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Molecular analysis of B-cell differentiation in selective or partial IgA deficiency

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SUMMARY

Selective IgA deficiency is the most common form of primary immunodeficiency, the molecular basis of which is unknown. To investigate the cause of selective IgA deficiency, we examined what stage of B-cell differentiation was blocked. DNA and RNA were extracted from three Japanese patients with selective IgA deficiency and three with a partial IgA deficiency. In selective IgA deficiency patients, $I\alpha$ germline transcript expression levels decreased and α circle transcripts were not detected. Stimulation with PMA and TGF- β 1 up-regulated $I\alpha$ germline and α circle transcripts. In some patients, IgA secretion was induced by stimulation with anti-CD40, IL-4 and IL-10. In partial IgA deficiency patients, $I\alpha$ germline, α circle transcripts and $C\alpha$ mature transcripts were detected in the absence of stimulation. Our findings suggest that the decreased expression level of $I\alpha$ germline transcripts before a class switch might be critical for the pathogenesis of some patients with selective IgA deficiency. However, in patients with a partial IgA deficiency, B-cell differentiation might be disturbed after a class switch.

Keywords selective IgA deficiency partial IgA deficiency germline transcripts circle transcripts TGF- β 1

INTRODUCTION

Selective IgA deficiency is a common form of primary immunodeficiency in Caucasians. However, there is a difference in frequency between the Caucasian and Asian populations (approximately 1 in 700 Caucasians and 1 in 18 500 Japanese being affected) [1,2]. Some IgA deficiency individuals have increased susceptibility to upper respiratory tract or gastrointestinal infections. Although the frequency of IgA deficiency is relatively high, the molecular basis of this disease is unknown and it is sometimes associated with deficiency of the IgG subclass or IgE and with common variable immunodeficiency [3–5]. Some IgG subclass deficiencies are caused by CH-gene deletions [6–8]. In addition, some cases of secondary IgA deficiency are caused by antiepileptic drugs [9], the others being associated with autoimmune disorders and malignancy. In patients with partial IgA deficiency whose serum IgA level is 2SD below normal levels [10], the serum IgA level increases with age. Therefore, it is conceivable that the mechanism underlying the IgA deficiency pathogenesis is heterogeneous [11].

B cells differentiate to IgA-bearing cells through a DNA recombination process that joins the $S\mu$ to the $S\alpha$ region with a deletion of the intervening sequence and this process is initiated

by $I\alpha$ germline transcripts. After switching, B cells normally differentiate from membrane IgA-bearing to IgA-secreting cells. The IgA deficiency may result from a defect or blockade at several levels, such as: 1) a structural gene defect; 2) impaired switching, which may be due to the lack of a specific switch recombinase, activation-induced cytidine deaminase (AID) [12], polymorphism, or accessibility of the S or I region; 3) failure of IgA-bearing B cells to differentiate into plasma cells; and 4) a defect at the transcriptional and/or at the post-transcriptional level [13]. There is an $S\mu/S\alpha$ fragment or $S\alpha/S\mu$ fragment of circular DNA in the IgA class switch recombination (CSR). Recently, Kinoshita *et al.* [14] examined whether isotype-specific transcripts are generated from I promoters located on excised circular DNA and found that isotype-specific I- $C\mu$ transcripts, termed circle transcripts, were produced only in cells that express AID and undergo CSR in mice. Kinetic analysis of circle transcripts showed that they disappeared more quickly after the removal of cytokine stimulation than germline transcripts, circular DNA, or AID expression. Thus, circle transcripts are a hallmark of active CSR. In this study, to investigate the pathogenesis of IgA deficiency, we examined what stage of B-cell differentiation was blocked in this protein deficiency.

METHODS

Patients

Patients 1, 2 and 3 had a primary selective IgA deficiency whose serum IgA level was below the detection limit; patients 4, 5 and 6

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had a partial IgA deficiency whose serum IgA level was above 5 mg/dL but 2SD below normal levels [10] at more than one year old, as shown in Table 1. Informed consent was obtained from all these patients or their parents.

Cell preparation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood of patients and control donors by gradient centrifugation in Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) [16]. PBMCs were suspended at a density of 10^6 /ml in an RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, L-glutamin (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 µg/ml). PBMCs (10^6 /ml) were cultured in the presence or absence of phorbol myristate acetate (PMA) (10 ng/ml) (Sigma Aldrich, St. Louis, MO, USA) and recombinant human-TGF- β 1 (1 ng/ml) (R & D systems, Inc., Wiesbaden, Germany) for 24 h. Further, PBMCs obtained from patients of the selective IgA deficiency were cultured in the presence or absence of anti-CD40, IL-4 and IL-10 for seven days.

DNA transfer blot analysis and sequencing of IgA constant region

Genomic DNA was purified from a polynuclear cell fraction with a Sepa Gene (Sanko Jyunyaku, Tokyo, Japan). DNA transfer blot analysis was performed according to a previous report using a C α 2 probe, which was a 2-kb Pst I fragment from ch. h. Ig α -25 [17].

The fragments of the I promoter region, exon1, exon2, exon3 and the membrane exon of the α 1 gene were amplified, ligated to a T-vector (Novagen, Madison, WI, USA) and sequenced using an ABI 377 DNA Sequencing System (Applied Biosystems, Indianapolis, IN, USA).

PCR amplification of α 1 hs1, 2 enhancer

DNA fragments, including the region of variable number of tandem repeats (VNTR), of the α 1 hs1, 2 enhancer were amplified with consensus-flanking primers and the cycling conditions were as follows: sense 5'-GGGTCTGGTCCCAAAGATGGC-3' and antisense 5'-TTCCAGGGGTCTGTGGGTCC-3' [18]; 94°C for 1 min, 64°C for 1 min and 72°C for 1 min for 40 cycles.

cDNA synthesis

RNA was extracted from PBMCs cultured in the presence or absence of PMA and TGF- β 1 for 24 h using an Isogen kit (Nippon Gene, Tokyo, Japan) and cDNA synthesis from 2 µg of RNA was performed using a cDNA synthesis kit according to the manufacturer's instructions.

Semiquantitative PCR analysis of I α germline transcripts

Figure 1 schematically shows the locations of oligomers used in the following experiments in the regions of JH, I α 1, C α 1 and C μ . PCR amplification of the I α germline transcripts was carried out using the primers and cycling conditions as follows. The sense primer was chosen from the 3' region of the I α 1 exon and the antisense primer was obtained from the 3' region of the C α 1 exon1 [19]. The following primers were used: IS, sense 5'-TGAGTGGACCTGCCATGA-3' (GenBank accession number-L04540), CA1, antisense 5'-CTGGGATTCGTGTAGT GCTT-3' (J00220) (Fig. 1). For unstimulated cDNA; 94°C for 1 min, 58°C for 1 min and 72°C for 1 min for 28, 32, 36, 40 cycles. For stimulated cDNA; 94°C for 1 min, 58°C for 1 min and 72°C for 1 min for 35 cycles. The plasmid containing a 337 bp cDNA fragment from I α germline transcripts was partially substituted with a 267 bp fragment from BLM cDNA [20] and was used as a competitor DNA. The PCR product of the wild type was 337 bp and that of the competitor was 287 bp. Each template contained 1 µl of cDNA from 2 µg of RNA extracted from PBMCs cultured in the presence of PMA and TGF- β 1 and one of fivefold dilutions of the competitor DNA.

