INTERNATIONAL JOURNAL OF MOLECULAR MEDICINE 12: 185-191, 2003

(24) A novel single-nucleotide substitution, Leu 467 Pro, in the interferon-γ receptor 1 gene associated with allergic diseases

MINAKO AOKI[1], EIKO MATSUI[1], HIDEO KANEKO[1], RYOSUKE INOUE[1], TOSHIYUKI FUKAO[1], MIZUHO WATANABE[1], TAKAHIDE TERAMOTO[1], ZENICHIRO KATO[1], KIYOTAKA SUZUKI[1], YASUYUKI SUZUKI[2], KIMIKO KASAHARA[1] and NAOMI KONDO[1]

Departments of [1]Pediatrics, and [2]Medical Education Development Center, Gifu University School of Medicine, Gifu, Japan

Received January 13, 2003; Accepted February 24, 2003

Abstract. We identified a novel heterozygous single-nucleotide substitution 1400 T→C (Leu 467 Pro) in the seventh exon of the interferon-γ receptor 1 (*IFNGR1*) gene. This substitution was detected in 6 of the 89 allergic patients but not in the 72 non-allergic subjects. There was a difference in the L467P frequency between the allergic patients and the non-allergic subjects (Fisher's exact test: p=0.033). The 6 patients with L467P have allergic diseases such as bronchial asthma and/or allergic rhinitis. Furthermore, a familial analysis for L467P revealed a linkage between allergic diseases and L467P. Serum IgE levels of the patients with L467P were higher than those of the non-allergic subjects (p=0.001). Our previous studies have been shown that interferon-γ (IFN-γ) production by PBMCs in the allergic patients was lower than that in the non-allergic subjects. In this study, although IFN-γ production in the allergic patients with L467P was equivalent to that in the non-allergic subjects, their serum IgE levels were high and they had allergic diseases. Our results suggest that some allergic patients have IFNGR dysfunction, and that L467P in the *IFNGR1* gene is one of candidate susceptibility genes for allergic diseases.

Introduction

Susceptibility to allergic diseases such as bronchial asthma (BA), atopic dermatitis (AD) and allergic rhinitis (AR) is determined by the interaction between an unknown number of genetic and environmental factors (1). Single-nucleotide substitutions in several genes have been reported to be associated with allergic diseases (2,3).

Interferon-γ (IFN-γ) plays an important role in allergic diseases. IFN-γ produced by Th1 cells inhibits Th2 responses through its binding to interferon-γ receptors (IFNGR1 and IFNGR2) and inhibits interleukin-4 (IL-4)-induced IgE production (4). Hence, a dysfunction of the IFN-γ signal pathway may lead to acceleration of Th2 responses.

The loci of the *IFNGR1* and *IFNGR2* genes are on chromosomes, 6q23-24 and 21q22, respectively, and both loci have been associated with allergic diseases based on a genome-wide search (5). We hypothesized that the *IFNGR1* and *IFNGR2* genes are candidate susceptibility genes for allergic diseases.

We previously reported that the serum IgE level was negatively correlated with IFN-γ production by peripheral blood mononuclear cells (PBMCs), and that IFN-γ production by interleukin-12 (IL-12)-stimulated PBMCs in an allergic group was lower than that in a non-allergic group (6,7). Moreover, in some allergic patients, the reduced IFN-γ production following stimulation with IL-12 was associated with the heterozygous interleukin-12 receptor ß2 (IL-12R ß2) mutation (8). We also reported that the predominant expression of 950delCAG of interleukin-18 receptor α (IL-18R α) chain cDNA was associated with reduced IFN-γ production following stimulation with interleukin-18 (IL-18) and with high serum IgE levels in some allergic patients (9,10).

Herein, we report that a novel single-nucleotide substitution 1400 T→C (Leu 467 Pro) in the *IFNGR1* gene is associated with allergic diseases.

Materials and methods

Patients and control subjects. Eighty-nine allergic patients (56 males and 33 females; mean age ± 1SD, 10.8±9.8 years old; BA: 44, AD: 22, AR: 15, BA and AD: 7, BA and AR: 1) and 72 non-allergic subjects (37 males and 35 females; mean age ± 1SD, 10.4±13.1 years old) were studied. The allergic patients were selected based on having major allergic diseases such as BA and/or AD and/or AR. The diagnosis of BA was made according to the criteria of the American Thoracic

Correspondence to: Dr Minako Aoki, Department of Pediatrics, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8705, Japan
E-mail: e2104001@guedu.cc.gifu-u.ac.jp

Abbreviations: BA, bronchial asthma; AD, atopic dermatitis; AR, allergic rhinitis; IFN-γ, interferon-γ; IL-12, interleukin-12; IL-18, interleukin-18; IFNGR, interferon-γ receptor; *IFNGR1* gene, interferon-γ receptor 1 gene; L467P, Leu 467 Pro; STAT1, signal transducer and activator of transcription 1

Key words: allergic disease, IgE, IFN-γ, bronchial asthma, allergic rhinitis, *IFNGR1* gene, single-nucleotide substitution

Table I. Sequence of oligonucleotides for PCR.

Primer		Sequence	Position	Size of the amplified fragment (bp)	Annealing Temp. (°C)
IFNGR1	1	Forward: 5'-TAGCAGCATGGCTCTCCTCTT-3' Reverse: 5'-CTGGATCTCACTTCCGTTCA-3'	-7-552	559	54
IFNGR1	2	Forward: 5'-TGTAAATGGAGACGAGCAGG-3' Reverse: 5'-CTTCACAGACCACCTCCTTT-3'	453-970	518	55
IFNGR1	3	Forward: 5'-CGTCATACCAGCCATTTTCC-3' Reverse: 5'-TTGGTGCAACTTAGCTGATC-3'	926-1490	565	54
IFNGR2	1	Forward: 5'-TGCGCTCGCCATGGCGGTTT-3' Reverse: 5'-GCCTTTGACCTGTTGGATTC-3'	-99-570	669	56
IFNGR2	2	Forward: 5'-TGATACCTCCACGGCCTTTT-3' Reverse: 5'-ATGGCTTGATCTCTTCCAGG-3'	498-1063	566	54

Society, that of AD according to the criteria of Hanifin (11) and that of AR according to the criteria of Skoner (12) and Dykewicz *et al* (13). The non-allergic subjects were healthy and did not have a history of allergic diseases. All of the subjects were randomly selected among patients in our hospital. Informed consent was obtained from all subjects or their parents.

Cell preparation. PBMCs were isolated from the heparinized blood of the non-allergic subjects and allergic patients by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden). PBMCs were suspended at a density of 10^6/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell culture. PBMCs (10^6/ml) were cultured in the presence or absence of 10 µg/ml phytohemagglutinin (PHA; Gibco BRL, Grand Island, NY, USA), 5 IU/ml recombinant human IL-12 (R&D Systems, Inc., Wiebaden, Germany) or 400 ng/ml recombinant human IL-18 for 24 h in a final volume of 1 ml in a round-bottom tube (Falcon 2059, Becton Deckinson Labware, Franklin Lakes, NJ, USA) at 37°C in a humidified atmosphere containing 5% CO_2. The recombinant human IL-18 used had been prepared in our laboratory using an *Escherichia coli* expression system (according to an unpublished method developed by Kato *et al*). The endotoxin level of the recombinant human IL-18 was <0.1 pg per µg of the protein.

Detection of single-nucleotide substitution in the IFNGR1 gene. RNA was extracted from PBMCs cultured in the presence of PHA for 24 h using an Isogen kit (Nippon Gene, Tokyo, Japan). Fragments of *IFNGR1* (Gene Bank accession no. J03143) and *IFNGR2* (Gene Bank accession no. U05877) cDNAs were amplified and sequenced using an ABI 377 DNA sequencer (Applied Biosystems, CA, USA). The conditions for the PCR and the used primers are shown in Table I.

Genomic DNA was extracted from neutrophils with a Sepagene kit (Sanko Junyaku, Tokyo, Japan). The seventh exon of the *IFNGR1* gene (Gene Bank accession no. AL050337) was amplified and sequenced using an ABI 377 DNA sequencer. For concise detection of L467P, we designed a modified PCR technique of introducing the *Eco*RII site in the mutant fragment using the following mismatch primers: a sense primer 5'-^{1323}AGGACAAGAGCTCATAACCG-3' and an anti-sense primer 5'-^{1418}TTACCGCTATCATCCCCA-3'. The PCR products were electrophoresed on a 20% polyacrylamide gel following *Eco*RII (Wako, Tokyo, Japan) digestion at 37°C for 1 h.

IgE assay. Plasma samples obtained from heparinized blood were stored at -30°C. Plasma IgE levels were determined by chemiluminescent enzyme immunoassay. The values obtained were regarded as the serum IgE levels. The levels of specific IgE antibodies for house dust, mite, cedar pollen, cow's milk and hen's egg were measured by the fluoroenzyme immunoassay using a Uni-Cap assay kit (Pharmacia, Uppsala, Sweden).

Assays for cytokine. Culture supernatants in the test tubes incubated for 24 h were spun to remove cells after the cultures. IFN-γ concentrations in supernatants of cell culture were measured with a human IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (Ohtsuka, Tokyo, Japan); the detection limit was 15.6 pg/ml.

Immunoprecipitation for phosphorylated STAT1. PBMCs of allergic patients and non-allergic subjects were stimulated with IFN-γ (10^2 IU/ml) or control culture medium for 15 min. Cells were lysed in a solution containing 1% Triton X-100, 150 mM NaCl, 20 mM Na_2PO_4, 1% aprotinin, 5 mM PMSF, 100 mM NaF and 2 mM Na_3VO_4, and were immunoprecipitated with mouse antisera for STAT1 (Santa Cruz Biotechnology). Precipitates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring the fractionated products to a nitrocellulose membrane, blots

were probed with antibody for phosphotyrosine. Equal loading of STAT1 was confirmed by stripping the same membranes and reprobing them with the antiserum for STAT1.

Statistical analyses. The significance of difference between groups was analyzed by the Mann-Whitney U test. Distribution of the genotype of L467P in the *IFNGR1* gene was analyzed by the Fisher's exact test. Probability (p) values <0.05 were considered statistically significant.

Results

L467P in the IFNGR1 gene. IFNGR1 and *IFNGR2* cDNAs from allergic patients were sequenced. We identified a novel heterozygous single-nucleotide substitution 1400 T→C (L467P) in *IFNGR1* cDNA in some of the allergic patients. This substitution was also detected in the genomic DNA (Fig. 1A). We determined the prevalence of L467P in the *IFNGR1* gene in the allergic patients and non-allergic subjects by direct sequencing (Fig. 1B) and confirmed it by the restriction enzyme assay (Fig. 1C). We designed a mismatch primer to introduce the *Eco*RII site into the mutant fragment. A fragment amplified by PCR was incubated at 37°C with *Eco*RII for 1 h and was electrophoresed on a 20% polyacrylamide gel. The normal and mutant alleles were detected as a 98-bp band and a 78-bp one, respectively (Fig. 1C).

L467P was found in 6 (6.7%) of the 89 allergic patients. However, L467P could not be detected in the 72 non-allergic subjects. There was a difference in the L467P frequency between the allergic patients and the non-allergic subjects (Fisher's exact test: p=0.033) (Table II). The clinical features of the allergic patients with L467P are shown in Table III. All the patients with L467P have allergic diseases.

Genetic analysis for L467P in atopic families. The linkage of L467P to allergic diseases was investigated in two families (Fig. 2). In family 1, II-4 was the proband with L467P and suffering from BA. L467P was inherited from the father (I-1) with AR. The elder brother (II-3) with BA and AR also had this substitution. In family 2, II-8 was the proband with BA. L467P was inherited from the father (I-5) with AR. There was a clear linkage between allergic diseases and L467P.

Associations of L467P and total serum IgE levels or specific IgE levels. The total serum IgE levels of the patients with L467P (mean, 128.8 IU/ml; range, 45.3-366.4 IU/ml) were higher than those of the non-allergic subjects (mean, 19.4 IU/ml; range, 6.8-56.2 IU/ml) (p=0.001). The total serum IgE levels of the patients without L467P (mean, 446.7 IU/ml; range, 75.3-2648.5 IU/ml) were higher than those of the non-allergic subjects (p<0.0001). There was no difference between IgE levels of the patients with L467P and those of patients without L467P (p=0.112) (Fig. 3). Five of the 6 patients with L467P had high levels of specific IgE for inhalant antigens such as house dust and mite (Table III).

