

厚生労働科学研究費補助金（免疫アレルギー疾患予防・治療研究事業）
分担研究報告書
アレルギー疾患の治療反応性予測因子の確立及びテラーメイド治療法の確立に関する研究

分担課題名：遺伝子情報の網羅的解析、および小児喘息におけるロイコトリエン受容体拮抗薬の
治療反応性予測遺伝子因子とテラーメイド治療開発

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研究要旨 近年、気管支喘息、アトピー性皮膚炎などアレルギー疾患の増加が大きな社会問題になっている。本研究の目的は、アレルギー疾患の病因・病態および治療反応性予測因子を系統的に検出できる遺伝子診断キットを中心とする診断システムを開発することおよび、その診断をもとにして各病因・病態に合致したテラーメイド治療法を確立することである。多数報告のみられるアレルギー関連遺伝子多型情報を、臨床レベルで活用するためには、多くのサンプルを迅速に処理する必要がある。これを実現するために、今回試みた遺伝子検出キットは、非常に有用であると考えられた。今後、感受性、特異性を上げるためには、どの遺伝子をどのように組み合わせるべきかについてさらに検討を加えたい。また、さらに、治療への応用として、得られた遺伝子情報を活用し、適切な治療薬を見い出していきたいと考える。

研究協力者

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A.研究目的

近年、気管支喘息、アトピー性皮膚炎などアレルギー疾患の増加が大きな社会問題になっている。本研究の目的は、アレルギー疾患の病因・病態および治療反応性予測因子を系統的に検出できる遺伝子診断キットを中心とする診断システムを開発することおよび、その診断をもとにして各病因・病態に合致したテラーメイド治療法を確立することである。このことにより、良好・適格な治療効果が得られ、さらには、QOLの向上につながると考えられる。

B.研究方法

① アレルギー（アトピー）の病因遺伝子群の系統的、多角的な解明に基づいて、アレルギーの系統的遺伝子診断キットをインバーダーアッセイ法を用いて開

発した。

② 遺伝子検索キットには、IgE産生抑制系のシグナル伝達系に存在するサイトカイン、およびその受容体、メディエーター産生段階としてアラキドン酸カスケードに存在する酵素の遺伝子などの多型を組み合わせた。

③ アレルギーの系統的遺伝子診断キットによる解明をもとに、抗アレルギー薬の中で、ロイコトリエン受容体拮抗薬投薬前後における尿中アラキドン酸排泄量の検討、喘息日誌における症状改善度、QOL票の記載によるQOLの改善について検討を加えた。

（倫理面への配慮）

研究対象者には本研究の内容、方法および予想される結果について十分に説明し十分な理解（インフォームドコンセント）を得た上で採血が行なわれた。また、倫理面でも、結果による不利益は全く生じないか、または配慮が充分になされることから問題がないと判断された。

C. 研究結果

- ① 遺伝子検出キットをインベーターアッセイ法を利用して構築した。
- ② アレルギー症状を有する症例 109 例、アレルギー症状のみられない対照症例 196 例について、解析を行った。IgE 産生に抑制的に働くサイトカイン、サイトカイン受容体の多型のうち、IFN- γ receptor1、IL-12 receptor β 1、IL-12 に存在する多型は、明らかに対照群よりアレルギー群で頻度が高かった。IL-13 多型に関しても A アリルをもつ症例は、対照群よりもアレルギー群で多くみられた (表 1)。

Gene	Mutation	Genotype	アレルギー群 N=109	コントロール群 N=196
IL-12R β 1	M365T	T/T	42	64
		T/C	47	98
		C/C	20	34
IL-12B	G3757T	C/C	37	60
		C/T	48	100
		T/T	24	36
IFN- γ R1	L467F	A/A	98	183
		A/G	11	33
		G/G	0	0
IL-4R α	V50I	G/G	40	71
		G/A	53	90
		A/A	16	35
IL-13	R110Q	G/G	39	95
		G/A	68	81
		A/A	10	20

- ③ ロイコトリエン受容体拮抗薬のうち、モンテルカスト投薬前後における尿中アラキドン酸代謝産物の変化について検討した。対象は、6 歳以上の気管支喘息患者とした。尿中の LTE4 排泄量をモンテルカスト投与前後で検討したところ、効果のみられた症例では、モンテルカスト内服 4 週後に著明に LTE4 排泄量が減少していた (図 1)。

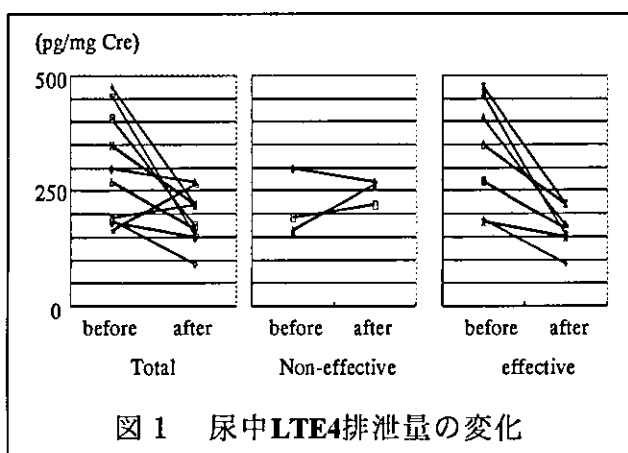


図 1 尿中 LTE4 排泄量の変化

TXB2 排泄量はモンテルカスト内服 4 週後に減少傾向にあった (図 2)。遺伝子多型との関連について検討中である。

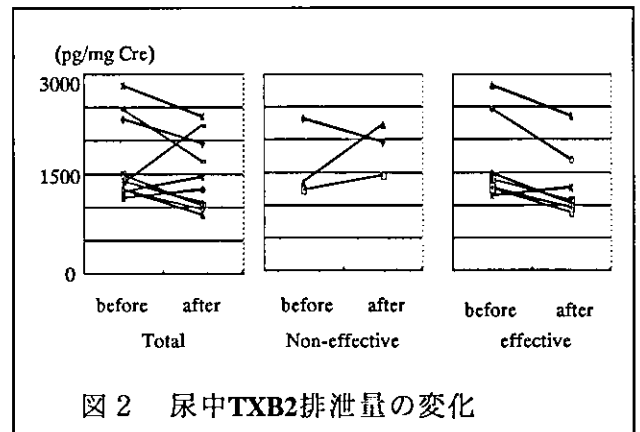


図 2 尿中 TXB2 排泄量の変化

D. 考察

増加を続けるアレルギー疾患患者の個々の病態を遺伝子レベルで整理し、分類することは、テーラーメイド医療を考える上で、非常に重要なことであると考えられる。多数報告のみられるアレルギー関連遺伝子多型情報を、臨床レベルで活用するためには、多くのサンプルを迅速に処理する必要がある。これを実現するために、今回試みた遺伝子検出キットは、非常に有用であると考えられた。今後、感受性、特異性を上げるためには、どの遺伝子をどのように組み合わせるべきかについてさらに検討を加えたい。また、さらに、治療への応用として、得られた遺伝子情報を活用し、適切な治療薬を見い出していきたいと考える。

