

# Induction of $\alpha 1$ and $\alpha 2$ gene expression in selective immunoglobulin A deficiency

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**Abstract.** Immunoglobulin A deficiency (IgAD) is the most common immunodeficiency, but the pathogenesis of most cases of IgAD is poorly understood. The gene and protein expression levels of members of the IgA subclasses in IgAD patients were analyzed by a reverse transcriptase (RT)-PCR method that could differentiate between  $\alpha 1$  and  $\alpha 2$  gene expression. Three selective, 5 partial and 2 secondary IgAD patients were examined. Peripheral blood mononuclear cells which were unstimulated or stimulated with TGF- $\beta 1$  and PMA for 24 h were cultured. The IgA1/IgA2 expression ratios were measured by zone densitometry. Three bands appeared (the  $\alpha 1$  and  $\alpha 2$  genes and a hetero-duplex formation), owing to the difference of 39 bases between  $\alpha 1$  and  $\alpha 2$  mRNAs. In the controls, there were no significant differences in the IgA1/IgA2 ratios between unstimulated and stimulated cells. In selective IgAD patients, both  $\alpha 1$  and  $\alpha 2$  gene expression was induced following stimulation, and  $\alpha 1$  gene expression was induced more dominantly than in the other IgAD patients following stimulation. Based on our results, suppression of  $\alpha 1$  gene expression may be related to the pathogenesis of IgAD.

## Introduction

Human immunoglobulin A (IgA) is the major type of secreted antibody consisting of two subclasses, IgA1 and IgA2. The ratios of IgA1/IgA2 in secretions vary, and the functions of IgA1 and IgA2 in immune response remain unclear (1,2).

IgA deficiency (IgAD) is the most common immunodeficiency. The prevalence in Caucasians is approximately 1 in 500, while the prevalence in the Japanese is much lower; approximately 1 in 18,000 (3-5). IgAD is associated with a variety of infections, allergies, autoimmune disorders, gastro-

intestinal diseases, malignancies, endocrinopathies, neurological diseases and genetic disorders (3,6). IgAD is often associated with other forms of immunoglobulin deficiencies, including IgG subclasses and IgE deficiency (7-10). Certain IgAD patients have an  $\alpha$  gene deletion, but the pathogenesis of some cases of IgAD is still poorly understood. Recently, it has become clear that certain common variable immunodeficiency and IgAD patients possess mutations in TNFRSF13B [encoding TACI (transmembrane activator and calcium-modulator and cyclophilin ligand interactor)] (11). The class switch disorder in IgA-producing B lymphocytes is one of the most important factors in IgAD patients (12). Asano *et al* (13) suggested that the decreased expression level of  $I\alpha$  germline transcripts before a class switch might be the cause of selective IgAD, and that B-cell differentiation might be disturbed after a class switch in partial IgAD patients. Husain *et al* (14) reported that the increased destruction of a subset of B cells is a cause of the inability of IgAD patients to produce IgA. Many studies have reported that certain cytokines, such as IL-4, IL-10, anti-CD40 and TGF- $\beta$ , play important roles in the production of IgA (15,16).

The molecular weights of the IgA heavy chain of the  $\alpha 1$  and  $\alpha 2$  genes are approximately 53 kD each, and the  $\alpha$ -chain constant region is encoded by three exons:  $C\alpha 1$ ,  $C\alpha 2$  and  $C\alpha 3$ .  $C\alpha 2$  includes a hinge region at its 5'-end. The hinge region of the  $\alpha 2$  gene has a deletion of 13 amino acids compared with that of the  $\alpha 1$  gene (1,17). The genes encoding  $\alpha 1$  and  $\alpha 2$  resemble each other closely and, in IgAD patients in particular, it has been difficult to make a quantitative analysis. In order to elucidate the pathogenesis of, and immunological reaction to, IgAD, we analyzed the gene expression of the IgA subclasses in IgAD patients. In this study, we devised a semiquantitative method in order to determine the gene expression levels of members of the IgA subclasses, and analyzed selective, partial and secondary IgAD patients. Using this method, the gene expression of the IgA subclasses could be analyzed in detail.

## Materials and methods

**Subjects.** As shown in Table I, we analyzed three selective IgAD patients (nos. 1, 2 and 3) with serum IgA levels below the detection limit ( $<5$  mg/dl), five partial IgAD patients (nos. 4, 5, 6, 7 and 8) with serum IgA levels  $>5$  mg/dl but  $-2$  standard deviations below the normal levels, and two

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Table I. Immunological data of patients.