Nested PCR analysis of α circle transcripts

Nested PCR analysis of α circle transcripts was carried out using the primers and cycling conditions as follows. The sense primer was chosen from the 3' region of the I α 1 exon and the antisense primer was obtained from the 3' region of the C μ . In the first round, the primers P1 and P4 were used at 95°C for 9 min in the denaturing step, 95°C for 1 min, 51°C for 1 min and 72°C for 2 min for 35 cycles. In the second round, the primers P2 (IS) and P3 were used at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min for 35 cycles. The primers were: P1, 5'-CACAGCCAGC

Table 1. Immunological data of patients

Patient no.	Sex	Age	Serum Ig (mg/dl)†			Surface Ig-bearing B cells (%)			IgG subclass (mg/dl)			
			IgG	IgA	IgM	IgG	IgA	IgM	IgG1	IgG2	IgG3	IgG4
Selective IgA deficiency												
1	M	6 years	1120 (630–1490)	<5 (45–258)	110 (72–305)	1	0	23	619	255	57.3	33.9
2	F	14 years	1750 (760–1680)	<5 (77–371)	259 (69–296)	1	1	13	630	625	35.1	18.4
3	F	7 years	1430 (660–1340)	<5 (51–279)	104 (73–310)	0	0	5	893	382	47.4	59.4
Partial IgA deficiency												
4	M	21 months	889 (460–1220)	13 (16–128)	69 (57–260)	1	1	3	466	98.4	13.5	16.2
5	F	16 months	465 (460–1220)	9 (16–128)	70 (57–260)	1	0	5	306	69.3	27.6	3.8
6	M	18 months	474 (460–1220)	10 (16–128)	83 (57–260)	2	0	11	295	97.0	40.0	4.4

†Normal range (2.5–97.5 percentile) of serum Ig are given in brackets where appropriate; they are from *Normal Range for Clinical Testing of Japanese Children* [15].

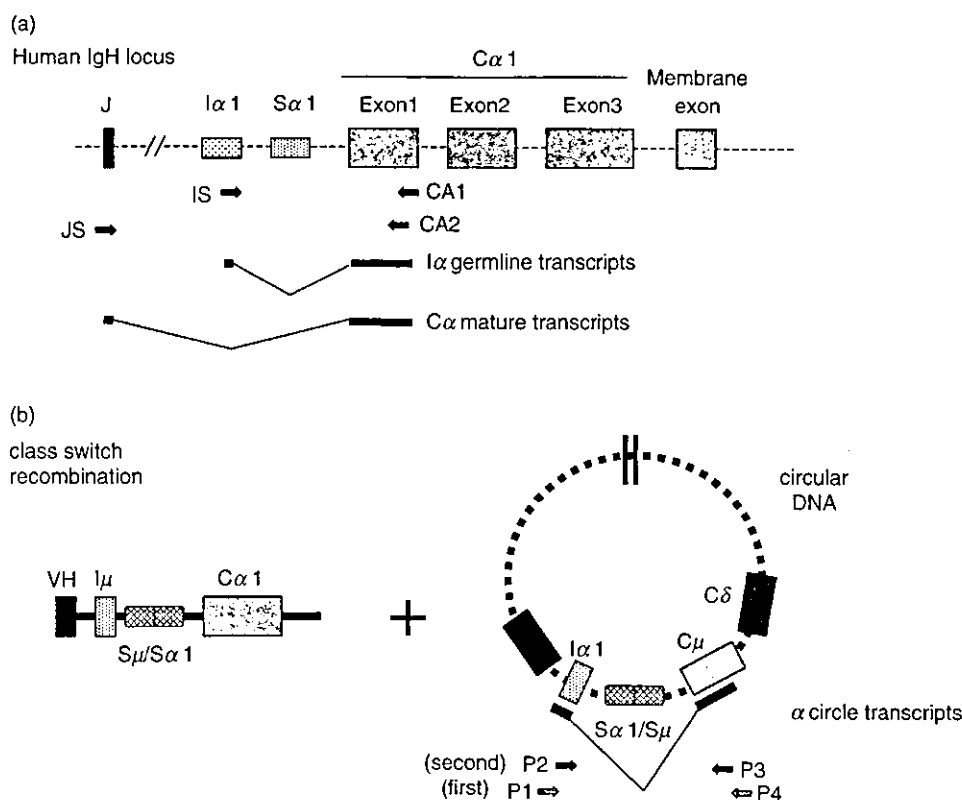


Fig. 1. (a) Schematic of PCR strategies. The human IgH locus after VDJ rearrangement is shown schematically at the top. Primers are indicated by arrows. The PCR fragments amplified from cDNA are indicated by thick lines with a V-shaped line representing splicing. (b) Schematic of PCR strategies for circle transcripts. The IgA class switch recombination is shown. Thick lines below circular DNA indicate exons of α circle transcripts connected with a V-shaped line representing splicing.

GAGGCAGAGC-3' (L04540), P2, 5'-TGAGTGGACCTGC CATGA-3' (L04540), P3, 5'-CGTCTGTGCCTGCATGACG-3' (X14940), P4, 5'-ACGAAGACGCTCACTTTGGG-3' (X14940) (Fig. 1).

PCR analysis of $C\alpha$ mature transcripts

PCR amplification of $C\alpha$ mature transcripts was carried out using the primers and cycling conditions as follows. The JH consensus sequence was used as the sense primer and the antisense primer was obtained from the 3' region of the $C\alpha$ 1 exon1. The following primers were used: JS, sense 5'-CCTGGTCAC CGTCTCCTCA-3' (L20778), CA2, antisense 5'-ACGTGGCAT GTCACGGACTT-3' (J00220) (Fig. 1); 94°C for 1 min, 59°C for 1 min and 72°C for 1 min for 32, 34, 36 and 38 cycles.

Assay of IgA secretion

The concentration of IgA in the supernatant of PBMCs cultured in the presence or absence of anti-CD40, IL-4 and IL-10 for seven days was assayed by an enzyme-linked immunosorbent assay kit (Cygnum Technologies, Southport, NC, USA).

RESULTS

Southern blot analysis of $C\alpha$ constant region

PstI-digested DNA samples from the six patients and one control subject were analysed using a $C\alpha$ 2 gene probe. Human $C\alpha$ genes are sufficiently homologous and detect both $C\alpha$ 1 and $C\alpha$ 2 genes.