IFN-γ production by PBMCs. To examine functional effects of L467P in the *IFNGR1* gene, we measured IFN-γ production by PBMCs following their stimulation with IL-12, IL-18 or PHA in all of the allergic patients and non-allergic subjects.

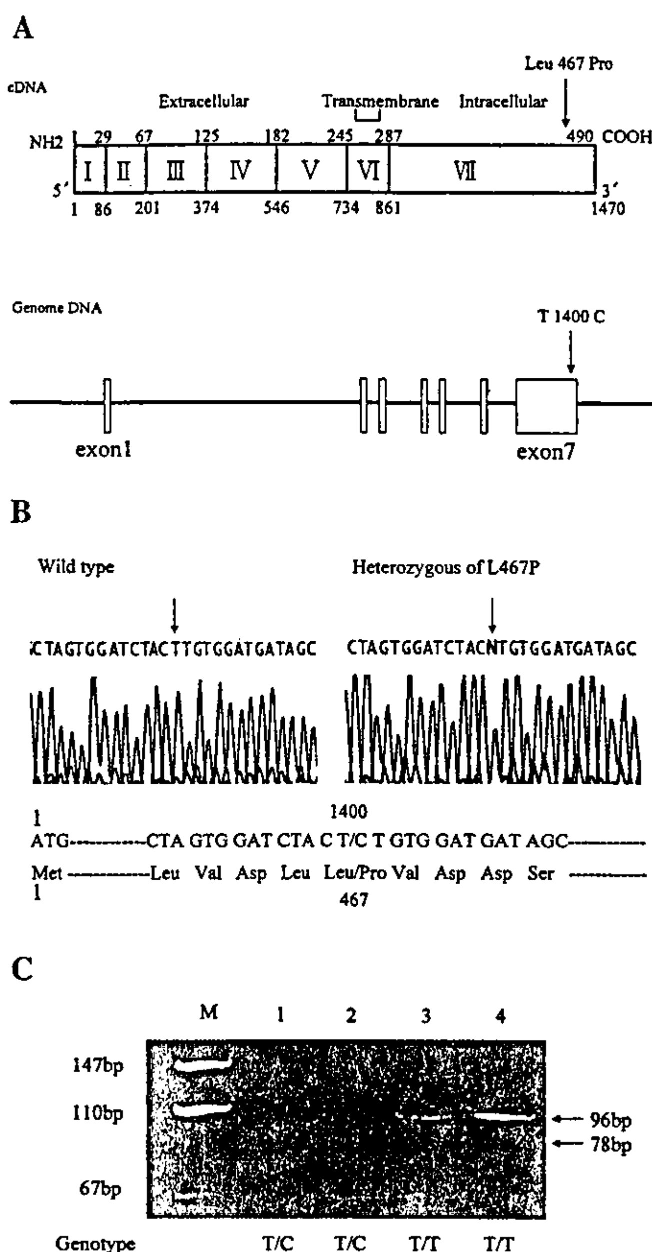

Figure 1. Detection of L467P in the *IFNGR1* gene. (A), Structure of *IFNGR1* gene and position of L467P. The *IFNGR1* gene consists of seven exons. Exon 1-exon 5 encode the receptor extracellular domain. Exon 6 encodes a small portion of the membrane proximal regions of the extracellular domain and the transmembrane domain. Exon 7 encodes the entire intracellular domain. L467P is located in exon 7 of the *IFNGR1* gene. (B), DNA sequence data of L467P. The data of a patient without L467P is shown on the left and the data of a patient with L467P is shown on the right. (C), Concise detection of L467P. We designed a mismatch primer to introduce the *Eco*RII site into the mutant fragment. A fragment amplified by PCR was incubated at 37°C for 1 h and was electrophoresed on a 20% polyacrylamide gel. The normal and mutant alleles were detected as a 98-bp band and a 78-bp one, respectively. Lanes 1-2, patients with L467P. Lanes 3-4, patients without L467P.

The IFN-γ production in the allergic patients without L467P was significantly lower than that in the non-allergic subjects (Fig. 4). Our previous studies have been shown that IFN-γ production in the allergic patients was lower than that in the non-allergic subjects. However, there was no difference in IFN-γ production between the allergic patients with L467P and the non-allergic subjects (Fig. 4).

Table II. Distribution of genotype of L467P in the *IFNGR1* gene.

Genotype	Non-allergic subjects (n=72) (%)	Allergic patients (n=89) (%)	Total (n=161) (%)	p-value
CTT (Leu)/CTT (Leu)	72 (100)	83 (93.3)	155 (96.3)	0.033[a]
CTT (Leu)/CCT (Pro)	0 (0)	6 (6.7)	6 (3.7)	
CCT (Pro)/CCT (Pro)	0 (0)	0 (0)	0 (0)	

[a]Statistical analyses were performed by the Fisher's exact test.

Table III. The clinical features of the allergic patients with L467P.

Patient	Allergic diseases	Serum IgE levels (IU/ml)	RAST scores				
			House dust	Mite	Cedar pollen	Cow's milk	Hen's egg
1	BA	340.5	4	4	5	0	0
2	BA	317.0	5	5	3	0	0
3	BA	150.0	4	4	0	0	0
4	BA	127.6	4	4	0	1	1
5	BA	115.0	5	4	0	0	0
6	BA, AR	19.0	0	0	0	0	0
Non-allergic subjects mean (range)		19.5 (6.8-56.2)					

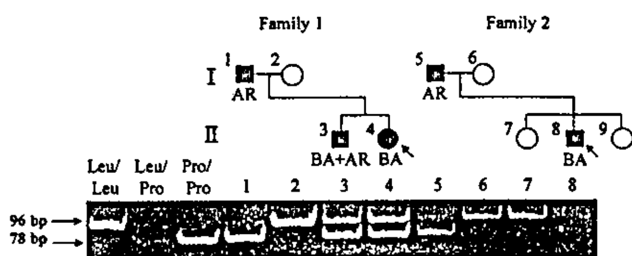

Figure 2. Genetic analysis for L467P in two atopic families. Family 1: II -4 was the proband with the heterozygous L467P and BA. Her father (I-1) had the homozygous L467P and severe AR. Her brother (II-3) with AR and BA also had the heterozygous L467P. Family 2: II-8 was the proband with the heterozygous L467P and BA. His father (I-5) had the heterozygous L467P and AR. We could not get informed consent from his sister (II-9).

Phosphorylation of STAT1 in PBMCs. The IFNGR signal is transduced by STAT1 phosphorylation. Therefore, we analyzed whether L467P in the *IFNGR1* gene affects phosphorylation of STAT1 in PBMCs. As shown in Fig. 5, IFN-γ stimulation (10^2 IU/ml) gave a similar level of phosphorylation of tyrosine residues in STAT1 in PBMCs from the allergic patients with L467P and non-allergic subjects. These data suggest that L467P in the *IFNGR1* gene have no effect on phosphorylation of STAT1 in PBMCs stimulated with IFN-γ.

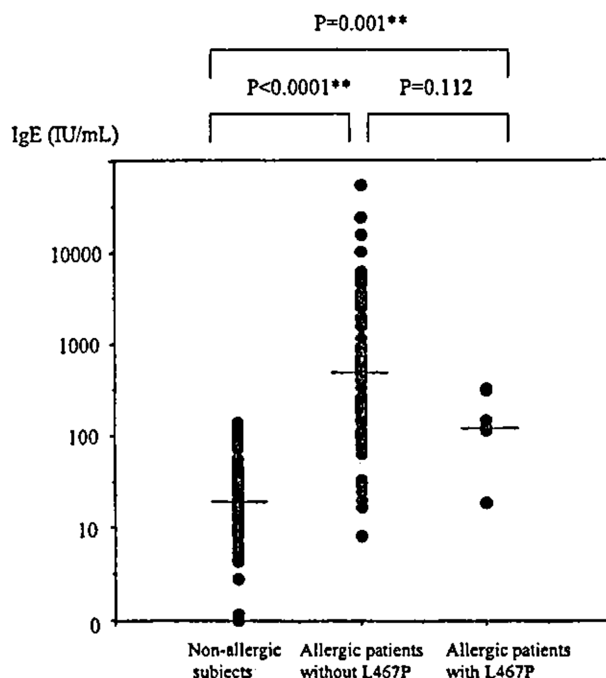

Figure 3. Relationship between L467P in the *IFNGR1* gene and total serum IgE levels. Serum IgE levels of the allergic patients with L467P were significantly higher than those of the non-allergic subjects without L467P (p=0.001**). Serum IgE levels of the allergic patients without L467P were significantly higher than those of the non-allergic subjects without L467P(p<0.0001**). There was no difference between IgE levels of the patients with L467P and those of patients without L467P (p=0.112). A bar indicates a genometric mean.

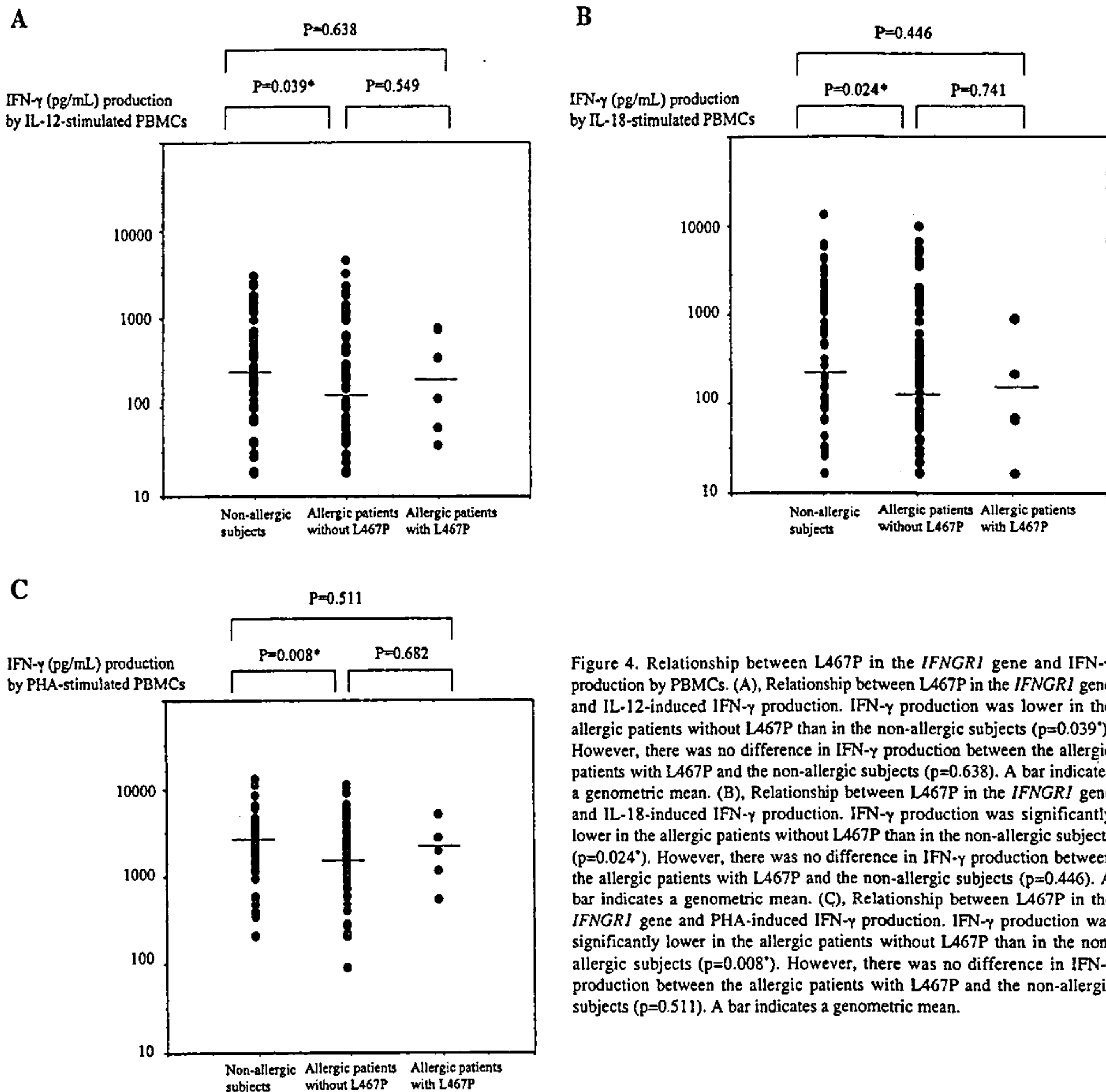

Figure 4. Relationship between L467P in the *IFNGR1* gene and IFN-γ production by PBMCs. (A), Relationship between L467P in the *IFNGR1* gene and IL-12-induced IFN-γ production. IFN-γ production was lower in the allergic patients without L467P than in the non-allergic subjects (p=0.039*). However, there was no difference in IFN-γ production between the allergic patients with L467P and the non-allergic subjects (p=0.638). A bar indicates a genometric mean. (B), Relationship between L467P in the *IFNGR1* gene and IL-18-induced IFN-γ production. IFN-γ production was significantly lower in the allergic patients without L467P than in the non-allergic subjects (p=0.024*). However, there was no difference in IFN-γ production between the allergic patients with L467P and the non-allergic subjects (p=0.446). A bar indicates a genometric mean. (C), Relationship between L467P in the *IFNGR1* gene and PHA-induced IFN-γ production. IFN-γ production was significantly lower in the allergic patients without L467P than in the non-allergic subjects (p=0.008*). However, there was no difference in IFN-γ production between the allergic patients with L467P and the non-allergic subjects (p=0.511). A bar indicates a genometric mean.