Patient no.	Gender	Age (years)	Serum level (mg/dl)			IgG subclass (mg/dl)			
			IgG	IgA	IgM	IgG1	IgG2	IgG3	IgG4
Selective IgA deficiency									
1	M	10	1363	<5	146	619	255.0	57.3	33.9
2	F	11	1640	<5	117	949	400.0	47.7	90.2
3	F	17	1261	<5	137	630	625.0	35.1	18.4
Partial IgA deficiency									
4	M	4	1223	17	120	933	<8.0	22.8	<3.0
5	F	3	1644	15	150	878	47.9	17.3	32.1
6	F	3	869	27	106	306	69.3	27.6	3.8
7	M	4	887	45	101	295	97.0	40.0	4.4
8	M	4	1624	8	100	1090	120.0	74.7	<3.0
Secondary IgA deficiency									
9	F	7	913	12	105	602	148.0	52.8	16.5
10	M	13	705	9	39	852	345.0	49.8	6.4

secondary IgAD patients (nos. 9 and 10) whose condition was caused by epileptic medication. Ten controls were also included in this study. We obtained informed consent from the patients, controls or their parents.

**Cell preparation and culture.** Peripheral blood mononuclear cells (PBMCs) were collected in heparin and separated by gradient centrifugation in Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) (18). Cells were suspended at a density of  $10^6/\text{ml}$  and cultured for 24 h in an RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Some of the PBMCs were stimulated with phorbol myristate acetate (PMA) (10 ng/ml) (Sigma Aldrich, St. Louis, MO, USA) and recombinant human TGF- $\beta$ 1 (1 ng/ml) (R&D Systems, Inc., Wiesbaden, Germany) for 24 h.

**cDNA synthesis and PCR amplification.** We extracted total RNA from PBMCs using an Isogen kit (Nippon Gene, Tokyo, Japan), and cDNA synthesis was carried out using 2  $\mu\text{g}$  of total RNA with oligo-dT and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). We used the following PCR primers, both of which were designed against the common sequence area of the  $\alpha$ 1 and  $\alpha$ 2 genes: sense 5'-CCT GGT CAC CGT CTC CTC A-3' (within the J exon; Gene Bank accession no. L20778) and antisense 5'-TCA CGC TCA GGT GGT CCT TG-3' (within the C $\alpha$  CH2 exon) (19). The PCR fragments included the CH1, hinge and CH2 regions, and their sizes were 532 bp for the  $\alpha$ 1 gene and 493 bp for the  $\alpha$ 2 gene. The PCR program was 35 or 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, using 1 or 2  $\mu\text{l}$  of cDNA as the template. The PCR products were run on 4% agarose gels for 120 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control.

**Zone densitometry analysis.** The IgA1/IgA2 gene expression ratios in stimulated cells were measured using zone densitometry. The peaks of each of the three bands were detected and measured. Half of the intensity of the heterozygous band was added to each of the  $\alpha$ 1 and  $\alpha$ 2 band intensities, and then the IgA1/IgA2 ratios were calculated. Subcloning was conducted according to the following steps. After electrophoresis the bands, including the  $\alpha$ 1 and  $\alpha$ 2 genes, were cut out, and DNA extraction was performed. We transformed the DNA fragments into a T-vector, then cultured and picked up the colonies. The plasmid DNA was extracted and digested to completion with *Eco*RI. We distinguished two isotypes,  $\alpha$ 1 and  $\alpha$ 2 genes, by the sizes of DNA bands on the gels; some of the  $\alpha$ 2 fragments were separated into two bands because one of the allotypes of the  $\alpha$ 2 gene, the A2m(2) allotype, had an *Eco*RI site (20).

**Quantification of IgA in plasma.** IgA was measured using enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated overnight at 4°C with goat anti-human IgA (Bethyl, Montgomery, TX), which was diluted to 1:100 with 0.05 M sodium carbonate, pH 9.6. After washing, the plates were incubated with standard serum and plasma dilutions. IgA was detected by horseradish peroxidase (HRP)-labeled goat anti-human IgA (Cappel, Organon Teknika, Turnhout, Belgium), which was diluted to 1:10,000 with 1% BSA, 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl and 0.05% Tween-20. The samples were tested repeatedly. The lower limit of IgA detection was 5 ng/ml.