As shown in Fig. 2a, the large deletion of the constant region on the $C\alpha$ genes was not detected in all subjects.

Genome sequence of $C\alpha$ constant region

To examine the mutation of the α heavy chain, PCR analysis was performed using the primers of the I promoter region, exon1, exon2, exon3 and the membrane exon of the α 1 gene. There were no mutations in these regions. The polymorphism of the $C\alpha$ 1 gene 882G→C (E175D) in exon2 was detected in patients 1, 3 and 5.

PCR amplification of α 1 hs1, 2 enhancer

We analysed the α hs1, 2 enhancer, which strongly regulates human IgH expression and is located within 12 kb downstream of both the human Ig $C\alpha$ 1 and $C\alpha$ 2 genes [18,21]. The α 1 hs1, 2 enhancer is located between $C\alpha$ 1 and $\phi\gamma$ (Fig. 2b). As shown in Fig. 2c, a fragment of the α 1 hs1, 2 enhancer was detected in all subjects. α 1 hs1, 2 has three variants with VNTR, namely α 1 A, α 1 B and α 1 C (including one, two and three repeats, respectively) [18]. The sizes of the PCR products were 462 bp, 515 bp and 568 bp for α 1 A, α 1 B and α 1 C, respectively. Controls 1 and 2, patients 2, 3 and 4 had the α 1 A/A allele, patient 1 had α 1 B/C, patient 5 had α 1 A/B and patient 6 had α 1 A/C.

Germline transcript expression in IgA deficiency

Germline transcripts are indispensable for the initiation of CSR. We examined the expression of $I\alpha$ germline transcripts using a

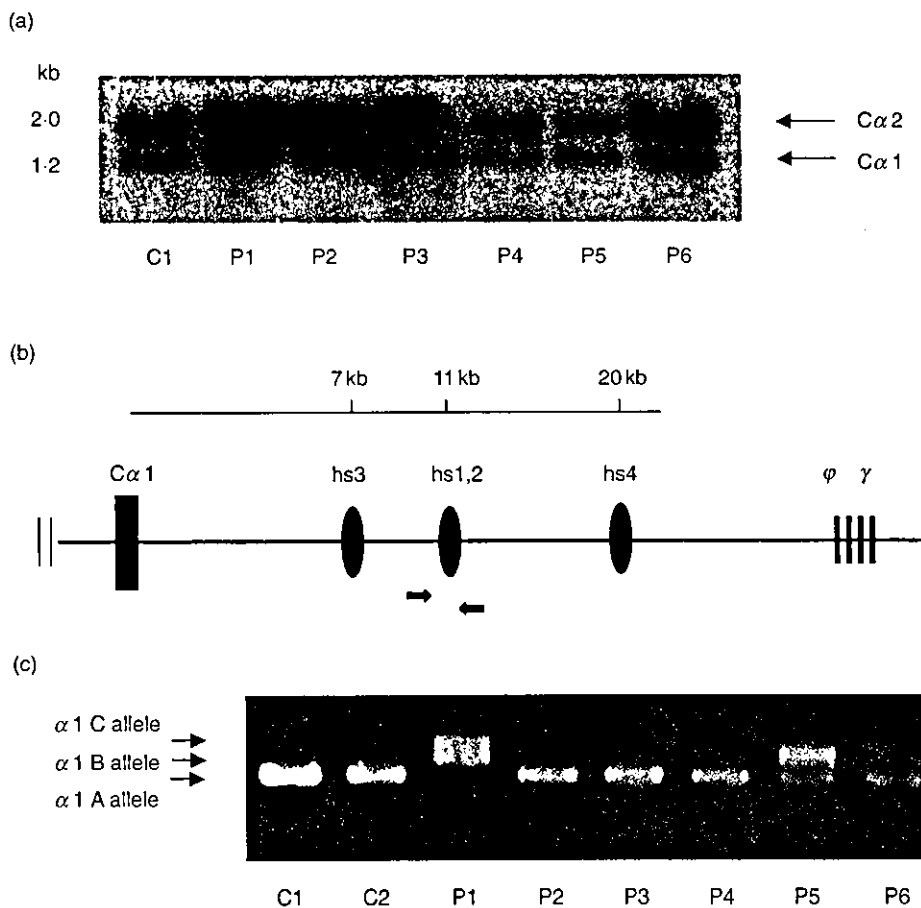


Fig. 2. (a) $C\alpha$ gene hybridization pattern. *Pst*I-digested DNA samples from control (C1) and patients (P1–P6) were analysed by Southern blot analysis. The probe was a 2-kb $C\alpha 2$ fragment. The length in kb of each $C\alpha$ gene, is indicated. (b) Schematic of PCR strategies for the detection of $\alpha 1$ hs1, 2 enhancer located between $C\alpha 1$ and $\phi\gamma$. The $\alpha 1$ hs3, $\alpha 1$ hs1, 2, and $\alpha 1$ hs4 fragments are located 7, 11, and 20 kb downstream of the $C\alpha 1$ gene, respectively (20). Primers are indicated by arrows. (c) PCR fragments of $\alpha 1$ hs1, 2 enhancer from genome DNA are shown. PCR products of sizes 462 bp, 515 bp and 568 bp for $\alpha 1$ A, $\alpha 1$ B and $\alpha 1$ C, respectively. C1 and C2, normal controls; P1–P6, patients 1–6.

semiquantitative PCR analysis. First, the expression of the $I\alpha$ germline transcripts of unstimulated PBMCs was examined by RT-PCR using different cycles and that expression from patients 1, 2 and 3 was not clearly detected even after 40 cycles were run and the expression levels were markedly lower than those in controls. In patients 4, 5 and 6, the expression levels were slightly lower than those in controls but the $I\alpha$ germline transcripts were detected at significant levels (Fig. 3a). Next, competitive PCR analysis was applied to measure the expression level of $I\alpha$ germline transcripts of PBMCs stimulated by PMA and TGF- β 1. In both controls and IgA deficiency patients, the target cDNA and competitor were almost equivalent between lane 3 and lane 4 (Fig. 3b). The $I\alpha$ germline transcripts of PBMCs from the selective and partial IgA deficiency patients were induced by PMA and TGF- β 1 at a level almost equal to those in controls. The $I\alpha$ germline transcripts of PBMCs from the selective and partial IgA deficiency patients were induced by PMA and TGF- β 1 at a level almost equal to those in controls.

Circle transcript expression in IgA deficiency

To determine whether the CSR from IgM to IgA could occur in the IgA deficiency patients, the expression of the α circle transcripts was examined. The α circle transcripts were generated

from $I\alpha$ promoters located on excised circular DNA and $C\mu$ (Fig. 1b). Because the circle transcripts were not clearly detected at first PCR (data not shown) even in controls, we performed nested PCR and the α circle transcripts were detected in controls, patients 4, 5 and 6, but not in patients 1, 2 and 3 (Fig. 4). However, the circle transcripts were induced in PBMCs from patients 1, 2 and 3 after stimulation with PMA and TGF- β 1.

