukotriene E_4; $11DTBX_2$, 11-dehydro-thromboxane B_2.

retention time of authentic LTE_4 were dried and resuspended in assay buffer, which was supplied in the Leukotriene C4/D4/E4 enzyme-immunoassay system (Amersham, Buckinghamshire, UK). Urinary LTE_4 concentrations determined by EIA were corrected for recovery of [^{3}H]-LTE_4. The urinary LTE_4 level was expressed as pg/mg creatinine.

Measurement of 11DTXB$_2$

The 11DTXB$_2$ was extracted from an acidified sample by adding an equal volume of octadecylsilyl silica powder (ODS) suspension (80 mg/mL in 40% ethanol) followed by mixing, centrifuging (at 2000 g for 3 min at room temperature) and either decanting or aspirating. The pellet was washed with an acidic alcohol solution and then with petroleum ether for deproteinizing and defatting. The 11DTXB$_2$ was eluted by ethyl acetate. The pooled ethyl acetate was evaporated to dryness with nitrogen gas. The dried residue, containing 11DTXB$_2$, was dissolved in the eluent (acetonitril : chloroform : acetic acid, 10 : 90 : 0.5, v/v/v) and applied to the open silica mini column (Bond Elute SI; VARIAN, Palo Alto, CA, USA). The column was washed with the eluent (acetonitril : chloroform : acetic acid, 20 : 80 : 0.5, v/v/v). The elution buffer, containing the 11DTXB$_2$, was dried with nitrogen gas and the amount of 11DTXB$_2$ was quantitated by RIA (11-Dehydrothromboxane B2 [^{125}I] RIA kit; Perkin Elmer Life and Analytical Sciences, Boston, MA, USA). The urinary 11DTXB$_2$ level was also expressed as pg/mg creatinine.

Statistical analyses

The Mann–Whitney unpaired *U*-test was used to compare controls and asthmatic children during the stable condition and the Wilcoxon paired test was used to compare asthmatic children during the stable condition and during an acute attack. Correlation was analyzed by Pearson correlation analysis. The percentage of changes was calculated using the following equation: % change = (level during stable condition – level during attack) × 100/level during attack. Data are expressed as the median (range) and $P < 0.05$ was considered significant.

RESULTS

Urinary LTE_4 and 11DTXB$_2$ levels

Urinary levels of LTE_4 and 11DTXB$_2$ were measured to define the potential roles of CysLTs and TXA_2 in children with bronchial asthma.

Leukotriene E_4 was measured by EIA. Urinary LTE_4 levels are plotted in Fig. 1. Urinary LTE_4 levels in asthmatic children during the stable condition (332 (128–965) pg/mg creatinine) was significantly higher ($P < 0.05$) than that of control subjects (233 (103–389) pg/mg creatinine; Fig. 1a). Comparing the different conditions of asthma, LTE_4 levels during an acute attack (476 (191–1100) pg/mg creatinine) were significantly higher ($P < 0.05$) than those during the stable condition (Fig. 1b).

11-Dehydro-thromboxane $B_2$was measured by RIA and was detectable in all urine samples. Urinary 11DTXB$_2$ levels are shown in Fig. 2. Urinary 11DTXB$_2$ levels in asthmatic children during the stable condition (1009 (46–6070) pg/mg creatinine) were significantly higher ($P < 0.05$) than those of control subjects (252 (41–716) pg/mg creatinine; Fig. 2a). However, there was no significant difference in 11DTXB$_2$ levels during an acute attack (1666 (110–5105) pg/mg creatinine) and during the stable condition (Fig. 2b).

Urinary levels of LTE_4 and 11DTXB$_2$ were observed during the stable condition, an acute attack and 2 days after treatment in nine asthmatic children (Fig. 3). Urinary LTE_4 increased from 373 pg/mg creatinine (range 183–556 pg/mg creatinine) during the stable condition to 546 pg/mg creatinine (range 280–1100 pg/mg creatinine) during an acute asthma attack and then decreased to 443 pg/mg creatinine (range 156–872 pg/mg creatinine) 2 days after treatment (Fig. 3a). In contrast, urinary 11DTXB$_2$ levels exhibited different patterns after an attack. Urinary 11DTXB$_2$ levels increased from 1009 pg/mg creatinine (range 131–2106 pg/mg creatinine) during the stable condition to 1285 pg/mg creatinine (range 166–3122 pg/mg creatinine) during an acute asthma attack and gradually decreased to 842 pg/mg creatinine (range 492–2708 pg/mg creatinine) 2 days after treatment. However, each patient showed variable levels of urinary 11DTXB$_2$ 2 days after treatment (Fig. 3b).

Correlations between urinary LTE_4 and 11DTXB$_2$

We assessed the relationship between LTE_4 and 11DTXB$_2$ in children with bronchial asthma (Fig. 4). No relationship was noted between these prostanoids in children with bronchial asthma or in the controls. In plots of changes from levels observed during an attack to

