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□ Review Article □

A Genetic Classification and a Tailor-made Medicine in Allergic Disorders

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INTRODUCTION

Allergic diseases such as bronchial asthma and atopic dermatitis develop by a combination of genetic and environmental factors. Several causative candidate genes of atopy have been reported for the genetic factors.

Recently treatment/management guidelines on bronchial asthma and many other disorders have been published, and they are utilized in clinical practice. However, the clinical features of the patients and the causes of the diseases vary. Therefore, personalized medical care (tailor-made medicine) is necessary for the improvement of QOL.

Here we present a new genetic classification of atopy (by Kondo N) and a tailor-made medicine in allergic disorders.

A GENETIC CLASSIFICATION OF ATOPY

Atopy is characterized by enhanced immunoglobulin E (IgE) responses to environmental antigens. IgE production is upregulated by Th2 cytokines, in particular, interleukin-4 (IL-4), and is downregulated by Th1 cytokines, in particular, interferon-γ (IFN-γ)(Fig. 1)[1]. IL-12, which is a cytokine that promotes cell-mediated Th1 responses and production of IFN-γ, is one of the important cytokines that downregulates IgE production. IL-18, originally known as an IFN-γ-inducing factor, is a recently cloned cytokine of approximately 18 kDa secreted by Kupffer cells of the liver and activated macrophages.[2] IL-18 strongly augments IFN-γ production by T lymphocytes, natural killer (NK) cell cytotoxicity and T lymphocyte proliferation.

Several linkage analyses and mutations for candidate genes of atopy, thai is enhanced IgE production, have been reported. In 1989, Cookson et al.[3] reported a linkage between IgE responses underlying asthma and rhinitis and chromosome 11q. Moreover, Shirakawa et al.[4] reported that a common variant of the β-subunit of the high-affinity IgE receptor (FcεRIβ) on chromosome 11, Ile 181 Leu within the 4th transmembrane domain, shows significant association with positive IgE responses. Several associations have been noted between atopy and genes on chromosome 5 cytokine cluster, including IL-4.[5,6] In 1998, Mitsuyasu et al.[7] reported that Ile 50 Val variant of IL-4 receptor α (IL-4Rα) chain upregulates IgE synthesis and is associated with atopic asthma. Moreover, Shirakawa et al.[8] noted genetic variants of IL-13.

Very recently, we found that reduced IFN-γ production by peripheral blood mononuclear cells (PBMCs) following stimulation with IL-12 or IL-18 is associated with the heterozygous IL-12 receptor β2 (IL-12Rβ2) chain gene or IL-18 receptor α (IL-18Rα) chain gene mutations in atopic subjects.[9,10]

Based on these reports and our results, we present a new genetic classification of atopy (by Kondo N) in Fig. 2. There are four categories of genes that control the expression of allergic disorders, which include ① antigen recognition ② IgE production (down-

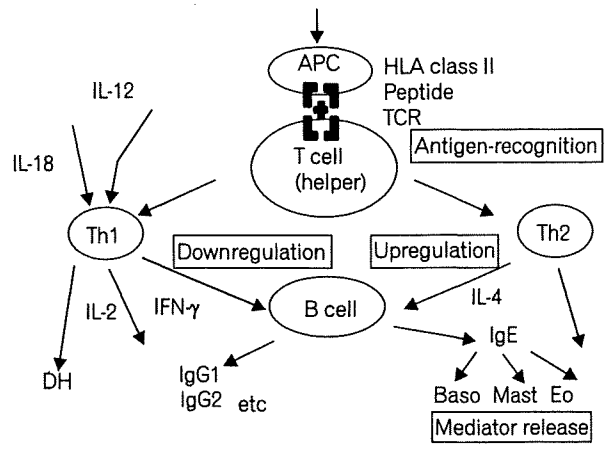

Fig. 1. Th1 and Th2 lymphocyte balance, and upregulation and downregulation of IgE production.

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regulation=brake and upregulation) ③ mediators production and release and ④ events on target organs.

The genetic screening kit, which is used to clarify an abnormal part in an individual patient in a short time, uses the invader method and, based on the allergic genetic classification mentioned above, it has been developed.

A TAILOR-MADE MEDICINE IN ALLERGIC DISORDERS

1. Tailor-made Medicine at Present

There are allergens, infections such as viruses, stress, meteoro-

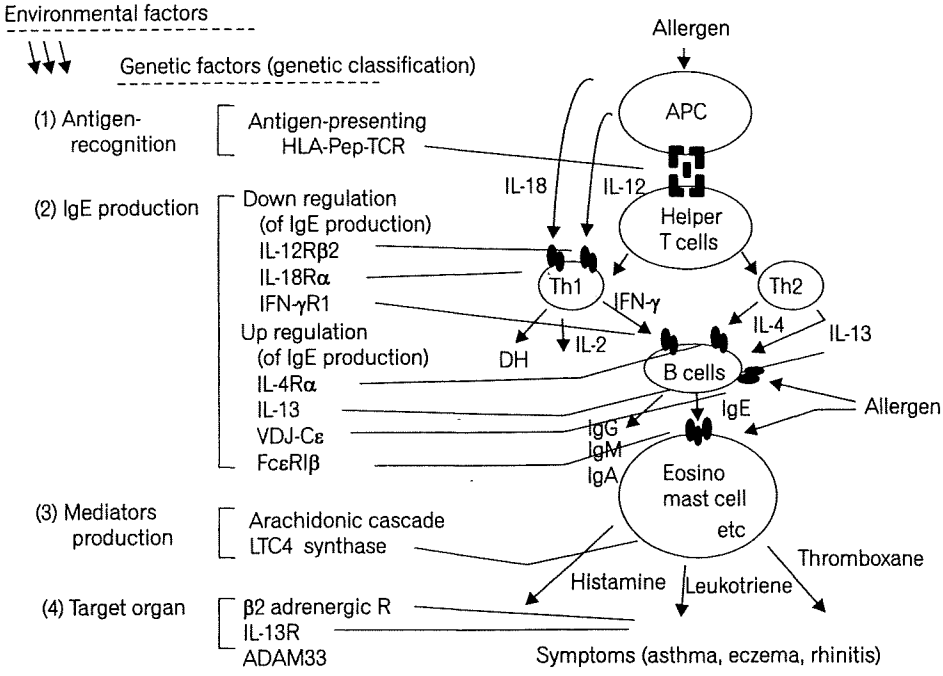

Fig. 2. Genetic factors and a new genetic classification of atopy (by Kondo N).

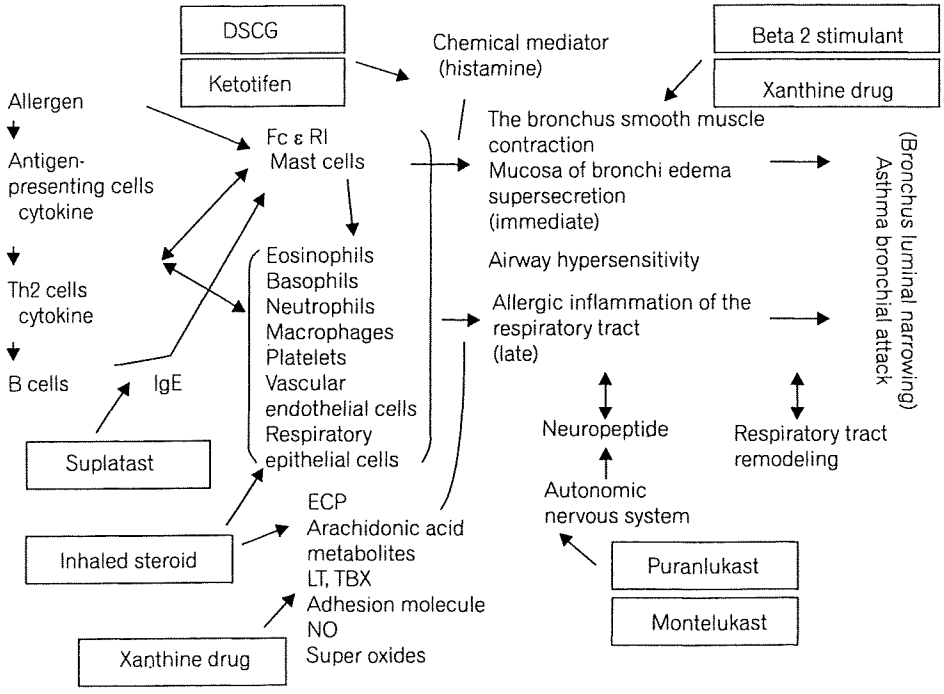

Fig. 3. Mechanisms of bronchial asthma attack and points of medicine.

logical changes and so on as environmental factors involved in allergic disorders such as bronchial asthma. Within the body, there are such factors as allergic immune reactions, airway hypersensitivities and autonomic nervous system. In the individual patient it is important to make clear which of these factors is dominant, and then tailor-made management should be carried out accordingly.

Bronchial asthma is one of the syndromes, namely, the bronchial asthma syndrome, which occurs due to various factors. There are several antiallergic agents for allergic syndromes but it is extremely important to know them well as tailor-made medicine. In other words, it is necessary to clarify the condition of the individual patient, and then to choose a medicine corresponding to it.