Mature transcript expression in IgA deficiency

We further examined the expression of $C\alpha$ mature transcripts, including both their membrane and secreted forms, by RT-PCR using different cycles. As shown in Fig. 5a, the expression levels of the $C\alpha$ mature transcripts of unstimulated PBMCs from patients 1, 2, 3 and 4 decreased. In patients 5 and 6, the expression levels of the $C\alpha$ mature transcripts decreased slightly compared to controls. The $C\alpha$ mature transcripts were induced by PMA and TGF- β 1 in patients 1 and 2. In patient 3, the $C\alpha$ mature transcripts were not markedly induced by PMA and TGF- β 1 stimulation (Fig. 5b).

IgA secretion induced by anti-CD40, IL-4 and IL-10

IgA secretion by CD40-activated PBMCs from patients with the selective IgA deficiency was examined. Anti-CD40, IL-4 and

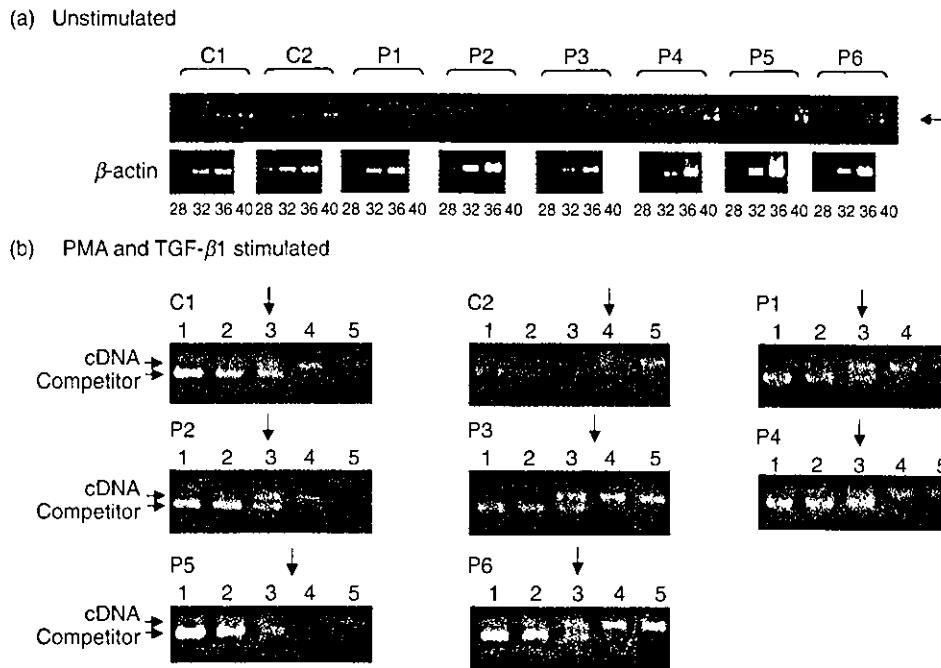
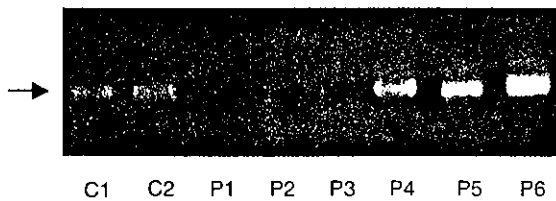


Fig. 3. (a) Expression of $I\alpha$ germline transcripts in unstimulated PBMCs. Semiquantitative determination using RT-PCR analysis. In each case, 28, 32, 36 and 40 cycles were run. β -actin was used as a control with a run of 28, 32 and 36 cycles. The position of target cDNA is indicated by an arrow. C1 and C2, normal controls; P1–P6, patients 1–6. (b) Competitive PCR of the expression of $I\alpha$ germline transcripts. Each template contained the same amount of cDNA synthesized from RNA extracted from PBMCs after stimulation with PMA and TGF- β 1 and one of fivefold dilutions of $I\alpha$ germline transcript competitor (lanes 1–5). Each equivalent point is indicated by an arrow. C1 and C2, normal controls; P1–P6, patients 1–6.

(a) Unstimulated



(b) PMA and TGF- β 1 stimulated

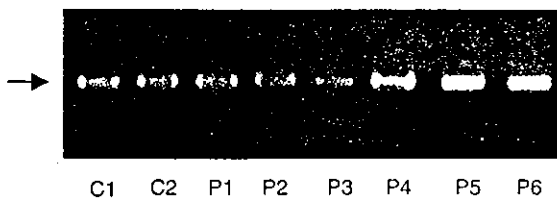


Fig. 4. Detection of α circle transcripts in PBMCs cultured without or with PMA and TGF- β 1. The second PCR fragments of α circle transcripts are shown and are indicated by arrows. C1 and C2, normal controls; P1–P6, patients 1–6.

IL-10 induced IgA secretion in PBMCs from control and patient 1. In patient 2, IgA secretion was slightly induced. However, in patient 3, it was not induced by the same stimulation (Fig. 5c).

DISCUSSION

In this study, we demonstrated the following in selective IgA deficiency patients: 1) $C\alpha$ genes were not deleted; 2) expression levels of $I\alpha$ germline transcripts of unstimulated PBMCs markedly decreased; 3) $I\alpha$ germline transcripts were induced by PMA and TGF- β 1; 4) α circle transcripts of unstimulated PBMCs were not detected; 5) α circle transcripts were detected after stimulation; 6) $C\alpha$ mature transcripts were induced by PMA and TGF- β 1; and 7) IgA secretion was induced by appropriate stimulation.

Thus, the decreased expression level of the $I\alpha$ germline transcripts is critical for the pathogenesis of the selective IgA deficiency in our patients and it is possible to induce IgA CSR in these patients. In partial IgA deficiency patients, although the expression levels of the $I\alpha$ germline transcripts were slightly lower than controls, α circle and mature transcripts were detected. The number of surface IgA-bearing B cells was low in all of the IgA deficiency patients. It is suggested that a defect of the membrane-bound IgA at the post-transcriptional level may cause low IgA production in partial IgA deficiency patients. The expression of the membrane-bound immunoglobulin is indispensable for the generation of efficient primary and secondary immunoglobulin responses [22]. In this study, there was no mutation of the alternative splice site for the membrane exon of the $C\alpha$ 1 gene.