E. 結論

アレルギー疾患の遺伝子診断キットを開発した。臨床へ応用するため、さらに、今後、診断、治療に着目した感受性、特異性の向上、薬理遺伝学的見地からの検討が必要である。

F. 健康危険情報

なし

G.研究発表

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H.知的財産権の出願、登録状況

1 特許取得

アレルギー素因を規定する遺伝子の検出法：

PBM101(特願 2004-276639)

アレルギーの病因遺伝子としてのロイコトリエンC4

合成酵素遺伝子変異の発見と臨床応用

(岐大研 14—111)

2、実用新案登録 特になし

3、その他 特になし

研究成果の刊行に関する一覧表

雑 註

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IV. 研究成果の刊行物・別冊

RNA editing of interleukin-12 receptor β 2, 2451 C-to-U (Ala 604 Val) conversion, associated with atopy

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Summary

Background The production of IgE in B lymphocytes is down-regulated by IFN- γ . IL-12 induces IFN- γ production by T lymphocytes and natural killer cells by binding to its specific receptor. RNA editing is a post-transcriptional modification.

Objective Here we show that the RNA editing of IL-12 receptor (R) β 2 is associated with atopy.

Methods Atopic patients and non-atopic healthy controls were studied. Fragments of IL-12R β 2 cDNA and genomic DNA were amplified and sequenced. Furthermore, the function of the IL-12R β 2 chain was investigated.

Results Sequence analysis of the cDNA clones representing IL-12R β 2 mRNA transcripts revealed a C-to-U conversion at nucleotide 2451 (Ala 604 Val) on exon 13 in some atopic patients. Surprisingly, sequence analysis of their genomic DNA showed no 2451 C-to-T (Ala 604 Val) mutation. We concluded that the observed C-to-U mismatch in the cDNA clone is due to a post-transcriptional modification, RNA editing. The C-to-U conversion was observed in 21 (20.6%) of 102 atopic patients, whereas this conversion was observed in only 4 (3.8%) of 104 non-atopic subjects ($P < 0.001$). IFN- γ production by peripheral blood mononuclear cells (PBMCs) stimulated with IL-12 in the subjects with the C-to-U conversion was significantly lower than that in the subjects without the C-to-U conversion. In atopic patients with the C-to-U conversion, PBMCs faintly showed the tyrosine phosphorylation of Stat4, and the IgE production by PBMCs was not suppressed by IL-12 whereas it was suppressed by IFN- γ .

Conclusions The RNA editing of IL-12R β 2, 2451 C-to-U (Ala 604 Val) conversion causes impairment of the IL-12 signal cascade and the subsequent reduction in IFN- γ production, resulting in the impaired down-regulation of IgE production. This is the first report indicating that atopy is associated with RNA editing.

Keywords atopy, IL-12 receptor β 2, RNA editing

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Introduction

Atopy is characterized by enhanced immunoglobulin E (IgE) responses to common environmental antigens and leads to clinical disorders such as asthma, eczema and rhinitis. IL-4 promotes a class switch to IgE in B lymphocytes and Th2 CD4⁺T lymphocyte differentiation [1]. IgE production by B lymphocytes is down-regulated by IFN- γ that is one of the Th1 cytokines [1]. IL-12 induces IFN- γ production by T lymphocytes and natural killer (NK) cells by binding to its specific receptor [2–4].

The receptor of IL-12 is composed of two distinct subunits, β 1 and β 2, that assemble to form a high-affinity IL-12 receptor (R) complex [5]. While the β 2 chain of the IL-12R is expressed only in Th1 lymphocytes, the β 1 chain is expressed in both Th1 and Th2 lymphocytes. Thus, the expression of

both β 1 and β 2 chains accounts for the responsiveness of T lymphocytes to IL-12 and mediates Th1 lymphocyte differentiation [6]. On binding to its receptor, IL-12 induces activation of specific members of the Stat family of transcription factors, which then translocate to the nucleus and bind to genomic promoter regions. Stat4 is particularly important in this respect, since Stat4-deficient mice manifest impaired IFN- γ production [7]. Furthermore, the phenotype of the IL-12p40-deficient mouse is similar to that of the Stat4-deficient mouse [8]. We reported that reduced IFN- γ production by peripheral blood mononuclear cells (PBMCs) following stimulation with IL-12 but not with phytohemagglutinin (PHA) is associated with the heterozygous IL-12 β 2 chain gene mutations, 1577 A-to-G (Arg 313 Gly), 2496 del 91, and 2799 A-to-G (His 720 Arg), in some atopic subjects [9].

RNA editing is a post-transcriptional modification that results in the generation of nucleotides within an RNA transcript that do not match the bases present within the genome [10]. Mammalian RNA editing events, often represented by cytidine-to-uridine (C-to-U) and adenosine-to-inosine

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(A-to-I) conversions, are predominantly mediated by base deaminations [10]. Here we show that the RNA editing of the IL-12R β 2, 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 μ g/mL PHA for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂ [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 β 2 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 β 2 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 β 2 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 β 2 genomic DNA were amplified by PCR with the sense primer 5'-GATGACAGCTCTGACAGCTG-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 β 2 and transfected Ba/F3 cell clones

The expression constructs encoding human wild-type IL-12R β 2 or variant-type (2451 C-to-U conversion) IL-12R β 2 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 β 2 or the variant-type IL-12R β 2. Then, the transfected Ba/F3 cells were cloned.

Flow cytometric analysis

IL-12R β 2 expressing Ba/F3 cell clones were detected by indirect immunofluorescence analysis using flow cytometry. Briefly, 10^6 cells in 100 μ L of staining buffer were incubated with 1 μ g/mL rat anti-hu IL-12R β 2(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 μ Ci [³H] thymidine per well 4 h before harvesting onto glass-fiber filters. [³H] 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 Na₂PO₄, 1% aprotinin, 5 mM PMSF, 100 mM NaF and 2 mM Na₃VO₄, 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% CO₂. 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 nucleotide 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			P-value
	n	-	+	
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.