Discussion

Through the linkage and association studies, a number of putative atopy genes have been identified. Some major Th2 cytokines and their receptor genes, such as the *IL-4 promoter, IL-4 receptor* and *IL-13* genes, were reported to be associated with allergic diseases or high serum IgE levels (3,14,15). On the other hand, Th1 cytokines and their receptor genes, such as the *IL-12 receptor, IL-18 receptor* and *IFN-γ* genes were also reported to be associated with allergic diseases (2,8,10,16,17).

IFN-γ, a major cytokine from Th1 cells, directly suppresses IgE synthesis (18,19). IFN-γ exerts its various biologic effects through its binding to the cell-surface ligand-binding chain IFNGR1 in combination with its signaling chain, IFNGR2 (20-23). There are common polymorphisms in the *IFNGR1* (Val 14 Met) and *IFNGR2* (Gln 64 Arg) genes (24,25). Gao *et al* reported that there is an association between such common polymorphisms and total serum IgE levels in the British population, but not in the Japanese population (24). Nakao *et al* also showed that such polymorphisms are not associated with atopic asthma in the Japanese population (25).

In this study, we identified a novel single-nucleotide substitution 1400 T→C (L467P) in the seventh exon of the *IFNGR1* gene. All patients who had L467P substitution suffered from BA or AR or both. These results showed that L467P in the *IFNGR1* gene might have associated with the phenotype of allergic diseases such as BA or AR. Furthermore, a familial analysis for L467P revealed a linkage between allergic diseases and L467P.

To examine the functional effects of L467P in the *IFNGR1* gene, we measured total serum IgE levels, specific IgE levels

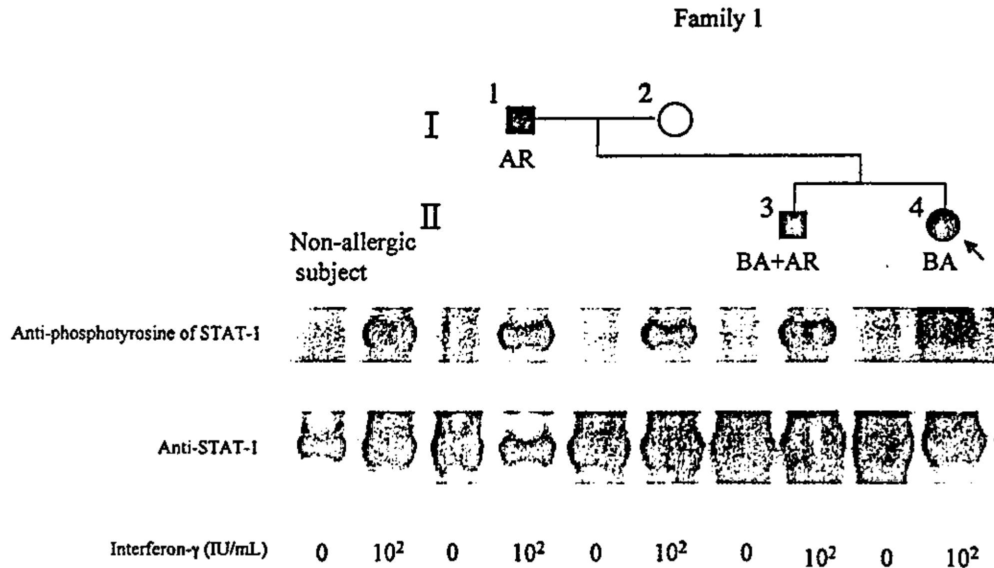

Figure 5. Phosphorylation of STAT1 in PBMCs. PBMCs from the patients of family 1 and non-allergic subject were cultured with or without IFN-γ (10^2 IU/ml) for 15 min. Cell lysates were immunoprecipitated with an anti-STAT1 antibody, and resolved by SDS-PAGE; fractionated products were transferred to a nitrocellulose membrane, and blotted with anti-phosphotyrosine of STAT1 (upper) or anti-STAT1 (lower) antibodies. A clear band was detected on the blots with the antibody against phosphotyrosine of STAT1 in PBMCs from the non-allergic subject stimulated with IFN-γ. The same bands were shown on the blots with the antibody against phosphotyrosine of STAT1 in PBMCs from the allergic patients with L467P stimulated with IFN-γ.

and IFN-γ production by PBMCs following stimulation with IL-12, IL-18 or PHA in all of the allergic patients and non-allergic subjects. L467P was found to be associated with high total serum IgE levels and high levels of specific IgE for inhalant antigens such as house dust and mite. Generally, IFN-γ production by PBMCs in the allergic patients was reported to be lower than that in the non-allergic subjects (6,7,9,26). A decrease in the IFN-γ production leads to the predominance of Th2 cells over Th1 cells, which leads to the inability of B-cells to suppress IgE production, subsequently resulting in an increase in the serum IgE levels. In this study, in spite of the IFN-γ production in the allergic patients with L467P was not lower than that in the non-allergic subjects, their serum IgE levels were high and they exhibited allergic symptoms. These data suggest that this heterozygous L467P in the *IFNGR1* may affects the cellular response to IFN-γ by the dominant negative effect (27) and causes an increased susceptibility to allergic diseases although sufficient IFN-γ production in some allergic patients.

The intracellular domain of the *IFNGR1* gene contains two functionally important regions (28). First, a membrane proximal region that includes an LPKS sequence that participates in the binding of JAK1 to IFNGR1 required for ligand processing and biologic responsiveness. Second, a region spanning the carboxyl-terminal 39 amino acids that are necessary for biologic responses; this region includes a YDKPH sequence that, when phosphorylated, forms the STAT1 docking site on IFNGR1. L467P exists in this functionally important region of the *IFNGR1* gene and is located only six amino acids downstream of the STAT1 docking site. Our study showed that the L467P in the *IFNGR1* gene does not affect STAT1 phosphorylation. Therefore, L467P may affect B-cell functions that are affected by IFN-γ stimulation via a route other than STAT1 phosphorylation (29,30). It is necessary to further study how L467P affects the IFNGR function.

Severe heterozygous frame-shift mutations (818del4 or 818delT) or homozygous missense mutations of the *IFNGR1* gene were identified as the cause of susceptibility to mycobacterial infection (MIM 209950) (31-33). These heterozygous frame-shift mutations of the *IFNGR1* gene were reported to suppress signal transduction of IFNGR1 by the dominant negative effect and cause an increased susceptibility to mycobacterial infection. L467P has not been reported to be associated with this disorder and, in this study, none of the patients with L467P were susceptible to mycobacterial infection. Different nucleotide substitutions in the *IFNGR1* gene may result in different disease phenotypes, such as immune deficiency and allergic diseases.

Our study showed that the *IFNGR1* gene might be one of the candidate susceptibility genes for allergic diseases and L467P in the *IFNGR1* gene is associated with allergic diseases.

References

1. Holgate ST: Genetic and environmental interaction in allergy and asthma. J Allergy Clin Immunol 104: 1139-1146, 1999.
2. Kondo N, Matsui E, Kaneko H, Fukao T, Teramoto T, Inoue R, Watanabe M, Aoki M, Kasahara K and Morimoto N: Atopy and mutations of IL-12 receptor beta 2 chain gene. Clin Exp Allergy 31: 1189-1193, 2001.
3. Mitsuyasu H, Izuhara K, Mao XQ, Gao PS, Arinobu Y, Enomoto T, Kawai M, Sasaki S, Dake Y, Hamasaki N, Shirakawa T and Hopkin JM: Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma. Nat Genet 19: 119-120, 1998.
4. Venkataraman C, Leung S, Salvekar A, Mano H and Schindler U: Repression of IL-4-induced gene expression by IFN-gamma requires Stat1 activation. J Immunol 162: 4053-4061, 1999.

5. Cookson W: The alliance of genes and environment in asthma and allergy. Nature 402: B5-B11, 1999.
6. Teramoto T, Fukao T, Tashita H, Inoue R, Kaneko H, Takemura M and Kondo N: Serum IgE level is negatively correlated with the ability of peripheral mononuclear cells to produce interferon gamma (IFN gamma): evidence of reduced expression of IFN gamma mRNA in atopic patients. Clin Exp Allergy 28: 74-82, 1998.
7. Matsui E, Kaneko H, Teramoto T, Fukao T, Inoue R, Kasahara K, Takemura M, Seishima M and Kondo N: Reduced IFN gamma production in response to IL-12 stimulation and/or reduced IL-12 production in atopic patients. Clin Exp Allergy 30: 1250-1256, 2000.
8. Matsui E, Kaneko H, Fukao T, Teramoto T, Inoue R, Watanabe M, Kasahara K and Kondo N: Mutations of the IL-12 receptor beta2 chain gene in atopic subjects. Biochem Biophys Res Commun 266: 551-555, 1999.
9. Shikano H, Kato Z, Kaneko H, Watanabe M, Inoue R, Kasahara K, Takemura M and Kondo N: IFN-gamma production in response to IL-18 or IL-12 stimulation by peripheral blood mononuclear cells of atopic patients. Clin Exp Allergy 31: 1263-1270, 2001.
10. Watanabe M, Kaneko H, Shikano H, Aoki M, Sakaguchi H, Matsui E, Inoue R, Kato Z, Kasahara K, Fukutomi O, Kondo T and Kondo N: Predominant expression of 950delCAG of IL-18R alpha chain cDNA is associated with reduced IFN-gamma production and high serum IgE levels in atopic Japanese children. J Allergy Clin Immunol 109: 669-675, 2002.
11. Hanifin JM: Atopic dermatitis. J Allergy Clin Immunol 73: 211-226, 1984.
12. Skoner DP: Allergic rhinitis: definition, epidemiology, pathophysiology, detection, and diagnosis. J Allergy Clin Immunol 108: S2-S8, 2001.
13. Dykewicz MS, Fineman S, Skoner DP, Nicklas R, Lee R, Blessing-Moore J, Li JT, Bernstein IL, Berger W, Spector S and Schuller D: Diagnosis and management of rhinitis: complete guidelines of the Joint Task Force on Practice Parameters in Allergy, Asthma and Immunology. Ann Allergy Asthma Immunol 81: 478-518, 1998.
14. Noguchi E, Shibasaki M, Arinami T, Takeda K, Yokouchi Y, Kawashima T, Yanagi H, Matsui A and Hamaguchi H: Association of asthma and the interleukin-4 promoter gene in Japanese. Clin Exp Allergy 28: 449-453, 1998.
15. Graves PE, Kabesch M, Halonen M, Holberg CJ, Baldini M, Fritzsch C, Weiland SK, Erickson RP, von Mutius E and Martinez FD: A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children. J Allergy Clin Immunol 105: 506-513, 2000.
16. Nieters A, Brems S and Becker N: Cross-sectional study on cytokine polymorphisms, cytokine production after T-cell stimulation and clinical parameters in a random sample of a German population. Hum Genet 108: 241-248, 2001.
17. Barnes KC, Freidhoff LR, Nickel R, Chiu YF, Juo SH, Hizawa N, Naidu RP, Ehrlich E, Duffy DL, Schou C, Levett PN, Marsh DG and Beaty TH: Dense mapping of chromosome 12q13.12-q23.3 and linkage to asthma and atopy. J Allergy Clin Immunol 104: 485-491, 1999.
18. Coffman RL and Carty J: A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma. J Immunol 136: 949-954, 1986.
19. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA and Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136: 2348-2357, 1986.
20. Pernis A, Gupta S, Gollob KJ, Garfein E, Coffman RL, Schindler C and Rothman P: Lack of interferon gamma receptor beta chain and the prevention of interferon gamma signaling in TH1 cells. Science 269: 245-247, 1995.
21. Marsters SA, Pennica D, Bach E, Schreiber RD and Ashkenazi A: Interferon gamma signals via a high-affinity multisubunit receptor complex that contains two types of polypeptide chain. Proc Natl Acad Sci USA 92: 5401-2405, 1995.
22. Bach EA, Tanner JW, Marsters S, Ashkenazi A, Aguet M, Shaw AS and Schreiber RD: Ligand-induced assembly and activation of the gamma interferon receptor in intact cells. Mol Cell Biol 16: 3214-3221, 1996.
23. Kotenko SV, Izotova LS, Pollack BP, Mariano TM, Donnelly RJ, Muthukumaran G, Cook JR, Garotta G, Silvennoinen O, Ihle JN and Pestka S: Interaction between the components of the interferon gamma receptor complex. J Biol Chem 270: 20915-20921, 1995.
24. Gao PS, Mao XQ, Jouanguy E, Pallier A, Doffinger R, Tanaka Y, Nakashima H, Otsuka T, Roberts MH, Enomoto T, Dake Y, Kawai M, Sasaki S, Shaldon SR, Coull P, Adra CN, Niho Y, Casanova JL, Shirakawa T and Hopkin JM: Non-pathogenic common variants of IFNGR1 and IFNGR2 in association with total serum IgE levels. Biochem Biophys Res Commun 263: 425-429, 1999.
25. Nakao F, Ihara K, Kusuhara K, Sasaki Y, Kinukawa N, Takabayashi A, Nishima S and Hara T: Association of IFN-gamma and IFN regulatory factor 1 polymorphisms with childhood atopic asthma. J Allergy Clin Immunol 107: 499-504, 2001.
26. Jung T, Witzak K, Dieckhoff K, Zachmann K, Heidrich S, Aversa G and Neumann C: IFN-gamma is only partially restored by co-stimulation with IL-12, IL-2, IL-15, IL-18 or engagement of CD28. Clin Exp Allergy 29: 207-216, 1999.
27. Dighe AS, Farrar MA and Schreiber RD: Inhibition of cellular responsiveness to interferon-gamma (IFN gamma) induced by overexpression of inactive forms of the IFN gamma receptor. J Biol Chem 268: 10645-10653, 1993.
28. Farrar MA, Fernandez-Luna J and Schreiber RD: Identification of two regions within the cytoplasmic domain of the human interferon-gamma receptor required for function. J Biol Chem 266: 19626-19635, 1991.
29. Gao PQ, Sims SH, Chang DC and Deisseroth AB: Interferon-gamma priming effects in the activation and deactivation of ISGF3 in K562 cells. J Biol Chem 268: 12380-12387, 1993.
30. Bluyssen HA, Muzaffar R, Vlieststra RJ, van der Made AC, Leung S, Stark GR, Kerr IM, Trapman J and Levy DE: Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. Proc Natl Acad Sci USA 92: 5645-5649, 1995.
31. Jouanguy E, Lamhamedi-Cherradi S, Lammas D, Dorman SE, Fondaneche MC, Dupuis S, Doffinger R, Altare F, Girdlestone J, Emile JF, Ducoulombier H, Edgar D, Clarke J, Oxelius VA, Brai M, Novelli V, Heyne K, Fischer A, Holland SM, Kumararatne DS, Schreiber RD and Casanova JL: A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. Nat Genet 21: 370-378, 1999.
32. Dorman SE and Holland SM: Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. J Clin Invest 101: 2364-2369, 1998.
33. Newport MJ, Huxley CM, Huston S, Hawrylowicz CM, Oostra BA, Williamson R and Levin M: A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. N Engl J Med 335: 1941-1949, 1996.