We present the mechanism for an asthma attack (Fig. 3). Chemical mediators such as histamine are produced after an allergen invasion, after which bronchial smooth muscular contraction, hypersecretion, and edema set in, which cause the immediate type of asthma attack. Moreover, various kinds of inflammatory cells contribute to allergic inflammation of the respiratory tract, upon which late and severe asthma attacks occur,

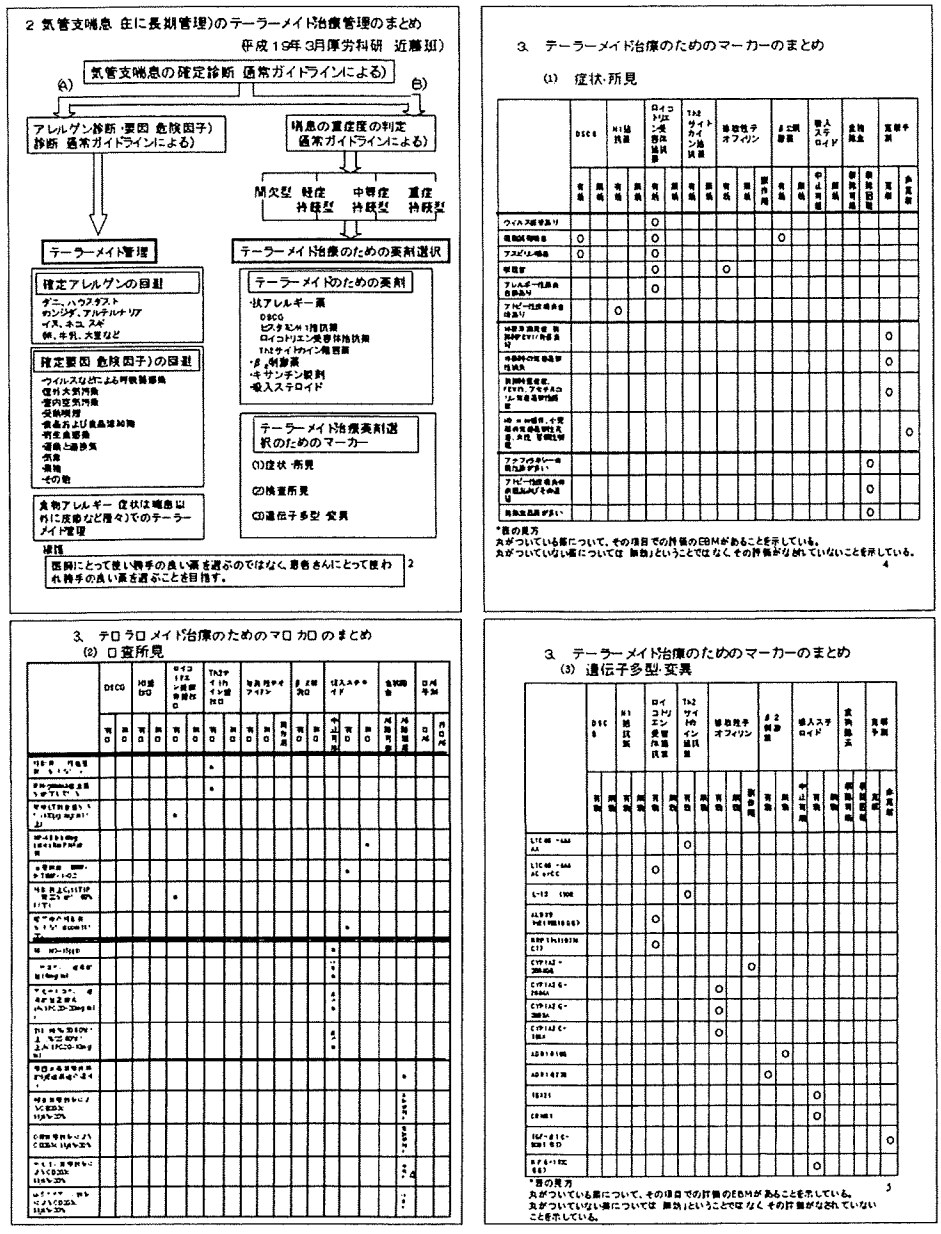

Fig. 4. The manual for the tailor-made medicine of bronchial asthma, edited by Kondo N, et al (Japanese edition. This will be translate to English).

in which cases, arachidonic acid cascades and leukotrienes are very active. In asthma attacks, it is necessary to judge which mechanism is dominant and to use specific anti-asthma agents against each mechanism for each patient. A beta 2 stimulant drug and a xanthine derivative are used for direct cancellation of respiratory tract constriction. For IgE production and immediate asthma attack control, DSCG and ketotifen are used and suplatast is used for Th2 cytokine control. Montelukast and pranlukast, antileukotriene receptors, show effectiveness in case of subjects with allergic inflammation due to arachidonic acid cascade and leukotriene action. General control of allergic imflammation requires steroid inhalation. Prolonged administration of low doses of a xanthine derivative induces antiallergic inflammation action.

The authors measured various kinds of parameters of patients who developed asthma attacks and clarified that the conditions of the patients varied with each individual patient - the IgE system is sometimes dominant, the arachidonic cascades is sometimes dominant, and so on. In the near future, tailor-made treatment will be done based on laboratory tests which analyze the conditions of patients.

2. The Near Future Prospects of Tailor-made Medicine

Based on the genetic classification of atopy and the genetic screening kits, the tailor-made medicine will be developed. Now, we are newly developing the manuals (guidelines) on the tailor-made medicine of bronchial asthma based on the detections for various symptoms, the various immune responses and the various causative candidate genes mutations or polymorphisms. Fig. 4 shows parts of the Japanese edition of the manual. For example, as one of the markers of symptoms, in viral induced asthma, antileukotriene receptors is effective. For example, as one of the markers of labolatory findings, in the poor IFN-γ production, Th2 cytokine controller is effective. For example, as one of the gene markers, in LTC4S-444 AA type, Th2 cytokine controller is effective and, in LTC4S-444 AA type, antileukotriene receptor is effective.

In the near future, the guidelines on the tailor-made medicine of bronchial asthma and allergic disorders will be available.

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Medical and Pharmaceutical Society for WAKAN-YAKU

Review

Current position of Japanese and Chinese medicine in regard to tailor-made medicine -From the viewpoint of allergic disorders-

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Allergic diseases such as bronchial asthma and atopic dermatitis develop by a combination of genetic and environmental factors. Recently treatment / management guidelines on bronchial asthma and many other disorders have been published, and they are utilized in clinical practice. However, the clinical features of the patients and the causes of the diseases vary. Therefore, personalized medical care (tailor-made medicine) is necessary for the improvement of QOL.

We described the current position of Japanese and Chinese medicine in regard to tailor-made medicine from the viewpoint of allergic disorders, including the results of research concerning genome and post-genome factors (pharmacogenomics, pharmacoprotemics and pharmacometabonomics) by the authors.

Key words Japanese and Chinese medicine, tailor-made medicine, allergic disorders.

Introduction

We describe the current position of Japanese and Chinese medicine in regard to tailor-made medicine from the viewpoint of allergic disorders.

Allergic diseases such as bronchial asthma and atopic dermatitis develop by a combination of genetic and environmental factors. Recently treatment / management guidelines on bronchial asthma and many other disorders have been published, and they are utilized in clinical practice. However, the clinical features of the patients and the causes of the diseases vary. Therefore, personalized medical care (tailor-made medicine) is necessary for the improvement of QOL.

First we describe tailor-made medicine for allergic diseases, including the results of research concerning genome and post-genome factors (pharmacogenomics, pharmacoproteomics and pharmacometabonomics) by the authors.

Tailor-made medicine at present

(1) Tailor-made management

There are allergens, infections such as viruses, stress, meteorological changes and so on as environmental factors involved in allergic disorders such as bronchial asthma. Within the body, there are such factors as allergic immune reactions, airway hypersensitivities and autonomic nervous system. In the individual patient it is important to make clear which of these factors is dominant, and then tailor-made management should be carried out accordingly.

(2) Tailor-made medicine for allergic reactions

Bronchial asthma is one of the syndromes, namely, the bronchial asthma syndrome, which occurs due to various factors. There are several antiallergic agents for allergic syndromes but it is extremely important to know them well as tailor-made medicine. In other words, it is necessary to clarify the condition of the individual patient, and then to choose a medicine corresponding to it.

We present the mechanism for an asthma attack (Figure 1). Chemical mediators such as histamine are produced after an allergen invasion, after which bronchial smooth muscular contraction, hypersecretion, and edema set in, which cause the immediate type of asthma attack. Moreover, various kinds of inflammatory cells contribute to allergic inflammation of the respiratory tract, upon which late and severe asthma attacks occur, in which cases, arachidonic acid cascades and leukotrienes are very active. In asthma attacks, it is necessary to judge which mechanism is dominant and to use specific anti-asthma agents against each mechanism for each patient. A beta 2 stimulant drug and a xanthine derivative are used for direct cancellation of respiratory tract constriction. For IgE production and immediate asthma attack

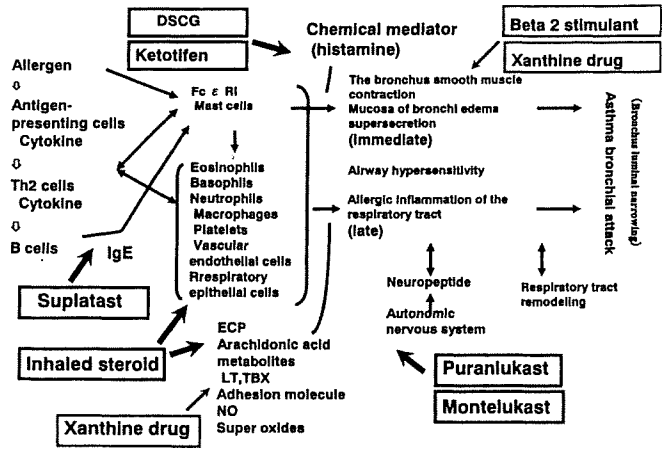

Figure 1 Mechanisms of bronchial asthma attack and points of medicine

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control, DSCG and ketotifen are used and suplatast is used for Th2 cytokine control. Montelukast and pranlukast, anti-leukotriene receptors, show effectiveness in cases of subjects with allergic inflammation due to arachidonic acid cascade and leukotriene action. General control of allergic inflammation requires steroid inhalation. Prolonged administration of low doses of a xanthine derivative induces antiallergic inflammation action.