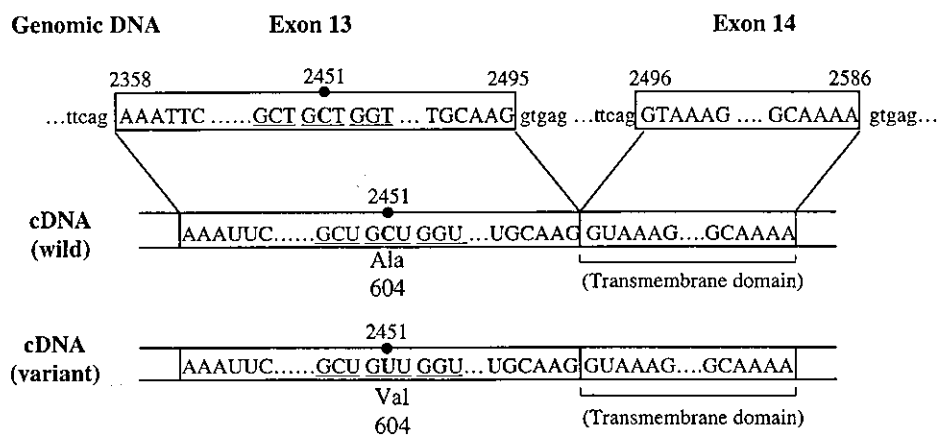


Fig. 1. Sequence analysis of genomic DNA and the cDNA of IL-12R β2 exons 13 and 14 and their flanking regions in non-atopic healthy controls and atopic patients. Sequence analysis of genomic DNA of the IL-12R β2 chain from any of the non-atopic healthy controls and any of the atopic patients showed no 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 IL-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 β 2 chain cDNA

	IFN- γ concentration (pg/mL)*		P-value†
	Without C-to-U conversion (n = 169)	With C-to-U conversion† (n = 25)	
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

*Geometric means are shown, and the ranges of SD are shown in parentheses. †at nucleotide 2451 (Ala 604 Val) in IL-12R β 2 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 β 2 chain

To investigate the expression of the wild-type or variant-type IL-12R β 2 chain, Ba/F3 cells were transfected by electroporation with wild-type IL-12R β 2 cDNA or variant-type IL-12R β 2 cDNA containing the C-to-U conversion at nucleotide 2451 in the PEF-BOS expression vector and then cloned [5]. The IL-12R β 2-chain-expressing cells were detected by flow cytometry using the anti-IL-12R β 2 antibody. The results revealed that the staining intensity and the percentage of cells expressing the IL-12R β 2 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 β 2 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 β 2 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 β 2 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 β 2 chain cDNA is associated with reduced signal transduction of IL-12 for IFN- γ production by PBMCs.

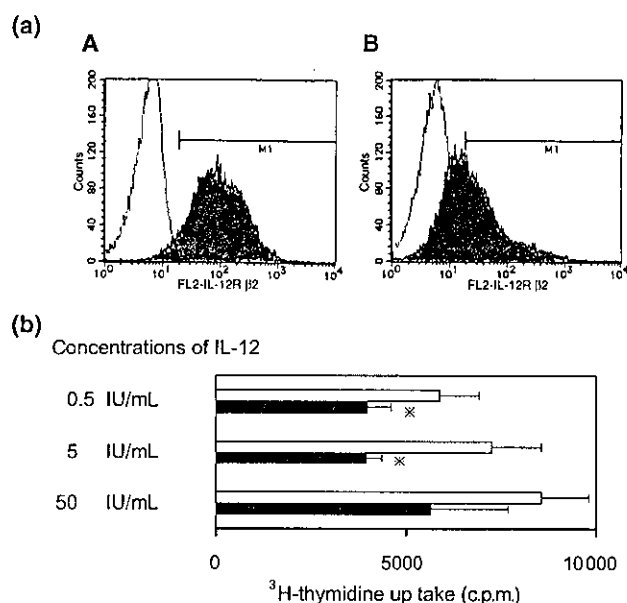


Fig. 2. (a) Expression of the IL-12R β 2 chain without or with the C-to-U conversion at nucleotide 2451 (Ala 604 Val) in Ba/F3 cell clones transfected with wild-type (A) or variant-type (B) IL-12R β 2 cDNA. 10^6 cells in $100 \mu\text{L}$ of staining buffer were incubated with $1 \mu\text{g/mL}$ rat anti-hu IL-12R β 2(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 β 2 expressing cells were detected by flow cytometry. The staining intensity and the percentage (50.1%) of cells expressing the IL-12R β 2 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 β 2 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 β 2 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 β 2 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 β 2 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 β 2 chain cDNA. In contrast, in the

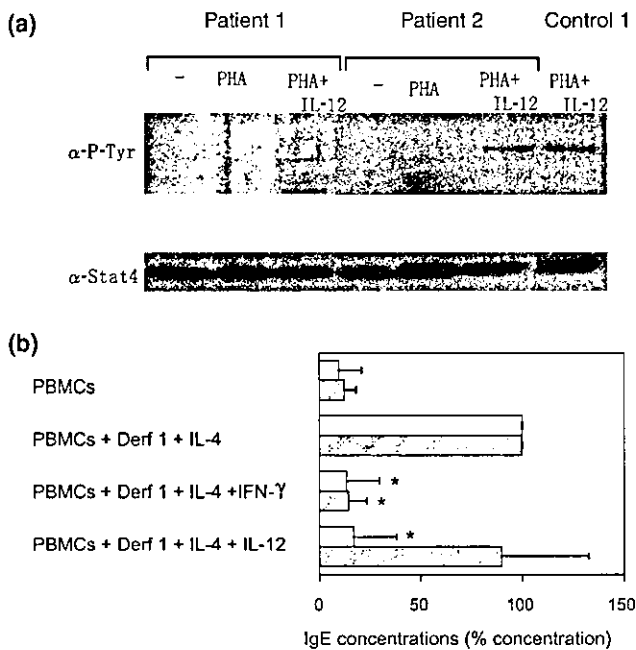


Fig. 3. (a) Tyrosine phosphorylation of Stat4 in PBMCs. PBMCs from the patients with or without the C-to-U conversion at nucleotide 2451 (Ala 604 Val) on the IL-12R β 2 chain and the control subject were cultured with PHA and IL-12. Cell lysates were immunoprecipitated with anti-Stat4, resolved by SDS-PAGE, 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 β 2 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 β 2 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 β 2, 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 β 1 chain gene causes impairment of salmonella and mycobacterial immunity [12, 13]. The development of Th1 lymphocytes is disturbed in IL-12 or IL-12R β 1 knockout mice [8, 14]. In this study, atopic subjects with RNA editing of IL-12R β 2, 2451 C-to-U (Ala 604 Val) conversion, did not exhibit any impairment of salmonella and

mycobacterial immunity. The IL-12R β 2 subunit, similar to the IL-12R β 1 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 β 1, which does not contain any tyrosine residues, the cytoplasmic region of IL-12R β 2 contains three tyrosine residues, suggesting an important role of the β 2 subunit in IL-12 signal transduction [5]. Presky et al. [5] reported that Ba/F3 cells transfected with IL-12R β 2 alone proliferates in response to human IL-12 although the role of endogenous mouse IL-12R β 1 in IL-12 signal transduction in these transfectants cannot be ruled out. Thus, IL-12R β 2 is different from IL-12R β 1 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 β 2 and the proliferative responses to IL-12 in variant-type IL-12R β 2 transfected Ba/F3 were lower than those of wild-type. The RNA editing of IL-12R β 2, 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 lymphocytes 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 β 2 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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Review Article

Genetic defects in downregulation of IgE production and a new genetic classification of atopy

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ABSTRACT

Atopic disorders, such as asthma, eczema and rhinitis, develop due to the interactions between genetic and environmental factors. Atopy is characterized by enhanced IgE responses to environmental antigens. The production of IgE is upregulated by Th2 cytokines, in particular interleukin (IL)-4, and downregulated by Th1 cytokines, in particular interferon (IFN)- γ . In the present review, we present the genetic factors responsible for IgE production and genetic defects in the downregulation (brake) of IgE production, especially in terms of IL-12 and IL-18 signaling, mutations of the IL-12 receptor β 2 chain gene and mutations of the IL-18 receptor α chain gene in atopy. Moreover, we newly present a genetic classification of atopy. There are four categories of genes that control the expression of allergic disorders, which include: (i) antigen recognition; (ii) IgE production (downregulation = brake; and upregulation); (iii) the production and release of mediators; and (iv) events on target organs. In the near future, this genetic classification will facilitate the development of tailor-made treatment.

