

ment.¹⁹ This huge difference in IC50 may reflect the type of inhibitory mechanism that is employed (Fig. 6). Interestingly, an intact form of h18-108 antibody, an IgG molecule, has a much higher affinity with a Kd of 0.64 nM and an IC50 of 5 nM.¹⁹ This improvement is most likely the result of its overall stability and the bivalency of the intact antibody molecule. Regardless of the affinity, these different molecules will be useful for determining the precise mechanism by which IL-18 receptor is activated or inhibited. Information gained from such studies will undoubtedly help us design potent therapeutic reagents.

A number of drugs targeting and neutralizing the deleterious effects of cytokines have been developed. Etanercept is a soluble fusion protein that is composed of two tumor necrosis factor alpha (TNF- α) receptors (p75) and a Fc fragment of a human IgG1 molecule; it has been shown to be effective and safe in patients with rheumatoid arthritis.³⁸ Recently, it was reported that the blockade of TNF- α by etanercept had efficacy in patients with asthma.^{39,40} In addition to the anti-TNF drugs, the role of IL-18 in allergy suggests that drugs that neutralize the effect of IL-18, such as monoclonal antibodies or IL-18BP, may also be useful for the management of allergy.⁷ The chemical characteristics of IL-18BP (a single polypeptide, a monomer, and with a low molecular weight of 25 kDa) support its use in clinical settings. Because of these characteristics, it is possible that its dosage could be lower than that of molecules, such as monoclonal antibodies and etanercept (multiple polypeptides, oligomer, high molecular weight of > 300 kDa) that are much bigger and more complex. Establishing a system that enables researchers to highly express the desired protein to study its structural mechanism could open avenues of research to develop new reagents and strategies for the treatment of various diseases, including allergy.

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The response of bovine beta-lactoglobulin-specific T-cell clones to single amino acid substitution of T-cell core epitope

Kondo M, Kaneko H, Fukao T, Suzuki K, Sakaguchi H, Shinoda S, Kato Z, Matsui E, Teramoto T, Nakano T, Kondo N. The response of bovine beta-lactoglobulin-specific T-cell clones to single amino acid substitution of T-cell core epitope.

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Cow's milk is one of the most common food allergens in the first year of life, with approximately 2.5% of infants experiencing an allergic reaction to it. Beta-lactoglobulin (BLG) is one of the major allergens in cow's milk. Previously, we reported that four of six T-cell clones (TCC) which were established from cow's milk allergy patients recognized BLGp97-117 as the core sequence and also recognized BLG in association with the human leucocyte antigen (HLA)-DRB1*0405 allele. Using two of these four TCCs, we evaluated the T-cell response to BLG peptides with single amino acid substitution or deletion and identified BLGp102-112 as the minimum essential region in BLGp97-117. In the alanine-scan assay, the proliferative responses of TCCs to pE108A disappeared, and the proliferative responses of TCCs to pC106A decreased. In the analog peptide proliferation assay, pY102S had retained some T-cell response to the two TCCs. Collecting these results, we propose a motif for the interaction between the HLA-DRB1*0405 allele and antigen peptide, and suggest that BLGp105-108 are important residues to retain the TCR/BLG-peptide/HLA complex. pY102A and pY102S are partial agonists for the T-cell receptor. These peptides might be considered as candidate peptides for the modification of the T-cell response to BLG in cow's milk allergy.

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Cow's milk allergy is one of the most common food allergies in young children, with approximately 2% to 2.5% of all infants experiencing allergic reactions to it. The majority of children out-grow their allergy to cow's milk before the age of 3 yr, but 15% of these infants with IgE-mediated cow's milk allergy retain their sensitivity into the second decade of life (1, 2).

Beta-lactoglobulin (BLG) is the major whey protein of the milk of the ruminants. It is

relatively resistant to acidic pH and to proteolytic enzymes, leaving its structure relatively unchanged during digestion and possibly allowing the passage of intact protein into the circulation (3). Moreover, it is not present in human milk and is considered to be a major allergen of cow's milk (3). IgE-binding regions of BLG have been identified. Selo et al. showed that the major IgE-epitopes of BLG appeared to be fragments p41–60, p102–124, and p149–162 (4).

The isotype switching of antigen-specific B cells to IgE is controlled by T cells that play a key role in the initiation of allergic symptoms, as well as in tolerance induction. Antigenic peptide-made

Abbreviations: BLG, beta-lactoglobulin; IFN-gamma, interferon gamma; PBMCs, peripheral blood mononuclear cells.

T-cell clone responses to peptides of single amino acid substitution

Table 1. Patients' profile and characterization of T-cell clones

	Sex	Age (yr)	IgE (IU/ml)	CAP-RAST		Antigen presenting HLA†	Recognition site of BLG peptide‡
				Milk	BLG		
YA	Female	1	1136.8	94	55.4	DRB1*0405	p97-117 (TDYKKYLLFCMENSAPAEQSL)
HA	Female	2	249	2.3	1.5	DRB1*0405	p97-117 (TDYKKYLLFCMENSAPAEQSL)

BLG, beta-lactoglobulin; RAST, radioallergosorbent test.

†Previously reported data (6).

complexes with HLA class II molecules are displayed on the surface of antigen-presenting cells for recognition by CD4⁺ T cells.

We have previously reported that the BLG peptide (BLGp97-117) was presented by HLA-DRB1*0405 using BLG-specific T-cell lines and clones (TCCs) obtained from patients with cow's milk allergy (5, 6). Four of six TCCs recognized the BLG peptide (BLGp97-117) presented by HLA-DRB1*0405, and suggested that HLA-DRB1*0405 is an immunoregulatory gene product for T-cell responses to BLG (6). We hypothesize that single amino acid substitutions on an allergen peptide carrying the T-cell epitope might modify the T-cell response to allergen. In this study, we identified the minimal core epitope of TCCs specific to BLG using BLG-derived peptides, and the analog peptides which modified the T-cell responses. We proposed a motif for the interaction between the HLA-DRB1*0405 allele and antigen peptide.

Material and methods

Subjects

As shown in Table 1, we obtained BLG-specific TCCs from two patients (YA and HA) with cow's milk allergy. Allergic symptoms such as eczema, urticaria, and wheezing occurred in less than 1 h after cow's milk ingestion in these patients.

Both of the TCC recognized BLGp97-117 as the core sequence and recognized BLG in association with the HLA-DRB1*0405 allele (6).

TCCs specific to BLG Culture

The TCCs were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 10% pooled AB, heat-inactivated normal human male plasma in 24-well flat-bottomed cultured plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). The plates were incubated at 37°C in 5% CO₂ humidified air. After 7–10 days, irradiated

(30 Gy) autologous peripheral blood mononuclear cells (PBMCs) (1.5×10^5 /well) pulsed with a BLG-derived peptide mixture (1 µM each for 5 h), human rIL-2 (50 U/ml) (Genzyme, Cambridge, MA, USA), and human rIL-4 (10 U/ml) (Biosource International, Camarillo, CA, USA) were added to the culture wells and the TCCs were maintained for another 7 days.