Clin Exp Allergy 2003; 33:1097–1102

(26) Association between interleukin-18 gene polymorphism 105A/C and asthma

S. Higa*, T. Hirano*, M. Mayumi†, M. Hiraoka†, Y. Ohshima†, M. Nambu‡, E. Yamaguchi§, N. Hizawa§, N. Kondo¶, E. Matsui¶, Y. Katada‖, A. Miyatake**, I. Kawase* and T. Tanaka*

*Department III of Internal Medicine, Osaka University Medical School, Osaka, †Department of Pediatrics, Fukui Medical University School of Medicine, Fukui, ‡Department of Pediatrics, Tenri Hospital, Nara, §First Department of Medicine, Hokkaido University School of Medicine, Sapporo, ¶Department of Pediatrics, Gifu University School of Medicine, Gifu, ‖Department of Allergy, Osaka Minami National Hospital, Osaka, and **Miyatake Asthma Clinic, Osaka, Japan

Summary

Background IL-18 has been shown to exert anti-allergic or allergy-promoting activities, but the existence of genetic polymorphisms in the coding regions of IL-18 gene has not been demonstrated.
Objective The aim of this study was to investigate whether polymorphism is present in the coding regions of the IL-18 gene and, if so, to further analyse the association between polymorphism and asthma in a case–control study.
Methods We screened the coding regions of the IL-18 gene for polymorphisms by using PCRsingle-stranded conformation polymorphism and direct sequencing of PCR products, followed by analysis of the association between polymorphism and asthma.
Results We identified one polymorphism (105A/C) in the coding regions. The frequency of the 105A allele was significantly higher in asthmatic patients than in controls ($P<0.01$; odds ratio (OR) = 1.83 (1.37–2.26)). Significant linkage disequilibrium was observed between the 105A/C and −137G/C polymorphisms in the 5′ flanking region of the IL-18 gene ($D=0.58$, $P<0.0001$). However, in asthmatic patients the 105A allele was not associated with either total serum IgE or IL-18 levels.
Conclusion The 105A/C polymorphism of the IL-18 gene may be associated with the pathogenesis of asthma.

Keywords asthma, IL-18, polymorphism
Submitted 5 August 2002; revised 15 January 2003; accepted 3 May 2003

Introduction

The IFN-γ-inducing cytokine IL-18 has been shown to exert innate as well as cellular immunity through both NK activation and Th1 induction by acting on the responding cell population [1–4]. It also has an inhibitory effect on IgE synthesis by directly acting on B cells when stimulated with IL-12 through the induction of endogenous IFN-γ [5]. In contrast, it was found that IL-18 directly stimulates IL-4 and histamine release from basophils [6], IL-13 production by NK and T cells [7], induces naïve T cells to differentiate into Th2 cells [8] and enhances IL-8 production by eosinophils [9]. These findings indicate that IL-18 has both anti-allergic and allergy-promoting effects [10].

Excess of IL-18 production was recently found in patients with allergic diseases, including asthma and atopic dermatitis [11–15]. Since IL-18 also increased in patients with other diseases in which a predominance of Th1 cells may play an important role [16–20], it remains to be clarified whether IL-18 has a positive or negative function at the onset or development of allergic diseases. In order to identify the genetic determinants for asthma, we examined whether or not polymorphisms occur in the coding regions of the IL-18 gene and, if so, what the relation is between polymorphism and asthma. In fact, one silent polymorphism (A/C) located at the 105th nucleotide initiating from ATG was identified in the coding regions of the IL-18 gene. The frequency of this polymorphism in controls significantly differed from that in patients with asthma, indicating an association of IL-18-105A/C with asthma.

Correspondence: Toshio Tanaka, Department III of Internal Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan. E-mail: ttanak@imed3.med.osaka-u.ac.jp

Materials and methods

Subjects

A total of 221 asthmatic children 1–18 years of age, 276 asthmatic adults 18–78 years old and 85 adult controls without any past or current allergic diseases, including asthma, atopic dermatitis and pollen's diseases, were enrolled in the study. Asthma was diagnosed on the basis of the American Thoracic Society guidelines. Classification of asthma severity was judged by global strategy for asthma management and prevention. Four hundred and seven asthmatic patients showed total IgE concentrations above 200 IU/mL or specific sensitization to major allergens such as

© 2003 Blackwell Publishing Ltd

house dust mite, dog and cat, and classified into atopic type of asthma. A full verbal and written explanation of the study was given to the parents of the asthmatic children or asthmatic adults and written informed consent was obtained. The study was approved by the relevant ethical committees in accordance with the guidelines for the management of genetic studies.

Screening for polymorphisms in the coding regions of the IL-18 gene

Total RNA was extracted from peripheral blood mononuclear cells by using RNAzol (Biotecx, Houston, TX, USA) according to the manufacturer's instructions. The extracted RNA was then dissolved in DEPC water and its concentration was measured with a spectrometer. The coding region of the IL-18 gene spanning exons 3–7 was amplified by RT-PCR according to the manufacturer's protocol (Perkin-Elmer Cetus Co., Norwalk, CT, USA). The cDNA was amplified by PCR in the presence of 180 kBq (α-^{32}P)-dCTP (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK). PCR conditions were as follows: 94 °C, 1 min; 63 °C, 1 min; and 72 °C, 1 min for 36 cycles. The primers used for amplification of the coding region of IL-18 spanning exons 3–7 were: sense, ATG-GCT-GCT-GAA-CCA-GTA-GA; anti-sense, AGC-TAG-TCT-TCG-TTT-TGA-ACA-G. The product was then digested with the *Msp*I restriction enzyme (Toyobo Co., Osaka, Japan) at 37 °C for 4 h and examined for the presence of polymorphisms by single-stranded conformation polymorphism (SSCP) analysis [21].

DNA sequencing

PCR products were purified from the gel and DNA sequencing was performed according to the protocol for the dye terminator cycle sequencing FS ready reaction kit (Applied Biosystems, Osaka, Japan). Briefly, the amplification of the coding region of IL-18 cDNA for sequencing was performed in 25 cycles of denaturation for 10 s at 96 °C, annealing for 5 s at 50 °C and extension for 4 min at 60 °C by using the dye terminator cycle sequencing FS ready reaction kit (ABI) and the 2400 Thermal cycle and 377 sequencer (Perkin-Elmer Cetus).

Genotyping

Genomic DNA was extracted from peripheral blood leucocytes by means of the QIA prep Spin (Qiagen Gmbh, Hilden, Germany). For the PCR, 100 ng of DNA was used, and for the detection of IL-18-105A/C polymorphism, primers (sense, TGT-TTA-TTG-TAG-AAA-ACC-TGG-AAT-T; anti-sense, CCT-CTA-CAG-TCA-GAA-TCA-GT) were designed. The partial coding region of the IL-18 gene was then amplified by PCR according to the manufacturer's protocol. PCR conditions were as follows: 94 °C, 1 min; 50 °C, 1 min; and 72 °C, 1 min for 40 cycles. The PCR product was digested with *Taq*I restriction enzyme (Toyobo) at 65 °C for 2 h and electrophoresed on the PAGE gel (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The gel was then stained with ethidium bromide and photographed. In order to examine the promoter regions, −607C/A and −137G/C for polymorphisms, previously reported methods and primers were employed [22]. The genotype of β2-adrenergic receptor (β2-ADR)-16 Arg/Gly was determined by using primers (sense, CGG-GAA-CGG-CAG-AGC-CTT-CTT-GCT-GGC-ACC-CAC-T; anti-sense, CCG-TCT-GCA-GAC-GCT-CGA-AC), and the PCR conditions were as follows: 94 °C, 1 min; 60 °C, 1 min; and 72 °C, 1 min for 40 cycles. The PCR product was digested with *Bbv*I (Toyobo) at 37 °C for 2 h.

Measurement of IL-18

Serum concentrations of IL-18 were measured by means of ELISA assays (IL-18 ELISA kit, MBL, Nagoya, Japan).

Statistical analysis

The frequencies of alleles were calculated by counting. Data were analysed with MedCalc and Arlequin software and the Hardy–Weinberg equilibrium was determined be means of the χ^2 goodness-of-fit test. Fisher's exact test was used to detect differences in allele distribution between the groups, and the exact-test-of-population differentiation was used to determine whether the genotypic composition of the groups was significantly heterogeneous. The odds ratio (OR) was calculated by means of logistic regression, and the confidence interval (CI) was calculated at the 95% level. The maximum likelihood estimates of relative linkage disequilibriums between 105A/C and −607C/A or −137G/C were calculated according to the methods of Thompson et al. [23]. Statistical significance was assumed for P-values less than 0.05.