Key words: atopy, IgE production downregulation, interferon- γ , interleukin-12 receptor β 2, interleukin-18 receptor α .

INTRODUCTION

Atopic disorders, such as asthma, eczema and rhinitis, develop due to the interactions between genetic and environmental factors. Atopy is characterized by enhanced IgE responses to environmental antigens. The production of IgE is upregulated by Th2 cytokines, in particular interleukin (IL)-4, and is downregulated by Th1 cytokines, in particular interferon (IFN)- γ .¹ Interleukin-12, which is a cytokine that promotes cell-mediated Th1 responses and the production of IFN- γ , is one of the important cytokines that downregulates IgE production. Interleukin-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 Interleukin-18 strongly augments IFN- γ production by T lymphocytes, natural killer (NK) cell cytotoxicity and T lymphocyte proliferation.

In the present review, we discuss the genetic factors responsible for IgE production and the genetic defects in the downregulation (brake) of IgE production in atopy. Moreover, we newly present a genetic classification of atopy.

DEVELOPMENT OF ALLERGIC DISEASES

A questionnaire was distributed in March 1991 to children under 16 years of age who were attending kindergarten or elementary or junior high school in two Japanese cities, namely Gifu, with a temperate climate, and Itoman, with a subtropical climate. The number of subjects analyzed was 1243 in Gifu and 1953 in Itoman. Multiple logistic regression analysis was performed using SAS (SAS Institute, Cary, NC, USA).

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Multiple logistic regression analysis showed that, in both cities, children of families with a history of allergy have a significantly higher risk (relative risk 3.58 and 4.22 for Gifu and Itoman, respectively) of contracting an allergic disease (Table 1). These results show that there is a genetic accumulation in the development of allergic disorders. Therefore, the development of allergic disorders is correlated with some genes. We think that multiple causative genes, but not a single gene, are correlated, because there are multiple pathogeneses of allergic reactions.

GENETIC FACTORS OF ENHANCED IGE PRODUCTION AND ATOPY

Serum IgE levels of atopic children were plotted against serum IgE levels of their parents (Fig. 1) and a good correlation was found ($P < 0.016$). Therefore, this indicates that IgE production shows genetic accumulation.³ Several linkage analyses and mutations for candidate genes of atopy (i.e. enhanced IgE production) have been

reported. In 1989, Cookson *et al.*⁴ reported a linkage between IgE responses underlying asthma and rhinitis and chromosome 11q. Moreover, Shirakawa *et al.*⁵

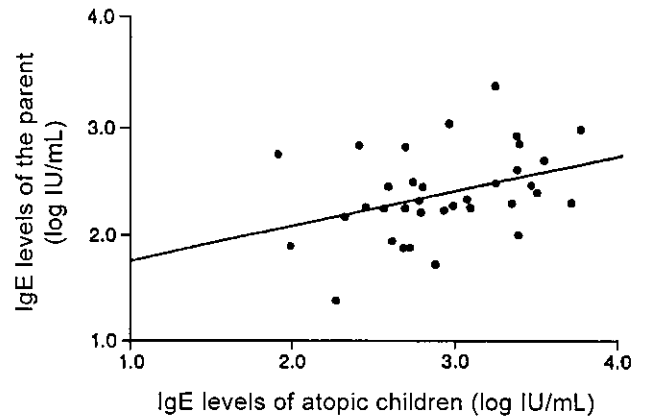


Fig. 1 Relationship between serum total IgE levels of atopic children and the IgE levels of their parents (the highest IgE level of two spouses was used). Children older than 6 years were selected. $y = 1.38 + 0.3461x$; $P < 0.016$.

Table 1 Genetic and environmental factors in relation to any allergic diseases as analyzed by multiple logistic regression

Independent variables	Relative risk (95% confidence interval)	
	Gifu (n = 1243)	Itoman (n = 1953)
Family history		
No	1	1
Yes	3.58 (2.17–5.91)	4.22 (2.91–6.12)
Sex		
Male	1	1
Female	0.93 (0.69–1.27)	0.60 (0.45–0.79)
Age (years)		
0–3	1.72 (0.87–3.40)	0.70 (0.27–1.82)
4–6	1.47 (0.93–2.31)	0.80 (0.44–1.46)
7–9	1.30 (0.81–2.07)	1.10 (0.75–1.62)
10–12	1.15 (0.71–1.85)	1.06 (0.72–1.56)
13–15	1	1
Structure of house		
Made of wood	1	1
Made of reinforced concrete	1.22 (0.87–1.72)	1.15 (0.75–1.78)
Apartment house	1.27 (0.66–2.42)	0.94 (0.60–1.48)
Flooring		
Wooden floor	1	1
Tatami	0.98 (0.64–1.49)	1.91 (1.08–3.38)
Carpet on tatami	1.17 (0.79–1.72)	1.65 (0.75–3.63)
Carpet on wooden floor	2.00 (1.17–3.42)	1.71 (0.91–3.23)
Pets		
No	1	1
Yes	0.88 (0.62–1.23)	0.81 (0.58–1.14)

reported that a common variant of the β -subunit of the high-affinity IgE receptor (Fc ϵ R1 β) on chromosome 11, Ile181Leu within the 4th transmembrane domain, shows significant association with positive IgE responses. Several associations have been noted between atopy and genes on the chromosome 5 cytokine cluster, including IL-4.^{6,7} In 1998, Mitsuyasu *et al.*⁸ reported that the Ile50Val variant of the IL-4 receptor α (IL-4R α) chain upregulates IgE synthesis and is associated with atopic asthma. Moreover, Shirakawa *et al.*⁹ noted genetic variants of IL-13. Very recently, we found that reduced IFN- γ production by peripheral blood mononuclear cells (PBMC) following stimulation with IL-12 or IL-18 is associated with heterozygous IL-12 receptor β 2 (IL-12R β 2) chain gene or IL-18 receptor α (IL-18R α) chain gene mutations in atopic subjects.^{10,11}

GENETIC DEFECTS IN THE DOWNREGULATION OF IGE PRODUCTION IN ATOPY

The production of IgE is upregulated by Th2 cytokines, in particular IL-4, and is downregulated by Th1 cytokines, in particular IFN- γ .¹ Interleukin-12 and IL-18 are the important cytokines that induce IFN- γ and downregulate IgE production (Fig. 2).

In this section, the genetic defects in the downregulation (brake) of IgE production, especially, in terms of IL-12 and IL-18 signaling, are discussed.