The $I\alpha$ germline transcripts are conceivably critical for the initiation of switching from $C\mu$ to $C\alpha$. In a previous study, it was reported that the $I\alpha$ germline transcripts were absent in peripheral B cells of IgA deficiency patients, suggesting the impairment of IgA switching [13]. However, it was also reported that the $I\alpha$ germline transcripts were detected in all of the IgA deficiency patients tested as well as in normal controls [23]. Consistent with

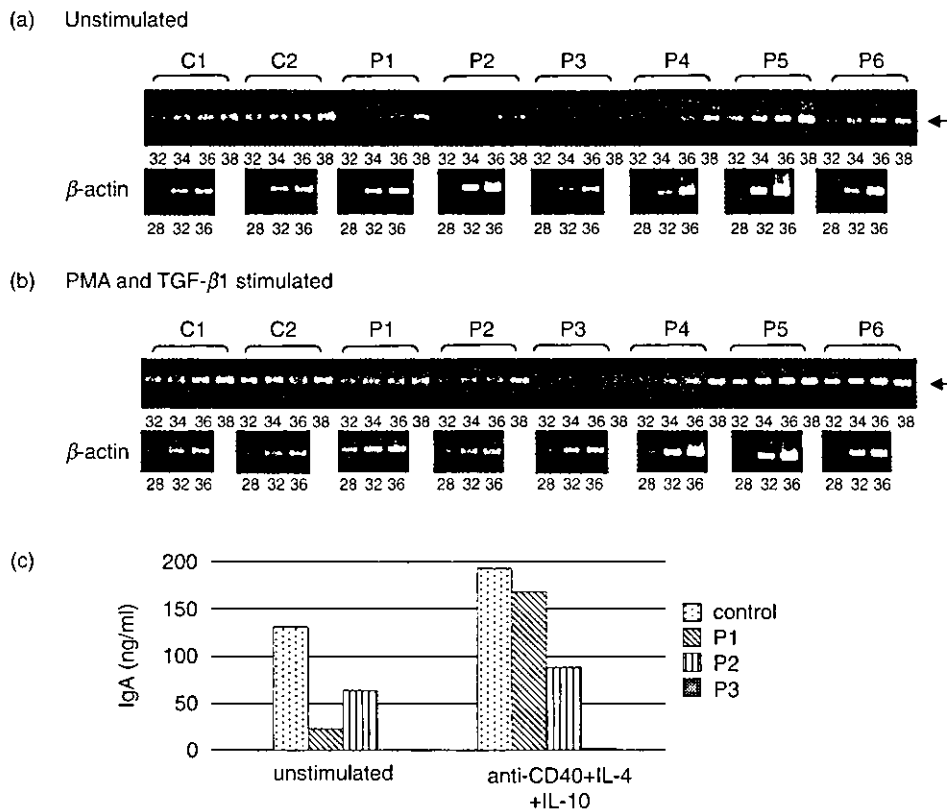


Fig. 5. Expression of $C\alpha$ mature transcripts in PBMCs cultured without (a) or with (b) PMA and TGF- β 1. Semiquantitative determination using RT-PCR analysis. In each case, 32, 34, 36 and 38 cycles were run. β -actin was used as a control with a run of 28, 32 and 36 cycles. The positions of target cDNA are indicated by arrows. C1 and C2, normal controls; P1–P6, patients 1–6. (c) IgA secretion was induced by activation of PBMCs. PBMCs (10^6 /mL) were cultured in the presence or absence of anti-CD40, IL-4, and IL-10 for seven days. Concentration of IgA in the supernatant of PBMCs was measured by an enzyme-linked immunosorbent assay. P1–P3, patients 1–3.

previous reports, our study revealed two different types of defects in B-cell differentiation – one was a decreased $C\alpha$ mRNA level in IgA-switched B cells and the other was a switching defect, which may be present in IgA deficiency patients.

It is possible that some stimulation corresponding to that with PMA and TGF- β 1 is reduced or blocked in selective IgA deficiency patients. In patients 1 and 2 but not 3, PMA and TGF- β 1 could induce the α germline and mature transcripts and CD40 and appropriate cytokines induced IgA production. Therefore, in patient 3, the PMA and TGF- β 1 pathways might be blocked, which were common signals in CD40 and cytokines, such as mitogen-activated protein kinase [24,25] and protein kinase C [26] signal transduction. In patient 1 and 2, distinct signal pathways between TGF- β 1 and CD40 might be disturbed in B cells. Muller *et al.* reported that the serum levels of TGF- β 1 in IgA deficiency patients were low [27]. In our cases, there was no difference in the level of TGF- β 1 in plasma among selective IgA deficiency patients, partial IgA deficiency patients and controls (data not shown).

In recent studies, many lines of evidence have been presented indicating that primary IgA deficiency is inherited and associated with a certain major histocompatibility complex-conserved haplotype mainly in populations of the western world [28–30]. However, there are only a few studies that show an association of haplotypes, such as [HLA-A1, B8, DR3] [30], with the IgA deficiency in Japanese patients [31]. In our study, the decreased

expression level of the $I\alpha$ germline transcripts is critical for the pathogenesis of the selective IgA deficiency in some patients. Partial IgA deficiency has distinct causes from those of the selective IgA deficiency. Since in most of the partial IgA deficiency patients the serum IgA level normalizes with age, the existence of suppressor factors for IgA B-cell differentiation may be assumed. In our cases, B-cell differentiation in selective IgA deficiency patients showed impairment before the CSR stage, while B-cell differentiation in partial IgA deficiency patients showed impairment after the CSR stage.

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Methylenetetrahydrofolate Reductase Polymorphism in Patients with Bronchial Asthma

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ABSTRACT

Objective: Bronchial asthma is a chronic inflammatory condition of the respiratory tract. The C677T mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene is reported to confer susceptibility to cardiovascular diseases and inflammatory conditions. We hypothesized that TT genotype of MTHFR may influence the development of bronchial asthma and thus examined the C677T polymorphism in our Japanese asthmatic patients.

Design: Clinical Investigation.

Methods: Genotypes for MTHFR were determined in 461 asthmatic patients (male/female ratio: 248/213) by the polymerase chain reaction and restriction fragment length polymorphism method and the results were compared with those obtained from 1430 healthy subjects (male/female ratio: 939/491).

Results: For the male population, the frequency of the TT genotype in asthmatic patients was significantly higher than in healthy subjects (16.9% vs. 11.0%, odds ratio = 1.65, 95% confidence interval: 1.12-2.44, P = 0.011). For the female population, the frequency of the TT genotype in atopic asthmatic patients was insignificantly higher than in non-atopic asthmatic patients (17.3% vs. 11.8%).

Conclusion: Our findings suggest that the TT genotype of MTHFR is a probable genetic risk factor for the development of bronchial asthma in Japanese males.

KEY WORDS

bronchial asthma, homocysteine, methylenetetrahydrofolate reductase, oxidative stress, polymorphism

INTRODUCTION

Bronchial asthma is a condition characterized by episodic reversible airway obstruction, airway hyperresponsiveness and allergic inflammation of the airway. Multiple inflammatory cells, cytokines and other mediators participate in the pathogenesis of bronchial asthma¹⁾.