Intracellular IFN-gamma and IL-4 staining of BLG-specific TCCs

The TCCs specific to BLG were cultured at a density of 2×10^6 cells/ml in an RPMI 1640 medium for 4 h at 37°C. During the 4-h incubation, the cells were stimulated with a combination of 25 ng/ml of phorbol 12-myristate 13-acetate (SIGMA, St Louis, MO, USA) and 2 µg/ml of ionomycin (SIGMA) in the presence of 10 ng/ml of Brefeldin-A (SIGMA). Then the cells were directly stained with a PC-5-conjugated anti-CD4 monoclonal antibody (Coulter-Immunotech, Marseille, France) for 15 min at room temperature, and fixed with a Fluorescence-activated cell sorting (FACS) Lysing Solution (Becton Dickinson, Mountain View, CA) for 10 min. After washing, they were pre-incubated with a FACS Permeabilizing Solution (Becton Dickinson) for 10 min and after washing again, they were incubated with FASTIMMUNE interferon (IFN)-gamma FITC/IL-4 PE (Becton Dickinson) for 30 min at room temperature. Flow cytometric analysis was performed using a FACS Calibur.

Synthesis of BLG-derived peptides

BLG-truncated peptides for searching the core sequences of BLG p97-117 (TDYKKYLLFCMENSAPAEQSL), BLG-derived peptides for analyzing alanine-scan mutant peptides, and BLG-derived analog peptides of 11 amino acid residues were synthesized using the multiple method based on the Fmoc strategy (5). The substitution of a single amino acid residue was done for each peptide. All peptides were purified by C18 reverse-phase HPLC (Gilson, Middleton, WI, USA).

Proliferative responses of the TCCs using BLG-derived peptides

The proliferative responses of the TCCs using BLG-derived peptides were assayed as described previously (5). Briefly, the TCCs (3×10^4 /well) were cultured in 96-well flat-bottomed culture plates in the presence of BLG-derived analog peptides (1 μ M), a soluble BLG peptide mixture (1 μ M each), BLG crude protein (25 or 2.5 μ g/ml) and 30 Gy-irradiated autologous PBMC (1.5×10^5 /well) for 72 h. Before the final 16-h period, 1 μ Ci per well of [3 H]TdR was added, and the incorporated radioactivity was measured by liquid scintillation counting (7).

Production of IL-4, IFN-gamma and IL-10 in supernatants of TCCs using BLG-derived peptides

The TCCs (3×10^4 cells/well in 96-well flat-bottomed culture plates) were cultured in the presence of a soluble BLG peptide mixture (1 μ M) and irradiated autologous PBMC (1.5×10^5 /well) for 56 hr. Culture supernatants of the TCCs were collected and stored in aliquots at -80°C until the determination of lymphokine concentrations. Enzyme-linked immunosorbent assay kits for detecting human IL-4 (Biosource International), IFN-gamma (Ohtsuka, Tokyo, Japan) and IL-10 (Biosource International) were used for quantification of the lymphokines in the supernatants, according to the manufacturers' instructions.

Binding images between BLGp102-112 and HLA-DRB1*0405 molecules

The binding images between the BLGp102-112 and HLA-DRB1*0405 molecules were displayed with RASMOL, which is the standard molecular graphics program (8).

Results

Intracellular IFN-gamma and IL-4 staining of BLG-specific TCCs

To characterize the TCCs (YA4, HA5.7), intracellular IFN-gamma and IL-4 were analyzed. YA4 showed an intracellular IL-4 single positive cell pattern and HA5.7 showed an IFN-gamma single positive cell pattern (data not shown). These results suggested that YA4 had the Th2 phenotype and HA5.7 had the Th1 phenotype.

Proliferative responses of the TCCs using BLG-truncated peptides

Previously, we reported that the TCCs recognized BLGp97-117 as the core sequence. To

define the minimum essential region in BLGp97-117, BLGp97-117 (TDYKKYLLFCMENSAP-PEQSL)-truncated peptides in the N and C termini were designed. The proliferative responses of the TCCs using these truncated peptides were compared (Fig. 1). Although BLGp102-112 (YLLFCMENSAP) induced proliferative responses of TCCs, BLGp103-112 and BLGp102-111 induced no proliferative responses in YA4 and HA5.7. Therefore, both YA4 and HA5.7 recognized BLG p102-112 (YLLFCMENSAP) as the minimal epitope. Proliferative responses and cytokine production of the TCCs using BLG-derived alanine-scan mutant peptides.

To predict the TCR/BLGp102-112/HLA complex structure, BLGp97-117-derived alanine-scan mutant peptides were designed. The proliferative responses of TCCs to pE108A disappeared, and the proliferative responses of TCCs to pC106A decreased (Fig. 2a). The cytokine production patterns are shown in Fig. 2b. In YA4, which had the Th2 phenotype, IL-4 production increased in proportion to its proliferative reaction. In HA5.7, which had the Th1 phenotype, IFN-gamma production increased in proportion to its proliferative reaction. IL-10 production increased according to its proliferative reaction in YA4 and HA5.7 (Fig. 2c).

Proliferative responses and cytokine production of the TCCs using BLG-derived analog peptides

Single amino acid substitution peptides of BLGp102-112 were designed. The proliferative responses of TCCs to these peptides were investigated. pY102S had retained some T-cell responses to both YA4 and HA5.7 (Fig. 3a). pY102S, pL103V and p104V had retained some T-cell responses to HA5.7. IFN-gamma and IL-4 production increased in proportion to their proliferative reaction, as observed in the case of the BLG alanine scan peptide (data not shown). BLG crude induced the IL-10 production of the clones of both YA4 and HA5.7 (Fig. 3b). pY102S showed some IL-10 production of YA4, not HA5.7. pY102S, pL103V, and p104V showed no IL-10 production of HA5.7.

Binding images between BLGp102-112 and HLA-DRB1*0405 molecules

We analyzed that the binding images between the BLGp102-112 and HLA-DRB1*0405 molecules based on the structural modeling. Amino acid residues of L104 and M107 on the BLGp102-112 could be fitted in the pocket of HLA-DRB1*0405 molecules, and F105 and E108 on

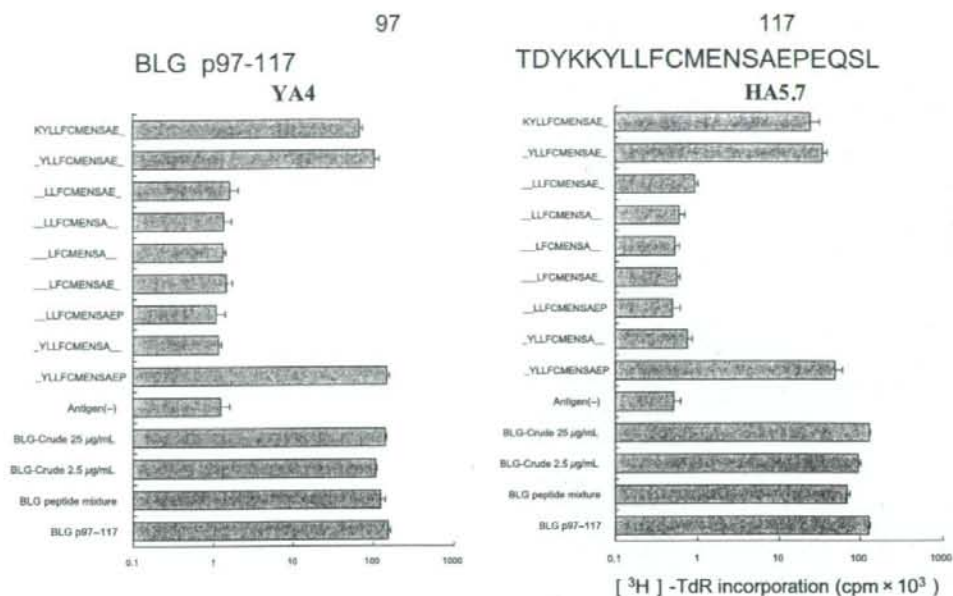


Fig. 1. T-cell clone responses to truncated beta-lactoglobulin (BLG) peptides. Peptides truncated from the N- or C-terminal were designed to define the minimal T-cell activation-inducing epitope. The proliferative responses of the TCCs using these truncated peptides are shown. Both TCCs (YA4 and HA5.7) recognized minimal peptide BLGp102-112 (YLLFCMENSAAE).

the BLGp102-112 could be presented on the surface of HLA-DRB1*0405 molecules.