**Quantification of IgA subclasses in plasma.** The levels of the IgA subclasses in plasma were measured using ELISA. For IgA1, coating was performed using a mouse monoclonal anti-IgA1 antibody (NI69-11), and detection of IgA1 was

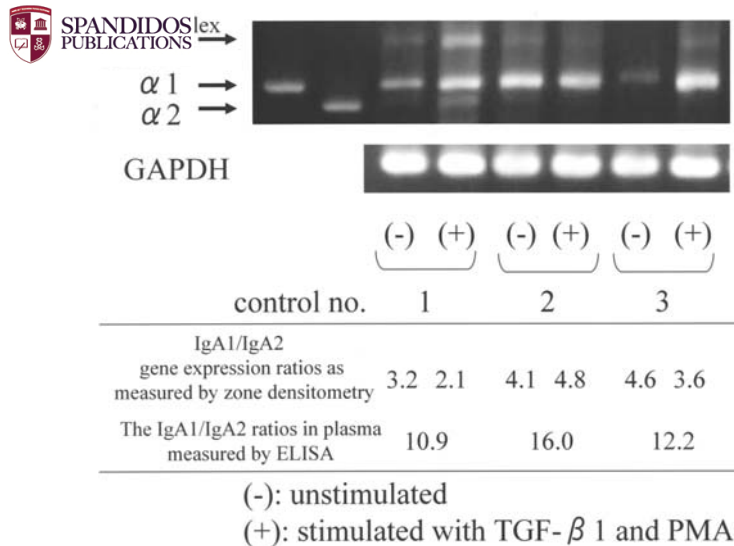


Figure 1. Expression of the  $\alpha 1$  and  $\alpha 2$  genes in healthy controls. PBMCs from three healthy controls were cultured for 24 h in the absence or presence of stimulation with TGF- $\beta$ 1 and PMA. After PCR, three bands appeared representing  $\alpha 2$  and  $\alpha 1$  gene expression and hetero-duplex formation, as indicated by arrows. GAPDH was used as a control. The IgA1/IgA2 expression ratios following stimulation were measured by zone densitometry, and the IgA1/IgA2 ratios in plasma were measured using ELISA.

performed using an HRP-labeled goat anti-human IgA antibody (Cappel, Organon Teknika) (21). For IgA2, coating was performed with goat anti-human IgA, and detection of IgA2 was performed using a mouse anti-human IgA2-HRP antibody (B3506B4) (22). ELISA plates were coated overnight at 4°C with mouse monoclonal anti-IgA1 (diluted to 1:200 with PBS-0.02% Tween-20) or goat anti-human IgA (diluted to 1:100 with PBS-0.02% Tween-20). After washing, plates were incubated with standard serum and plasma dilutions. IgA1 was detected using goat anti-human IgA-HRP antibody

(diluted 1:10,000 with PBS-0.02% Tween-20), and IgA2 was detected using mouse anti-human IgA2-HRP antibody (diluted to 1:1000 with PBS-0.02% Tween-20). Samples were tested repeatedly. The lower limits of IgA1 and IgA2 detection were 5 and 1  $\mu$ g/ml, respectively.

**Statistical analysis.** Significant differences between two groups were analyzed by paired t-tests. The correlation coefficients were determined by Pearson's product-moment correlation coefficient.  $p < 0.05$  was considered to be statistically significant.

## Results

**PCR amplification of  $\alpha 1$  and  $\alpha 2$  gene expression in control PBMCs.** RT-PCR analysis was performed using primer pairs that amplified both  $\alpha 1$  and  $\alpha 2$  mRNAs and could distinguish between them owing to the deletion of 39 bases in the hinge region of the  $\alpha 2$  mRNA. As shown in Fig. 1, the controls displayed an intense  $\alpha 1$  band and a less intense, shorter  $\alpha 2$  band in all three PCR conditions. Another band with less electrophoretic mobility than the  $\alpha 1$  band was determined to be a hetero-duplex formation from the  $\alpha 1$  and  $\alpha 2$  fragments, because the subcloning of this band yielded clones of both  $\alpha 1$  and  $\alpha 2$  fragments. The expression of the  $\alpha 1$  and  $\alpha 2$  genes in the healthy controls is shown in Fig. 1. The gene expression of the PCR products tended to be enhanced more strongly when cells were stimulated with TGF- $\beta$ 1 and PMA. In all of the healthy controls,  $\alpha 1$  gene expression was dominant. The IgA1/IgA2 expression ratios were measured by zone densitometry in the healthy controls and were found to vary (1.1-4.8) among the controls. There were no significant differences in the IgA1/IgA2 expression ratios of unstimulated cells and cells stimulated with TGF- $\beta$ 1/PMA ( $p > 0.05$ ). To confirm the IgA1/IgA2 ratios as analyzed by zone densitometry, we counted the number of colonies from the PCR products. The