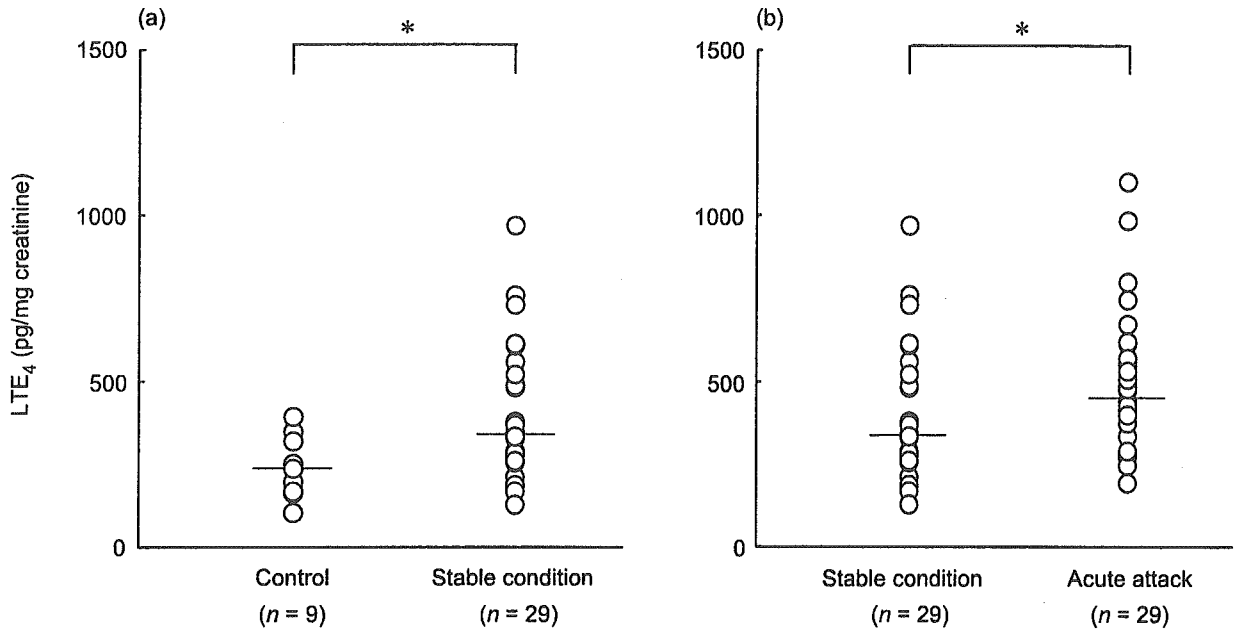

Fig. 1 (a) Urinary leukotriene E_4 (LTE_4) levels in asthmatic children during the stable condition (median 332 pg/mg creatinine; range 128–965 pg/mg creatinine) and in controls (median 233 pg/mg creatinine; range 103–389 pg/mg creatinine). (b) Urinary LTE_4 levels in asthmatic children during an acute asthma attack (median 476 pg/mg creatinine; range 191–1100 pg/mg creatinine) and during the stable condition (median 332 pg/mg creatinine; range 128–965 pg/mg creatinine). Horizontal bars indicate median values. $^*P < 0.05$.

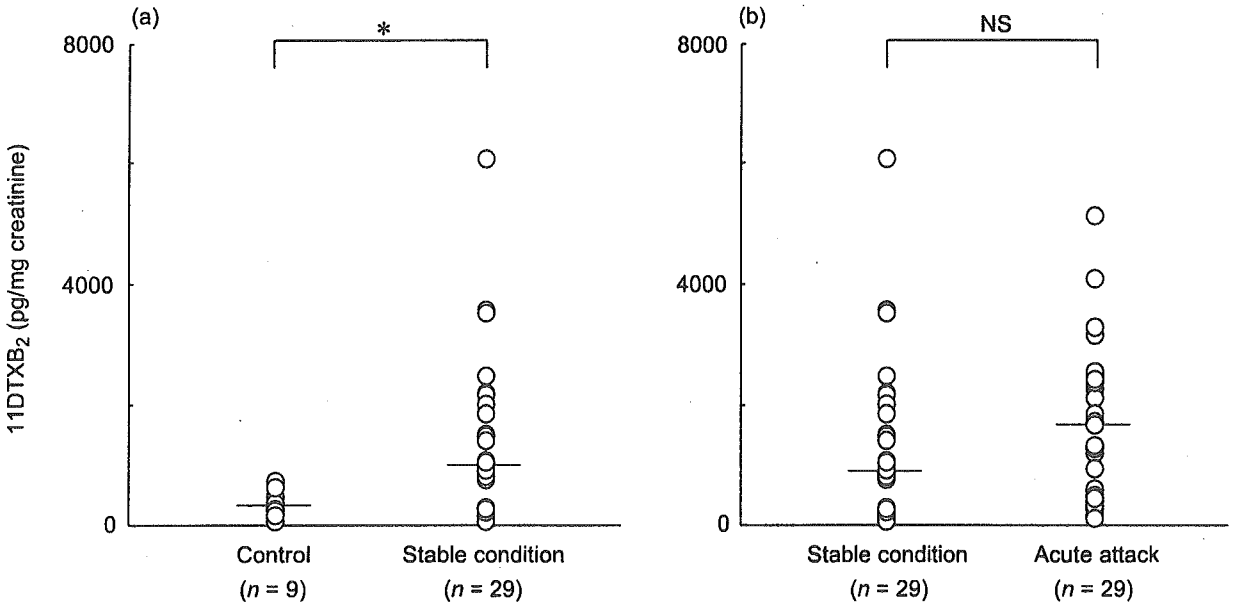

Fig. 2 (a) Urinary 11-dehydro-thromboxane B_2 ($11DTXB_2$) levels in asthmatic children during the stable condition (median 1009 pg/mg creatinine; range 46–6070 pg/mg creatinine) and in controls (median 252 pg/mg creatinine; range 41–716 pg/mg creatinine). (b) Urinary $11DTXB_2$ levels in asthmatic children during an acute asthma attack (median 1666 pg/mg creatinine; range 110–5105 pg/mg creatinine) and during the stable condition (median 1009 pg/mg creatinine; range 46–6070 pg/mg creatinine). Horizontal bars indicate median values. $^*P < 0.05$.

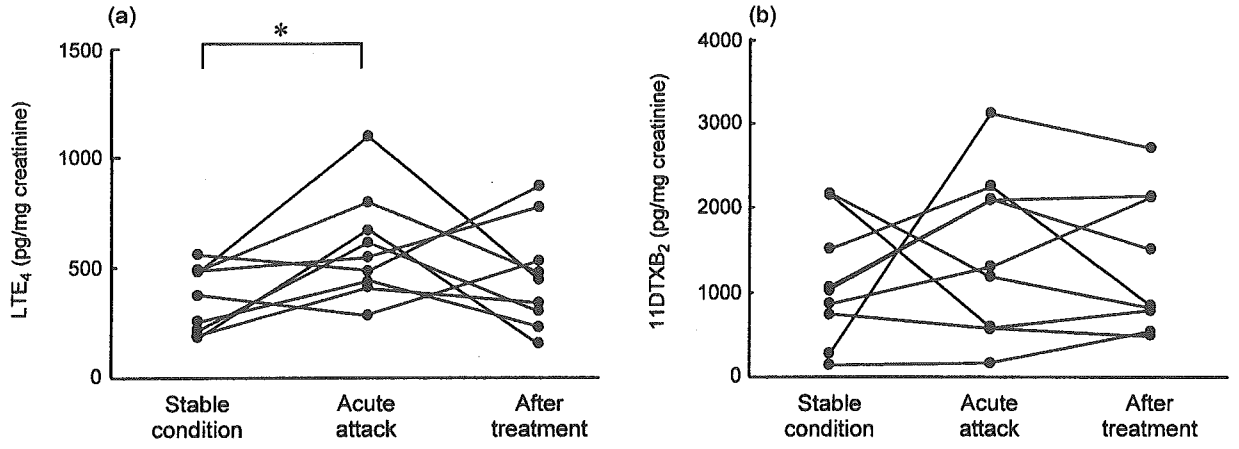

Fig. 3 Urinary leukotriene E_4 (LTE_4) and 11-dehydro-thromboxane B_2 ($11DTXB_2$) levels in nine children with bronchial asthma during the stable condition, an acute asthma attack and 2 days after treatment. (a) Urinary LTE_4 levels increased from a median of 373 pg/mg creatinine (range 183–556 pg/mg creatinine) during the stable condition to 546 pg/mg creatinine (range 280–1100 pg/mg creatinine) during an acute asthma attack, decreasing again to 443 pg/mg creatinine (range 156–872 pg/mg creatinine) 2 days after treatment. (b) Urinary $11DTXB_2$ levels were apt to increase from a median of 1009 pg/mg creatinine (range 131–2166 pg/mg creatinine) during the stable condition to 1285 pg/mg creatinine (range 166–3122 pg/mg creatinine) during an acute asthma attack and then decrease slowly to 842 pg/mg creatinine (range 492–2708 pg/mg creatinine) 2 days after treatment. $^*P < 0.05$.