CDR3 loops of TCCs (HA5.7) were demonstrated to be 'QKV' in a previous study (6). This sequence is composed of an amide-bearing residue, a positively charged residue, 'K', and a hydrophobic residue, 'Q'. The negatively charged residue, E108, interacted electrically with the 'K' of the CDR3 loops. The hydrophobic residue, F105, and the 'V' of the CDR3 loops interacted with each other (Fig. 4).

Discussion

YA had an immediate type milk allergy and the food challenge test was positive. Her main symptom was urticaria and a skin rash after milk ingestion. HA had immediate and non-immediate type milk allergy. She had atopic dermatitis, a symptom which worsens after milk ingestion. The YA4 clone had a Th2 phenotype and the HA5.7 clone had a Th1 phenotype. In these cases, the clone phenotype was suitable for each clinical symptom. Two TCC (YA4 and HA5.7) needed a minimum of peptide BLGp102-112 (YLLFCMENSAAE) presented by HLA-DRB1*0405 to proliferate. By using BLG-derived alanine-scan mutant peptides, the

proliferative responses of the TCCs to pE108A disappeared, and those to pC106A decreased. Cytokine secretions of TCCs decreased in proportion to their proliferative responses. Considering the binding images between the BLGp102-112 and HLA-DRB1*0405 molecules based on the structural design reported above, E108 on the BLGp102-112 is the key residue in the proliferation of TCCs.

The major safety concern regarding food allergen immunotherapy has been addressed by engineering 'hypoallergenic' forms of major allergenic food proteins. These mutated ('engineered') major food proteins have lost their ability to bind to IgE but have retained the ability to interact with T cells (9). In previous literature, the tolerogenic peptide size is around 20 amino acids (10-14). These tolerogenic peptide sizes are in accord with the fact that peptides with 12 to 20 amino acids presented with HLA complex class II molecules on the surface of antigen-presenting cells are recognized by T cells (15). The presence of T-cell epitopes is essential for tolerogenic peptides as immunomodulation is mainly induced by T cells (16). In our cases, the analog peptides that change amino acids pY102A and pY102S have some T-cell response to TCCs. pY102A induced some IL-10 production in YA4

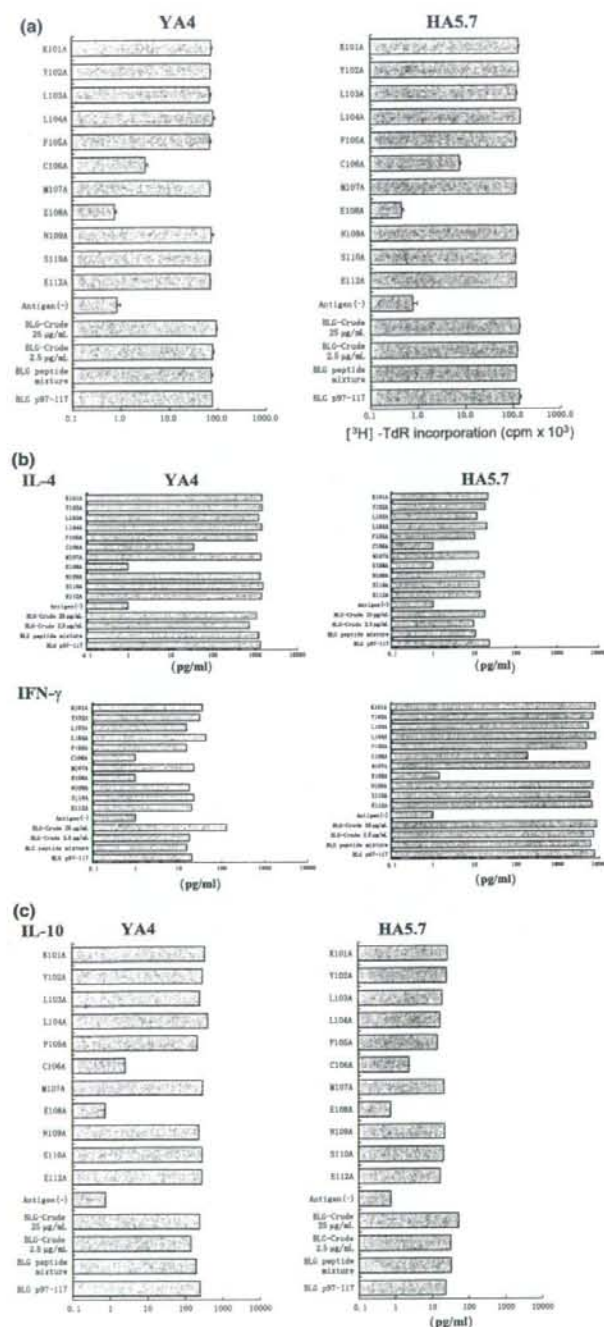


Fig. 2. T-cell clone (TCC) responses to beta-lactoglobulin (BLG) alanine-scan mutant peptides. BLGp97-117-derived alanine-scan mutant peptides were designed. Each polypeptide has 21 amino acid residues from number 97 to 117 and contains one amino acid substitution for alanine. The BLG peptide mixture is a mixture of synthetic peptides overlapping each other by 11 residues (5). BLG crude is a BLG protein (25 or 2.5 µg/ml). (a) The proliferative responses of the TCCs against BLGp97-117-derived alanine-scan mutant peptides were shown. (b) IL-4 and IFN-γ production of the TCCs against BLGp97-117-derived alanine-scan mutant peptides was shown. (c) IL-10 production of the TCCs against BLGp97-117-derived alanine-scan mutant peptides was shown.

(Fig. 3b). It has been reported that IL-10 induced T-cell anergy and played an important role in the induction and maintenance of antigen-specific T-cell tolerance (17). These results show that

pY102A and pY102S might have potential as immunoregulatory peptides.