Interleukin-12 and IL-12R

Interleukin-12, which is produced by activated antigen-presenting cells, is a cytokine that consists of two disulfide-linked subunits, p35 and p40. Interleukin-12

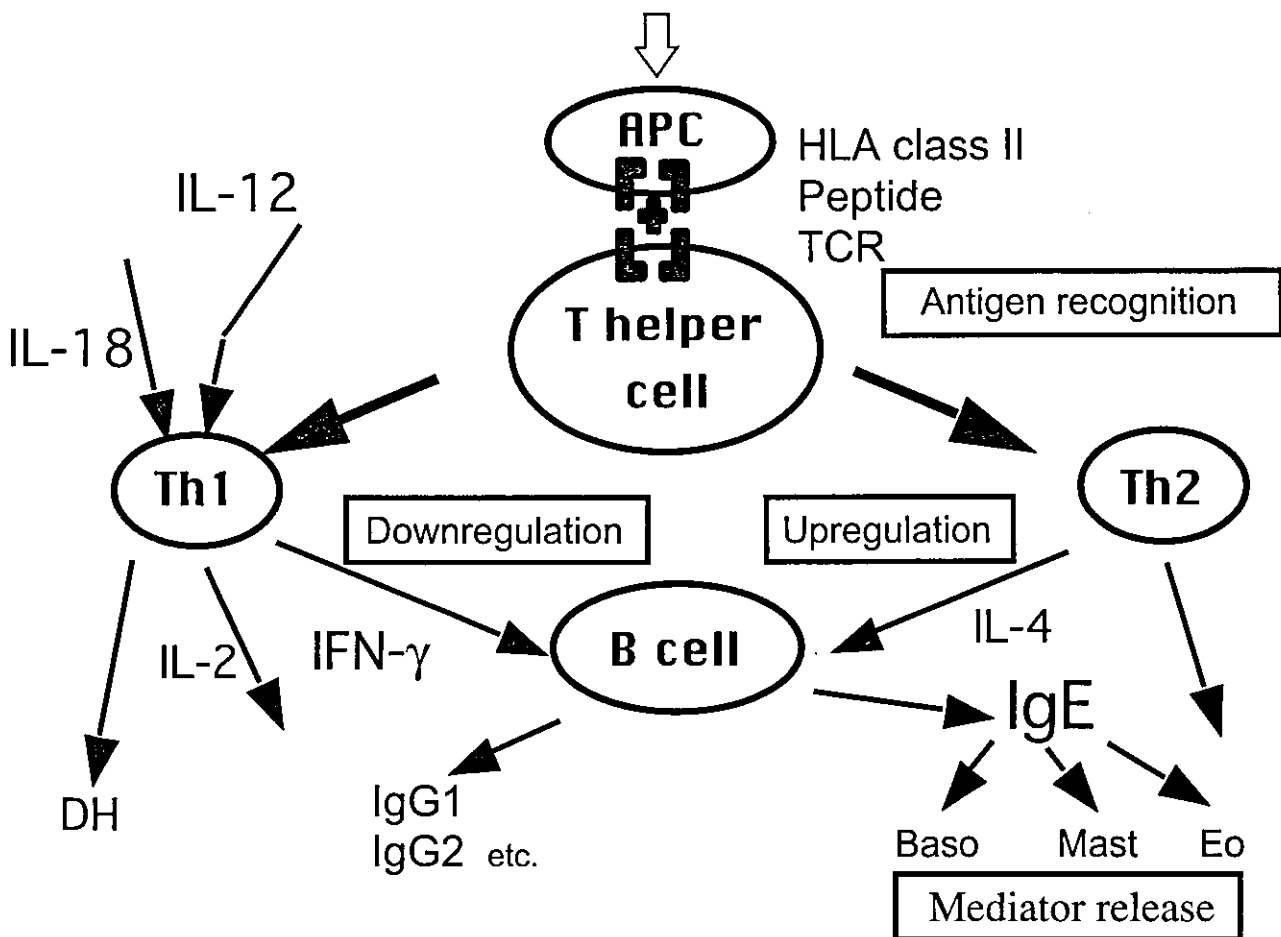


Fig. 2 The Th1 and Th2 lymphocyte balance and upregulation and downregulation of IgE production. IL, interleukin; DH, delayed-type hypersensitivity; IFN, interferon; APC, antigen-presenting cell; HLA, human leukocyte antigen; TCR, T cell receptor; Baso, basophils; Mast, mast cells; Eo, eosinophils.

plays a central role in promoting Th1-type immune responses and, thus, cell-mediated immunity.¹²⁻¹⁵ Interleukin-12 also induces IFN- γ production by T lymphocytes and NK cells.¹⁶⁻¹⁸ The receptor for IL-12 (IL-12R) is composed of two distinct subunits, β 1 and β 2¹⁹ (Fig. 3). Although the β 2 chain of the IL-12R is expressed only in Th1 lymphocytes, the β 1 chain is expressed in both Th1 and Th2 lymphocytes.²⁰ The IL-12R β 1 chain does not contain any cytoplasmic tyrosine residues, whereas the cytoplasmic region of the IL-12R β 2 chain contains three tyrosine residues. This suggests that the β 2 subunit plays an important role in IL-12 signal transduction. Interleukin-12 induces activation of specific members of the signal transducers and activators of transcription (Stat) family of transcription factors and it has been shown that Stat4-deficient mice manifest impaired production of IFN- γ ²¹ and the phenotype of the IL-12-p40-deficient mouse is similar to that of the Stat4-deficient mouse.¹⁵ Therefore, Stat4 is particularly important. Interleukin-12 induces rapid tyrosine phosphorylation of Stat4 and the formation of nuclear complexes capable of binding to DNA sequences, such as the Stat4-binding site.^{21,22}

Interleukin-18 and IL-18R

A variety of biological functions have been associated with human IL-18, including the induction of the proliferation of activated T lymphocytes, enhancement of NK cytotoxicity, induction of the production of IFN- γ and granulocyte-macrophage colony stimulating factor (GM-CSF), and promotion of a Th1 response.^{2,23-25} The

activity of IL-18 is via an IL-18R complex. This IL-18R complex is composed of a binding chain termed IL-18R α , a member of the IL-1R family previously identified as the IL-1R-related proteins, and a signaling chain, also a member of the IL-1R family. The IL-18R complex recruits the IL-1R-activating kinase and tumor necrosis factor (TNF)-associated factor 6, which phosphorylates nuclear factor (NF)- κ B-inducing kinase, with subsequent activation of NF- κ B²⁶⁻²⁸ (Fig. 4).