The authors measured various kinds of parameters of patients who developed asthma attacks and clarified that the conditions of the patients varied with each individual patient -- the IgE system is sometimes dominant, the arachidonic cascade is sometimes dominant, and so on. In the near future, tailor-made treatment will be done based on laboratory tests which analyze the conditions of patients.

(3) Tailor-made management for stress and infection

Stress and viral infections become dominant in some patients. The authors are investigating a gene of the dopamine D4 receptor in patients who show symptoms easily caused by stress. Medical treatment training is very important for such patients. Furthermore, there are patients in whom asthma attacks are easily induced by respiratory syncytial viral and influenza viral infections. It is important for these patients to be treated against such viral infections.

Current position and role of Japanese and Chinese medicine in regard to tailor-made medicine and its development

As with tailor-made medicine, we ascertain each patient's condition in regard to Japanese and Chinese medicine and know both well. YO-shou: A patient's condition can be in a cheerful state, In-shou: a state without spirit, Jitsu-shou: a state with physical strength, Kyo-shou: a weak state without physical strength, and we ascertain whether or not the following symptoms have appeared: Kan: pain from cold, unidentified arthralgia, abdominal pain, hypothermia; Netsu: a slight fever, night sweating, epistaxis; Kyo: anorexia, gastrointestinal disorder, tire easily; Jitsu: good appetite, constipation; and in regard to the mind (Ki): orthostatic syncope (Kikyo), psychosomatic disease (Kitai); blood (Ketsu): adolescent malaise, painful menses; water (Sui): lassitude, mental impairment, dizziness, headache, diarrhea, tire easily. We identify and know these possible factors well and are in accord with the personalized medical treatment of the West (Figure 2, Table 1).

The future prospects of tailor-made medicine

(1) Allergic classification and development of a genetic screening kit and tailor-made treatment -- Pharmacogenomics based on genetic analysis

We genetically classified allergies (atopy) as in Figure 3, based on results reported abroad and our own results: (a) antigen presentation (b) IgE-producing (c) mediator-producing (d) target organ. Of these four stages, the authors reported gene mutations in the antigen presentation stage and IgE-producing inhibition. In regard to the latter, there were IL-12R beta 2-chain mutations,[1-3] IL-12R beta 1-chain mutations, IL-18R α chain abnormality of the genes, and IFN-γ R1 mutations. Furthermore, a mutation in the gene of the leukotriene synthase (-444 polymorphism of the LTC4 synthase) has been reported.

The genetic screening kit, which is used to clarify an abnormal part in an individual patient in a short time, uses the invader method and, based on the allergic genetic classification mentioned above, it has been developed, and the details of the corresponding tailor-made treatment for each patient have been worked out.

The development of a new medicine is as necessary as using an existing remedy properly from such a viewpoint, i. e., tailormaking it. We show this next.

(2) Tailor-made treatment development prospects -- Pharmacoproteomics based on elucidation by structural biomedicine

We mainly elucidated the three-dimensional structure of

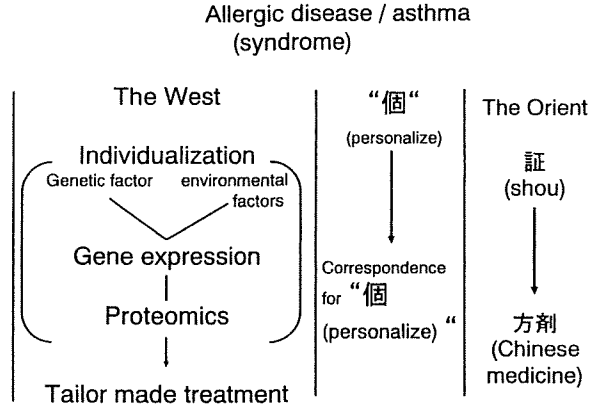

Figure 2 Treatmennt

Table 1 Roles of Japanese and Chinese medicine

(1) Order (tailor) made medicine-shou (証)
(2) Correction of imbalance of immune system, immunomodulator, modulator

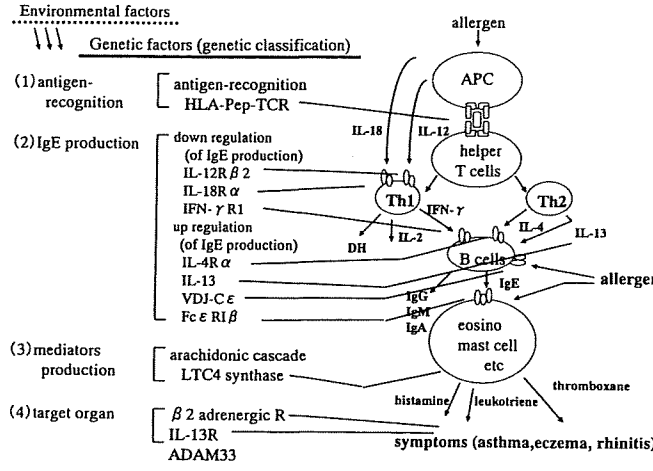

Figure 3 Genetic factors and genetic classification in allergy (by Kondo N)

proteins, and studied the system to understand the structural biology. We understand the 3 dimensions of protein dysfunction in sickness from a three-dimensional structural abnormality viewpoint and aim at applying this to medical care treatment or, more so, to prevention. We, so to speak, aim at studying the system which we call structural biomedicine (structure biological medicine -by authors).

Based on protein structure information, development of a remedy will be made thereafter.

(3) Tailor-made treatment development prospects -- Pharmacometabonomic analysis based on elucidation of genetic molecular ecological medicine

Allergic disorders are developed by a combination of genetic factors and environmental factors. From such a viewpoint, we are investigating the direct relationship between genes and environmental factors in allergies.

For example, it became clear that IFN-γ production and IFN-γ gene expression decreased due to viral infection. IFN-γ production also decreased as PH decreased. In this way the decrease in IFN-γ is induced by both genetic and environmental factors.

Tailor-made medicine is possible on the basis of this pharmacometabonomic viewpoint[4]--environmental and genetic analysis.

Th1 / Th2 imbalance and hygiene hypothesis, and current position of Japanese and Chinese medicine

There are many epidemiologic reports concerning hygiene hypothesis.

For the development of allergic disorders, IgE production and the Th1 and Th2 imbalance are important. These are induced by both genetic and environmental factors. Moreover, this Th1 and Th2 balance may be regulated by regulatory T cells, IL-10 and so on.

In innate immunity, childhood infections such as endotoxin, LPS stimulation through toll-like receptors (TLRs) may induce regulatory T cells and IL-10 production, and these may regulate the Th1/Th2 balance in the acquired immune system (Figure 4). As a result, this prevents the development of allergic disorders. If these stimulations are not

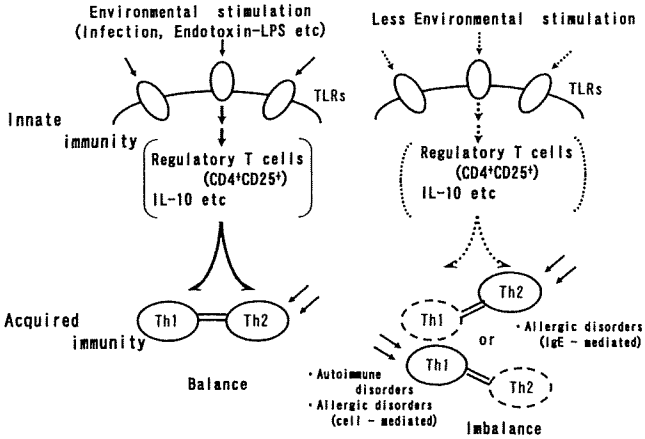

Figure 4 Hygiene hypothesis and Th1 · Th2 imbalance

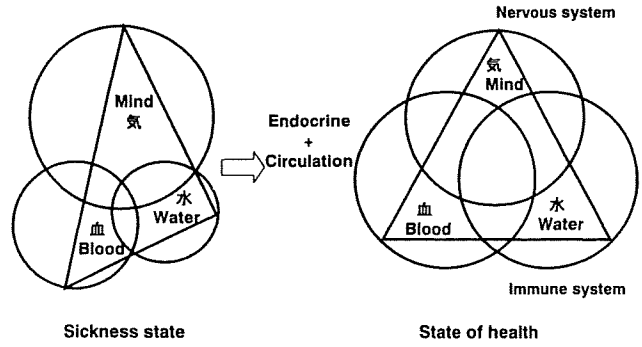

Figure 5 Balance of mind (ki, 気), blood (ketsu, 血) and water (sui, 水)

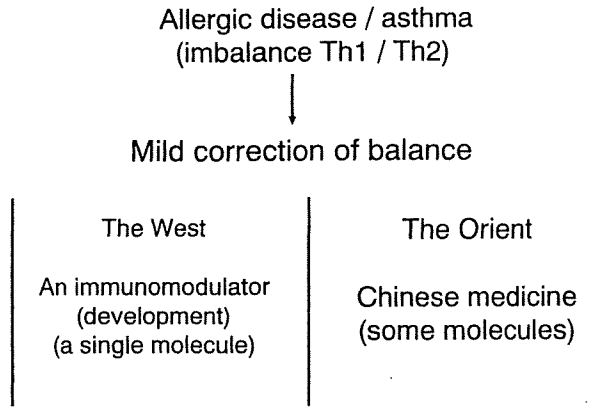

Figure 6 Treatmennt

enough, the result is that the development of regulatory T cells is also not enough, so this can not regulate the Th1 / Th2 balance and can not protect against the development of allergic diseases.