In this study, alanine-scan was performed because it lacks the side chain of the native

T-cell clone responses to peptides of single amino acid substitution

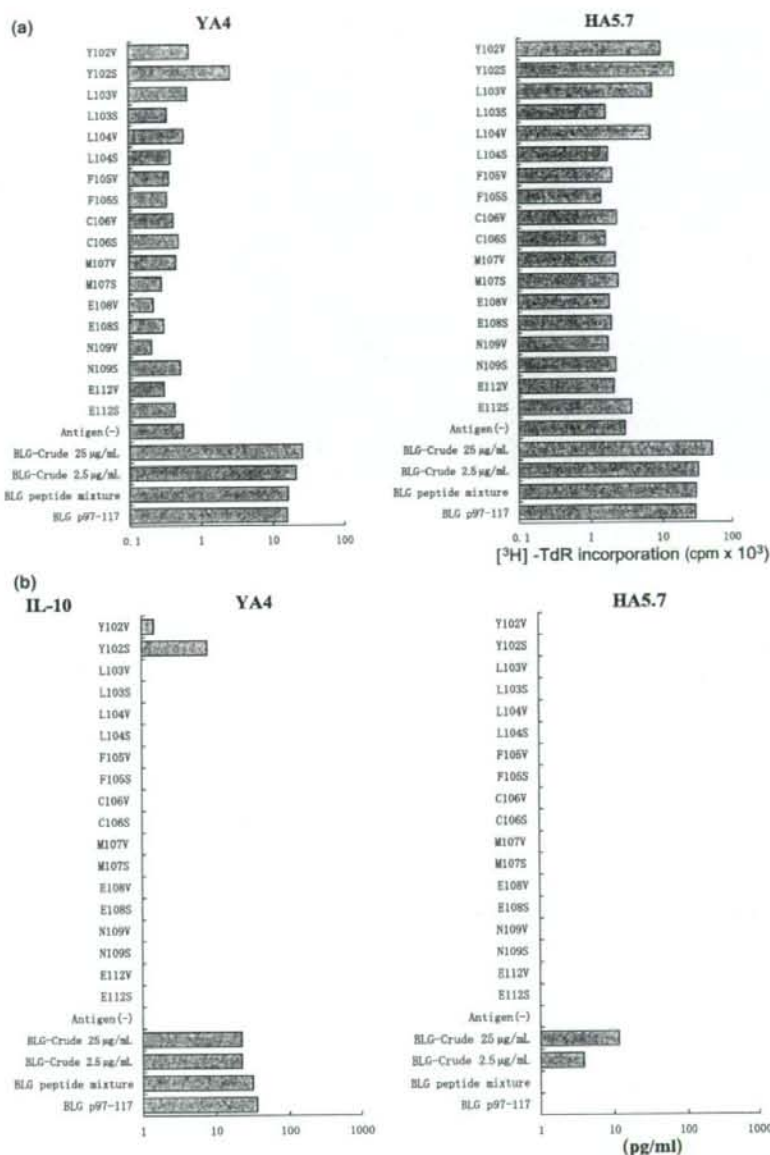


Fig. 3. T-cell clone (TCC) responses to beta-lactoglobulin (BLG) analog peptides. Single amino acid substitution peptides of BLGp102-112 were designed. Each polypeptide has 11 amino acid residues from number 102 to 112 and contains one amino acid substitution, as indicated in Fig. 3. (a) Proliferative responses of the TCCs to single amino acid substituted peptides of BLG p102-112 are shown. (b) IL-10 production of the TCCs to single amino acid substituted peptides of BLG p102-112 is shown.

amino acid beyond the β carbon and thus can assess the impact of its absence on binding affinity. It does not alter significantly the main-chain conformation and does not impose extreme electrostatic or steric effects.

In conclusion, this study has identified the T-cell minimal core epitope, predicted the TCR/

BLG-peptide/HLA complex structure, and searched for the immunomodulated peptides. The p105-108 substitutions are important to retain the TCR/BLG-peptide/HLA complex structure. Our data suggest that peptides with a mutated core epitope might be considered as candidate peptides for the modification

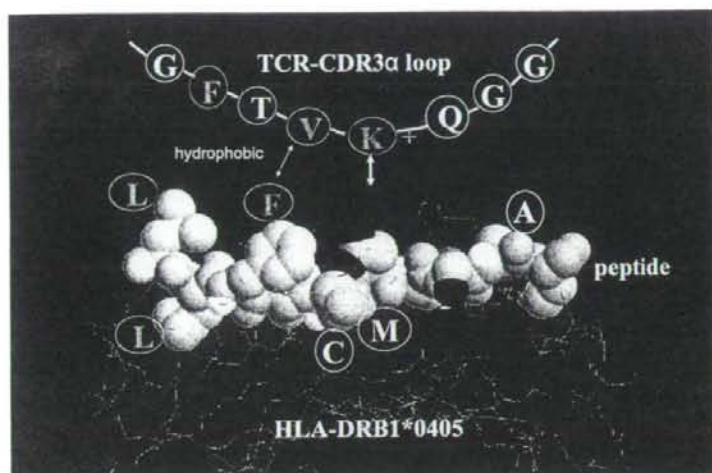


Fig. 4. Binding images between BLGp102-112 and HLA-DRB1*0405 molecules based on structural modeling. The CDR3 loops of the TCCs (HA5.7) were demonstrated to be 'QKV'. The negatively charged residue pE108 and the positively charged residue 'K' of the CDR3 loops interacted. The hydrophobic residue pF105 and 'V' of the CDR3 loops interacted with each other.

of the T-cell response to BLG in cow's milk allergy.

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Various Expression Patterns of $\alpha 1$ and $\alpha 2$ Genes in IgA Deficiency

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ABSTRACT

Background: IgA deficiency (IgAD) is the most common immunodeficiency, however the pathogenesis in most cases of IgAD is unknown. There are 2 subclasses of IgA, IgA1 and IgA2, and its heavy chains are encoded by 2 different genes, the $\alpha 1$ and $\alpha 2$ genes. To investigate the molecular pathogenesis of IgA deficiency, it is important to evaluate each of the expressions of IgA1 and IgA2 separately.

Methods: In this study, we report on the reverse transcriptase (RT)-PCR method in which $\alpha 1$ and $\alpha 2$ mRNAs can be separately evaluated. This method is based on electrophoretic separation using the difference of 39 bases between $\alpha 1$ and $\alpha 2$ mRNAs. Three selective, 5 partial and 2 secondary IgAD patients were examined.

Results: In the 3 selective IgAD patients, no $\alpha 1$ or $\alpha 2$ mRNA expression was detected. In the 5 partial IgAD patients, various $\alpha 1$ and $\alpha 2$ mRNA expression patterns were found. One of the partial IgAD patients showed only $\alpha 2$ gene expression, but not $\alpha 1$ gene expression, and was found to show an $\alpha 1$ gene deletion together with $\gamma 2$ and ϵ gene deletions. His plasma IgA2 level was within the normal range.

Conclusions: Patients with an $\alpha 1$ gene deletion can be considered as having partial IgAD. Using this method, we identified the second case of $\alpha 1$ gene deletion in Japan, and classified IgAD patients on the basis of $\alpha 1$ and $\alpha 2$ expression.

KEY WORDS

gene expression, IgA subclasses, partial IgA deficiency, selective IgA deficiency, $\alpha 1$ gene deletion

ABBREVIATIONS

IgA, immunoglobulin A; IgAD, immunoglobulin A deficiency; RT, reverse transcriptase; CVID, common variable immunodeficiency; TAC1, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; SD, standard deviation; PBMCs, peripheral blood mononuclear cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; BSA, bovine serum albumin; PBS, phosphate-buffered saline

INTRODUCTION

Human immunoglobulin A (IgA) is the most abundant immunoglobulin in secretions. IgA has 2 subclasses, IgA1 and IgA2. The ratios of IgA1 : IgA2 are approximately 9 : 1 in serum and 6 : 4 in saliva.¹ In mucosal tissue, IgA synthesis greatly exceeds that of other immunoglobulin classes. These 2 subclasses play important roles in the first line of defense, and the amount ratio of these molecules in secretions varies.