Results

A novel polymorphism, 105A/C, in the coding regions of the IL-18 gene

It has been recently shown that IL-18 concentrations in the sera from patients with asthma and atopic dermatitis are elevated [11–15]. As shown in Fig. 1, we could confirm that the serum IL-18 levels for the patients with asthma were higher than for controls. Specifically, the average level of

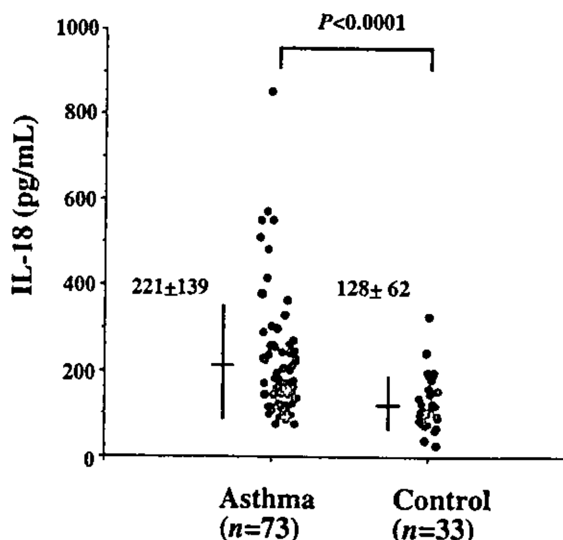

Fig. 1. Increased serum levels of IL-18 in patients with asthma.

serum IL-18 for patients with asthma was 221 pg/mL, which was significantly higher than for the controls (128 pg/mL) ($P<0.0001$). In order to determine whether this up-regulation of IL-18 levels in asthmatic patients was associated with genetic polymorphisms or whether IL-18 might play a positive or negative role at the onset of asthma, we examined the coding regions of the IL-18 gene for the existence of polymorphisms. The translational coding region spanning exons 3–7 was amplified through RT-PCR of 30 different samples. The existence of polymorphisms was then verified by SSCP analysis. Fig. 2 shows the results. A predominant band was obtained from most of the samples, as well as another, more slowly migrating band from some of them. The sequence analysis of the PCR products identified a novel polymorphism (A/C) at the 105th site counting from ATG, but this polymorphism was silent.

IL-18-105A allele was associated with asthma

Next, the frequency of this polymorphism was studied in 497 asthmatic patients and 85 controls by PCR as shown in Fig. 2. Table 1 demonstrates that the frequency of the A allele for patients with asthma was 0.891, which was significantly higher than that for controls (0.818) ($P<0.01$). The OR of carrying one of the two A alleles vs. none was calculated as 1.83. This OR increased substantially to 1.92 for the data from asthmatic children (Table 2). This result demonstrates that the IL-18-105A/C polymorphism is associated with asthma. In order to exclude that this significance is due to the chance of population admixture, allele frequencies of β2-ADR-16-Arg/Gly were measured. The frequency of Arg allele for patients with asthma was similar to that from controls. We also examined the association of IL-18-105A/C polymorphism with the current severity of asthma and with atopic status. However, we failed to observe an association between the genotype and severity or atopic status (Table 2).

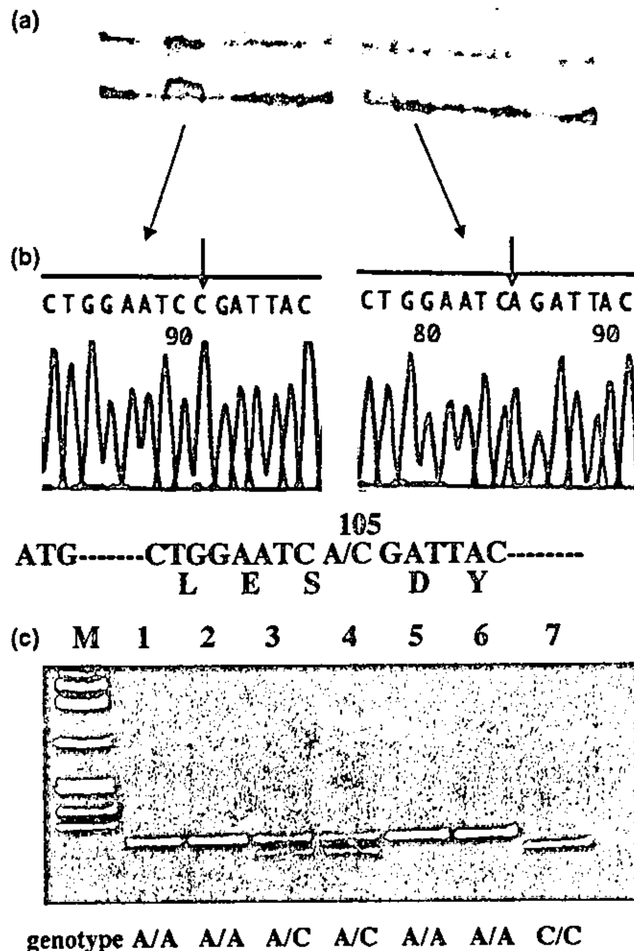

Fig. 2. IL-18-105A/C polymorphism RT-PCR-SSCP analysis of coding regions of the IL-18 gene. The coding region spanning exons 3–7 was amplified by RT-PCR and then digested with *Msp*I. The products were then electrophoresed. Direct sequencing of the PCR products determined the existence of polymorphism located at the 105th site, A/C. Genotyping of IL-18-105A/C by RFLP-PCR.

Linkage disequilibrium between 105A/C and –137G/C promoter polymorphism

Polymorphisms in the promoter region of the IL-18 gene have recently been identified [22]. The changes from C to A at position –607 (–607C/A) and from G to C at position –137 (–137G/C) were found to affect the transcription of the IL-18 gene. We next tried to determine whether there was a linkage disequilibrium between the coding polymorphism 105A/C and –607C/A or –137G/C. Table 3 shows the pairwise analysis of the polymorphisms, indicating a significant disequilibrium between 105A/C and –137G/C, and between 105A/C and –607C/A. A linkage disequilibrium between 105A/C and –137G/C ($D=0.575$, $P<0.0001$) was more significantly observed than that between 105A/C and –607C/A ($D=0.283$, $P=0.0034$). Finally, we verified the association of the genotype of 105A/C with serum IgE levels and IL-18 levels for asthmatic patients, but as shown in Table 4, no significant relation could be detected.

Discussion

In this study, we identified one polymorphism, 105A/C, in the coding regions of the IL-18 gene and demonstrated the association between this polymorphism and asthma. The OR was 1.83 for the asthmatic phenotype when the allele was A. Case–control studies are sometimes susceptible to the population admixture, but the frequency of Arg in patients with asthma was similar to that from controls. These results suggest that IL-18 may play a role in the pathogenesis of asthma, although it remains to be determined as to how the polymorphism influences the pathogenesis. The linkage disequilibrium between 105A and –137G suggests that the 105A allele may be associated with the up-regulation of IL-18

Table 1. Allele and genotype frequencies of IL-18-105A/C polymorphisms in patients with asthma and controls

Allele	Asthma ($n=497$)	Control ($n=85$)	Genotype	Asthma ($n=497$)	Control ($n=85$)
A	0.891	0.818	AA	0.792	0.682
C	0.109	0.182	AC	0.197	0.271
$P=0.0062$			CC	0.010	0.047
			$P=0.0089$		

Table 2. Allele frequencies of IL-18-105A/C and β2-ADR-16-Arg/Gly in asthmatic adults and children (upper panel) and allele frequencies of IL-18-105A/C in asthmatic patients classified by severity and atopics (lower panel)

Allele	Control	Asthma	Adult	Children
IL-18–105A/C				
A	139	886	490	396
C	31	108	62	46
Odds ratio		1.83	1.76	1.92
(CI)		(1.37–2.26)	(1.10–2.82)	(1.18–3.10)
P-value		0.0062	0.0172	0.0089
β2-ADR-16				
Arg	71	484	269	215
Gly	81	506	283	223
Odds ratio		1.09	1.08	1.10
(CI)		(0.78–1.53)	(0.75–1.55)	(0.76–1.59)
P-value		0.6169	0.6588	0.6135

Table 2a

		Severity of asthma			Type of asthma	
Allele	Control	Intermittent	Mild to moderate	Severe	Atopics	Non-atopics
A	139	523	206	77	722	164
C	31	64	26	9	92	16
Odds ratio		1.82	1.76	1.91	1.75	2.29
(CI)		(1.43–2.31)	(1.01–3.08)	(0.87–4.18)	(1.11–2.75)	(1.20–4.37)
P-value		0.0110	0.0460	0.1058	0.0129	0.0104

Table 3. Combined genotype frequencies of IL-18-105A/C, IL-18-137G/C and IL-18-607C/A

	105A/C			
	A/A	A/C	C/C	
– 137G/C				
G/G	59	0	0	$P<0.0001$
G/C	1	17	1	$n=84$
C/C	0	0	6	$D=0.575$
– 607C/A				
C/C	16	0	0	$P=0.0034$
C/A	28	10	1	$n=83$
A/A	15	7	6	$D=0.283$

Linkage disequilibrium between 105A/C and – 137G/C was more significantly observed than that between 105A/C and – 607C/A.

Table 4. Serum levels of IgE and IL-18 from patients with asthma

	IgE (IU/mL)	IL–18 (pg/mL)
Genotype		
A/A	869+1852 ($n=360$)	220±148 ($n=57$)
A/C	1176±2973 ($n=87$)	224±102 ($n=16$)
C/C	1274±1176 ($n=4$)	ND

Serum levels of IgE and IL-18 were compared with the IL-18-105A/C genotype.
Results are indicated as means±SD. There was no significant difference.
ND = Not determined.

expression through the –137G allele, although we did not find an increase in the serum IL-18 levels of asthmatic patients with the AA haplotype of the 105 polymorphism. A previous report regarding polymorphisms in the promoter region showed that the –137G allele enhanced the transcriptional activity of the IL-18 gene when cells were stimulated with PMA plus ionomicin, but that there were no significant differences in promoter activity between –137G and –137C alleles in the absence of stimulants [22]. Alternatively, it is possible that 105A/C, although quiescent, directly affects the modulation of transcription of the gene or RNA stabilization [24] or that 105A/C is linked to another polymorphism, which alters the functional property of IL-18 or its gene regulation. Further studies are required to clarify these points.

Recently, several lines of *in vitro* and *in vivo* studies have generated contradictory results about the function of IL-18 in the pathophysiology of allergic diseases [10]. The activities of IL-18, including induction of IFN-γ synthesis by T cells or NK cells and of Th1 induction [1–3] as well as inhibition of IgE production by B cells when expressed together with IL-12 [5], show that this cytokine may be a negative regulator in view of the pathogenesis of allergic diseases. However, IL-18 has also been reported to induce the release of both histamine and IL-4 from basophils [6], and cause Th2 differentiation [8]. The effect of IL-18 on the asthma mouse model thus seems to be paradoxical. For instance, administration of IL-18 was found to enhance Ag-induced eosinophils recruitment into the trachea and bronchoalveolar lavage fluid of sensitized mice *in vivo* [25], and to increase Th2 cytokines and airway eosinophilia in a mouse model of allergic asthma [26]. In

contrast, coadministration of IL-12 and IL-18 was shown to inhibit antigen-induced airway hyper-responsiveness, eosinophilia and serum IgE levels [27], and IL-18 deficiency to cause enhancement of allergen-induced eosinophilia accumulation [28]. Although the reasons for such contradictory results remain to be determined, it is likely that they are related to the responding cells or to whether or not IL-18 together with IL-12 acts on the cells. On the other hand, changes in IL-18 properties or expression through genetic polymorphisms could lead to the exacerbation or suppression of allergic responses.

Polymorphisms in candidate genes have been identified and associated with asthma [29, 30]. The IL-18 gene is located at chromosomal region 11q23 [31]. Previous studies of genome-wide research for asthma have demonstrated that the region of 11q21–24 was linked to asthma phenotype, specific IgE to a group of common aeroallergens and positive skin test to house dust mite [32, 33]. Candidate genes in this region include matrix metalloproteinase genes 1, 3 and 8, and the δ, ε and γ subunits of CD3. Moreover, the newly identified IL-18-105A/C polymorphism and/or the previously identified IL-18-137G/C polymorphism can be considered to be one of such polymorphisms associated with asthma. We expect that our observations are going to contribute to an understanding of the pathophysiology of asthma and result in the establishment of predictive markers for asthma.

Acknowledgements

This investigation was supported by the Pollution-related Health Damage Compensation and Prevention Association of Japan, Ministry of Education, Science and Culture of Japan, the Ministry of Health and Welfare of Japan.