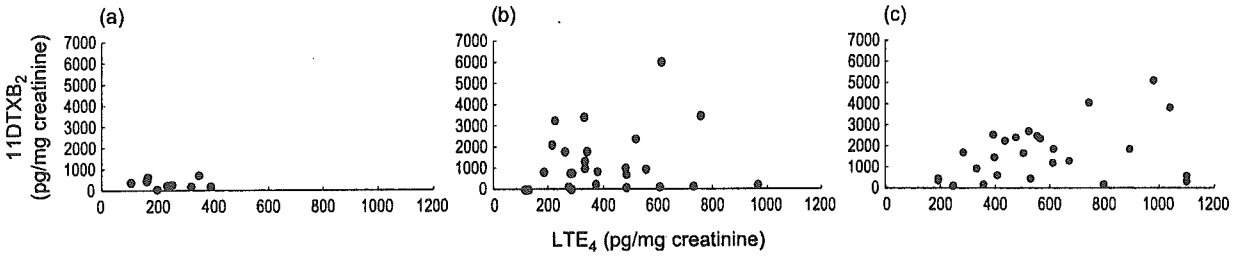

Fig. 4 Relationship between urinary leukotriene E_4 (LTE_4) and 11-dehydro-thromboxane B_2 ($11DTXB_2$) levels. No relationship was noted between these prostanoids in (a) control subjects, (b) asthmatic children while in the stable condition and (c) asthmatic children during an acute asthma attack.

levels during the stable condition, the changes in LTE_4 were not related to changes in $11DTXB_2$ in children with bronchial asthma (Fig. 5). Neither gender, age, serum IgE nor eosinophil count had any relationship with urinary levels of LTE_4 or $11DTXB_2$ (data not shown). One patient (no. 5) had high eosinophil counts (1344/μL during the stable condition; 871/μL during an acute attack; and 2567/μL when he felt better 2 days after treatment). However, the eosinophil count did not correlate with urinary levels of LTE_4 or $11DTXB_2$. There was no significant correlation between urinary levels of LTE_4 and the severity of asthma; however, the severity of asthma in patients with high levels of urinary LTE_4 were classified as 'moderate persistent' or 'severe persistent'.

Discussion

Cysteinyl leukotrienes and TXA_2 are considered to play important roles in the pathogenesis of bronchial asthma. The relationship between urinary LTE_4 and $11DTXB_2$ in the pathogenesis of asthma has been reported by several investigators;[2–5,8,11,13,20] because of the instability of CysLTs and TXA_2, the end-products of the cascade were determined. However, most studies have been

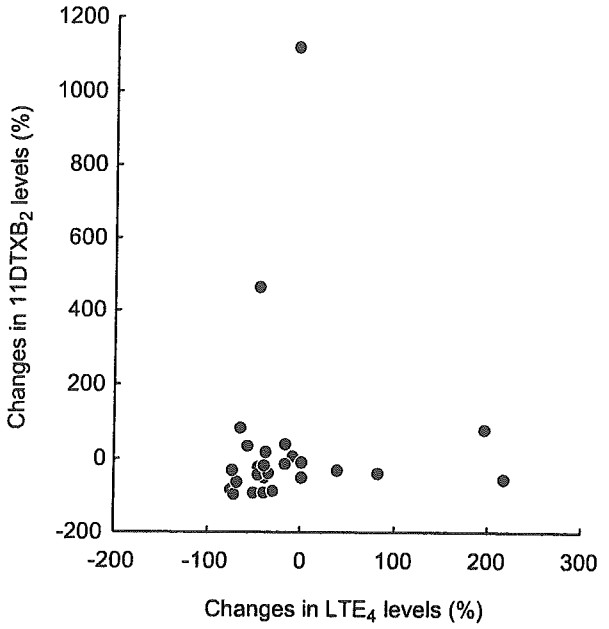

Fig. 5 Changes (%) in leukotriene E_4 (LTE_4) levels did not correlate with changes (%) in 11-dehydro-thromboxane B_2 ($11DTXB_2$) levels in children with bronchial asthma. The percentage change was calculated as follows: (level during stable condition – level during attack) × 100/level during attack.

performed in adults. In the present study, we have demonstrated the relationship between urinary LTE_4 and $11DTXB_2$ in children with bronchial asthma.

In the present study, urinary LTE_4 levels in children with bronchial asthma during the stable condition were significantly higher than in control children. In addition, urinary LTE_4 levels in children during an acute asthma attack were higher than during the stable condition. Asano *et al.*[5] also demonstrated that patients with mild to moderate asthma excrete LTE_4 in the urine at a greater rate than control subjects. Taylor *et al.*[4] revealed that urinary LTE_4 was significantly higher in asthma patients after antigen challenge than in control subjects. The results of the present study are consistent with previous findings in adult asthmatic patients.[3,4,9,11,17,20]

In the present study, urinary $11DTXB_2$ levels were higher in children with bronchial asthma than in controls. Unlike LTE_4, urinary $11DTXB_2$ levels did not increase markedly during an acute attack.

Oosaki *et al.*[3,20] reported on variations in urinary levels of these mediators in patients with spontaneous asthma attacks who were monitored for 3 days and whose state improved. The study of Oosaki *et al.*[3,20] showed that urinary levels of LTE_4 were significantly higher during the attack and returned to control levels once the patient's state had improved. In the present study, the urinary levels of these prostanoids were measured in asthmatic children during the stable condition, during an acute attacks and 2 days after treatment. In eight children, urinary LTE_4 levels increased during an acute attack and decreased 2 days after treatment. One patient (no. 8) exhibited a different pattern of urinary LTE_4 excretion: levels decreased during an acute attack and then increased when she felt better 2 days after treatment. However, the urinary $11DTXB_2$ levels in this patient increased during an acute attack and then decreased 2 days after treatment. This patient had atopic-type bronchial asthma and was treated with theophyline, steroid inhalant, DSCG and a β_2-adrenergic receptor agonist. Before she was enrolled in the study, she had been treated with an LTRA for 5 weeks. However, LTRA treatment had little effect on her asthma. Urinary $11DTXB_2$ levels tended to increase during an asthma attack and persisted 2 days after treatment. Similar to the findings of the present study, Oosaki *et al.* have shown that the median level of urinary $11DTXB_2$ was highest during the 3rd hospital day in atopic-type patients and during the 2nd hospital day in non-atopic-type patients.[3]

In the present study, urinary levels of LTE_4 and $11DTXB_2$ were slightly higher than those reported previously.[2–5,8,11,13,20] Osamura *et al.* had reported that urinary levels of $11DTXB_2$ were significantly high between 1 and 3 years after birth and that they tended to decrease gradually with age thereafter.[21] Because all our subjects were children (1–15 years of age), this may explain why the urinary levels of $11DTXB_2$ were slightly higher in the present study than those reported previously.