Selective IgA deficiency (IgAD) is the most common immunodeficiency, and has been found with a

frequency ranging from 0.03 to 0.3%. The prevalence differs according to ethnic groups, with a lower frequency in the Japanese population, namely, 1/18,000 people.^{2,3} Most IgAD patients remain healthy, but some suffer from a variety of infections, allergies, autoimmune disorders, gastrointestinal diseases, malignancies, endocrinopathies, neurological diseases, and genetic disorders.^{2,4} The pathogenesis of IgAD has not yet been completely clarified. IgAD has been found to be associated with IgG2, IgG4, and IgE deficiencies.⁵⁻⁸ The class switch disorder in IgA-producing B lymphocytes is one of the most important factors in IgAD patients.⁹ Asano *et al.* suggested

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Table 1 Immunological data of patients

Patient No.	Sex	Age	Serum level (mg/dl)			IgG subclass (mg/dl)			
			IgG	IgA	IgM	IgG1	IgG2	IgG3	IgG4
Selective IgA deficiency									
1	M	10 years	1363	< 5	146	619	255	57.3	33.9
2	F	11 years	1640	< 5	117	949	400	47.7	90.2
3	F	17 years	1261	< 5	137	630	625	35.1	18.4
Partial IgA deficiency									
4	M	4 years	1223	17	120	933	< 8.0	22.8	< 3.0
5	F	3 years	1644	15	150	878	47.9	17.3	32.1
6	F	3 years	869	27	106	306	69.3	27.6	3.8
7	M	4 years	887	45	101	295	97.0	40.0	4.4
8	M	4 years	1624	8	100	1090	120	74.7	< 3.0
Secondary IgA deficiency									
9	F	7 years	913	12	105	602	148	52.8	16.5
10	M	13 years	705	9	39	852	345	49.8	6.4

that decreased expression levels of α germline transcripts before a class switch may be the cause of selective IgAD, and B-cell differentiation might be disturbed after a class switch in partial IgAD patients.¹⁰ Husain *et al.* reported that the increased destruction of a subset of B cells is the cause for the inability to produce IgA in IgAD patients.¹¹

The association between IgAD and common variable immunodeficiency (CVID) has been discussed,³ and it was reported recently that some CVID and IgAD patients have mutations in TNFRSF13B (encoding TACI; transmembrane activator and calcium-modulator and cyclophilin ligand interactor).^{12,13}

The molecular weights of the IgA1 and IgA2 heavy chains are both approximately 53 kD. The $\alpha 1$ and $\alpha 2$ genes show about 97% of its identity.¹⁴ This high homology between them makes it difficult to analyze the $\alpha 1$ and $\alpha 2$ genes separately. The α -chain constant region of both $\alpha 1$ and $\alpha 2$ genes is encoded by 3 exons. The hinge region of the human α chain is encoded at the beginning of the second exon. The hinge region of the $\alpha 2$ gene shows a deletion of 39 nucleotides (corresponding to 13 amino acids) when compared with that of the $\alpha 1$ gene.¹⁵ To clarify the pathogenesis and immunological reactions of IgAD clear, we analyzed $\alpha 1$ and $\alpha 2$ gene expression in IgAD patients. In this study, we devised a new method to determine the expression levels of the $\alpha 1$ and $\alpha 2$ genes, and analyzed selective, partial and secondary IgAD patients.

METHODS

SUBJECTS

As shown in Table 1, we analyzed 3 selective IgAD patients (patients number 1, 2 and 3) with serum IgA levels below the detection limit (<5 mg/dl), 5 partial IgAD patients (patients number 4, 5, 6, 7, and 8) with serum IgA levels above 5 mg/dl but having more

than 2 standard deviations (SDs) below the normal level, and 2 secondary IgAD patients (patients number 9 and 10) whose conditions were caused by epileptic medication. Ten controls were also included in this study. Two of the 10 controls were child volunteers under 16 years of age, and the other 8 were adult volunteers 16 years or older. We obtained informed consent from the patients, controls, and parents of minors.

CELL PREPARATION

Peripheral blood mononuclear cells (PBMCs) were collected in heparin and separated by gradient centrifugation in Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).¹⁶ The cells were suspended at a density of 10^6 /ml and incubated for 24 hours in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l l-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin.¹⁰ In comparison with non-cultured cells, cells cultured for 24 hours showed higher levels of $\alpha 1$ and $\alpha 2$ gene expression (unpublished data).

cDNA SYNTHESIS AND PCR AMPLIFICATION

We extracted total RNA from PBMCs using an Iso-gen kit (Nippon Gene, Tokyo, Japan), and cDNA synthesis was carried out using 1 or 2 μ g of total RNA with oligo-dT and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). We used the following PCR primer pair, which was targeted to the common sequence area of the $\alpha 1$ and $\alpha 2$ genes: sense 5'-CCTGGTCACCGTCTCCTCA-3' (placed at the J exon; Gene Bank accession number-L20778) and antisense 5'-TCACGCTCAGGTGGTCTTG-3' (placed at the C α CH2 exon)¹⁷ (Fig. 1A). The PCR fragments included the CH1, hinge and CH2 regions, and the size was 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