In Japanese and Chinese medicine, the balance of mind / blood / water is important. It is not only for the immune system but also the nervous system and total balance of every living body (Figure 5).

There are immunomodulators to correct the imbalance of Th1 and Th2 in the West. Japanese and Chinese medicine also maintain the balance (Figure 6).

Conclusion

Chinese medicine for shou: the individual state), is the same as tailor-made medicine in the West.

We also correct imbalances of the immune system by an immunomodulator. This is also one of the roles of Chinese medicine.

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ORIGINAL ARTICLE

Lymphocyte Responses to Chymotrypsin- or Trypsin V-Digested β-Lactoglobulin in Patients with Cow's Milk Allergy

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Chymotrypsin- or trypsin V- (a mixture of trypsin and chymotrypsin) digested β-lactoglobulin (BLG) peptides were prepared and were confirmed to have much less immunoglobulin (Ig)G and IgE reactivity compared with intact BLG by IgG inhibition enzyme-linked immunosorbent assay and IgE dot blotting. The lymphocyte responses to intact BLG and these peptides were examined using peripheral blood mononuclear cells (PBMCs) from 10 patients with cow's milk allergy. The PBMCs from most patients had lower lymphocyte responses to chymotrypsin- and trypsin V-digested BLG peptides than those to intact BLG. However, PBMCs from one and two patients retained significant proliferative responses to both peptides and to only the former peptide, respectively. Interferon-γ production stimulated by chymotrypsin-digested peptides was still detectable in all five patients tested. Chymotrypsin-digested BLG reduced IgE reactivity but still induced some lymphocyte responses.

Cow's milk is one of the most common food allergens in the first year of life, with approximately 2 to 2.5% of infants experiencing allergic reactions to it. The majority of children outgrow their allergy to cow's milk before the age of 3 years, but 15% of infants with immunoglobulin (Ig)E-mediated cow's milk allergy retain their sensitivity into the second decade.[1,2]

The therapy for food allergy is a problem that is still to be resolved. The first therapeutic approach to patients with cow's milk allergy is elimination from the diet of cow's milk proteins. However, this is not always easy because cow's milk is an essential constituent of the diet or can be found in other foods as a hidden allergen. Moreover, elimination from the diet may cause nutritional imbalance.

Specific allergen immunotherapy has been shown to be effective in modulating allergic responses in diseases such as rhinitis and asthma.[3,4] However, the ability of whole cow's milk to crosslink mast cell-bound IgE, resulting in anaphylactic reaction, has limited the application of rush immunotherapy with intact cow's milk.

A possible immunotherapeutic approach to cow's milk allergy would be the use of hydrolyzed or enzymatically digested peptides of cow's milk, which can induce immunomodulation by T-cell response but which do not cause IgE-mediated reactions. Even the use of hydrolyzed or digested peptides can cause IgE-mediated reactions if IgE epitopes are still present in the digested peptides. On the other hand, T-cell epitopes may not be retained by hydrolysis or digestion. In the latter case, no immunomodulation is expected.

Generally, IgE antibodies to the various allergen components in cow's milk proteins (such as casein and whey proteins) are present in patients with cow's milk allergy. One of the major allergens in cow's milk is β-lactoglobulin (BLG). It has no homologous counterpart in human milk. In rodents, partially hydrolyzed whey protein and trypsin-digested BLG induced specific immunologic tolerance to BLG.[5,6] These data in mice encouraged us to use enzymatically digested peptides of BLG to induce immunologic tolerance in patients with cow's milk allergy.

In this study, two kinds of BLG peptides digested by chymotrypsin alone or trypsin V (a combination of chymotrypsin and trypsin) were prepared and reduced IgE reactivity was confirmed. For the first step of a possible

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immunotherapy using these peptides, we analyzed the proliferative response of peripheral blood mononuclear cells (PBMCs) to intact BLG and to these digested peptides in 10 patients with cow's milk allergy.

Materials and Methods

Subjects

Fourteen healthy children (age 4 months–12 years; mean age 3.9 years) without cow's milk allergy and 10 children with cow's milk allergy (age 1–6 years; mean age 4.1 years) were studied. Allergic symptoms such as urticaria, erythema, and wheezing occurred in less than 1 hour after cow's milk ingestion in these allergic patients (Table 1). The diagnosis of allergy to cow's milk was based on clinical symptoms after ingestion, including an open challenge test and cow's milk-specific IgE measured by CAPRAST (Sweden Diagnostics). Cow's milk proteins were eliminated from the diets of these patients at the time of investigation.

Preparation of Enzyme-Digested BLG Peptides

The digested peptide fragments were prepared from BLG (Lot 51 H7210, Sigma, St Louis, MO) or edible BLG (WPI, Auckland, New Zealand) as follows. BLG extracts (5 mg/mL) were incubated for 6 hours at 40°C with trypsin V (Biocon Japan, Nagoya, Japan) or chymotrypsin (MP Biomedicals, Irvine, CA) at a final enzyme concentration of 0.1 mg/mL. After digestion, the enzymes were inactivated by incubation for 10 minutes at 100°C.

Sodium Dodecyl Sulphate-Polyacrylamide gel Electrophoresis Analysis of Cow's Milk, BLG, and Peptides

Twenty-microlitre samples of cow's milk (5 μg), BLG (1 μg), and the digested BLG fragments (1 μg) were electrophoresed on a 10 to 20% gradient sodium dodecyl sulphate (SDS)—polyacrylamide gel at 20 mA of constant current until the tracking dye reached the bottom of the gel. The polypetides were visualized by Coomassie blue staining.

High-Performance Liquid Chromatography Gel Filtration of the Digested Peptides

The preparations were subjected to high-performance liquid chromatography gel filtration to analyze the molecular weight distribution of the digested peptides. The preparations were applied to TSKgelG3000PW$_{XL}$ (TOHO, Tokyo, Japan) and eluted with 0.1% trifluoroacetic acid and 55% acetonitrile. The column was calibrated using standard proteins for molecular weights as follows: α-casein (24.5 kD), BLG (18.3 kD), α-lactalbumin (14.2 kD), aprotinin (6.5 kD), β-chain of insulin (3.5 kD), angiotensin II (1.0 kD), and glutathione (0.3 kD). The molecular weight of the peptides was estimated using a calibration curve, plotting the logarithm of the molecular weight against retention time.

IgG Inhibition Enzyme-Linked Immunosorbent Assay

A 96-well microtitre plate (Maxisorp, NUNC, Denmark) was coated with 100 μL of intact BLG at 1 μg/mL in 0.05 M

Table 1. Characterization of Allergic Patients

							CAP-RAST Values	
Patient	*Sex*	*Age (yr)*	*Symptoms Caused by Cow's Milk Intake*	*BA*	*AD*	*IgE (IU/mL)*	*Milk*	*BLG*
1	M	3	Erythema	−	+	2,200	0.78	0.37
2	F	2	Vomiting, wheezing	+	+	5,000	>100.0	2.0
3	M	5	Wheezing	+	−	1,500	67.0	12.0
4	M	6	Wheezing, urticaria	+	−	88	4.0	<0.34
5	F	4	Wheezing	+	−	120	0.92	<0.34
6	M	4	Wheezing, urticaria	+	+	600	1.2	0.52
7	M	6	Wheezing, urticaria	+	+	510	>100.0	5.2
8	F	2	Wheezing, urticaria	+	−	470	16.0	<0.34
9	M	1	Erythema	+	+	190	10.0	0.86
10	M	3	Wheezing, urticaria	+	+	100	13.0	1.0

AD=atopic dermatitis; BA=bronchial asthma; BLG=β-lactoglobulin; IG=immunoglobulin.

sodium bicarbonate (pH 9.0) overnight at room temperature. After washing four times with 0.1% Tween 20 in phosphate-buffered saline (PBST), blocking was done with 0.4% goat serum for 90 minutes at room temperature. After washing four times with PBST, the wells were incubated with rabbit anti-BLG antisera (50 μL, 1:64,000 dilution) and 50 μL of serially diluted samples (intact BLG, enzyme-digested polypeptides) for 90 minutes at room temperature. After washing four times with PBST, the wells were incubated with a peroxidase-conjugated anti-rabbit IgG antibody (goat) (1:10,000 dilution). After washing four times with PBST, the plate was developed with a peroxidase substrate buffer. After 15 minutes, the reaction was stopped with 25 μL of 4N H_2SO_4 and the OD was measured at 490 nm. The percentage of inhibition was calculated as (total reactivity − remaining reactivity after absorption) × 100/total reactivity.

IgE Dot Blotting

Twenty micrograms of protein was applied onto nitrocellulose filter paper (0.2 μm, BioRad, CA). After washing and blocking with 5% bovine serum albumin (BSA) in PBS, the membranes were incubated with sera from controls who had no specific IgE for cow's milk or BLG or patient 2, who had specific IgE for BLG (1:20 dilution). The membranes were then treated with alkaline phosphatase-conjugated monoclonal antihuman IgE (GE-1, Sigma, 1:500 dilution) and colour-developed by a 5-bromo-4-chloro-3-indol phosphate and nitro blue tetrazolium solution (Sigma).