References

1 Okamura H, Tsutsui H, Komatsu T et al. Cloning of a new cytokine that induces INF-γ production by T cells. Nature 1995; 378:88–91.
2 Tsutsui H, Nakanishi K, Higashino K, Okamura H, Miyazawa Y, Kaneda K. IFN-γ-inducing factor upregulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. J Immunol 1996; 156:3967–73.
3 Okamura H, Tsutsui H, Kashiwamura S, Yoshimoto T, Nakanishi K. Interleukin-18 (IL-18) a novel cytokine that augments both innate and acquired immunity. Adv Immunol 1998; 70:281–312.
4 Dinarello CA. IL-18: a TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. J Allergy Clin Immunol 1999; 103:11–24.
5 Yoshimoto T, Okamura H, Tagawa A, Nakanishi K. Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-γ production from activated B cells. Proc Natl Acad Sci USA 1997; 94:3948–53.
6 Yoshimoto T, Tsutsui H, Tominaga K et al. IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. Proc Natl Acad Sci USA 1999; 96:13962–6.
7 Hoshino T, Wiltrout RH, Young HA. IL-18 is a potent coinducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. J Immunol 1999; 162:5070–7.
8 Yoshimoto T, Mizutani H, Tsutsui H et al. IL-18 induction of IgE: dependence on $CD4^+$ T cells, IL-4 and STAT6. Nature Immunol 2000; 1:132–7.
9 Wang W, Tanaka T, Okamura H et al. Interleukin-18 enhances the production of interleukin-8 by eosinophils. Eur J Immunol 2001; 31:1010–6.
10 Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. Annu Rev Immunol 2001; 19:423–4.
11 Tanaka H, Miyazaki N, Oashi K et al. IL-18 might reflect disease activity in mild and moderate asthma exacerbation. J Allergy Clin Immunol 2001; 107:331–6.
12 Wong CK, Ho CY, Ko FWS et al. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-γ, IL-4, IL-10 and IL-13) in patients with allergic asthma. Clin Exp Immunol 2001; 125:177–83.
13 El-Mezzein REH, Matsumoto T, Nomiyama H, Miike T. Increased secretion of IL-18 *in vitro* by peripheral blood mononuclear cells of patients with bronchial asthma and atopic dermatitis. Clin Exp Immunol 2001; 126:193–8.
14 Tanaka T, Tsutsui H, Yoshimoto T et al. Interleukin-18 is elevated in the sera from patients with atopic dermatitis and from atopic dermatitis-model mice, NC/Nga. Int Arch Allergy Immunol 2001; 125:236–40.
15 Higashi N, Gesser B, Kawana S, Thestrup-Pedersen K. Expression of IL-18 mRNA and secretion of IL-18 are reduced in monocytes from patients with atopic dermatitis. J Allergy Clin Immunol 2001; 108:607–14.
16 Yamada G, Shijubo N, Shigehara K, Okamura H, Kurimoto M, Abe S. Increased levels of circulating interleukin-18 in patients with advanced tuberculosis. Am J Respir Crit Care Med 2000; 161:1786–9.
17 Shigehara K, Shijubo N, Ohmichi M et al. Increased levels of interleukin-18 in patients with pulmonary sarcoidosis. Am J Respir Crit Care Med 2000; 162:1979–82.
18 Kanai T, Watanabe M, Okazawa A et al. Interleukin 18 is a potent proliferative factor for intestinal mucosal lymphocyte in Crohn's disease. Gastroenterology 2000; 119:1514–23.
19 Nicoletti F, Di Marco R, Mangano K et al. Increased serum levels of interleukin-18 in patients with multiple sclerosis. Neurology 2001; 57:342–4.
20 Kawashima M, Yamamura M, Taniai M et al. Levels of interleukin-18 and its binding inhibitors in the blood circulation of patients with adult-onset Still's disease. Arthritis Rheum 2001; 44:550–60.
21 Hayashi K. PCR-SSCP: a method for detection of mutations. Genet Anal Tech Appl 1992; 9:73–9.
22 Giedraitis V, He B, Huang W-X, Hillert J. Cloning and mutation analysis of the human IL-18 promoter: a possible role of polymorphisms in expression regulation. J Neuroimmunol 2001; 112:146–52.
23 Thompson EA, Deeb S, Walker D, Motulsky AG. The detection of linkage disequilibrium between closely linked markers: RFLPs at the AI-CIII apolipoprotein genes. Am J Hum Genet 1988; 42: 113–24.
24 Du YZ, Dickerson C, Aylsworth AS, Schwartz CE. A silent mutation, C924T (G308G), in the L1CAM gene results in X linked hydrocephalus (HSAS). J Med Genet 1998; 35:456–62.
25 Kumano K, Nakao A, Nakajima H et al. Interleukin-18 enhances antigen-induced eosinophil recruitment into the mouse airways. Am J Respir Crit Care Med 1999; 160:873–8.
26 Wild JS, Sigounas A, Sur N et al. IFN-gamma-inducing factor (IL-18) increases allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma. J Immunol 2000; 164:2701–10.
27 Hofstra CL, Van Ark I, Hofman G, Kool M, Nijkamp FP, Van Oosterhout AJ. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsivesess, eosinophilia, and serum IgE levels. J Immunol 1999; 161:5054–60.

28 Kodama T, Matsuyama T, Kuribayashi K et al. IL-18 deficiency selectively enhances allergen-induced eosinophilia in mice. J Allergy Clin Immunol 2000; 105:45–53.
29 Cookson WOCC. Asthma genetics. Chest 2002; 121:7S–13S.
30 Palmer LJ, Cookson WOCM. Using single nucleotide polymorphisms as a means to understanding the pathophysiology of asthma. Respir Res 2001; 2:102–12.
31 Baysal BE, Willett-Brozick JE, Taschner PEM et al. A high-resolution integrated map spanning the SDHD gene at 11q23: a 1.1-Mb BAC contig, a partial transcript map and 15 new repeat polymorphisms in a tumour-suppressor region. Eur J Hum Genet 2001; 9:121–9.
32 Xu J, Meyers DA, Ober C et al. Genomewide screen and identification of gene–gene interactions for asthma-susceptibility loci in three U.S. populations: collaborative study on the genetics of asthma. Am J Hum Genet 2001; 68:1437–46.
33 Koppelman GH, Stine OC, Xu J et al. Genome-wide search for atopy susceptibility genes in Dutch families with asthma. J Allergy Clin Immunol 2002; 109:498–506.

British Journal of Dermatology 2003; **149**: 39–45.

Cutaneous Biology

Administration of anti-interleukin 18 antibody fails to inhibit development of dermatitis in atopic dermatitis-model mice NC/Nga

S.HIGA, M.KOTANI,* M.MATSUMOTO,* A.FUJITA,* T.HIRANO, M.SUEMURA,† I.KAWASE AND T.TANAKA

Department III of Internal Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita City, Osaka 565-0871, Japan
**Research and Development Center, Sunstar Incorporation, Osaka, Japan*
†Nissay Hospital, Osaka, Japan

Accepted for publication 28 November 2002

Summary

Background Interleukin (IL)-18 has been shown to activate basophils to produce histamine and IL-4 and to induce naive T cells to differentiate into T-helper (Th) 2 cells. However, when expressed together with IL-12, IL-18 induces Th1 cell development and inhibits IgE synthesis. Previously we reported that serum IL-18 levels were elevated in the sera from atopic dermatitis-model mice NC/Nga, prior to the onset and during the development of dermatitis.
Objectives We studied whether neutralization of IL-18 activity might affect dermatitis in NC/Nga mice, to investigate the role of IL-18 on dermatitis.
Methods NC/Nga mice were given weekly anti-IL-18 antibody starting at 5 weeks of age to 13 weeks and development of dermatitis, scratching behaviour and serum IgE concentrations were evaluated.
Results Continuous injections of anti-IL-18 antibody failed to inhibit the onset and development of dermatitis and IgE elevation. The treatment, rather, tended to lead to an exacerbation of dermatitis and scratching behaviour. In addition, the administration of anti-IL-18 antibody did not ameliorate the responsiveness of lymphocytes to IL-4, which was previously demonstrated as an immunological abnormality in the mouse.
Conclusion This study demonstrates that, at least in NC/Nga mice, IL-18, although excessively expressed before the onset of dermatitis, shows antiallergic actions.

Key words: atopic dermatitis, interleukin-18, NC/Nga mouse

The interferon (IFN)-γ-inducing cytokine, interleukin (IL)-18, has been shown to exert innate immunity and cellular immunity through both natural killer (NK) cell activation and helper T cell (Th)1 induction by acting on the responding cell population.[1–3] Also it has an inhibitory activity on IgE synthesis by directly acting on B cells when given with IL-12 through the induction of endogenous IFN-γ.[4] In contrast it was found that IL-18 directly stimulates IL-4 and histamine release from basophils,[5] IL-13 production by NK and T cells[6] and induces naive T cells to differentiate into Th2 cells.[7] These evidences indicate that IL-18 shows both antiallergic and allergy-promoting activities.[8]

In a previous study, we evaluated the serum levels of IL-18 in patients with atopic dermatitis (AD) and in the AD model, NC/Nga mice,[9,10] to examine whether IL-18 might be associated with the immunological abnormality of AD.[11] In NC/Nga mice serum IL-18 levels were found to increase before the onset of dermatitis and elevation of IgE and remained elevated during the development of dermatitis. In this study we investigated whether neutralization of IL-18 through continuous injection of anti-IL-18 antibody would affect dermatitis as well as IgE elevation in NC/Nga.

Correspondence: Toshio Tanaka.
E-mail: ttanak@imed3.med.osaka-u.ac.jp

© 2003 British Association of Dermatologists

Materials and methods

Culture medium, lymphokines and antibodies

RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Gibco), 2-mercaptoethanol (0·05 mmol L^{-1}), L-glutamine (2 mmol L^{-1}), penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}) was used as culture medium. Lipopolysaccharide (LPS) W extract from *Escherichia coli* 0111:B4 was obtained from Difco Laboratories Inc. (Detroit, MI, U.S.A.). Recombinant mouse IL-2, IL-4 and IL-12 were purchased from Genzyme (Boston, MA, U.S.A.). Rat antimouse IL-4, anti-CD3, anti-CD28, fluorescein (FITC)-conjugated anti-CD8, anti-CD4, anti-CD45R/B220, anti-I-A^b, anti-I-A^d monoclonal antibodies were purchased from PharMingen (San Diego, CA, U.S.A.). Neutralizing rabbit anti-IL-18 antibody,[1,12] kindly provided by Dr Okamura (Hyogo College of Medicine), was prepared from sera of rabbits immunized with murine recombinant IL-18. A dose of 200 μg of anti-IL-18 antibody completely blocked IFN-γ-inducing activity of 50 ng of IL-18 in spleen cells stimulated with concanavalin A.[1] Rabbit IgG was purchased from PharMingen and was used as a control antibody. For detection of cytokines, the pair of antibodies of rat-antimouse IL-4 and rat-anti-IFN-γ (antibodies was purchased from PharMingen. The studies were approved by the Institutional Review Board.

Evaluation of the development of dermatitis in NC/Nga

Four-week-old NC/Nga mice (SPF grade) were obtained from Japan SLC Inc. (Shizuoka, Japan) and housed at 23 ± 3 °C in 55 ± 15% relative humidity with 12/12 h of light/dark cycle (light on 07·00–19·00). To neutralize endogenously produced IL-18, five mice were injected intraperitoneally with 100 μg of rabbit antimouse IL-18 antibody before the onset of dermatitis on weekly intervals starting at 5 weeks of age through 13 weeks. The administration of rabbit antimouse IL-18 antibody caused a reduction of serum levels of IL-18 from 268 ± 6 pg mL^{-1} to undetectable levels (< 25 pg mL^{-1}) in NC/Nga mice. As control, five mice were injected with 100 μg of rabbit IgG. The severity of dermatitis was assessed once a week with the following scoring system by observers who were blinded to the intervention. Symptoms were evaluated by skin dryness, eruption and wounds on three parts of the body: ear, face and head, and back. Each symptom was graded from 0 to 3 (no symptoms, 0; mild, 1; moderate, 2; and severe, 3). Blood was taken at 14 weeks from retro-orbital vessels and serum was obtained by centrifugation at 1000 **g** for 5 min at 4 °C and stored at − 80 °C until use. The serum IgE level was measured by means of an enzyme immunoassay (Yamasa Shouyu Co. Ltd, Chiba, Japan). The frequency of scratching behaviour of 13-week-old NC/Nga mice, such as scratching of the nose, ears and dorsal skin with the hind paws, was measured during a 20-min period. Licking of the belly and dorsal skin during grooming was disregarded. Each occurrence of scratching of the head, neck, dorsal skin, ears and nose was scored to obtain the maximum score.