Various Expression Patterns of α Genes in IgAD

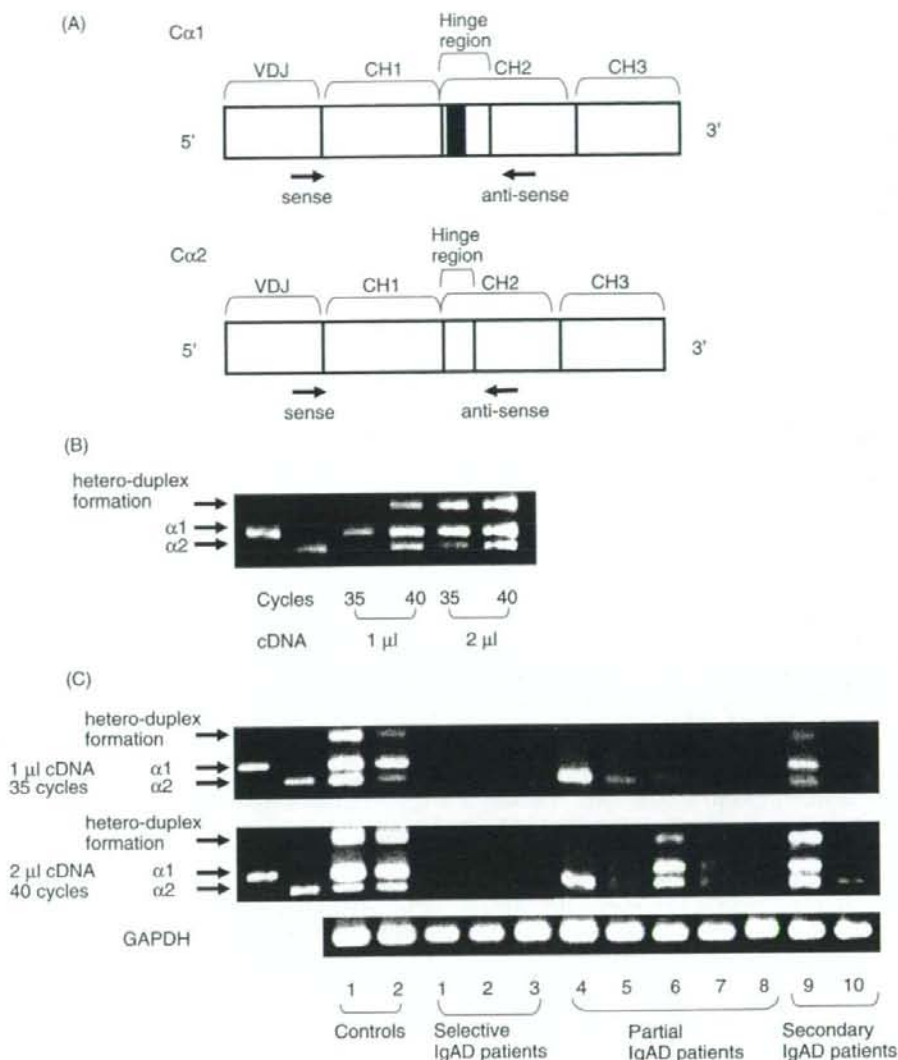


Fig. 1 Structure and expression of the $\alpha 1$ and $\alpha 2$ genes. (A) The primer pair was targeted to the common sequence area of the $\alpha 1$ and $\alpha 2$ genes. These figures show mature $C\alpha$ gene transcripts. The arrows indicate the positions of the primers. The black box indicates the deletion of 39 bases. VDJ: variable diversity joining region, CH: constant heavy chain. (B) RT-PCR analysis of RNA extracted from the PBMCs of a control subject, starting from 1 μ l or 2 μ l of cDNA and using 35 or 40 cycles. The two lanes at the left show PCR products amplified from the template DNA, which are T-vectors containing the IgA1 (lane 1) or IgA2 (lane 2) genes. (C) RT-PCR analysis of RNA extracted from the PBMCs of control subjects and IgAD patients. The $\alpha 1$ fragment, $\alpha 2$ fragment, and hetero-duplex formation are indicated by arrows. GAPDH was used as a control.

for 1 minute, 60°C for 1 minute, and 72°C for 1 minute using 1 or 2 μ l of cDNA. The PCR products were run on 4% agarose gels for 120 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a

control.

DNA EXTRACTION AND PCR AMPLIFICATION

Genomic DNA was purified from polymorphonuclear

cells using Sepa Gene (Sanko Junyaku, Tokyo, Japan). We used the following PCR primer pair, which was targeted at the common sequence area of the $\alpha 1$ and $\alpha 2$ genes: sense 5'-TGACCAGCTCAGGCCATCTCT-3' and antisense 5'-CTTTGCAAACAGAGCACTGA-3'. The PCR program was 40 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. Four percent agarose gel electrophoresis was performed for 120 minutes. The other primer pairs were as follows: for amplification of the *Ce* gene, sense 5'-A CCTTCAGCGGTAAGAGAGGG-3' and antisense 5'-T GGGAACGTACCTGCACACTT-3', (the PCR program was 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute); for amplification of the *Cy2* gene, sense 5'-ATCTCTTCTCAGCACCACCT-3' and antisense 5'-CGTGGCACTCATTTACCCGGA-3', (the PCR program was 35 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute); for the *Cp* gene, sense 5'-ATGTGTGTCCCCGGTGAGTGA-3' and antisense 5'-AGGGCCACCCCTGTGAACAGA-3', (the PCR program was 35 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute).

QUANTIFICATION OF IgA SUBCLASSES IN PLASMA

The levels of the IgA subclasses in plasma were measured by enzyme-linked immunosorbent assay (ELISA). For IgA1, coating was performed with a mouse monoclonal anti-IgA1 (NI69-11), and detection of IgA1 was performed with horseradish peroxidase (HRP)-labeled goat anti-human IgA (Cappel, Organon Teknika, Turnhout, Belgium).¹⁸ For IgA2, coating was performed using goat anti-human IgA (BETHYL, Montgomery, TX, USA), and detection of IgA2 was performed using mouse anti-human IgA2-HRP (B3506B4).¹⁹ ELISA plates were coated overnight at 4°C with mouse monoclonal anti-IgA1 (diluted to 1 : 200 with 0.05 M sodium carbonate, pH 9.6) or goat anti-human IgA (diluted to 1 : 100 with 0.05 M sodium carbonate, pH 9.6). The plates were washed then incubated with standard serum and plasma dilutions. IgA1 was detected using goat anti-human IgA-HRP (diluted to 1 : 10000 with 1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]-0.02% Tween 20), and IgA2 was detected using mouse anti-human IgA2-HRP (diluted to 1 : 1000 with 1% BSA in PBS-0.02% Tween 20). The samples were tested repeatedly. The lower limits of IgA1 and IgA2 detection were 5 ng/ml and 1 µg/ml, respectively.

RESULTS

PCR AMPLIFICATION OF $\alpha 1$ AND $\alpha 2$ GENE EXPRESSION

RT-PCR analysis was performed using primer pairs that amplified both $\alpha 1$ and $\alpha 2$ mRNAs and could distinguish $\alpha 2$ mRNA from $\alpha 1$ mRNA taking advantage of the deletion of 39 bases in the hinge region of the $\alpha 2$ gene. Various PCR conditions were tested, and

the optimal conditions were deemed to be 2 µl of cDNA and 40 cycles of amplification (Fig. 1B). Control samples gave an intense $\alpha 1$ band and a less-intense, shorter $\alpha 2$ band in all 4 PCR conditions. Another band with less electrophoretic mobility than the $\alpha 1$ band was determined to be a hetero-duplex formation of the $\alpha 1$ and $\alpha 2$ fragments, because the subcloning of this band yielded clones of the $\alpha 1$ and $\alpha 2$ fragments. Figure 1C shows $\alpha 1$ and $\alpha 2$ gene expression in 10 IgAD patients. In 3 selective IgAD patients (patients number 1, 2 and 3), no expression of the $\alpha 1$ and $\alpha 2$ genes was detected. Three partial IgAD patients (patients number 5, 6 and 7) and 2 secondary IgAD patients (patients number 9 and 10) showed $\alpha 2$ and $\alpha 1$ gene expressions; however, patient No. 4 showed only $\alpha 2$ gene expression, but no $\alpha 1$ gene expression. One partial IgAD patient (No. 8) showed no bands in this RT-PCR analysis.

PLASMA IgA LEVELS IN CONTROLS AND IgAD PATIENTS

Plasma IgA1 and IgA2 levels were assayed separately (Table 2). The IgA1 levels were much higher than the IgA2 levels in the 10 controls. In the 3 selective IgAD patients, plasma IgA1 and IgA2 levels were very low. One partial IgAD patient (patient No. 4) had a normal IgA2 level, but no detectable IgA1. This finding is in accordance with the result of this patient showing no $\alpha 1$ gene expression in PBMCs. Although the IgA2 level of patient No. 5 was below the threshold, other patients with partial IgAD (patients number 6, 7, 8) showed various IgA1 and IgA2 levels although these levels were much lower than that of the control group.

PCR AMPLIFICATION OF THE IMMUNOGLOBULIN GENES OF IgAD PATIENTS

In patient No. 4, $\alpha 1$ gene expression was not detected and no IgA1 protein was detected in his plasma. As shown in Table 1, the plasma levels of the IgG subclasses showed that IgG2 and IgG4 levels were below the detection limits. Hence, we carried out PCR amplification of the genomic $\alpha 2$ and $\alpha 1$ genes, together with the μ , $\gamma 2$ and ϵ genes. As shown in Figure 2, no PCR products were detected for the $\alpha 1$, $\gamma 2$ and ϵ genes in patient No. 4, whereas they were clearly detected in 2 control and other IgAD patients. A large genomic deletion of the A1-GP-G2-G4-E genes can be proposed as the molecular basis of IgAD in patient No. 4.