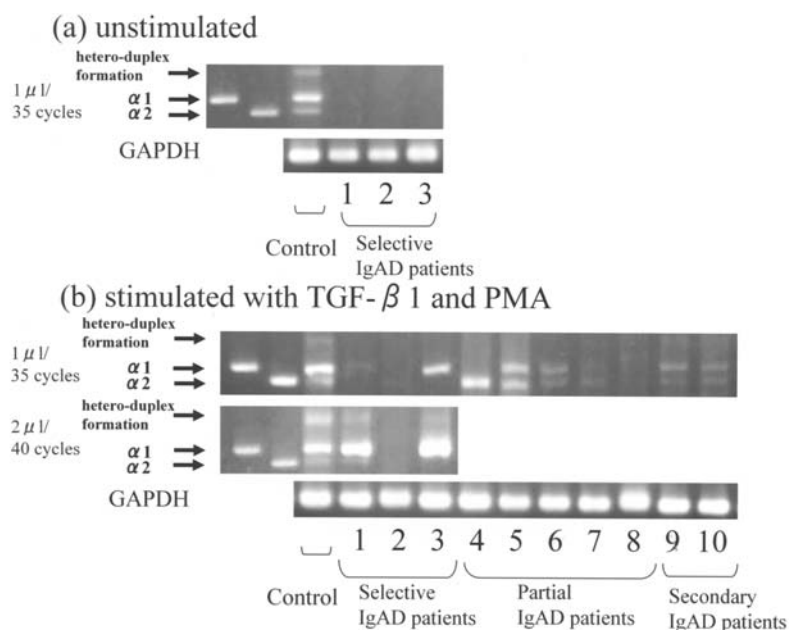


Figure 2. Expression of the  $\alpha 1$  and  $\alpha 2$  genes in IgAD patients. PBMCs from IgAD patients were cultured for 24 h in the absence (a) or presence (b) of stimulation with TGF- $\beta$ 1 and PMA. In each case, 1 or 2  $\mu$ l of cDNA was used as a template and 35 or 40 cycles were run. GAPDH was used as a control.

number of colonies correlated with the IgA1/IgA2 ratios determined by zone densitometry (data not shown).

**PCR amplification of  $\alpha 1$  and  $\alpha 2$  gene expression in IgAD patients.** Expression of the  $\alpha 1$  and  $\alpha 2$  genes in the IgAD patients is shown in Fig. 2. Stimulation of cells with TGF- $\beta$ 1/PMA induced expression of mature transcripts in some selective IgAD patients. No selective IgAD patients showed any bands without stimulation, while selective IgAD patients (nos. 1 and 3) showed two or three bands following stimulation. Some partial IgAD patients (nos. 5, 6 and 7) and secondary IgAD patients (nos. 9 and 10) showed  $\alpha 2$ ,  $\alpha 1$  and heteroduplex gene expression following stimulation, while patient no. 4 showed only  $\alpha 2$  gene expression. This patient was found to have a deletion of  $\alpha 1$ ,  $\gamma 2$ ,  $\gamma 4$  and  $\epsilon$  genes, as in a previously reported case (10). Using this method, we identified the second case of  $\alpha 1$  gene deletion in Japan. Partial IgAD patient no. 8 showed no bands using this RT-PCR method. The IgA1/IgA2 ratios of IgAD patients analyzed by zone densitometry are shown in Table II. ND (not detected) in Table II means that gene expression was not detected or that the peaks were too faint to detect when analyzed by zone densitometry. The expression levels of both the  $\alpha 1$  and  $\alpha 2$  genes relative to GAPDH expression in IgAD patients were suppressed when compared with the controls (data not shown). In selective IgAD patients, both  $\alpha 1$  and  $\alpha 2$  gene expression was induced following stimulation. In particular,  $\alpha 1$  gene expression was more dominant than that of  $\alpha 2$  in these patients when compared with the other IgAD patients and healthy controls.

**IgA, IgA1 and IgA2 concentration in the controls and IgAD patients.** The levels of IgA1 and IgA2 proteins in plasma are shown in Table III. ND (not detected) in Table III means that levels were below the detection limit. In all controls, the

Table II. IgA1/IgA2 gene expression ratios measured by zone densitometry.