Suzuki *et al.*[2] reported that no significant relationship was observed between urinary LTE_4 and $11DTXB_2$ in asthmatic patients. Oosaki *et al.*[3] also examined the relationship in changes (%) between these two metabolites; however, they noted no significant difference. In the present study, consistent with results of previous studies, no relationship was observed between urinary LTE_4 and $11DTXB_2$ in children with bronchial asthma. In addition, changes (%) in LTE_4 levels were not associated with $11DTXB_2$ levels in children with bronchial asthma. This suggests that increases in the levels of these two metabolites are not correlated with one another.

Neither gender, age, serum IgE nor eosinophil count revealed any relationship with urinary levels of LTE_4 or $11DTXB_2$. Eosinophils play an important role in the

pathogenesis of bronchial asthma and the eosinophil count is correlated with the clinical severity of the disease.[22] However, there are few studies referring to the correlation between eosinophil count and urinary levels of LTE_4 or $11DTXB_2$. There was no significant correlation between urinary levels of LTE_4 and the severity of asthma; however, the severity of the asthma in patients with high levels of urinary LTE_4 tended to be classified as 'moderate persistent' or 'severe persistent'.

In conclusion, we have shown significantly higher levels of urinary LTE_4 and $11DTXB_2$ in asthmatic children during the stable condition. These findings strongly suggest that the arachidonate cascade metabolites CysLTs and thromboxanes play certain roles in the pathogenesis of bronchial asthma in children. According to the differential changes in urinary levels of these metabolites during an acute attack, we suppose that an imbalance in the metabolism arises between the 5-lipoxgenase pathway and the cyclooxygenase pathway. The measurement of LTE_4 and $11DTXB_2$ in urine samples, which is a safe and easily available method of estimating the synthesis and release of the mediator in children, would be useful in understanding the pathogenesis of bronchial asthma.

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Induction of granulocyte-macrophage colony-stimulating factor by a new cytokine, ML-1 (IL-17F), via Raf I-MEK-ERK pathway

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Background: ML-1 (IL-17F) is a recently discovered cytokine, and its function remains elusive. GM-CSF is a crucial cytokine for the maturation of various cell types and regulates allergic airway inflammation.

Objective: The functional effect of ML-1 in the expression of GM-CSF was investigated.

Methods: The levels of gene and protein expression in normal human bronchial epithelial cells (NHBEs) in the presence or absence of various kinase inhibitors or, in some cases, of a Raf1 dominant-negative mutant were determined by RT-PCR and ELISA, respectively. Western blotting was performed to investigate kinase activation.

Results: The results showed first that ML-1 induces, in a time-dependent and dose-dependent manner, the gene and protein expression for GM-CSF NHBEs, which are associated with activation of Raf1 and MAP kinase kinase (MEK) kinases. Selective MEK inhibitors, PD98059 and U0126, and Raf1 kinase inhibitor I significantly inhibited ML-1–induced GM-CSF production. Furthermore, overexpression of Raf1 dominant-negative mutants inhibited IL-17F–induced GMCSF expression. The combination of PD98059 and Raf1 kinase inhibitor I completely blocked GM-CSF production, whereas 2 protein kinase C inhibitors, Ro-31-7549 and GF109203X, and a phosphatidylinositol 3-kinase inhibitor, LY294002, showed no inhibitory effect.

Conclusion: These findings suggest that ML-1 induces GM-CSF expression through the activation of the Raf1-MEK–extracellular signal-regulated kinase 1/2 pathway. (J Allergy Clin Immunol 2004;114:444-50.)

Key words: Bronchial epithelial cells, ERK1/2, GM-CSF, MAP kinase, Raf1

Abbreviations used
DMSO: Dimethyl sulfoxide
ERK: Extracellular signal-regulated kinase
MEK: MAP kinase kinase
NF-κB: Nuclear factor κB
NHBE: Normal human bronchial epithelial cell
PI3K: Phosphatidylinositol 3-kinase
PKC: Protein kinase C

Airway allergic inflammation is characterized by activation and migration of various inflammatory cells, such as eosinophils, granulocytes, and macrophages. The accumulation of these cell types causes bronchoconstriction, lung damage, and fibrosis. Bronchial epithelial cells play an important role in the regulation of airway inflammation through their ability to express a wide range of proinflammatory cytokines, including GM-CSF.[1] Investigation of the regulatory pathway involving modulation of airway epithelium by cytokines is thus important to uncover the pathogenic mechanisms of airway inflammation.

GM-CSF plays a pivotal role in inflammatory and immunologic processes. Release of GM-CSF in the airway can mediate acute inflammatory responses as well as initiate and perpetuate local immune responses. Elevated levels of GM-CSF have been well demonstrated in bronchoalveolar lavage fluid, endobronchial biopsy, and sputum samples from asthmatics.[2-4] Elevated levels of GM-CSF, mainly derived from epithelial cells, have been demonstrated to increase eosinophil activation and survival in asthmatics.[5-7] Bronchial asthma is an inflammatory disease of the airways characterized by eosinophil infiltration of the lung and airway hyperreactivity. GM-CSF stimulates the recruitment and activation of eosinophils via β-integrin–mediated adhesion to epithelial and endothelial cells[8] and prolongs eosinophil survival via inhibition of apoptosis.[9-11] Moreover, mouse models of asthma and diesel-induced hyperresponsiveness have demonstrated an association between epithelial cell-derived GM-CSF and airway hyperresponsiveness.[12,13] These findings suggest that GM-CSF is 1 of the critical cytokines in the pathogenesis of allergic airway inflammation, and airway epithelium plays a pivotal role in causing and perpetuating airway inflammation via the induction of GM-CSF. However, the regulatory mechanisms of GM-CSF expression in airway epithelium have not been well understood.

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Recently, we and others independently discovered a novel cytokine, ML-1[14] (IL-17F[15,16]), belonging to the IL-17 gene family, but its function and signaling pathways remain to be defined. ML-1 is expressed in activated $CD4^+$ T cells, basophils, and mast cells, 3 important cell types involved in allergic inflammation.[14] Significantly, upregulated ML-1 gene expression is seen at sites of allergen challenge in the airways of asthmatics,[14] but its involvement in pulmonary allergic inflammation is unclear. To gain further understanding of the function and signaling pathways of ML-1, the role of ML-1 in the expression of GM-CSF was investigated. In this article, we provide evidence that ML-1 is able to induce GM-CSF expression in bronchial epithelial cells, involving the activation of the Raf1–MAP kinase kinase (MEK)–extracellular signal-regulated kinase (ERK) 1/2 signaling pathway.