LONGITUDINAL CHANGE IN THE SERUM IgA LEVEL OF PATIENT NO. 4

The immunological data of 2 families of patients with IgG2-IgG4-IgA1-IgE deficiency, including patient No. 4, are shown in Table 3. The serum IgA levels of patient No. 4 over time are shown in Table 4. The patient's IgA levels remained at a level more than 2 SDs

Table 2 The levels of IgA subclasses and IgA1/IgA2 ratios in plasma as measured by ELISA

Patient No.	Serum IgA levels (mg/dl)	Plasma IgA1 and IgA2 levels (mg/dl)		IgA1/IgA2 ratio	mRNA expression		Genes		
		IgA1	IgA2		$\alpha 1$	$\alpha 2$	$\alpha 1$	$\alpha 2$	
Selective IgA deficiency									
1	< 5	2.48 \pm 0.26	ND [†]	NC [‡]	- [§]	-	+ [¶]	+	
2	< 5	ND	ND	NC	-	-	+	+	
3	< 5	2.16 \pm 0.74	ND	NC	-	-	+	+	
Partial IgA deficiency									
4	17	ND	13.67 \pm 1.39	NC	-	+	-	+	
5	15	2.71 \pm 0.36	ND	NC	+	+	+	+	
6	27	8.28 \pm 0.52	0.91 \pm 0.25	8.40	+	+	+	+	
7	45	29.29 \pm 6.83	4.88 \pm 0.54	6.97	+	+	+	+	
8	8	6.28 \pm 0.67	0.86 \pm 0.45	8.87	-	-	+	+	
Secondary IgA deficiency									
9	12	8.59 \pm 1.77	1.17 \pm 0.36	6.56	+	+	+	+	
10	9	0.56 \pm 0.09	0.18 \pm 0.03	3.03	+	+	+	+	
Controls (n = 10)		128.02 \pm 36.98	19.39 \pm 8.93	7.96 \pm 4.14					

[†] ND : undetected, [‡] NC : not calculated.

[§] + : detected, [¶] - : undetected.

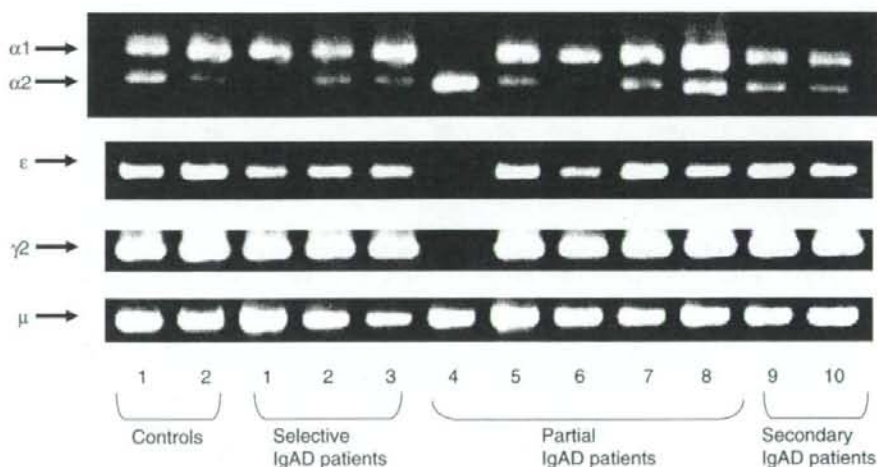


Fig. 2 PCR analysis of immunoglobulin genes in IgAD patients. PCR analysis of $\alpha 1$, $\alpha 2$, ϵ , $\gamma 2$ and μ genes was performed in control subjects and IgAD patients. Two different sized bands for the α gene appeared, corresponding to the $\alpha 1$ and $\alpha 2$ genes, as indicated by arrows.

below the normal value, but above 5 mg/dl. The immunological data of his family members are shown in Family 1 (Table 3). The patient's older brother was also found to have the same gene deletion. His serum IgA level was 21 mg/dl, and he also showed a pattern of partial IgAD. The serum IgA levels of another patient with IgG2-IgG4-IgA1-IgE deficiency and her family members are shown as Family 2 (Table 3).⁸ Both the patient and her older sister had the same deletions of the A1-GP-G2-G4-E genes. The serum IgA level of the patient's sister was 18 mg/dl and she

also showed a pattern of partial IgAD.

DISCUSSION

The serum IgA subclass levels of IgAD patients, particularly selective IgAD patients, are very difficult to measure by ELISA; thus, there have been few reports on the levels of serum IgA subclasses in IgAD patients. The $\alpha 1$ and $\alpha 2$ gene expression levels are low in most IgAD patients, and there have been no previous reports on the gene expression levels of the IgA subclasses in these patients.²⁰⁻²³ Hummelshoj *et al.*

Table 3 Immunological data of patient no. 4 and 2 families of patients with IgG2-IgG4-IgA1-IgE deficiency

Family 1							
	Age	Serum level					
		mg/dl					IU/ml
		IgA	IgG1	IgG2	IgG3	IgG4	IgE
Patient No. 4	4	17	933	< 8.0	22.8	< 3.0	< 2.0
Father	45	119	659	451	58.0	19.0	70.0
Mother	41	201	730	224	25.4	14.2	190.0
Brother	11	21	952	< 8.0	23.3	< 3.0	< 2.0
Family 2							
	Age	Serum level					
		mg/dl					IU/ml
		IgA	IgG1	IgG2	IgG3	IgG4	IgE
Patient	1	< 5	996	< 8.0	10.4	< 3.0	< 5.0
Father	27	161	720	281	13.7	14.1	214.8
Mother	26	180	794	248	16.7	8.2	4.2
Sister	3	18	645	< 8.0	15.1	< 3.0	< 5.0

Table 4 Longitudinal change in the serum IgA levels of patient no. 4

	20m	25m	30m	38m	56m	57m
Serum IgA level (mg/dl)	11	12	11	11	20	17
Normal value [†]	1year	2y	3y	4y		
Mean (\pm 2SD)	49 (16–128)	60 (20–149)	74 (25–174)	89 (31–202)		

[†] data on normal values according to reference 28.

reported on the expression of germline transcripts of the $\alpha 1$ and $\alpha 2$ genes in IgAD patients.²⁴ The germline transcripts were induced during stimulation with TGF- β , however their levels were lower than those of control subjects. Our report shows the expression of mature transcripts of the $\alpha 1$ and $\alpha 2$ genes in partial IgAD patients. In the 3 selective IgAD patients, no expression of $\alpha 1$ and $\alpha 2$ genes was detected, presumably because the expression level was too low. In 2 of them, only plasma IgA1 levels could be measured, and in 1 of them, neither IgA1 nor IgA2 levels were unable to be measured. Patient No. 8 showed no mRNA expression of IgA subclasses, but exhibited detectable levels of plasma IgA1 and IgA2 levels. It might be assumed that the pathogenesis of partial IgAD in patient No. 8 is similar to that in selective IgAD patients. As shown in Table 2, patients number 1, 2, 3 and 8 showed no $\alpha 1$ or $\alpha 2$ gene expression, while patients number 5, 6, 7, 9 and 10 showed both $\alpha 1$ and $\alpha 2$ gene expression. There is a possibility that the mechanism underlying IgA deficiency may be different between these 2 groups. The plasma IgA2 levels were unable to be measured in 1 partial IgAD patient; however, $\alpha 2$ gene expression was seen in this patient using our method. This method is effective for determining whether partial IgAD patients lack ex-

pression of the $\alpha 1$ and $\alpha 2$ genes when their plasma IgA, IgA1 and IgA2 levels are under the detection limit.