Several lines of evidence suggest that oxidative stress contributes to airway inflammation and epithelial damage and to alterations in the immune system, and that individuals with lowered antioxidant capacity are at increased risk for developing asthma^{2,3)}. Therefore, genetic, environmental and dietary factors that diminish antioxidant defenses could potentially increase the vulnerability to oxidative stress and thus the risk for developing asthma. Accordingly, study of

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genetic polymorphism related to oxidative stress may be of help to our understanding of the complex pathophysiology of asthma, and could lead to the development of new and effective management protocols for this common disease.

There is general interest in hyperhomocysteinemia as a risk factor for vasculopathy, which is thought to exert its effects through oxidative damage^{4,5}. Recently, a common C to T mutation at nucleotide position 677 (C677T) has been identified in the gene coding for 5,10-methylenetetrahydrofolate reductase (MTHFR), which is involved in the remethylation of homocysteine to methionine^{6,7}. The C677T mutation causes a valine-for-alanine substitution, which decreases MTHFR activity and tends to be associated with elevated blood homocysteine levels. Other studies suggest that the TT genotype of MTHFR is significantly associated with coronary artery disease and ischemic stroke^{8,9}. It has also been reported that the TT genotype may represent a genetic risk factor for the development and aggravation of inflammatory conditions such as inflammatory bowel disease, Kawasaki disease and chronic glomerulonephritis¹⁰⁻¹³.

Based on the above considerations, we hypothesized that the TT genotype of MTHFR may influence the development of bronchial asthma and thus examined the C677T polymorphism in Japanese asthmatic patients. This is the first report to demonstrate the association between MTHFR C677T mutation and development of bronchial asthma.

METHODS

Subjects

We studied 461 Japanese patients with bronchial asthma who had been seen at the outpatient clinics of Fukui Medical University Hospital (Fukui), Tenri Hospital (Nara), Osaka University Hospital (Osaka), Hokkaido University Hospital (Hokkaido), Gifu University Hospital (Gifu) and their affiliated hospitals. They included 248 males and 213 females and their age ranged from 1 to 85 years (mean 33 years; median 28 years). The diagnosis of asthma was based on the criteria of the National Asthma Education and Prevention Program, Expert Panel Report II¹⁴. Atopy was defined by the presence of high levels of specific serum IgE (≥ 0.35 KU_A/l) to at least one aeroallergen (such as house dust mite) using the CAP radioallergosorbent test fluoro enzyme immunoassay system (Pharmacia Upjohn Diagnostics, Tokyo, Japan)¹⁵. As age-matched healthy controls, 1430 subjects (939 males and 491 females) were derived from studies conducted by our group ($n = 339$)¹¹ (unpublished results) and other groups ($n = 1091$)^{8,16,17}. Informed consent was obtained from our subjects (461 patients and 339 healthy controls) and/or their parents before blood samples were collected. This study protocol was approved by the ethics committees of all the hospitals that participated in the present study.

Genetic analysis

Genomic DNA was extracted from peripheral-blood leukocytes. Identification of the C677T mutation in the MTHFR gene was performed by polymerase chain reaction

(PCR) using primers described by our group¹¹: 5'-TGAAG-GAGAAGGTGTCTGCGGA-3' and 5'-AGGACGGT-GCGGTGAGAGTG-3' (Toagosei, Tsukuba, Japan). PCR thermal cycling conditions were a 10-min denaturation period at 94°C and 43 cycles of the following: 94°C for 1 min, 55°C for 30 s and 72°C for 1 min. This was followed by a 10-min extension at 72°C (Program Temp Control System PC-700, ASTEC, Fukuoka, Japan). The amplified products were digested with *HinfI* (TaKaRa Biomedicals, Ohtsu, Japan) at 37°C for 4 hours. The *HinfI*-treated PCR fragments were electrophoresed in 5% polyacrylamide gel and visualized with ethidium bromide. The C to T mutation creates a *HinfI* recognition site that leads to digestion of the 198-bp PCR product into 175- and 23-bp fragments. Heterozygote subjects show three fragments (198 bp, 175 bp and 23 bp) and a homozygous C to T mutation results in the production of two fragments of 175 bp and 23 bp.

Although we did not examine blood homocysteine levels in our subjects, data from another group on healthy Japanese individuals showed that the mean homocysteine level was significantly higher in subjects with the TT genotype (15.7 μ M) than in those carrying the CC (11.6 μ M) or CT (11.8 μ M) genotype¹⁸.

Statistical analysis

The analysis of genotype involved comparing the number of subjects carrying the TT genotype with the number of subjects carrying the other genotypes. Statistical analysis was also performed on the numbers of C677 alleles and T677 alleles. The differences were examined by the chi-squared test or Fisher's exact test where appropriate. Statistical significance was inferred when the P value was < 0.05.

RESULTS

The genotype frequencies of MTHFR in healthy subjects and asthmatic patients are listed in Table 1. The frequencies of the C677T polymorphism in healthy subjects were CC: CT: TT = 579 (40.5%): 681 (47.6%): 170 (11.9%). The frequency of the TT genotype was higher in both male and female asthmatic patients (16.9% vs. 11.0%, 16.0% vs. 13.6%, respectively) and the difference was significant for the male population (odds ratio = 1.65, 95% confidence interval: 1.12-2.44, $P = 0.011$). The allele frequency of the T mutation was significantly higher in male asthmatics than in male healthy controls (41.3% vs. 34.3%; odds ratio = 1.35, 95% confidence interval: 1.10-1.65, $P = 0.004$).

According to the criteria described in the METHODS section, 378 patients (82%) were classified as "atopic" asthmatics and 83 (18%) were "non-atopic" asthmatics. The distribution of the MTHFR C677T polymorphism was examined according to atopy (Table 2). For the female population, the frequencies of the TT genotype and the T677 alleles were higher in atopic asthmatics than those in non-atopic asthmatics (17.3% vs. 11.8%, 40.7% vs. 34.3%, respectively), but the differences were statistically insignificant.

Table 1. Genotype distribution of the MTHFR gene in healthy subjects and asthmatic patients

	MTHFR		
	CC	CT	TT
Healthy subjects			
Male (n = 939)	398 (42.4%)	438 (46.6%)	103 (11.0%)
Female (n = 491)	181 (36.9%)	243 (49.5%)	67 (13.6%)
Asthmatic patients			
Male (n = 248)	85 (34.3%)	121 (48.8%)	42 (16.9%)*
Female (n = 213)	80 (37.6%)	99 (46.5%)	34 (16.0%)

MTHFR, 5,10-methylenetetrahydrofolate reductase. Numbers in parentheses represent percentage of individuals.

*P = 0.011 vs. corresponding healthy subjects.