Antigen-Induced Proliferative Responses of the PBMCs

PBMCs were isolated from heparinized blood from control donors and patients by gradient centrifugation in Ficoll-Paque (Pharmacia AB, Uppsala, Sweden). PBMCs were cultured with BLG or the digested peptides at a concentration of 20 μg/mL at 37°C in a 5% CO_2-humidified atmosphere for 5 days. Proliferative responses to food antigens were performed as previously described.[7] Briefly, these assays were performed in triplicate in 96-well, flat-bottomed microtitre plates (Nunclon, Roskike, Denmark) by using 2×10^5 cells per well in a total volume of 200 μL. The culture medium consisted of RPMI 1640 (Sanko Junyaku Co., Ltd, Tokyo, Japan) supplemented with 10% pooled human AB serum (Cambrex Bio Science Walkersville Inc., Walkersville, MD), L-glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL). Proliferation was measured by [3H]-thymidine incorporation (0.5 μCi/well) during the last 16 hours of culture. Proliferation response was measured as the stimulation index (SI) by using the following formula: counts per minute (cpm) incorporated into antigen-stimulated cultures/cpm incorporated into medium control.

Antigen-Induced Interferon-γ Production

Culture supernatants of PBMCs stimulated with intact BLG or the digested peptides for 5 days, as described above, were spun to remove PBMCs and the supernatants were frozen at −30°C until assay. Interferon-γ (IFN-γ) concentration was measured with the use of a human IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (JIMRO, Takasaki, Japan); the detection limit was 15.6 pg/mL.

Statistical Analysis

Student's *t*-test was used to determine significant differences in the SI between healthy controls and subjects with cow's milk allergy.

Results

Subjects

As shown in Table 1, we analyzed 10 patients with cow's milk allergy. Allergic symptoms such as urticaria, erythema, and wheezing occurred in less than 1 hour after cow's milk ingestion in these patients. All patients had cow' milk-specific IgE, and seven of them had BLG-spec : IgE, examined by CAP-RAST.

Preparation of BLG Peptides Digested by Chymotrypsin or Trypsin V

We focused on the allergenicity of BLG and made two kinds of BLG peptides. We used chymotrypsin and trypsin V to make BLG peptides. Trypsin V is a mixture of chymotrypsin and trypsin. Figure 1 shows the digestive sites of BLG polypeptides by these enzymes. Digestion with chymotrypsin resulted in small peptides ranging from 3- to 31-amino acid residues whereas digestion with trypsin V produced smaller peptides ranging from 1- to 20-amino acid residues. As expected, these digested polypeptides were hardly visualized by 10 to 20% gradient SDS-polyacrylamide gel electrophoresis (Figure 2A). Figure 2B

Chymotrypsin

LIVTQTMKGLDIQKVAGTW↓Y↓SLAMAASDISLLDAQSAPLRVY↓
VEELKPTPEGDLEILLQKW↓ENDECAQKKIIAEKTKIPAVF↓KIDA
LNENKVLVLDTDY↓KKY↓LLF↓CMENSAEPEQSLVCQCLVRTPEV
DDEALEKF↓DKALKALPMHIRLSF↓NPTQLEEQCHI

Trypsin V

LIVTQTMK↓GLDIQK↓VAGTW↓Y↓SLAMAASDISLLDAQSAPLR↓VY↓
VEELK↓PTPEGDLEILLQK↓W↓ENDECAQK↓K↓IIAEK↓TK↓IPAVF↓K↓
IDALNENK↓VLVLDTDY↓K↓K↓Y↓LLF↓CMENSAEPEQSLVCQCLVR↓
TPEVDDEALEK↓F↓DK↓ALK↓ALPMHIR↓LSF↓NPTQLEEQCHI

Figure 1. β-Lactoglobulin peptide fragments generated by chymotrypsin or trypsin V digestion. Digestive sites by chymotrypsin and trypsin are shown by *arrows* and *open arrows*, respectively. Trypsin V is a mixture of chymotrypsin and trypsin.

shows the results of gel filtration column chromatography of intact BLG and its digested peptides by these proteases. Both preparations retained polypeptides with a molecular weight of around 1.6 kD. We used these peptides for further analyses.

IgG and IgE Binding Capacity of BLG Polypeptides

We first confirmed the reduced antigenicity of these BLG polypeptides by IgG inhibition ELISA using rabbit anti-BLG antisera (Figure 3A). Intact BLG effectively inhibited binding of the anti-BLG antibody in a dose-dependent manner, and both chymotrypsin-digested peptides and trypsin V-digested peptides had similar inhibitory capacities, but they inhibited binding of the anti-BLG antibody much less than intact BLG. As shown in Figure 2A, the digested peptides were too small to separate in SDS-PAGE, so we employed IgE dot blot analysis instead of IgE immunoblot, using the controls' and patient 2's sera. As shown in Figure 3B, IgE binding capacity was reduced in chymotrypsin-digested peptides and trypsin V-digested peptides, compared with intact BLG.

Antigen-Induced Proliferative Responses of the PBMCs

As shown in Figure 4, the SI with BLG at concentrations of 20 μg/mL in PBMCs from 14 healthy controls without

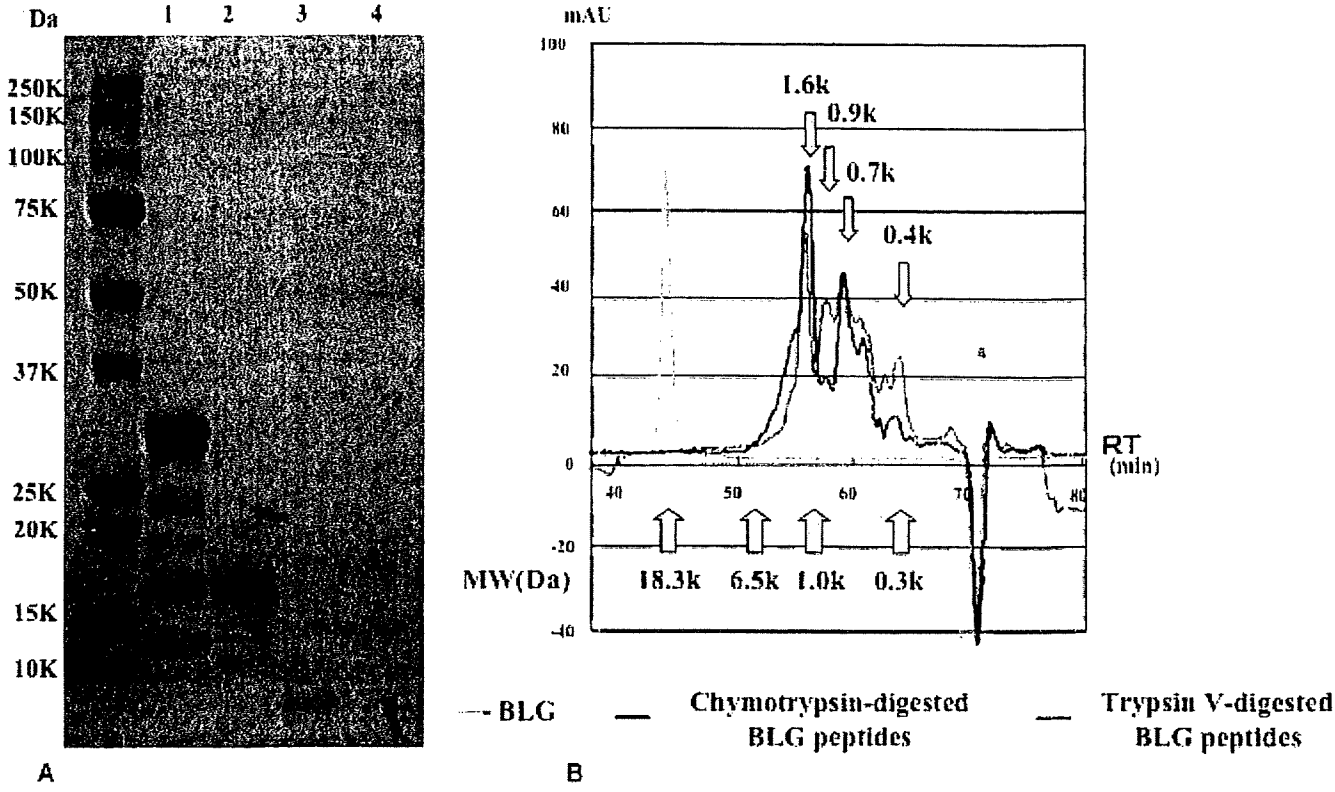

Figure 2. Characterization of digested β-lactoglobulin (BLG) peptides. *A*, Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis analysis using a 10 to 20% gradient SDS-polyacrylamide gel. Polypeptides were visualized by Coomassie blue staining. Lane 1, 5 μg of cow's milk protein; lane 2, 1 μg of BLG; lane 3, 1 μg of chymotrypsin-digested BLG peptides; lane 4, 1 μg of trypsin V-digested BLG peptides. *B*, Gel filtration analysis of BLG and its digested peptides. Calculated molecular weights for several peaks of peptides are shown above the peaks.