Preparation of CD4+ T cells and B cells

Spleen cells from NC/Nga, BALB/c or C3H/He mice were suspended at a concentration of 2 × 10^6 per mL in RPMI 1640 containing 5 mmol L^{-1} ethylenediamine tetraacetic acid and 2% fetal bovine serum. The cell suspension was incubated with FITC anti-I-A^b (or anti-I-A^d), FITC anti-B220 and FITC anti-CD8 for 30 min at 4 °C on a turning wheel. The cells were then washed twice and resuspended with magnetic beads coated with sheep anti-FITC antibodies (Advanced Magnetics, Cambridge, MA, U.S.A.). Cells that bound antibody were depleted by three rounds of exposure to a magnetic field. The residual cells were collected, washed twice, resuspended in culture medium and used as purified CD4+ T cells. B cells were prepared from spleen cells by negative selection by using FITC anti-CD4 and FITC anti-CD8.

Differentiation of CD4+ T cells into T-helper 1 or 2 cells

Induction of Th1 or Th2 differentiation from CD4+ T cells was done according to the protocol.[13] Briefly, CD4+ T cells (4 × 10^5 cells $well^{-1}$) were incubated with plate-bound anti-CD3 (1 μg mL^{-1}) plus anti-CD28 antibodies (1 μg mL^{-1}) with or without IL-4 (50 ng mL^{-1}) or anti-IL-4 antibody (10 μg mL^{-1}) for Th2 differentiation, or with or without IL-12 (20 ng mL^{-1}) plus anti-IL-4 antibody (10 μg mL^{-1}) for Th1 differentiation. Two days later, IL-2 (10 ng mL^{-1}) was added into the culture and further incubated for an additional 4 days. Viable cells were then obtained by Ficoll-Hypaque centrifugation, washed and re-stimulated with immobilized anti-CD3 antibody plus anti-CD28 antibody. Supernatants were collected at 24 h, and the IL-4 and IFN-γ concentrations were measured by enzyme-linked immunosorbent assay (ELISA).

IgE induction by lymph node B cells

B cells (10^6 cells mL^{-1}) were incubated with LPS (20 μg mL^{-1}) and IL-4 (50 ng mL^{-1}) for 6 days. Supernatants were harvested and IgE concentrations were determined by ELISA as described before.[14]

Statistical analysis

Statistical analysis was performed using the Student's *t*-test and $P < 0.05$ was considered significant.

Results

Continuous neutralization of interleukin-18 exacerbates dermatitis in NC/Nga mice

The NC/Nga mice spontaneously develop severe dermatitis which histologically mimics human AD and shows high IgE elevation with ageing under conventional circumstances beyond the age of 8 weeks.[9] Representative skin and its histological features are shown in Figure 1. Previously we reported increased serum IL-18 levels in NC/Nga mice with evident dermatitis (151–938 pg mL^{-1}) and in NC/Nga mice with no apparent dermatitis (452–876 pg mL^{-1}) but that those in BALB/c and C57/BL6 mice were less than 50 pg mL^{-1}.[11] To evaluate the role of IL-18 on the onset or the development of dermatitis, NC/Nga mice were continuously injected intraperitoneally with either neutralizing anti-IL-18 antibody (100 μg) or rabbit IgG (100 μg) as a control weekly from 5 weeks (before the onset of dermatitis) to 13 weeks of age, as serum IL-18 elevation was found from 5 weeks to 6 weeks of age. The skin symptoms were examined weekly. In the control mice, dermatitis appeared at 9 weeks of age and increased in severity gradually with ageing (Fig. 2). The mean of the skin severity score reached 3·8 at 14 weeks of age. Surprisingly, the administration of neutralizing anti-IL-18 antibody did not prevent the onset of the skin lesions nor inhibit their development. Rather, this treatment tended to lead to the exacerbation of dermatitis with a skin severity score of 9·3 at 14 weeks of age, although the difference was statistically not significant ($P = 0.08$). At the age of 13 weeks, the number of scratching behaviours for 20 min was counted in all mice. The average number of scratching in the control mice was 67 whereas that of the anti-IL-18 antibody-treated mice was 123·8 (Fig. 2). Subsequently, the serum IgE levels at the age of 14 weeks were also measured. Figure 2 shows that the average IgE from control mice or from anti-IL-18 antibody-treated mice showed 30·5 or 41·2 μg mL^{-1}, respectively, and there was no statistically significant difference in the IgE levels between the two groups.

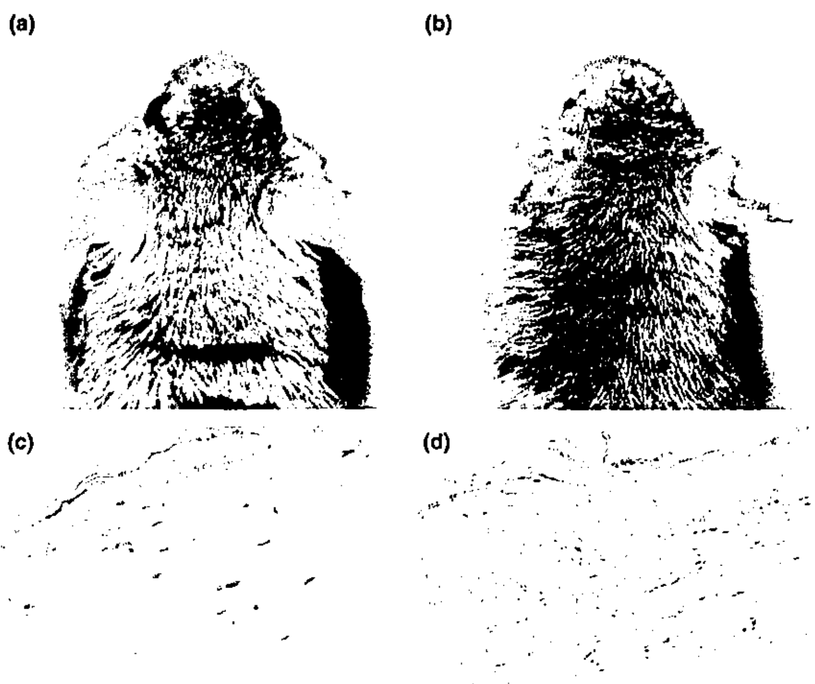

Figure 1. Clinical and histological features of NC/Nga skin: (a,c) aged 10 weeks under specific pathogen-free circumstances; (b,d) aged 10 weeks under conventional circumstance. Histology shows haematoxylin and eosin-stained section of the ear. Original magnification × 10.

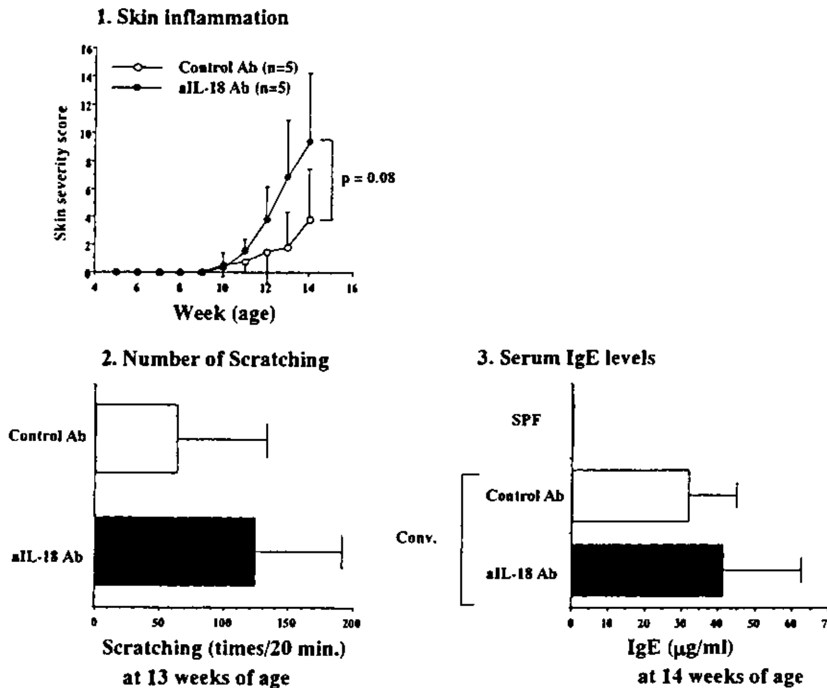

Figure 2. Administration of anti-interleukin (IL)-18 antibody does not suppress severity of dermatitis and scratching behaviour. Five NC/Nga mice were administered intraperitoneally with rabbit antimouse IL-18 antibody (100 μg) or rabbit IgG (100 μg) weekly from 5 weeks to 13 weeks of age. Skin lesions were evaluated weekly by artificial skin severity score. At 13 weeks of age the number of scratching behaviours in 20 min was counted. The serum IgE levels at 14 weeks of age or from mice grown under specific pathogen free (SPF) conditions were measured by enzyme-linked immunosorbent assay. The data from five mice were shown as the mean ± SD.

Continuous injection of anti-interleukin-18 antibody failed to ameliorate interleukin-4 responsiveness of lymphocytes in NC/Nga mice

The mechanisms through which NC/Nga mice develop dermatitis and IgE elevation under conventional circumstances remain unknown. But recently it was reported that responsiveness of B cells to IL-4 was exaggerated, leading to excess production of IgE.[15] Indeed IgE synthesis by purified B cells from three different NC/Nga mice was, as reported previously, dramatically upregulated in response to LPS and IL-4, irrespective of the presence of dermatitis, when compared with B cells from BALB/c or C3H/He mice (Fig. 3). One representative result of three experiments was shown in the figure. The differentiation of CD4+ T cells into IL-4 producers was excessively promoted in NC/Nga in response to anti-CD3 plus IL-4 stimulation, whereas that into IFN-γ producers remained similar to that of control mice (Fig. 3). It remained to evaluate whether IL-4 responsiveness in NC/Nga mice might be associated with overproduction of IL-18. Spleen cells from dermatitis-prone NC/Nga (grown under specific pathogen-free circumstances), control antibody-injected or anti-IL-18 antibody-injected NC/Nga mice were obtained. IgE synthesis by B cells and the differentiation activity of CD4+ T cells into IL-4 producers were examined. The weekly administration of anti-IL-18 antibody did not affect IgE production by purified B cells stimulated with LPS and IL-4 nor the differentiation process of CD4+ T cells into Th2 cells by anti-CD3 plus anti-CD28 antibodies and IL-4 (Fig. 4). Thus IL-4 responsiveness in NC/Nga mice is not associated with overproduction of IL-18.

Discussion

In addition to our previous report,[11] it has recently been demonstrated that transgenic mice expressing caspase 1 or IL-18 in keratinocytes spontaneously developed AD-like skin disease with elevation on the active form of IL-18 in serum.[16,17] Thus, the role of IL-18 on the onset or development of skin lesions, or on IgE elevation in NC/Nga mice was examined. We showed that *in vivo* neutralization of IL-18 activity failed to suppress the onset and the development of skin lesions, IgE elevation and IL-4 responsiveness of lymphocytes in NC/Nga mice. Indeed this treatment appeared to lead to an enhancement in the severity of dermatitis and scratching behaviour, indicating that IL-18 might inhibit an exacerbation of dermatitis in this model mouse.