METHODS

Cell culture and human recombinant ML-1

Normal human bronchial epithelial cells (NHBEs) were purchased from Clonetics (San Diego, Calif) and cultured in bronchial epithelial basal medium (Clonetics) containing 0.5 ng/mL human recombinant epidermal growth factor, 52 μg/mL bovine pituitary extract, 0.1 ng/mL retinoic acid, 0.5 μg/mL hydrocortisone, 5 μg/mL insulin, 10 μg/mL transferrin, 0.5 μg/mL epinephrine, 6.5 ng/mL tri-iodothyronine, 50 μg/mL gentamicin, and 50 μg/mL amphotericin-B (Clonetics). The cells were cultured for no more than 3 passages before the analysis. In the case of the experiments using Raf1 dominant-negative mutants, a bronchial epithelial cell line, BEAS-2B, was used instead of NHBE for efficient transfection and was cultured in Hanks' F12/Dulbecco modified Eagle medium (Biofluids, Rockville, Md) with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin (Life Technologies-BRL, Gaithersburg, Md). Human recombinant ML-1 was generated as described previously.[14] Endotoxin levels were tested by using Kinetic-QCL Chromogenic Limulus amebocyte lysate (Bio Whittaker, Walkersville, Md). Endotoxin levels were undetectable. The cells were treated with ML-1 (10 and 100 ng/mL) for various periods.

Gene expression of GM-CSF

Total RNA was extracted by using RNeasy (Qiagen, Chatsworth, Calif) from 1×10^6 cells at 1, 2, 4, 12, and 24 hours after stimulation with 10 and 100 ng/mL ML-1. cDNAs were synthesized from 500 ng of total RNA in the presence of Moloney murine leukemia virus reverse transcriptase (1 U/reaction; Sigma, St Louis, Mo), oligo (dT) primer, and reaction buffer at 42°C for 90 minutes, followed by PCR. The sequences of PCR primers for GM-CSF were forward, 5′-GTGGCCTGCAGCATCTCT-3′, and reverse, 5′-AAAGGGGATGACAAGCAGAA-3′; and for G3PDH were forward, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. The amplification reaction was performed for subsaturating cycles with denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds. The expected size for GM-CSF was 375 bp and for G3PDH was 450 bp. The PCR products were detected by ethidium bromide staining and quantified by video densitometry with Image 1.61 software (National Institutes of Health Public Software, National Institutes of Health, Bethesda, Md). The level of GM-CSF gene expression was quantified by calculating the ratio of densitometric readings of the band intensity for GM-CSF and G3PDH from the same cDNA sample. The values are expressed as means ± SDs (n = 3 experiments). In all cases, the induction of GM-CSF by ML-1 was clearly evident, allowing quantitative comparison by RT-PCR.

Protein levels of GM-CSF

GM-CSF protein levels in the supernatants and cell lysates of ML-1–stimulated cells were determined with a commercially available ELISA kit (Biosource, Camarillo, Calif) according to the manufacturer's instructions. Cell supernatants were harvested from cultures in the absence or presence of 10 or 100 ng/mL ML-1 at 2, 6, 12, 24, or 48 hours after stimulation. The amount of secreted GM-CSF was determined by the ELISA and expressed as the amount recovered per 10^6 cells. Cells corresponding to the supernatant samples described were lysed in 0.5 mL Nonidet P-40 lysis buffer (20 mmol/L Tris [pH 7.4], 4 mmol/L EDTA, 1 mmol/L phenylmethanesulfonyl fluoride, 100 mg/mL aprotinin, 200 mg/mL leupeptin, 50 mmol/L NaF, 5 mmol/L $Na_4P_2O_7$, and 1% Nonidet P-40 [all Sigma]). The GM-CSF concentration of cell lysate was reported as the amount recovered per 10^6 cells. The values are expressed as means ± SDs (n = 6 experiments).

ML-1–induced Raf1 and MEK activation and the effect of inhibitors

For analysis of activation of the Raf1-MEK-ERK1/2 pathway, NHBEs were treated with ML-1 (100 ng/mL) and in some cases with or without the treatment with a Raf1 kinase inhibitor I or the vehicle control (Me_2SO), for 1 hour. The final concentration of Me_2SO did not exceed 0.1% (vol/vol). After treatment and lysis of the cells, the cellular extracts (1×10^6 cell equivalents/lane) were suspended with an equal volume of 2 times loading buffer (0.1 mol/L Tris-HCl, pH 6.8; 4% SDS; 0.005% bromphenol blue; and 20% glycerol) containing 2-mercaptoethanol (0.7 mol/L), subjected to 4% to 20% Tris-glycine gel electrophoresis (NOVEX, San Diego, Calif), and probed with various antibodies: rabbit anti-MEK1/2 antibody, antiphospho-MEK1/2 antibody (Cell Signaling Technology, Beverly, Mass), anti-Raf1 antibody, and antiphospho-Raf1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), followed by incubation with peroxidase-linked donkey anti-rabbit Ig antibody (Amersham Biosciences, Piscataway, NJ) and visualized with enhanced chemiluminescence.

For analysis of the effects of kinase inhibitors, the cells were treated in the presence or absence of the following kinase inhibitors at varying doses: MEK1/2 inhibitors, PD98059 (2′′-amino-3′-methoxyflavone; Calbiochem, La Jolla, Calif) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis[o-aminophenylmercapto] butadiene; New England Bio Labs, Beverly, Mass); p38 inhibitor, SB202190 (4-[4-fluoro-phenyl]-2-[4-hydroxyphenyl]-5-[4-pyridyl] 1H-imidazole; Calbiochem); a Raf1 kinase inhibitor I (5-iodo-3-[(3,5-dibromo-4-hydroxyphenyl) methylene]-2-indolinone; Calbiochem); a c-Jun N-terminal kinase inhibitor, SP600125 (Anthra [1,9-cd]pyrazol-6[2H]-one; Calbiochem); protein kinase C (PKC) inhibitors, Ro-31-7549 (2-[1-(3-aminopropyl) indol-3-yl]-3-[1-methylindol-3-yl] maleimide acetate; Calbiochem) and GF109203X (3-[1-(dimethylaminopropyl) indol-3-yl]-4-[indol-3-yl] maleimide, 3-[1-(3-[dimethylamino]propyl)-1H-indol-3-yl]-4-[1H-indol-3-yl]-1H-pyrrole-2,5-dione; Sigma); a phosphatidylinositol 3-kinase PI3K inhibitor, LY294002 (2-[4-morpholinyl]-8-phenyl-4H-1-benzopyran-4-one; Calbiochem); and a vehicle control, dimethyl sulfoxide (DMSO; Me_2SO) for 1 hour before treatment with ML-1 (100 ng/mL). The cells and cell supernatants were harvested at 2 and 24 hours after stimulation for analyses of RT-PCR and ELISA, respectively. The final concentration of DMSO did not exceed 0.1% (vol/vol). GM-CSF gene expression and protein levels in the supernatants were determined as described. The values are expressed as means ± SDs (n = 4 experiments).