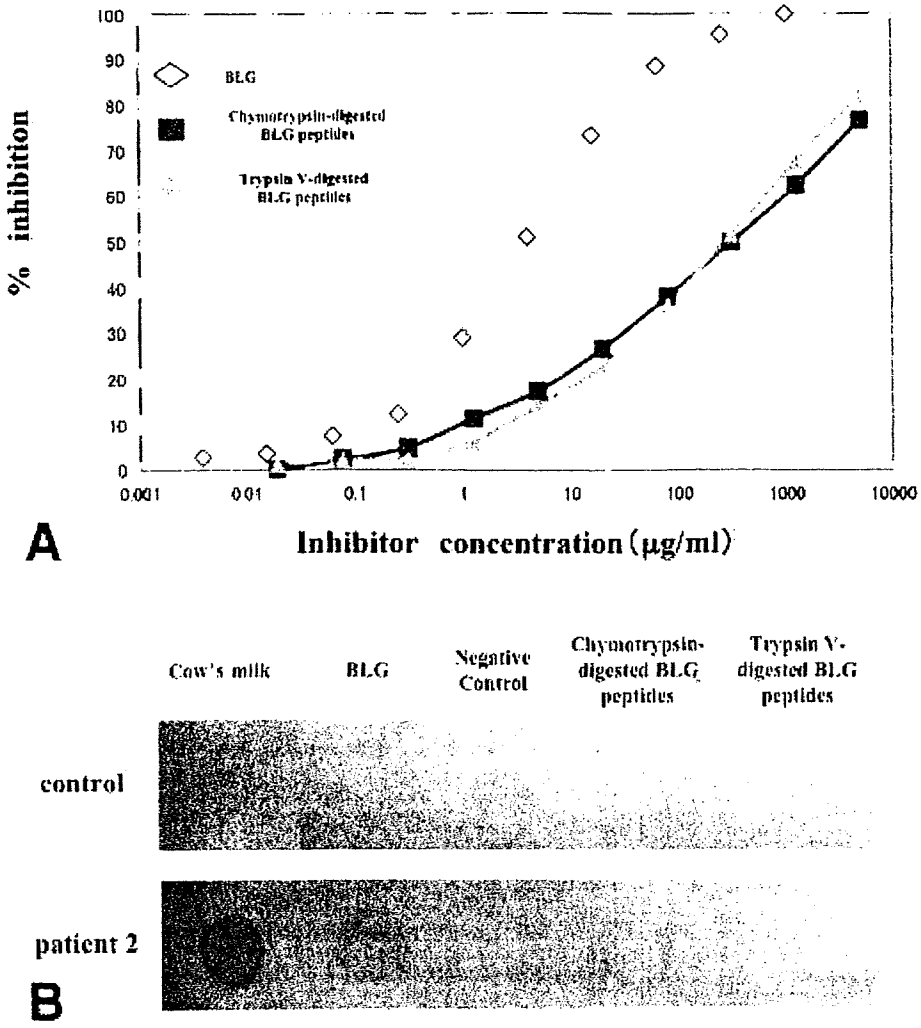

Figure 3. Evaluation of reduced B-cell epitopes in digested peptides. *A*, Immunoglobulin (Ig)G inhibition enzyme-linked immunosorbent assay (ELISA). Various amounts of intact β-lactoglobulin (BLG), chymotrypsin-digested BLG polypeptides, or trypsin V-digested polypeptides were co-incubated as inhibitors with rabbit anti-BLG antisera in ELISA assay. *B*, IgE dot blotting. Twenty micrograms of cow's milk, BLG, extensively hydrolyzed BLG (as a negative control), chymotrypsin-digested BLG polypeptides, and trypsin V-digested BLG polypeptides were applied onto nitrocellulose filter paper. Control and patient 2's sera were used as first antibodies in IgE dot blotting.

cow's milk allergy was 1.36 ± 0.42. PBMCs from 3 healthy controls and 10 patients with milk allergy were stimulated by intact BLG and the digested peptides. The SI with BLG was significantly higher in PBMCs from all patients than those from healthy controls. These results indicate that the proliferative response to BLG was present even in PBMCs from patients with cow's milk allergy whose CAP-RAST score for BLG was zero. The SI with chymotrypsin-digested BLG peptides was much lower than that with intact BLG in PBMCs from all patients except for patient 8. The SI with chymotrypsin-digested peptides in PBMCs from only patients 7, 8, and 9 was significantly higher than that in PBMCs from healthy controls. Moreover, the SI with trypsin V-digested polypeptides tended to be similar to or lower than that with chymotrypsin-digested ones. PBMCs from patients 7 and 9 retained a significantly high SI with chymotrypsin-digested BLG peptides but not with trypsin V-digested BLG polypeptides. PBMCs from patient 8 retained a significantly high SI with trypsin V-digested BLG peptides, as well as chymotrypsin-digested ones.

IFN-γ Production from Stimulated PBMCs

IFN-γ production from PBMCs stimulated with intact BLG or chymotrypsin- or trypsin V-digested BLG peptides were also examined (Figure 5). Supernatants of the culture media from 5 of the 10 patients were available for this experiment. IFN-γ production by either stimulation was under a detection limit in culture supernatants from a healthy control. IFN-γ production was also not detected in supernatants with no stimulation but was induced in supernatants with intact BLG stimulation from all patients tested. IFN-γ production by stimulation with the chymotrypsin-digested peptides was less than with intact BLG but was still at detectable levels in supernatants from all patients tested. IFN-γ production by stimulation with

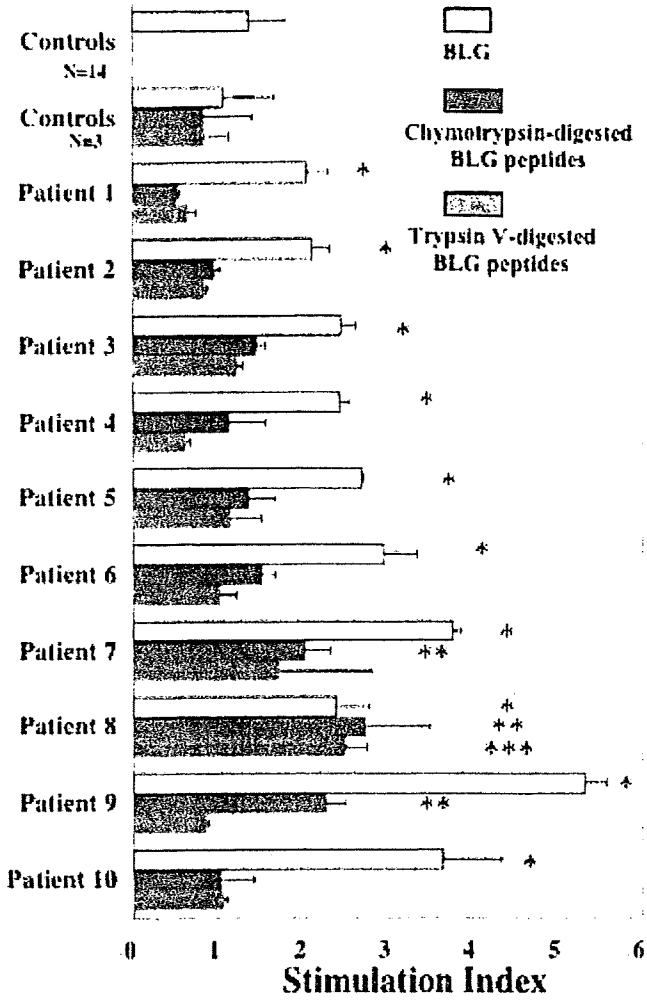

Figure 4. Peripheral blood mononuclear cells' (PBMCs) proliferative response to β-lactoglobulin (BLG) and digested peptides. PBMCs from 10 patients with cow's milk allergy were stimulated by BLG or digested peptides at a concentration of 20 μg/mL for 5 days. Proliferation was measured by [3H]-thymidine incorporation during the last 16 hours of culture. Proliferative response is shown as stimulation index. *, **, and *** indicate significantly higher stimulation index by stimulation with intact BLG, chymotrypsin-digested peptides, and trypsin V-digested peptides, respectively, than that in healthy controls ($p < .05$).

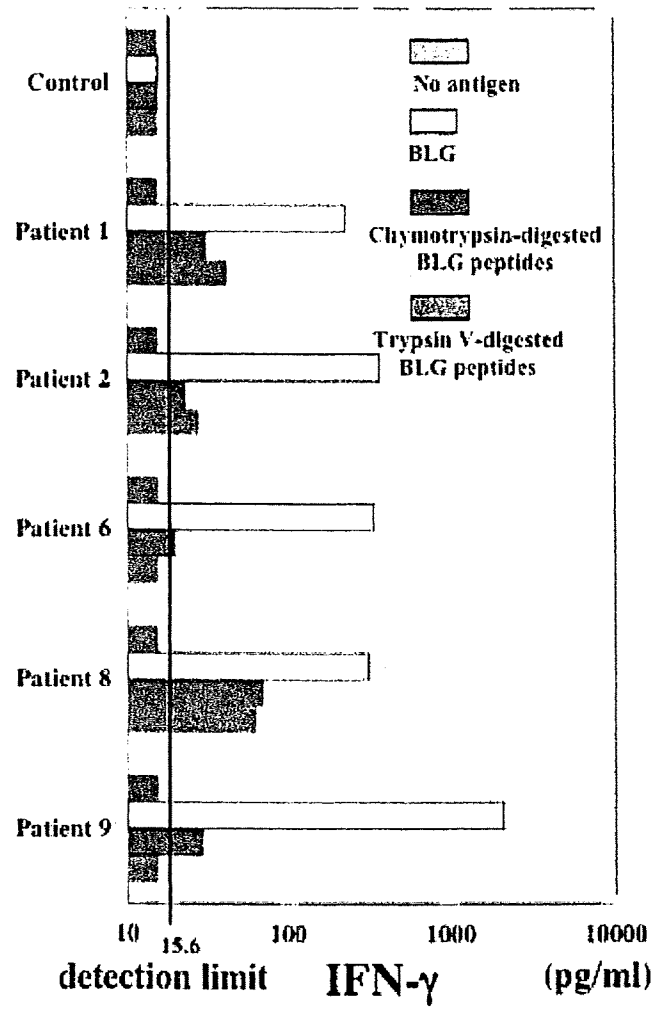

Figure 5. Interferon-γ (IFN-γ) production from peripheral blood mononuclear cells (PBMCs) stimulated by β-lactoglobulin (BLG) and digested peptides. PBMCs from 5 of the 10 patients with cow's milk allergy were stimulated by BLG or digested peptides at a concentration of 20 μg/mL for 5 days. IFN-γ was assayed in the supernatants of cultured PBMCs.

trypsin V-digested peptides was under a detection limit in supernatants from patients 6 and 9.