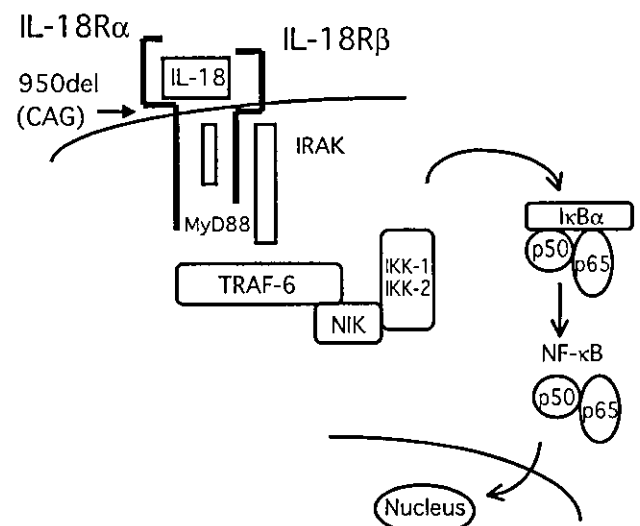


Fig. 4 Interleukin (IL)-18 signaling. IL-18R α , IL-18R β , IL-18 receptor α and β chains, respectively; IKK-1, *ikk-2*, I κ B α kinases 1 and 2, respectively; NF- κ B, nuclear factor- κ B; NIK, NF- κ B-inducing kinase; TRAF-6, tumor necrosis factor receptor-associated factor 6; IRAK, IL-1 receptor-associated kinase.

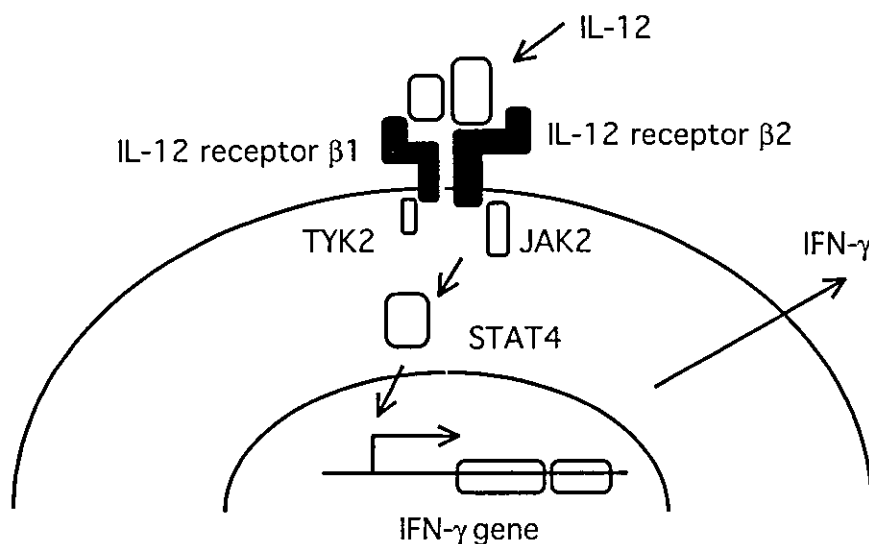


Fig. 3 Interleukin (IL)-12 signaling. TYK2, tyrosine kinase 2; JAK2, Janus kinase 2; STAT4, signal transducers and activators of transcription 4; IFN, interferon.

Interferon- γ production by IL-12 or IL-18 in atopy

We examined the production of IFN- γ in PBMC of atopic patients and healthy controls following stimulation with IL-12 or IL-18.^{10,11} The PBMC of non-atopic healthy controls showed adequate IFN- γ production following stimulation with either IL-12 or IL-18. Although the concentrations of IFN- γ in IL-18-stimulated PBMC were

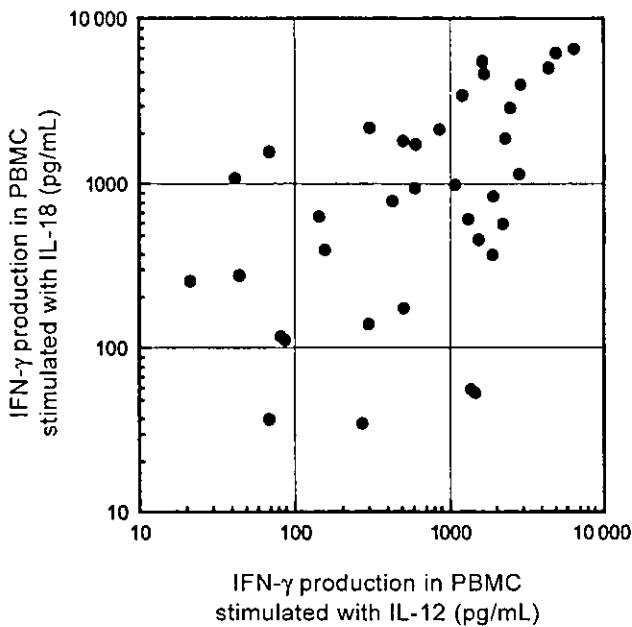


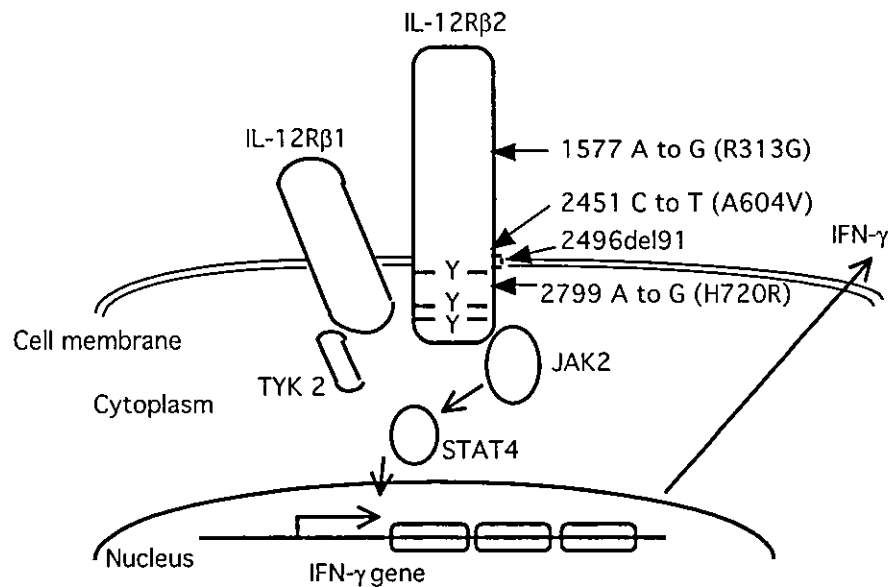
Fig. 5 Interferon (IFN)- γ production in peripheral blood mononuclear cells (PBMC) stimulated with interleukin (IL)-12 or IL-18 (see text for details).

correlated with those of IL-12-stimulated PBMC in atopic patients, there were cases showing different responses to IL-12 and IL-18, as shown in Fig. 5. The production of IFN- γ following stimulation with IL-12 (or IL-18) was poor, but IL-18 (or IL-12) stimulation elicited detectable IFN- γ production in some atopic patients. The discrepancy in IFN- γ production following stimulation with IL-12 or IL-18 suggests a disturbance in the IL-12 or IL-18 signal cascade in these patients.