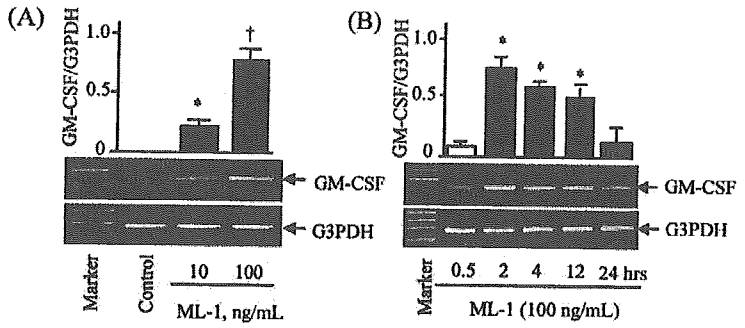

FIG 1. A, Induction of GM-CSF gene expression by different doses of ML-1. *$P < .05$ was considered significant versus control. †$P < .05$ was considered significant versus 10 ng/mL ML-1–stimulated cells. **B,** Time course study of GM-CSF gene expression. The values are expressed as means ± SDs (n = 3). *$P < .05$ was considered significant versus the intensity of 0.5-hour time point.

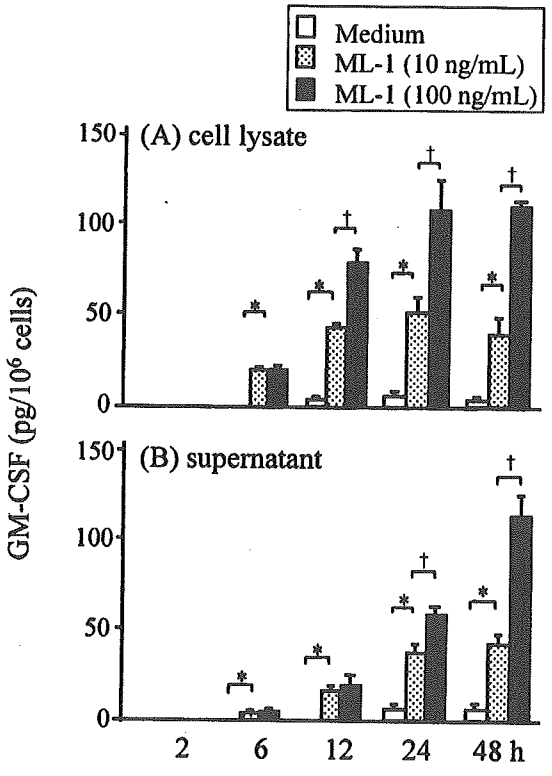

FIG 2. GM-CSF protein levels in lysates **(A)** and supernatants **(B)** of NHBEs. The values are expressed as means ± SDs (n = 6). *$P < .05$ was considered significant versus control. †$P < .05$ was considered significant versus 10 ng/mL ML-1–stimulated cells.

Overexpression of Raf1 dominant-negative mutant in BEAS-2B cells

The plasmid encoding pCMV-RafS621A vector (dominant-negative mutant of Raf1) cloned into pCMV and a control vector were purchased from Clontech (Palo Alto, Calif). The plasmids were prepared by using the Qiagen plasmid DNA preparation kit. NHBEs were difficult to use for transfection experiments. An epithelial cell line, BEAS-2B, was used instead. The cells were cultured on 100-mm plates and were transfected by an Effectene Reagent (Qiagen) according to the manufacturer's instructions. The cells were selected with 500 ng/mL Geneticin (G418; Gibco/BRL, Grand Island, NY). After selection, the cells were seeded into 6-well culture plates. The cells were near confluent, and the cells and cell supernatants were then harvested at 2 and 24 hours after stimulation with 100 ng/mL ML-1 for analyses of RT-PCR and ELISA, respectively. GM-CSF gene expression and protein levels in the supernatants were determined as described. The values are expressed as means ± SDs (n = 3 experiments).

Data analysis

The statistical significance of differences was determined by ANOVA. The values are expressed as means ± SDs from independent experiments. Any difference with P values $< .05$ was considered. When ANOVA indicated a significant difference, the Scheffe F test was used to determine the difference between groups.

RESULTS

To examine whether ML-1 was able to induce GM-CSF expression, NHBEs were stimulated with varying doses of ML-1, and the levels of gene expression and protein production in cell lysates and supernatants were analyzed. The results showed first that although GM-CSF gene expression was not seen in control culture, ML-1 induced, in a dose-dependent manner, the expression of GM-CSF at the 2-hour time point (Fig 1, *A* and *B*). In the time course experiments, ML-1–induced GM-CSF gene expression peaked at the 2-hour time point in ML-1 (100 ng/mL)–treated NHBEs (Fig 1, *C* and *D*) and returned to baseline 24 hours after stimulation. To analyze the protein expression for GM-CSF, NHBEs were cultured in the absence or presence of varying concentrations of ML-1 at 5 different time points. GM-CSF proteins were not detected in the absence of ML-1 in NHBEs. The levels of GM-CSF in cell lysates were significantly increased 6 hours after stimulation with 10 and 100 ng/mL ML-1 and reached plateau at the 12-hour and 24-hour time points, respectively (Fig 2, *A*), whereas the levels of secreted GM-CSF were significantly elevated 24 hours after stimulation and were increased further at 48 hours (Fig 2, *B*).