Taken together, lymphocyte responses to chymotrypsin-digested peptides were lower than those to intact BLG in all patients with cow's milk allergy, but lymphocyte responses were still retained significantly in some patients.

Discussion

In this article, we focused on one of the major allergens, BLG, in cow's milk, made chymotrypsin- or trypsin V-digested BLG peptides. Then we confirmed reduced IgE reactivity and analyzed the lymphocyte responses to these peptides compared with the responses to intact BLG in patients with cow's milk allergy as the first step in immunotherapy with enzyme-digested BLG peptides.

Most cases of immediate hypersensitivity to cow's milk are mediated by IgE specific to cow's milk constituents. After a patient with immediate cow's milk hypersensitivity ingests cow's milk, the allergens cross-link cow's milk-specific IgE bound to mast cells or basophils to induce the release of multiple mediators involved in immediate hypersensitivity reactions. The conservative therapeutic approach to such patients is the elimination of cow's milk proteins from the diet.

The progressive therapy for cow's milk allergy is to induce tolerance by immunomodulation. Oral desensitization using intact cow's milk was reported in a few patients with cow's milk allergy.[8] It took 4 to 8 months with increasing doses of milk intake. During the desensitization process, some mild side effects, such as angioedema and worsening of atopic dermatitis, were reported. We also performed oral desensitization with a similar protocol and experienced some reactions of immediate hypersensitivity during the therapy (unpublished observation). The ability of whole cow's milk to cross-link mast cell-bound IgE, resulting in anaphylactic reactions, has limited the application of rush immunotherapy with intact cow's milk.

Another possible immunotherapeutic approach to cow's milk allergy would be the use of hydrolyzed or enzymatically digested peptides of cow's milk constituents, which can induce immunomodulation by T-cell response but which do not cause IgE-mediated reactions. There are several formulas for milk allergy. However, the concept for these formulas is quite different from our concept. These formulas were made so as not to induce allergic reactions, so extensive hydrolysis was done to destroy the T-cell epitope and the B-cell epitope. There are several lines of evidence for the effectiveness of such enzyme-digested polypeptides. Chymotrypsin treatment of rye grass pollen induced potent T-cell responses but no B-cell responses in a murine model.[9] Pepsin-derived fragments of BSA, which preserved T-cell epitopes, favoured immune suppression rather than the helper T-cell function.[10] The clinical relevance of this approach was also reported in ragweed-sensitive patients.[11] In this study, pepsin-digested ragweed extract was as effective as crude ragweed in the treatment of ragweed-sensitive patients and the immediate skin test activity of the peptic fragments was 1,000-fold less than that of the original crude ragweed. The digested ragweed extract was more effective than the original intact ragweed in relieving clinical symptoms caused by ragweed.

In experimental models, induction of oral tolerance to cow's milk proteins using hydrolyzed peptides of BLG was investigated in detail.[12,13] In Balb/c mice, the lengths of potentially tolerogenic trypsin-digested BLG peptides were distributed between 8 and 23 amino acids.[12] Feeding of partially hydrolyzed formulas has been demonstrated to allow the induction of oral tolerance in a rat experimental model whereas extensively hydrolyzed formulas could not.[13] In the literature, the tolerogenic peptide size is around 20 amino acids.[14–16] These tolerogenic peptide sizes are in accord with the fact that peptides with 12 to 20 amino acids presented with human leukocyte antigen (HLA) complex class II molecules on the surface of antigen-presenting cells are recognized by T cells.[17] The presence of T-cell epitopes is essential for tolerogenic peptides because immunomodulation is induced by T cells.[18]

We chose BLG as a target protein and chymotrypsin as a digestive protease. BLG is one of the major allergens in cow's milk, and its molecular size is smaller than that of casein. Chymotrypsin digestion gives six peptides with 12- to 22-amino acid residues, which may be presented with the HLA type II molecule on antigen-presenting cells and which hence have possible tolerogenic capacities, as discussed above, although a 31-amino acid peptide, an 11-amino acid peptide, two 3-amino acid peptides, and one amino acid are also generated (see Figure 1). Trypsin digestion gives smaller peptides than chymotrypsin digestion (see Figure 1). For the comparison, we also used trypsin V-digested BLG peptides. Since trypsin V contains a mixture of trypsin and chymotrypsin, the resultant peptides were smaller than peptides digested by chymotrypsin or trypsin.

Lymphocyte proliferative response is a useful tool for the evaluation of food allergy, especially food-sensitive atopic dermatitis.[7] This response requires both T cells (predominantly CD4 lymphocytes) and monocytes as antigen-presenting cells.[19] Since lymphocyte proliferative response measures T-cell proliferation, which responds to interaction among HLA class II peptide (T-cell epitope) T-cell receptors, this assay is commonly used for the evaluation of T-cell epitopes.[20–25] IFN-γ is a cytokine produced by T lymphocytes, which are stimulated by interleukin-12 secreted from antigen-presenting cells. IFN-γ secretion from PBMCs is also commonly used for the evaluation of T-cell response to food allergens and their peptides.[26–28]

PBMCs from 10 patients with cow's milk allergy had a significantly higher proliferative response to BLG than those from healthy controls. We first had expected that most PBMCs from these patients could also have given a significant proliferative response to chymotrypsin-digested peptides. However, chymotrypsin digestion reduced lymphocyte proliferation compared with intact BLG in 9 of the 10 patients, and only 3 of them showed significant proliferation. Trypsin V digestion, as expected, reduced a proliferation response more than chymotrypsin digestion. IFN-γ production from PBMCs with no stimulation was under a detection limit, but IFN-γ production from PBMCs stimulated by the chymotrypsin-digested peptides was detectable in all of the five patients available for this assay. PBMCs from patients 1, 2, and 6 did not show a significant proliferative response to the

chymotrypsin-digested peptides but had detectable IFN-γ production with stimulation by chymotrypsin-digested peptides. These facts suggested that chymotrypsin digestion reduced lymphocyte responses but still retained some T-cell responses in some patients with cow's milk allergy.

In a previous study, we demonstrated that T-cell clones specific to BLG (YA4, HA5.7), which were established from patients with cow's milk allergy, needed, as a minimum, peptide BLGp102–112 (YLLFCMENSAE) when presented with HLA-DRB1*0405 to proliferate.[21] Unfortunately, chymotrypsin digestion does not retain this T-cell epitope. This may be one of the reasons why lymphocyte responses to chymotrypsin-digested BLG peptides became lower than those to BLG.

Finally, evaluation of the residual B-cell epitope in chymotrypsin-digested BLG is necessary for the application of immunomodulation therapy with the peptides. We performed inhibition ELISA using rabbit anti-BLG antisera and IgE dot blotting using the sera of patients who had BLG-specific IgE. These experiments clearly showed reduced B-cell epitopes in the digested polypeptides. However, the most reliable evaluation of the absence of the B-cell epitope would be the skin-prick test. We are planning to perform this test prior to clinical application of chymotrypsin-digested BLG peptide therapy.

In conclusion, we made chymotrypsin- or trypsin V-digested BLG peptides and analyzed the lymphocyte responses (predominantly T-cell responses) to these peptides. Chymotrypsin digestion decreased the lymphocyte responses compared with intact BLG but retained significant responses in PBMCs from some patients with cow's milk allergy. Hence, chymotrypsin-digested BLG peptides are a possible tool for immunomodulation therapy in some patients with cow's milk allergy,

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A novel polymorphism, E254K, in the 5-lipoxygenase gene associated with bronchial asthma

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Abstract. Cysteinyl-leukotrienes are important pro-inflammatory mediators in bronchial asthma (BA) and are derived from arachidonic acid by the action of 5-lipoxygenase. We identified a novel polymorphism, c.760 G>A (E254K), in exon 6 of the 5-lipoxygenase gene (*5-LO*). This substitution was detected in 11 out of 180 patients with BA, but not in any of the 150 non-allergic subjects. The frequency of c.760 G>A showed a significant difference between BA and non-allergic subjects (P=0.0007). The c.760 G>A polymorphism existed at the surface edge of the C-terminal catalytic domain, and the E-to-K substitution changed the charge of the side chain from negative to positive. Thus, our results suggest that E254K in the *5-LO* might be associated with BA.

Introduction

Bronchial asthma (BA) is a multifactorial genetic disease (1). Cysteinyl-leukotrienes (cys-LTs) play an important pro-inflammatory role in both early- and late-phase asthmatic responses (2). Cys-LTs constitute a class of potent biological mediators of inflammation and anaphylaxis. 5-lipoxygenase is an essential enzyme which catalyzes the first committed steps in the biosynthetic pathway leading to the production of cys-LTs (3-6). The actions of 5-lipoxygenase result in the sequential conversion of arachidonic acid to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and then to leukotriene (LT) A4.

The *5-LO* is located on chromosome 10q11.2 (7). In several studies, the addition of an Sp-1 binding motif (-GGG CGG-) or the deletion of one or two Sp-1 binding motifs in the *5-LO* core promoter, has been associated with reduced gene expression (8,9). In addition, the 5-lipoxygenase-activating protein (FLAP) promoter gene polymorphisms (21A repeat and 18A repeat) were reported to be associated with BA (10). Recent evidence demonstrated that up-regulation of *5-LO* and FLAP mRNAs might be involved in the increased leukotriene synthesis and play an important role in the pathogenesis of BA (11).

In this study we identified single-nucleotide polymorphisms (SNPs) in the *5-LO* and researched the relationship between SNPs in the *5-LO* and BA.