Role of mutations of the IL-12R β 2 chain gene in atopy

Recently, it was shown that homozygous nonsense mutation of the IL-12R β 1 chain gene resulted in impairment of immunity against *Salmonella* and mycobacteria.²⁹ Moreover, IL-12R β 1-knock out mice showed impaired development of Th1.³⁰ In a previous study,¹⁰ sequence analysis of the cDNA of IL-12R β 2 revealed three types of distinct genetic mutations (2496del91, 1577 A to G (R313G), 2799 A to G (H720R)) in some atopic patients (Fig. 6). Reduced production of IFN- γ by PBMC following stimulation with IL-12, but not IL-18, is associated with heterozygous IL-12R β 2 chain cDNA mutations in atopic subjects. In these atopic patients, a heterozygous IL-12R β 2 chain cDNA mutation results in decreased tyrosine phosphorylation of Stat4 and subsequently reduced production of IFN- γ following stimulation with IL-12. Such reduced production of IFN- γ could cause insufficient suppression of accelerated IgE production in B lymphocytes by IL-4, resulting in the elevation of serum

Fig. 6 Interleukin (IL)-12 signaling and mutations of IL-12 receptor β (IL-12R β) 2 chain gene. R, arginine; G, glycine; H, histidine; Y, tyrosine (2451 C to T: by RNA editing); TYK2, tyrosine kinase 2; JAK2, Janus kinase 2; STAT4, signal transducers and activators of transcription 4.



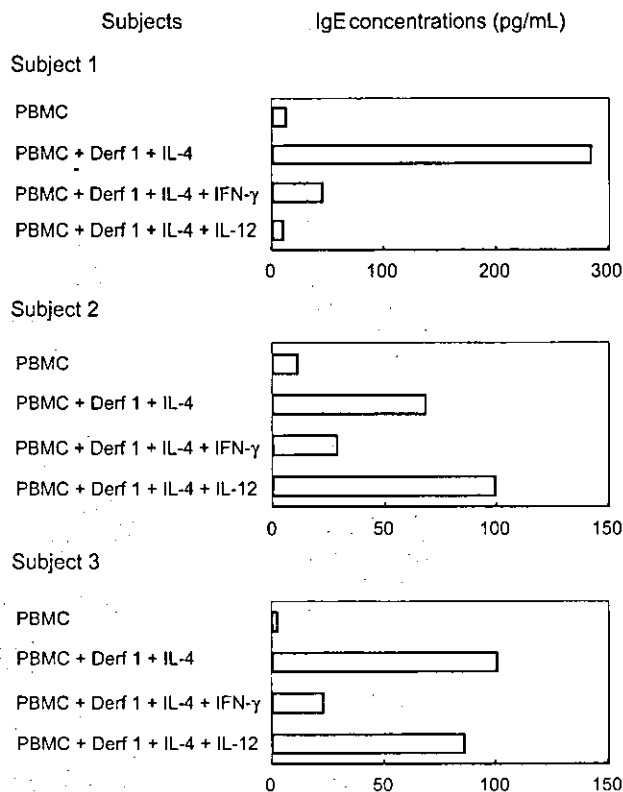


Fig. 7 Suppression of IgE production of peripheral blood mononuclear cells (PBMC; see text for details). The PBMC (10^6 /mL) were cultured with Derf 1 (0.5 μ g/mL; Asahi, Tokyo, Japan) and interleukin (IL)-4 (500 U/mL; Genzyme, Cambridge, MA, USA) in culture test tube for 14 days. Moreover, interferon (IFN)- γ (100 U/mL) or IL-12 (5 IU/mL) was added to these PBMC cultures. Culture supernatants were obtained after the cultures. The concentration of IgE in culture supernatants was measured by ELISA. Subject 1, an atopic patient without mutations of the IL-12R β 2 chain gene; subject 2, an atopic patient with 91del of the IL-12R β 2 chain gene; subject 3, an atopic patient with a missense mutation of the IL-12R β 2 chain gene.

IgE levels (Fig. 7). The 2496del91 mutation of IL-12R β 2, which is found all over the transmembrane portion, causes premature termination. The heterozygous missense mutations, 1577 A to G (R313G) and 2799 A to G (H720R), may lead to changes in the conformational structure. Moreover, these heterozygous mutations may play a role via a dominant negative effect. At least, these patients with heterozygous mutations of IL-12R β 2 chain cDNA have not exhibited impairment of immunity against *Salmonella* and mycobacteria.

The balance between IFN- γ -producing Th1 lymphocytes and proallergic Th2 lymphocytes is important. Heterozygous mutations of IL-12 β 1 or β 2 may result in

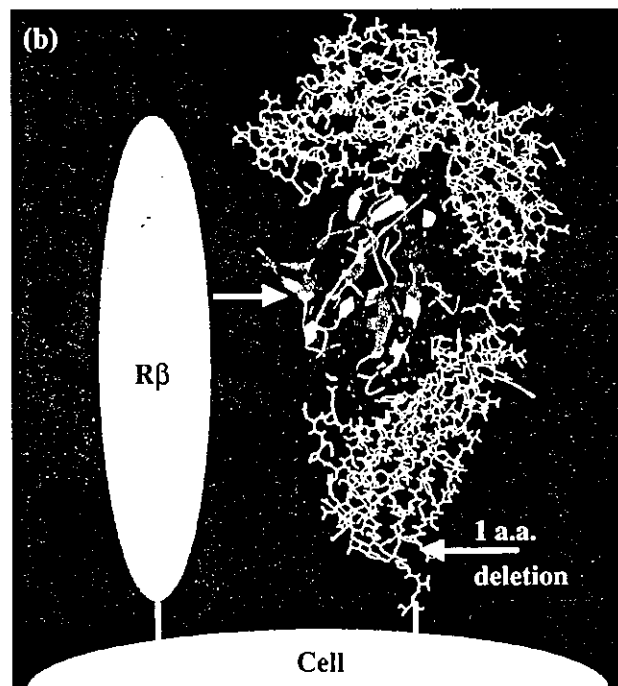
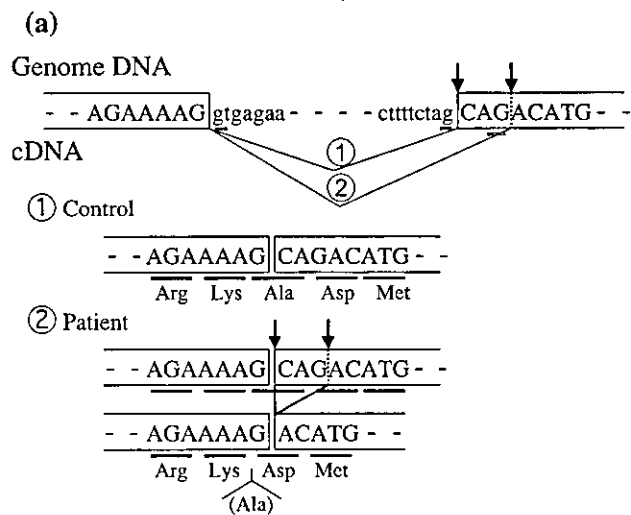