Bronchial epithelial cells are exposed to numerous environmental stimuli, which can activate intracellular

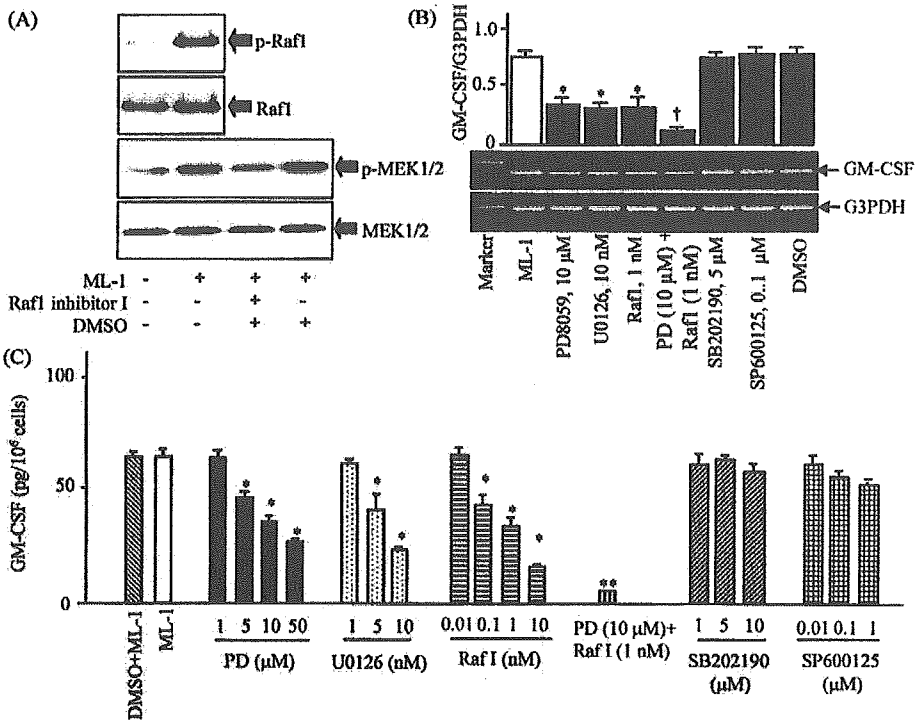

FIG 3. A, Western blotting analysis of Raf1 and MEK activation. The results shown are representative of 3 separate experiments. Effect of kinase inhibitors on GM-CSF gene (B) and protein (C) expression in NHBEs. The values are expressed as means ± SDs (n = 4 experiments). $^{*}P < .05$ was considered significant versus ML-1–stimulated cells. $^{\dagger}P < .05$ was considered significant versus single inhibitor.

signaling cascades, leading to upregulated gene expression and a wide range of responses, such as cytokine expression, proliferation, and apoptosis.[17] The mitogen-activated protein kinase family is likely to be central to these processes because they are known to regulate intracellular signal transduction in response to many agonists, including growth factors, cytokines, hormones, oxidants, and environmental stress factors.[18] To investigate ML-1–mediated signaling events leading to the induction of GM-CSF, the activation of the Raf1-MEK-ERK1/2 pathway was investigated. First, Western blotting analysis revealed activation of Raf1 and MEK1/2 in ML-1–stimulated cells (Fig 3, *A*), and preincubation of the cells with a Raf1 kinase inhibitor I (1 nmol/L) for 1 hour diminished the activation of MEK1/2 in comparison with that seen in the vehicle control culture (Fig 3, *A*). Furthermore, analysis of various kinase inhibitor effect on ML-1–induced GM-CSF expression showed that pretreatment of the cells for 1 hour with each of the selective MEK inhibitors, PD98059 (10 μmol/L), U0126 (5 nmol/L), and Raf1 kinase inhibitor I (1 nmol/L), significantly decreased the levels of ML-1–induced GM-CSF gene expression in NHBEs (Fig 3, *B*). As shown in Fig 3, *C*, 1 hour pretreatment of the cells with PD98059, U0126, and Raf1 kinase inhibitor I significantly attenuated, in a dose-dependent manner, the production of GM-CSF, whereas 1 hour pretreatment of the cells with vehicle alone (0.05% DMSO) did not affect GM-CSF release. In

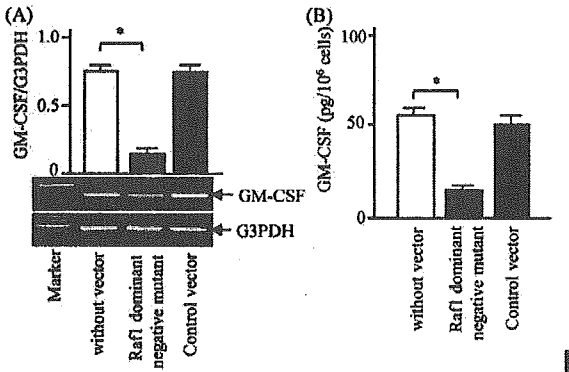

FIG 4. Effect of overexpression of Raf1 dominant-negative mutants on GM-CSF gene (A) and protein (B) expression in BEAS-2B cells. The values are expressed as means ± SDs (n = 3). $^{*}P < .05$ was considered significant versus the intensity of ML-1–stimulated cells without vector.

addition, the protein levels of GM-CSF were unchanged in ML-1–treated cells in the presence of a p38 kinase inhibitor, SB202190, and a JNK inhibitor, SP600125, even at a dose of 10 μmol/L and 1 μmol/L, respectively (Fig 3, *C*). Although induction of GM-CSF is partially inhibited by PD98059, U0126, or Raf1 kinase inhibitor I even at relatively high doses (50 μmol/L, 10 nmol/L, and 10 nmol/L, respectively), the combination of 10 μmol/L

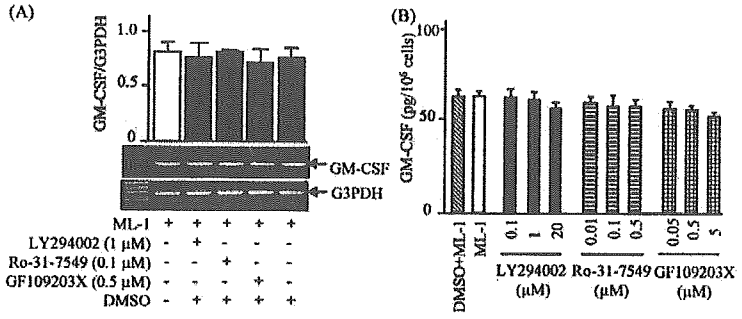

FIG 5. Effect of PD98059, U0126, Raf1 kinase inhibitor I, SB202190, and SP600125 on GM-CSF gene (A) and protein (B) expression in NHBEs.

PD98059 and 1 nmol/L Raf1 kinase inhibitor I inhibited, to a greater degree, the production of GM-CSF in NHBEs (Fig 3, *B* and *C*). The total number of cells and cell viability at the end of the culture period for each experiment did not differ among all culture conditions as determined by trypan blue exclusion assay, suggesting that the inhibition of ML-1–induced GM-CSF expression did not result from cytotoxicity.