Table 2. Genotype distribution of the MTHFR gene in patients with atopic and non-atopic asthma

	MTHFR		
	CC	CT	TT
Atopic asthmatic patients			
Male (n = 216)	72 (33.3%)	107 (49.5%)	37 (17.1%)
Female (n = 162)	58 (35.8%)	76 (46.9%)	28 (17.3%)
Non-atopic asthmatic patients			
Male (n = 32)	13 (40.6%)	14 (43.8%)	5 (15.6%)
Female (n = 51)	22 (43.1%)	23 (45.1%)	6 (11.8%)

MTHFR, 5,10-methylenetetrahydrofolate reductase. Numbers in parentheses represent percentage of individuals.

DISCUSSION

Advances in asthma management are likely to depend on a better understanding of how genetic and environmental factors influence susceptibility to, and outcome in, this disease. The implication of the MTHFR C677T polymorphism in the pathogenesis of bronchial asthma is entirely novel. The MTHFR gene is located on chromosome 1 at 1p36.3. The complementary DNA sequence is 2.2 kilobases long and consists of 11 exons^{6,7}. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine. In recent years, accumulating evidence suggests that mild to moderate hyperhomocysteinemia and its genetic promoter, MTHFR C677T mutation, may be associated with the development and progression of cardiovascular and inflammatory diseases⁶⁻¹³.

Reactive oxygen species (ROS) are implicated in the cellular toxicity of hyperhomocysteinemia^{4,5}. ROS, including superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH), are generated during oxidation of the sulfhydryl group of homocysteine. Excessive O₂⁻ inactivates nitric oxide (NO) with the formation of the adduct peroxynitrite (ONOO⁻). These oxidants are thought to account for the homocysteine-induced cytotoxicity. Furthermore, homocysteine both inhibits glutathione peroxidase activity *in vitro* and leads to a marked reduction in mRNA levels for the intracellular isoform; the inhibition of glutathione peroxidase is unique to homocysteine compared with other biologic thiols. Homocysteine significantly decreases the intracellular thiol redox state, as measured by the ratio [glutathione]/[glutathione disulfide], through these oxidative mechanisms.

The reported frequency of homozygotes for the muta-

tion, having the TT genotype, varies in different populations from one geographic area to another (ranging from 5 to 20%)^{6,7}. Our results showed that among Japanese healthy subjects the frequency of the TT genotype was 11.9% (170/1430), which was comparable to that in Caucasians.

The present study is the first to demonstrate the association between the TT genotype and development of bronchial asthma in male subjects. The mechanism(s) of the predisposition of the TT genotype to asthma remains speculative. The contribution of oxidative stress induced by this genotype is one possibility. Oxidative stress has been implicated in the initiation and worsening of asthma^{2,3}. Increased release of ROS has been reported in exhaled condensates¹⁹ and from circulating neutrophils and monocytes, bronchoalveolar lavage cells of patients with asthma²⁰. ROS can cause cellular damage by oxidizing membrane lipids, proteins and nucleic acids. Furthermore, oxidative stress can activate the transcription factor, nuclear factor kappa-B (NF- κ B), and previous studies reported high expression of this factor in airway cells of patients with asthma^{21,22}. Increased NF- κ B expression and DNA binding may underlie the increased expression of several inflammatory proteins in the asthmatic airway, including tumor necrosis factor- α , interleukin (IL)-1 β ; RANTES, eotaxin, macrophage chemotactic protein-1, macrophage inflammatory protein-1 α ; granulocyte-macrophage colony stimulating factor; inducible NO synthase, inducible cyclooxygenase; and intercellular adhesion molecule-1, vascular cell adhesion molecule-1^{21,25}. It seems reasonable to consider that these changes create feed-forward amplifying loops that could form the basis for the development and progression of the chronic inflammatory process in asthma and that the MTHFR TT genotype enhances these pathological events especially in male subjects.

It is of note that more males than females develop asthma during childhood, probably because of narrower air-

ways, increased airway tone and possibly higher IgE in boys¹⁴⁾. For the male asthmatic patients, the frequency of the TT genotype in children was insignificantly lower than in adults (patients aged < 16 years: 15.3% [20/131] vs. patients aged ≥ 16 years: 18.8% [22/117]). In addition, the frequency of the TT genotype in atopic asthmatics was almost comparable to that in non-atopic asthmatics (17.1% vs. 15.6%) (Table 2).

It needs to be taken into account that some proportion of the healthy controls reported by other groups (n = 1091)^{8,16,17)} may have allergic diatheses including bronchial asthma. The prevalence of asthma in Japanese children and adults is reported to be 3%²⁶⁾. If these individuals of the above healthy controls (3% of the total of 1091 subjects) were assumed to have the same distribution of the C677T polymorphism as the patient group, the frequency of the TT genotype would remain higher in both male and female asthmatics than that of the corresponding "non-asthmatic" healthy controls (16.9% vs. 10.8%, 16.0% vs. 13.6%, respectively). Under this condition, the difference would remain significant for the male population (odds ratio = 1.68, 95% confidence interval: 1.14-2.47, P = 0.007).

Atopy is a significant predisposing factor for the development of asthma¹⁴⁾. Certain genetic and environmental factors drive the development of a Th2 lymphocyte-predominant immune response, which is associated with atopy and IgE-mediated inflammation. Th2 lymphocytes generate cytokines, including IL-4, IL-5 and IL-13, which play a primary role in B cell switching to IgE synthesis. In contrast, Th1 lymphocytes, which play a primary role in interferon- γ production, inhibit B-cell IgE synthesis. Th1- and Th2-type cytokines reciprocally regulate the Th1/Th2 immune responses. Recent *in vitro* studies have suggested that oxidative environments may act differentially on activated human Th cells by inhibiting Th1 cytokine production but promoting the expression of Th2 cytokines; thiol antioxidants such as N-acetyl-L-cysteine and glutathione decrease IL-4 production in human T cells and IgE production by B cells^{27,28)}. Moreover, glutathione depletion in antigen presenting cells inhibits Th1 cytokines and/or favors Th2 responses²⁹⁾. In studies using human B cell lines, Yanagihara *et al.*³⁰⁾ showed that N-acetyl-L-cysteine regulates the IgE isotype switching by inhibiting the activation of NF- κ B. It was expected that altered redox status induced by the TT genotype may drive the development of the Th2-predominant immune responses to environmental stimuli, which is associated with atopy and allergic inflammation in the airway. For the female population, the frequency of the TT genotype in atopic asthmatics was higher than in non-atopic asthmatics, although the difference was statistically insignificant. Since the number of non-atopic asthmatics was rather small, further studies using larger population samples may be required to confirm this contention.