Fig. 8 (a) 950delCAG in interleukin (IL)-18 receptor α (IL-18R α) chain cDNA and (b) model of the ternary complex of IL-18R β : IL-18 : IL-18R α .

impairment of the downregulation (brake) of IgE production, whereas homozygous mutations of IL-12 β 1 or β 2 may lead to an obvious impairment of Th1-type cell-mediated immunity in addition to impairment of the downregulation of IgE production. The results of our study¹⁰ indicate that atopic diseases are caused, in part, by impairment of the IL-12 signal cascade, which downregulates IgE production, and that the mutation of

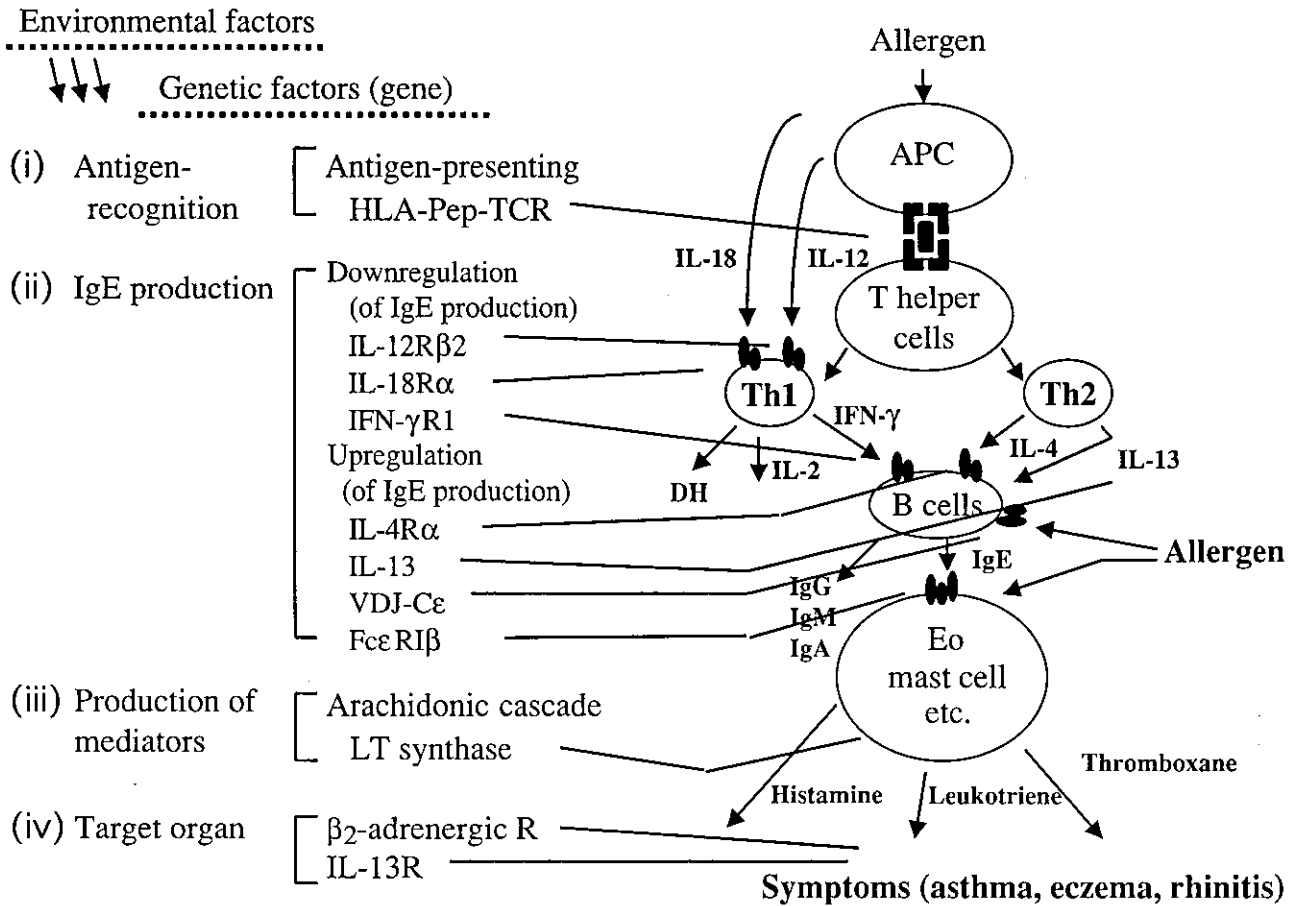


Fig. 9 A new genetic classification of atopy and genetic ecologic medicine in allergy (designed by N Kondo, 2002). HLA-Pep-TCR, human leukocyte antigen-peptide-T cell receptor; IL, interleukin; IFN, interferon; VDJ-Cε, variable diversity joining-ε constant region; LT, leukotriene; R, receptor; APC, antigen-presenting cell; DH, delayed-type hypersensitivity; Eo, eosinophils.

the IL-12β2 chain gene is one of the causative genes for atopy.

Role of mutation of the IL-18Rα chain gene in atopy

The IL-18Rα chain cDNA of atopic patients was sequenced.¹¹ We identified a three-base deletion of the IL-18Rα chain cDNA (950delCAG), which was generated by alternative splicing, as determined on the basis of genomic sequence data for the IL-18Rα chain gene (Fig. 8). Peripheral blood mononuclear cells with the predominant expression of 950delCAG significantly showed reduced IFN-γ production after IL-18 stimulation. There was a significant difference in the expression pattern of the IL-18Rα chain transcript between atopic patients and non-atopic controls. According to these

results, the dominant expression of the 950delCAG transcript of IL-18Rα chain cDNA, which was associated with reduced IFN-γ production following IL-18 stimulation and high serum IgE levels, predisposes to some atopic diseases.

Role of mutation of the IFN-γR1 chain gene in atopy

We identified a novel heterozygous single-nucleotide substitution 1400 T to C (Leu467Pro) in the seventh exon of the IFN-γR1 chain gene.³¹ This substitution was detected in six of 89 allergic patients, but not in 72 non-allergic subjects. There was a difference in the Leu467Pro frequency between allergic and non-allergic subjects (*P* < 0.05). Serum IgE levels of allergic patients with Leu467Pro were higher than those of non-allergic

subjects ($P < 0.001$). These results suggest that Leu467Pro in the IFN- γ R1 chain gene is one of the candidate susceptibility genes for atopic diseases.

GENETIC CLASSIFICATION OF ATOPY

Recently, mutations or genetic polymorphisms of several genes, such as those encoding the Fc ϵ R1 β ,⁵ IL-4R α subunit⁸ and IL-13,⁹ have been reported as the probable causative genes of atopy, which is characterized by enhanced IgE production. Based on these reports and our results, we present a new genetic classification of atopy in Fig. 9. There are four categories of genes that control the expression of allergic disorders, which include: (i) antigen recognition; (ii) IgE production (downregulation = brake; and upregulation); (iii) the production and release of mediators; and (iv) events on target organs. In the near future, this genetic classification will facilitate the development of tailor-made treatment.

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