The involvement of Raf1 kinase in ML-1–induced GM-CSF expression was further confirmed by the use of a Raf1 dominant-negative mutant with serine to alanine substitution at position 621. The results showed that overexpression of Raf1 dominant-negative mutants in BEAS-2B cells significantly inhibited ML-1–induced GM-CSF gene expression (Fig 4, *A*) and protein release (Fig 4, *B*), whereas the cells transfected with a control vector showed no significant change in the level of GM-CSF expression (Fig 4). These results suggest, therefore, the importance of the Raf1-MEK-ERK signaling pathway in ML-1–induced GM-CSF expression. It has been demonstrated that both PKC and PI3K are linked to the MAP kinase pathway, and that signaling from receptor tyrosine kinases to ERK1/2 is dependent on Ras and Raf1. Further, PKC has been shown to be a key activator of the Raf1/mitogen-activated protein kinase cascade at multiple steps that result in upregulation of ERK1/2. In our experiment, however, selective PKC inhibitors, Ro-31-7549 (0.1 μmol/L) and GF109203X (0.5 μmol/L), did not decrease the level of ML-1–induced GM-CSF gene expression in NHBEs (Fig 5, *A*). Similarly, no significant inhibitory effect was seen on ML-1–induced GM-CSF protein production when the inhibitors were used (Fig 5, *B*). Moreover, a PI3K inhibitor, LY294002, showed no significant inhibition of ML-1–induced GM-CSF gene expression in NHBEs (Fig 5, *A*) and protein production in NHBEs (Fig 5, *B*) when LY294002 (0.1-20 μmol/L) was used.

DISCUSSION

In this report, we provide evidence that ML-1 (IL-17F) is able to induce GM-CSF expression in primary bronchial epithelial cells through the activation of the Raf1-MEK-ERK1/2 pathway. ML-1 is derived from activated $CD4^+$ T cells, basophils, and mast cells, which are important regulatory cells for allergic airway inflammation.[14] The ability of ML-1 to induce epithelium-derived GM-CSF raises a strong possibility that ML-1–induced GM-CSF is involved in allergic inflammation. In our previous report, the ML-1 gene is upregulated in the bronchoalveolar lavage cells from asthmatic patients after segmental allergen challenge.[14] The current study lends support for a role of ML-1 in the airway inflammatory responses via the induction of GM-CSF.

The results of the current study demonstrate the involvement of the Raf1-MEK-ERK signaling pathway in ML-1–induced GM-CSF expression. It is of interest to note that the induction of IL-6 and IL-8 by ML-1 is dependent on the activation of ERK1/2, but not p38 and JNK,[19] suggesting that ML-1 is able to induce multiple cytokines via the same signaling pathway. Also, ERK1/2 may serve as a pivotal signaling molecule for IL-17 family members, such as IL-17A and ML-1 (IL-17F), and would be a potential therapeutic target for IL-17 family-associated airway diseases. Further, our pharmacologic studies showed that neither PKC nor PI3K is involved in ML-1–induced GM-CSF expression. This is in contrast to the finding that TNF-α induces GM-CSF expression via activation of PKC and subsequent activation of ERK1/2 kinases in NHBEs.[20] It is noted also that a PKC inhibitor, Ro-31-7549, does not affect IL-17A–induced IL-6 and IL-8 expression in bronchial epithelial cells.[21] Another PKC inhibitor, GF-109203X, also does not block diesel exhaust particle–induced GM-CSF expression.[22] Furthermore, a selective PI3K inhibitor, LY294002, does not affect IL-17A–induced IL-6 and IL-8 in bronchial epithelial cells.[23] Our data also showed that LY294002 did not inhibit ML-1–induced GM-CSF expression. Activated Ras promotes cell survival in epithelial cells through activation of PI3K and Akt/PKB, and it is known that at high dose (20 μmol/L), LY294002 induces apoptosis.[24] In the current study, the cell number and viability were not altered in the presence of 20 μmol/L of LY294002 (data not shown). This is likely a result of different culture conditions and cell types used. However, we cannot fully exclude the involvement of other molecules, such as RalGDS and PLC-ε, because recent

reports have demonstrated that Ras is able to activate these signaling molecules besides Raf1.[25,26] These results demonstrate, therefore, that multiple functional pathways exist in the regulation of GM-CSF expression.

To date, the upstream signaling pathway of Ras-Raf1-MEK-ERK1/2 induced by ML-1 is unclear. There have been few reports concerning the upstream signaling pathway of GM-CSF expression. However, the results of the current study suggest that Raf1 is predominantly associated with the activation of the MEK-ERK1/2 pathway. Therefore, we concluded that the Raf1-MEK-ERK1/2 pathway is a central upstream pathway of ML-1–induced GM-CSF expression in NHBEs. Further study is needed to identify functionally the putative receptor for ML-1 and its proximal signaling pathways. Also, the downstream signaling pathway is currently unclear. GM-CSF is known to be regulated by a transcription factor, nuclear factor κB (NF-κB).[27] IL-17A is known to activate NF-κB in chondrocytes and intestinal epithelial cells.[28] Because of high homology between IL-17A and ML-1, it is possible that ML-1 is able to activate NF-κB in the downstream signaling pathway. However, we could not detect the activation of NF-κB by ML-1 in NHBEs (data not shown), suggesting an NF-κB–independent pathway, for which further investigation is needed.

Bronchial epithelial cells are able to release GM-CSF in response to physiologic stimuli relevant for asthma, including dust mite proteolytic allergens, human rhinovirus 16, respiratory syncytial virus, and histamine.[29,30] Furthermore, bronchial epithelial cells have been demonstrated to secrete GM-CSF in response to several cytokines, such as TNF-α, IL-1β, IL-4, and IL-13, which are strongly involved in allergic response.[31,32] A substantial amount of GM-CSF is produced in the airway epithelium after antigen challenge in asthmatic subjects, and GM-CSF appears to be the major cytokine responsible for eosinophil survival in the bronchoalveolar lavage fluid of symptomatic asthmatic subjects.[33] In addition, GM-CSF acts as a cofactor for superoxide production and degranulation[33,34] and elicits multiple effects on other components of the immune systems, including dendritic cells, which initiate and perpetuate allergic inflammation.[35] Furthermore, GM-CSF also has multiple effects on neutrophil function, including increasing 5-lipooxygenase level, tyrosine phosphorylation, aggregation, superoxide anion production, degranulation, cytotoxicity, and adhesion to vascular endothelial cells.[36] Finally, GM-CSF stimulates monocyte survival and differentiation into mature macrophages, which are the largest cell population in the airways of patients with allergic inflammatory diseases.[37,38] Hence, these data strongly suggest that ML-1 shows multiple biological activities via the induction of GM-CSF, adding to a growing list of cytokines involved in the regulation of allergic inflammation.

In conclusion, this study reports that ML-1 induces GM-CSF via the activation of the Raf1-MEK-ERK1/2 pathway. These results suggest that ML-1 is involved in allergic inflammation via the induction of GM-CSF. ML-1 may cause and perpetuate airway inflammation at multiple steps. Furthermore, the control of the Raf1-MEK-ERK1/2 pathway is an attractive pharmacotherapeutic strategy for inhibition of ML-1–induced airway immunologic and inflammatory responses.

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