In conclusion, our results suggest that the MTHFR TT genotype is a probable genetic risk factor for the development of bronchial asthma, possibly through the oxidative mechanism, in male Japanese subjects. Although further investigations are required to clarify the molecular mechanisms governing whether a Th1 or Th2-immune response predominates in the hyperhomocysteinemic state, the findings presented here should provide new insights into the pathophysiology of bronchial asthma. The homocysteine-lowering effects of folate and vitamins B₆ and B₁₂ have been anticipated for individuals carrying the TT genotype⁷⁾. The other relevant subject of exploration may be the inves-

tigation of the possible favorable effects of these vitamins on the development and worsening of asthma.

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Characterization of T-cell Clones Specific to Ovomuroid from Patients with Egg-White Allergy

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Summary

Background: Allergic reactions to foods are particular problems for infants and young children. Ovomuroid (OM) is one of the major allergens found in egg-white. We previously established several T-cell clones (TCCs) specific to OM in non-polarizing conditions from 4 patients (TM and YN are immediate-type, IH and YT are non-immediate-type) with egg-white allergy. We characterized their reactive epitopes, antigen-presenting molecules (HLA class II), and usage of TCR alpha and beta genes and the CDR3 loop sequence.

Objective: The objective of this study was to characterize these seven clones (TM1.3, TM1.4, YN1.1, YN1.5, IH3.1, IH3.3 and YT6.1) for cytokine production patterns and cell-surface-marker phenotypes.

Methods: We measured the production of cytokines, namely, interleukin (IL)-4, IL-5 and interferon- γ (IFN- γ) by stimulation with ovomuroid peptides, and stained intracellular IL-4 and IFN- γ , and determined cell-surface markers using anti-interleukin-12 receptor (IL-12R) β 1, anti-IL-12R β 2 and anti-interleukin-18 receptor α (IL-18R α).

Results: Most TCCs secreted both IL-4 and IFN- γ in response to the OM peptide mixture, but the secretion patterns were variable; an IFN- γ dominant pattern was seen in IH3.1 and YT6.1, an IFN- γ > IL-4 pattern in TM1.3 and TM1.4, an IL-4 > IFN- γ pattern in YN1.5. In intracellular IFN- γ and IL-4 staining, IFN- γ single-positive cells were predominant in TM1.3, TM1.4, IH3.1 and YT6.1 and IFN- γ and IL-4 double-positive cells were predominant in YN1.1, YN1.5 and IH3.3. All TCCs were IL-12R β 1-positive, and TM1.3, IH3.1, IH3.3 and YT6.1 were both IL-12R β 2- and IL-18R α -positive. TM1.4 and YN1.1 were both IL-12R β 2- and IL-18R α -negative. Based on these results, TM1.3 and TM1.4, IH3.1 and YT6.1 had a Th1 character predominantly and YN1.1, YN1.5, and IH3.3 possessed a Th0 character predominantly.

Conclusions: The phenotypes of TCCs were not in accord with their clinical manifestations. TCCs established from patients with immediate-type hypersensitivity had either the Th1 or Th0 phenotype as well as those with non-immediate-type hypersensitivity.

Key Words

AD (atopic dermatitis), IFN- γ (interferon gamma), IL-12R β 2 (interleukin-12 receptor beta 2), IL-18R α (interleukin-18 receptor alpha), OM (ovomucoid), TCC (T cell clone), Th (helper T cell)

Abbreviations used:

AD: atopic dermatitis

FITC : Fluorescein isothiocyanate

HLA: Human leukocyte antigen

IFN- γ : Interferon gamma

IL: Interleukin

IL-12R : IL-12 receptor

IL-18R : IL-18 receptor

OM : Ovomucoid

PBMC : Peripheral blood mononuclear cell

PE : phycoerythrin

RAST: Radioallergosorbent test

rIL : recombinant Interleukin

TCC : T cell clone

TCL : T cell line

TCR: T cell receptor

Th: helper T cell

Introduction

Allergic reactions to foods are particular problems for infants and young children and present a wide spectrum of clinical reactions, including cutaneous, gastrointestinal and respiratory symptoms as well as systemic anaphylactic symptoms. It is also known that food allergies are more prevalent in children due to an immature gastrointestinal epithelial membrane barrier that allows more proteins through the barrier and into circulation (1). Among various food antigens, the hen's egg, particularly its egg-white, is one of the most common causes of food allergy in young children. One of the major components of egg-white, comprising approximately 10% of the total egg-white proteins, is ovomucoid (OM) (2), and it has been reported to play a more important role in the pathogenesis of allergic reactions to egg-white than other egg- white proteins (3,4).

Interferon- γ (IFN- γ) production characterizes the Th1 subset while interleukin (IL)- 4 production characterizes the Th2 subset (5). The differentiation into either Th1 or Th2 cells is a crucial step that determines the direction of subsequent adaptive immune responses. Interleukin-12 receptor (IL-12R) β 2 mRNA synthesis is restricted to Th1 cells (6,7), and the mRNA expression of Interleukin-18 receptor α (IL-18R α) was observed only in the Th1 clones (8). We recently established OM-specific TCCs from four Japanese atopic dermatitis (AD) patients with egg-white allergy (9). In that study, all the TCCs specific to OM exhibited the CD4⁺ phenotype. These clones were established in non-polarizing conditions, it was of interest to see whether they had Th1 or Th2 characters in relation to clinical phenotypes. We determined the cytokine production patterns and cell-surface-marker phenotypes of TCCs specific to OM.

Materials and Methods

Subjects

Clinical information on the four patients whose TCCs have been established was reported previously (9). The diagnosis of allergy to hen's egg-white was based on clinical symptoms, hen's egg-white challenge test results and CAP-RAST (10) against hen's egg-white and OM. All the patients had AD symptoms. Patient TM and YN presented immediate-type hypersensitivity symptoms, such as systemic urticaria and severe coughing, which occurred within thirty minutes after the antigen challenge, and their levels of total IgE and CAP-RAST for egg-white and OM were high. Patient IH and YT had presented non-immediate symptoms, such as systemic eczema, which occurred more than two hours after antigen challenge, and their levels of total IgE and CAP-RAST against egg-white and OM were low(11).

TCC culture

OM-specific TCC establishment was reported previously (9). These TCCs were cultured in an RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM of L-glutamine, 100 units/ml of penicillin, 100 mg/ml of streptomycin, and 10% pooled, heat-inactivated normal human male plasma in 24-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, N.J.). The plates were incubated at 37°C in 5% CO₂ humid air. After 7-9 days, irradiated (30 Gy) autologous PBMC (1.5 x 10⁵/well) pulsed with OM peptide mixture (1 mM each for 5 hr), human recombinant interleukin (rIL)-2 (50 U/ml) (Genzyme, Cambridge, MA), and human rIL-4 (10 U/ml) (Biosource International, Camarillo, CA) were added only to the culture wells. Thus, the TCCs were maintained for another 7 days.

Production of IL-4 and IFN- γ in supernatants of TCCs