Recent studies on the expression of various cytokines in AD skin have shown that the development of skin lesions results from sequential activation of Th2 and Th1 cells.[18] In acute lesions, a predominant expression of Th2 cytokines is found but in chronic eczematous lesions IFN-γ is preferentially expressed in the localized T cells.[19] The underlying causes for the initial accu-

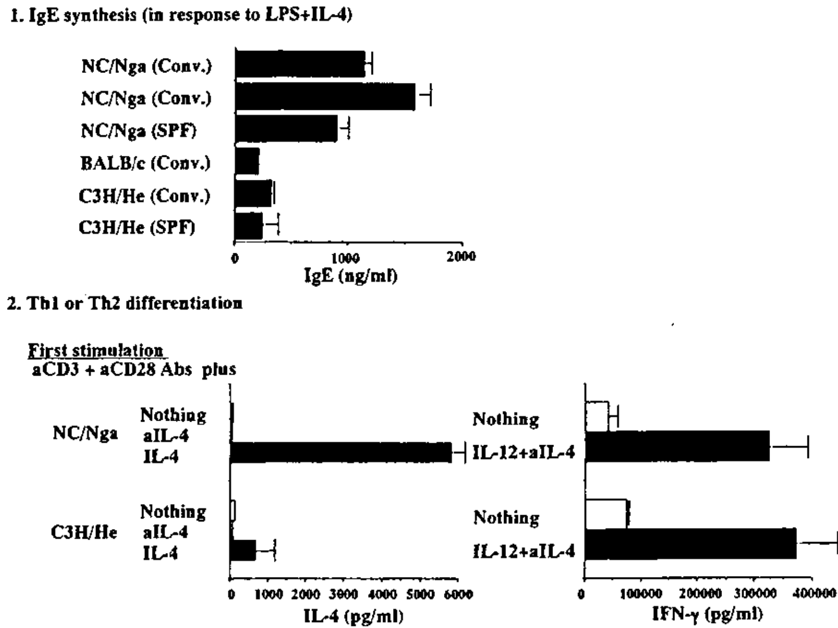

Figure 3. Responsiveness of interleukin (IL)-4 in NC/Nga mice. (1) Purified B cells (10^6 cells mL^{-1}) from one BALB/c, two C3H/He or three NC/Nga mice grown under conventional (Conv.) or specific pathogen free (SPF) conditions were incubated with lipopolysaccharide (20 μg mL^{-1}) and IL-4 (50 ng mL^{-1}) for 6 days and supernatants were harvested. The IgE concentrations in the supernatants from two wells were measured by enzyme-linked immunosorbent assay (ELISA). (2) Purified CD4+ T cells from NC/Nga or C3H/He were stimulated with plate-bound anti-CD3 (1 μg mL^{-1}) plus anti-CD28 (1 μg mL^{-1}) antibodies in the absence or presence of IL-4 (50 ng mL^{-1}) or anti-IL-4 antibody (10 μg mL^{-1}) for T-helper (Th) 2 development, or in the absence or presence of IL-12 (20 ng mL^{-1}) plus anti-IL-4 antibody (10 μg mL^{-1}) for Th1 development. Two days later, IL-2 (10 ng mL^{-1}) was added into the culture and further incubated for an additional 4 days. After the cells were washed, they were re-stimulated with plate-bound anti-CD3 plus anti-CD28 for 1 day. IL-4 and interferon-γ concentrations in the culture supernatants from three wells were then measured by ELISA. One representative result of three experiments was shown.

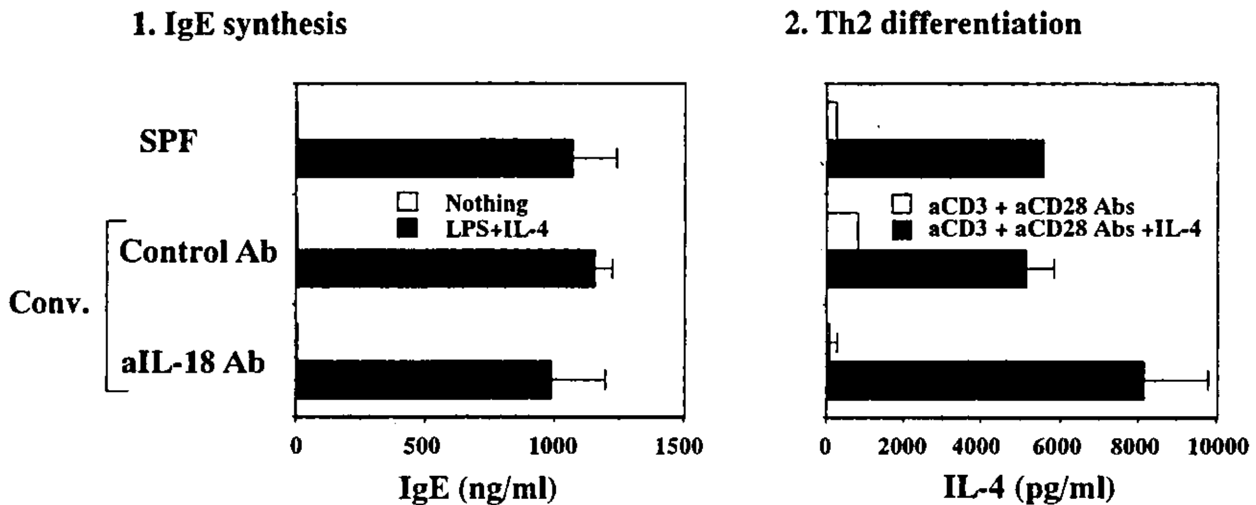

Figure 4. Administration of anti-interleukin (IL)-18 antibody does not affect IL-4 responsiveness in NC/Nga mice. (1) Purified B cells (10^6 cells mL^{-1}) from five NC/Nga mice raised under specific pathogen free (SPF) conditions, from five control antibody-injected NC/Nga mice, or from five anti-IL-18 antibody-injected NC/Nga mice were incubated with lipopolysaccharide (20 μg mL^{-1}) and IL-4 (50 ng mL^{-1}) for 6 days and supernatants were harvested. The IgE concentrations in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA). (2) Purified CD4+ T cells from three NC/Nga mice raised under SPF conditions, from three control antibody-injected NC/Nga mice, or from three anti-IL-18 antibody-injected NC/Nga mice were stimulated with plate-bound anti-CD3 (1 μg mL^{-1}) plus anti-CD28 (1 μg mL^{-1}) antibodies in the absence or presence of IL-4 (50 ng mL^{-1}). Two days later, IL-2 (10 ng mL^{-1}) was added into the culture and further incubated for an additional 4 days. After the cells were washed, the T cells were re-stimulated with plate-bound anti-CD3 plus anti-CD28 for 1 day. IL-4 concentrations in the culture supernatants were then measured by ELISA.

mulation of Th2 cells in the cutaneous lesions are unknown. Although it was anticipated that IL-18 might be the candidate molecule for this induction, the administration of anti-IL-18 antibody failed to prevent the onset of dermatitis. Thus it is possible that at least in this mouse model, IL-18 may not be directly associated with the initiation of spontaneous dermatitis (and accumulation of Th2 cells) and that IL-18 shows antiallergic actions in NC/Nga mice. Habu *et al.* recently reported that treatment of conventional NC/Nga mice either with IFN-γ, IL-12 or IL-18 from 4 weeks of age substantially inhibited the elevation of the serum IgE, IL-4 levels and onset of dermatitis.[20]

However, IL-18 appears to be related to the development of dermatitis. The process of the shift from Th2 to Th1 dominance in the lesions is also unknown but it is speculated that IL-12 from skin invading cells such as macrophages or eosinophils leads to the activation of Th1 cells in this process.[18] It seems likely that IL-18, although produced continuously, is involved in this process. The mechanisms by which excessive IL-18 is produced and its source in NC/Nga mice are unknown at the present time. Moreover, it is demonstrated that IL-4 has opposing functions, depending on the stage of immune activation.[21] Besides its well-known Th2-promoting properties, IL-4 is able, specifically at the stage of dendritic cell activation, to induce IL-12 production, indicating that the timing of intervention with cytokines is critical. As it was reported that keratinocytes and Langerhans cells are IL-18 producers,[22,23] analyses of the expression of cytokines including IL-4, IFN-γ, IL-12 and IL-18 during the development are required to investigate the pathogenesis of AD.

In summary, administration of a neutralizing anti-IL-18 antibody to NC/Nga mice *in vivo* does not suppress the onset of dermatitis and rather IL-18 may control the exacerbation of the dermatitis in this model. It is also not known if the effect on the lesions is direct or indirect. The establishment of IL-18-, IL-12- or IFN-γ-deficient NC/Nga mice will answer this problem.

Acknowledgments

This investigation was supported in part by the Ministry of Education, Science and Culture of Japan, the Ministry of Health and Welfare of Japan, and the Pollution-related Health Damage Compensation and Prevention Association of Japan. We thank Dr Okamura for his generous gift of anti-IL-18 antibody, Dr Nakanishi and Dr Yoshimoto for helpful advice and Dr Barsoumian for critical review of the manuscript.

References

1 Okamura H, Tsutsui H, Komatsu T *et al.* Cloning of a new cytokine that induces IFN-γ production by T cells. *Nature* 1995; **378**: 88–91.

2 Okamura H, Tsutsui H, Kashiwamura S *et al.* Interleukin-18 (IL-18) a novel cytokine that augments both innate and acquired immunity. *Adv Immunol* 1998; **70**: 281–312.

3 Dinarello CA. IL-18: a TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J Allergy Clin Immunol* 1999; **103**: 11–24.

4 Yoshimoto T, Okamura H, Tagawa A *et al.* Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon γ production from activated B cells. *Proc Natl Acad Sci USA* 1997; **94**: 3948–53.

5 Yoshimoto T, Tsutsui H, Tominaga K *et al.* IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proc Natl Acad Sci USA* 1999; **96**: 13962–6.

6 Hoshino T, Wiltrout RH, Young HA. IL-18 is a potent coinducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. *J Immunol* 1999; **162**: 5070–7.

7 Yoshimoto T, Mizutani H, Tsutsui H *et al.* IL-18 induction of IgE: dependence on $CD4^+$ T cells, IL-4 and STAT6. *Nature Immunol* 2000; **1**: 132–7.

8 Nakanishi K, Yoshimoto T, Tsutsui H *et al.* Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* 2001; **19**: 423–74.

9 Matsuda H, Watanabe N, Geba GP *et al.* Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int Immunol* 1997; **9**: 461–6.

10 Kotani M, Matsumoto M, Fujita A *et al.* Persimmon leaf extract and astragalin inhibit development of dermatitis and IgE elevation in NC/Nga mice. *J Allergy Clin Immunol* 2000; **106**: 159–66.

11 Tanaka T, Tsutsui H, Yoshimoto T *et al.* Interleukin-18 is elevated in the sera from patients with atopic dermatitis and from atopic dermatitis-model mice, NC/Nga. *Int Arch Allergy Immunol* 2001; **125**: 236–40.

12 Kawakami K, Qureshi MH, Zhang T *et al.* IL-18 protects mice against pulmonary and disseminated infection with *Cryptococcus neoformans* by inducing IFN-gamma production. *J Immunol* 1997; **159**: 5528–34.

13 Seder RA, Gazzinelli R, Sher A *et al.* Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci USA* 1993; **90**: 10188–92.

14 Tanaka T, Chu CC, Paul WE. An antisense oligonucleotide complementary to a sequence in Iγ2b increases γ2b germline transcripts, stimulates B cell DNA synthesis, and inhibits immunoglobulin secretion. *J Exp Med* 1992; **175**: 597–607.

15 Matsumoto M, Ra C, Kawamoto K *et al.* IgE hyperproduction through enhanced tyrosine phosphorylation of Janus kinase 3 in NC/Nga mice, a model for human atopic dermatitis. *J Immunol* 1999; **162**: 1056–63.

16 Yagi R, Nagai H, Iigo Y *et al.* Development of atopic dermatitis-like skin lesions in STAT6-deficient NC/Nga mice. *J Immunol* 2002; **168**: 2020–7.

17 Konishi H, Tsutsui H, Murakami T *et al.* IL-18 contributes to the spontaneous development of atopic dermatitis-like inflammatory skin lesion independently of IgE/stat6 under specific pathogen-free conditions. *Proc Natl Acad Sci USA* 2002; **99**: 11340–5.

18 Grewe M, Bruijnzeel-Koomen CA, Schopf E *et al.* A role for Th1 and Th2 cells in immunopathogenesis of atopic dermatitis. *Immunol Today* 1998; **19**: 359–61.
19 Grewe M, Gyufko K, Schopf E *et al.* Lesional expression of interferon-gamma in atopic eczema. *Lancet* 1994; **343**: 25–6.
20 Habu Y, Seki S, Takayama E *et al.* The mechanism of a defective IFN-γ response to bacterial toxins in an atopic dermatitis model, NC/Nga mice, and the therapeutic effect of IFN-γ, IL-12, or IL-18 on dermatitis. *J Immunol* 2001; **166**: 5439–47.
21 Biedermann T, Zimmermann S, Himmelrich H *et al.* IL-4 instructs TH1 responses and resistance to *Leishmania major* in susceptible BALB/c mice. *Nature Immunol* 2001; **2**: 1054–60.
22 Sugaya M, Nakamura K, Tamaki K. Interleukin 18 and 12 synergistically upregulates interferon-gamma production by murine dendritic epidermal T cells. *J Invest Dermatol* 1999; **113**: 350–4.
23 Naik SM, Cannon G, Burbach GJ *et al.* Human keratinocytes constitutively express interleukin-18 and secrete biological active interleukin-18 after treatment with pro-inflammatory mediators and dinitrochlorobenzene. *J Invest Dermatol* 1999; **113**: 766–72.