Materials and methods

Patients and non-allergic subjects. One hundred and eighty BA patients (105 males and 75 females, mean age 8.4±7.6 years of age) and 150 non-allergic subjects (90 males and 60 females, mean age 9.5±8.2 years of age) were studied. The diagnosis of BA was made according to the criteria of the American Thoracic Society. The non-allergic subjects were healthy and did not have a history of allergic diseases. All of the subjects were randomly selected from patients attending our hospital. Informed consent was obtained from all individuals or from their parents.

Detection of SNPs in 5-LO. Neutrophils were collected from heparinized blood. Genomic DNA was extracted from neutrophils with a Sepagene kit (Sanko Junyaku, Tokyo, Japan). The fourteen exons of *5-LO* were amplified using the PCR technique and sequenced using an ABI 3100 DNA auto-sequencer (Applied Biosystems, CA) in certain individuals with BA (n=16) and certain non-allergic subjects (n=14). For further study, the E254K substitution was detected in all individuals with BA (n=180) and non-allergic subjects (n=150), and the other three silent polymorphisms (c.21 C>T, c.270 G>A, c.1728 A>G) were detected in 60 individuals with BA and 60 non-allergic subjects. The primer details for the PCR used in the detection of these polymorphisms are shown in Table I.

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Abbreviations: SNP, single-nucleotide polymorphism; 5-LO, 5-lipoxygenase gene; FLAP, 5-lipoxygenase-activating protein; BA, bronchial asthma; cys-LTs, cysteinyl-leukotrienes; PBMCs, peripheral blood mononuclear cells; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; ICM, Internal Coordinate Mechanics; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; c., cDNA

Key words: single-nucleotide polymorphism, 5-lipoxygenase gene, bronchial asthma, leukotrienes

Table I. Primer details for PCR used to detect 4 polymorphisms.

Primer	Sequence	Amplified product size	Annealing temp (°C)
c.21 C>T FP	5'CGCCATGCCCTCCTACAC3'	150 bp	56
c.21 C>T RP	5'CCACGCTCGAAGTCGTTGTA3'		
c.270 G>A FP	5'GTGCCACAGCAGCATACCT3'	401 bp	55
c.270 G>A RP	5'CCTGCACAGCAGTGTCATTC3'		
c.760 G>A (E254K) FP	5'CCTGGTAGAGCGGGTCATGAATC3'	179 bp	62
c.760 G>A (E254K) RP	5'ACCTCCTGCTCCAAGGGGAGCT3'		
c.1728 A>G FP	5'GAAAGAGGATGGACGGACTG3'	295 bp	55
c.1728 A>G RP	5'CTCGTTTTCCTGGAACTGGC3'		

FP, forward primer; RP, reverse primer.

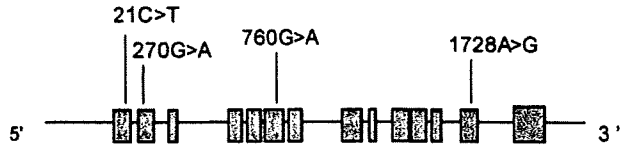

Figure 1. Gene structure and polymorphisms investigated in the *5-LO*. The positions marked were found in polymorphisms in the Japanese population.

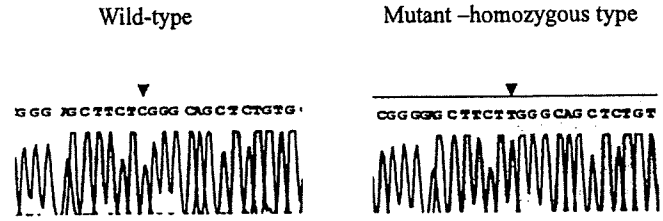

Figure 2. Big Dye terminator DNA sequence data of E254K in the reverse chain of *5-LO*. The data of a patient with E254 (wild-type) are shown on the left and the data of a patient with K254 (mutant-homozygous type) are shown on the right.

Measurement of urinary LTE4. Urine samples from 14 non-allergic subjects and 16 individuals with BA (13 without E254K and 3 with E254K), were stored at -80°C, and analyzed within 1 month of collection. The urinary creatinine level was determined by a creatinine test kit (Pure Auto S CRE-L, Daiichi-kagaku, Tokyo, Japan). Urinary LTE4 concentrations determined by EIA were corrected for recovery of [^{3}H]-LTE4. The urinary LTE4 level was expressed as pg/mg of creatinine.

Measurement of LTB4 production from neutrophils. Neutrophils were collected from heparinized blood in 14 non-allergic subjects and 16 individuals with BA (13 without E254K and 3 with E254K), and 2x10^6 cells/ml were cultured in an RPMI-1640 medium with 15% fetal calf serum, after stimulation with 1 μM ionomycin. At 0, 15 and 30 min after adding ionomycin, we aspirated 1 ml of the culture medium and stored it at -80°C for the measurement of LTB4. We used the LTB4 Immunoassay (R&D Systems Inc., Minneapolis, MN) to quantify the LTB4 concentration in a supernatant from the cultured ionomycin-stimulation neutrophils (12). The samples were applied to the C18 reverse-phase column, and were measured by ELISA.

Relative expression of 5-lipoxygenase mRNA. PBMCs were isolated from the heparinized blood of the 14 non-allergic subjects and 16 individuals with BA (13 without E254K and 3 with E254K) by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden) and stored at -80°C for the extraction of mRNA. We quantified the relative expression of 5-lipoxygenase mRNA by real-time PCR. The real-time PCR was carried out with a Light Cycler instrument (Roche, Mannheim, Germany) by using the Light Cycler SYBR-Green I RNA Master Kit (Roche). Each PCR cycle included denaturation at 95°C for 10 sec, primers annealing at 57°C for 10 sec, and a final extension at 72°C for 8 sec. The cDNA was amplified using the following primers: sense primer 5'actgg aaacacggcaaaaac3' in exon 3 and anti-sense primer 5'tcac ggggtaaatccttgtg3' in exon 4. The size of the PCR product was 96 bp and the intron size between these two primers was >26 kb (7). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for real-time quantitative PCR and we analyzed the relative expression of 5-lipoxygenase mRNA by the $2^{-\Delta\Delta Ct}$ method (13).

Homology structural model of 5-lipoxygenase E254 and K254. An initial homology model structure of 5-lipoxygenase was made by a FUGUE (http://www-cryst.bioc.cam.ac.uk/~fugue/) server. Coral 8R-lipoxygenase (2fnq) was selected as the most homologous template of 5-lipoxygenase from the Protein Data Bank (www.rcsb.org/pdb). The target-template identity rate is 39%. On the basis of this initial structural model, we optimized the structure and performed energy minimization using Internal Coordinate Mechanics (ICM) Pro version 3.3 (MolSoft). Furthermore, we generated the mutational form of this structure using the same software package. The validity of these structural models was evaluated by Ramachandran plot. The surface electrostatic potentials of these wild and mutant structures were calculated using Molmol software.

Table II. Allele and genotype frequencies of *5-LO* silent SNPs.

	Non-allergic subjects (n=60)	Bronchial asthma patients (n=60)	P-value
Allele frequency			
c.21 C>T			
C	108 (0.9)	110 (0.917)	
T	12 (0.1)	10 (0.083)	0.824
c.270 G>A			
G	115 (0.958)	119 (0.992)	
A	5 (0.042)	1 (0.008)	0.213
c.1728 A>G			
A	113 (0.942)	116 (0.967)	
G	7 (0.058)	4 (0.033)	0.539
Genotype frequency			
c.21 C>T			
CC	48 (0.8)	52 (0.867)	
CT	12 (0.2)	6 (0.100)	0.463
TT	0	2 (0.033)	
c.270 G>A			
GG	55 (0.917)	59 (0.983)	
GA	5 (0.083)	1 (0.017)	0.207
AA	0	0	
c.1728 A>G			
AA	53 (0.883)	56 (0.933)	
AG	7 (0.117)	4 (0.067)	0.529
GG	0	0	

Table III. Allele and genotype frequencies of *5-LO* missense SNP.

	Non-allergic subjects (n=150)	Bronchial asthma patients (n=180)	P-value
Allele frequency			
c.760 G>A			
G	300 (1)	348 (0.967)	
A	0	12 (0.033)	0.0007
Genotype frequency			
GG	150 (1)	169 (0.9400)	
GA	0	10 (0.0560)	0.0170
AA	0	1 (0.0056)	

Statistical analyses. Allele and genotype frequencies were calculated for each locus and tested for Hardy-Weinberg equilibrium. Distribution of the genotype of E254K in the *5-LO* was analyzed by Fisher's exact test. Probability (P) values <0.05 were considered statistically significant. The significance of difference was analyzed by the two-sample t-test.

Results

Polymorphisms in the 5-LO. We identified 4 SNPs in the *5-LO* in individuals with BA (Fig. 1). Three SNPs were silent polymorphisms: c.21 C>T (exon 1), c.270 G>A (exon 2) and c.1728 A>G (exon 13). There were no differences in the frequencies of the three SNPs between individuals with BA and non-allergic subjects (Table II).

One SNP was a missense polymorphism c.760 G>A, and the amino acid at 254 changed from Glu (E) to Lys (K) (Fig. 2). We determined the prevalence of c.760 G>A (E254K) in the *5-LO* of individuals with BA and non-allergic subjects. This SNP was found in 11 (0.061) out of the 180 individuals with BA. One was homozygous AA and 10 were heterozygous GA (2 out of the 10 were brother and sister). The mutant allele frequency was 0.033 in 180 individuals with BA. However, the mutant allele could not be detected in any of the 150 non-allergic subjects (Table III). There was a significant difference in the E254K frequency between