Patient no.	IgA1/IgA2 gene ratios	
	Stimulation	
	(-)	TGF- $\beta$ 1/PMA
Selective IgA deficiency		
1	ND	3.91 $\pm$ 0.55
2	ND	ND
3	ND	7.04 $\pm$ 1.22
Partial IgA deficiency		
4		No $\alpha 1$ gene transcripts
5		2.55 $\pm$ 1.74
6		1.72 $\pm$ 0.49
7		1.11 $\pm$ 0.27
8		ND
Secondary IgA deficiency		
9		1.50 $\pm$ 0.29
10		1.17 $\pm$ 0.10
Controls (n=10)	2.56 $\pm$ 1.22	2.35 $\pm$ 1.20
ND, not detected.		

IgA1 levels in plasma were dominant. The IgA1/IgA2 gene expression ratios following stimulation measured by zone densitometry were strongly correlated with those of plasma levels measured using ELISA in the controls (n=10, r=0.917, t=6.53, p<0.05) (Fig. 1).

Table III. IgA subclass levels and the IgA1/IgA2 ratios in plasma as measured using ELISA.

Patient no.	Plasma IgA, IgA1 and IgA2 levels (mg/dl)			IgA1/IgA2 ratios
	IgA	IgA1	IgA2	
Selective IgA deficiency				
1	2.13±0.01	2.48±0.26	ND	NC
2	ND	ND	ND	NC
3	4.00±0.59	2.16±0.74	ND	NC
Partial IgA deficiency				
4	9.18±2.87	ND	13.7±1.39	NC
5	5.16±0.21	2.71±0.36	ND	NC
6	10.11±0.13	8.28±0.52	0.91±0.25	8.40
7	37.46±8.36	29.29±6.83	4.88±0.54	6.97
8	6.53±0.52	6.28±0.67	0.86±0.45	8.87
Secondary IgA deficiency				
9	11.23±1.91	8.59±1.77	1.17±0.36	6.56
10	0.54±0.06	0.56±0.09	0.18±0.03	3.03
Controls (n=10)	154.01±37.89	119.61±34.69	20.44±8.63	7.96±4.14

ND, not detected; NC, not calculated.





The serum IgA subclass levels of IgAD patients, especially selective IgAD patients, are very difficult to measure using ELISA, so there have been few reports concerning them. The expression levels of the  $\alpha 1$  and  $\alpha 2$  genes in most IgAD patients are low, and there have been no reports concerning the levels of mature transcripts of the IgA subclasses in patients (23-26). Wang *et al* (27) concluded that the cause of IgAD is a defect in the transcriptional factors important for post-switch  $\text{Ca}$  gene transcription or a lack of signals for activation of the  $\text{Ca}$  gene in IgA-switched cells. According to Hummelshoj *et al* (16),  $\alpha 1$  and  $\alpha 2$  germline transcripts in IgAD patients were induced by the stimulation of certain types of cytokines, such as TGF- $\beta 1$  and IL-4. Such stimulation can lead to the induction of germline transcripts and mature transcripts in IgAD patients. In our report, using stimulation of cells with TGF- $\beta 1$  and PMA, we induced expression of both  $\alpha 1$  and  $\alpha 2$  transcripts in cells from IgAD patients and measured the IgA1/IgA2 ratios. In the healthy controls, there were no significant differences in the IgA1/IgA2 ratios with or without stimulation. In the IgAD patients, the gene expression levels of both IgA subclasses were suppressed. Expression of the  $\alpha 1$  gene was induced more dominantly than that of the  $\alpha 2$  gene following stimulation in selective IgAD patients. Kitani and Strober (28) reported that *Staphylococcus aureus*, Cowan I and TGF- $\beta 1$  induce mature  $\text{Ca}1$  transcripts, but do not induce  $\text{Ca}2$  mature transcripts. Based on our results, suppression of  $\alpha 1$  gene expression may be involved in the pathogenesis of selective IgAD. However, there was a discrepancy in the IgA1/IgA2 ratios based on gene expression levels and those based on protein expression levels. This discrepancy might have been caused by post-transcriptional modifications leading to, for example, protein or mRNA stability. The other possibility is that the production of IgA protein might have been different in peripheral circulating IgA-switched B cells and locally accumulated IgA-switched B cells, such as mucosal tissue. Secreted and membrane-localized IgA can not be distinguished by this method, and we are now investigating methods for the separation of membrane and secretory transcripts in each IgA subclass.

Our method was effective for detecting mature transcripts of IgA subclasses in cases whose serum IgA levels were under the detection limit. Additional patient analysis is needed to clarify the mechanism of the pathogenesis of IgAD.

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