Asano *et al.* reported detectable levels of the $C\alpha$ mature transcripts by RT-PCR using the same sense primer we employed and an antisense primer located in the $C\alpha$ CH1 region.¹⁰ Using our method, no $\alpha 1$ or $\alpha 2$ gene expression was detected in 3 selective IgAD patients. There was a difference in the annealing temperatures used (59°C in the study of Asano *et al.*), and different PCR conditions may result due to changes in the sensitivity of the assay.

IgAD patients are often associated with IgG subclass deficiency. In our study, 2 IgAD patients (patients number 4 and 8) had IgG subclass deficiency in the serum. Patient No. 4 showed deletions of the $\gamma 2$ and ϵ genes, and was considered to have a large genomic deletion of the $C\alpha 1$, $\Psi C\gamma$, $C\gamma 2$, $C\gamma 4$ and $C\epsilon$ genes, showing the same observations reported previously in a patient.⁸ In Japan, only 1 case has ever been reported. Beard *et al.*²⁵ reported that IgA-IgG2-IgG4 deficiency occurs in 4% of 73 IgAD patients. Deletions of the A1-GP-G2-G4-E genes are the most common deletions of the Ig heavy chain locus.²⁶ There could be more patients with an $\alpha 1$ gene deletion who might have partial IgAD. Partial IgAD is

often transient,²⁷ however, patients who have a deletion of the α gene will not show transient IgAD and will exhibit continuous partial IgAD.

We also classified the IgAD patients on the basis of the expression of $\alpha 1$ and $\alpha 2$ genes. Selective IgAD patients showed no $\alpha 1$ or $\alpha 2$ mRNA expression and extremely low protein levels, while secondary IgAD patients showed both mRNA and protein expressions of IgA subclasses. Partial IgAD can be classified into 3 patterns as follows: cases showing reduced levels of $\alpha 1$ and $\alpha 2$ gene expression; cases showing no $\alpha 1$ gene expression but normal $\alpha 2$ gene expression; and cases showing no $\alpha 1$ or $\alpha 2$ gene expression.

It is sometimes difficult to determine the pathogenic mechanisms operative in IgAD patients on the basis of serum IgA levels. Variations in the pathogenesis of IgAD patients might become better understood by examining the protein and mRNA expression levels of IgA subclasses.

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Genetic alterations in medulloblastoma from mice with defective DNA double strand break repair.

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Abstract

Inactivation of homologous recombination (HR) or non-homologous end-joining (NHEJ) predisposes to a spectrum of tumor types. Here, we have inactivated DNA double-strand break repair (DSBR) proteins, DNA Ligase IV (Lig4), Xrcc2 and Brca2 or combined Lig4/Xrcc2 during neural development using Nestin-cre. In all cases, inactivation of these repair factors, together with p53 loss, lead to rapid medulloblastoma formation. Genomic analysis of these tumors showed recurring chromosomal 13 alterations via chromosomal loss or translocations involving regions containing *Ptch1*. Sequence analysis of the remaining *Ptch1* allele showed a variety of inactivating mutations in all tumors analyzed, highlighting the critical tumor suppressor function of this hedgehog-signaling regulator. We also observed genomic amplification or upregulation of either N-Myc or cyclin D2 in all medulloblastomas. Additionally, Pten was also selectively targeted in tumors arising after disruption of HR. Thus, our data illustrate the central requirement for *Ptch1* tumor suppressor activity in preventing medulloblastoma and reveal other fundamental events required for cerebellar granule cell transformation.

Introduction.

Repair of DNA double strand breaks (DSBs) can occur via non-homologous end joining (NHEJ) or homologous recombination (HR). A distinguishing feature between these pathways is the requirement of HR for a sister chromatid present in the S/G2 phase of replicating cells to provide an error-free template for DNA repair (1, 2). Multiple factors coordinate HR and amongst these, Xrcc2 and Brca2 are critical (3, 4). In contrast to HR, NHEJ is an error-prone repair mechanism that enzymatically modifies the two ends of a DNA break so that they are compatible for direct ligation (5-8). Of the factors important for NHEJ, a DNA ligase IV (Lig4)/Xrcc4 complex facilitates ligation of broken non-compatible DNA ends. During development of the central nervous system (CNS), HR is required for prevention of genomic instability in proliferative progenitor cells while NHEJ molecules are critical in post-mitotic neurons to maintain genomic integrity (9), and their inactivation can perturb development of the nervous system, leading to neurodegeneration, microcephaly or brain tumors (10).

Medulloblastoma is the most common malignant pediatric brain tumor, and often arises from the granule neuron progenitors (GNPs) in the external germinal layer (EGL) of the developing cerebellum (11, 12), and many medulloblastoma models have a very similar gene expression profile to GNPs (13-17). Several human syndromes, such as Turcot (*APC* germline mutation), Gorlin (*PTCH1* germline mutation), Fanconi anemia complementation group D1 (*BRCA2* germline mutation) or Nijmegen Breakage (*NBS1* hypomorphic mutation), predispose to medulloblastoma (12, 18, 19). Additionally, mutations of other genes involved in the sonic hedgehog (SHH) pathway such as *SUFU*, *SMOH* or the Wingless (*WNT*) pathway such as *AXIN1* or β -*CATENIN* have also been found in sporadic human medulloblastomas, highlighting the importance of these pathways for preventing cancer (20). Mutations of the p53 pathway also occur in many sporadic medulloblastomas (14, 21, 22).

Consistent with the above, 10%-15% of *Ptch1* heterozygote mice develop medulloblastoma by 8 months of age, an event that is associated with inactivation of the remaining *Ptch1* allele (23-25). Loss of *p53* or *Ptch2* significantly increases the incidence of medulloblastoma in *Ptch1*^{+/-} mice (24, 26). Although *p53*-null mice are not predisposed to develop medulloblastoma (27, 28), *p53* loss is a prerequisite in many models of medulloblastoma (15-17, 26, 29-31). Similar radiation susceptibility in *Ptch1*^{+/-} mice suggests that DNA damage in the cerebellum strongly predisposes to tumorigenesis (32). Inactivation of proteins involved in NHEJ such as *Lig4*, *Xrcc4* and *Ku80* or HR such as *Xrcc2* or *Brca2* also leads to medulloblastoma formation in a *p53*-deficient background (9, 30, 31, 33, 34).

In order to further examine the genesis of medulloblastoma, we used neural-specific inactivation of conditional mutants for DNA DSB repair with associated *p53* inactivation. Remarkably, we found that *Ptch1* was specifically lost in all DNA repair-deficient medulloblastomas, and this involvement was also associated with limited other cytogenetic rearrangements that also underpinned these tumors. Therefore, *Ptch1* tumor suppressor activity is uniquely required to prevent transformation in cerebellar granule neurons, perhaps by restraining replication stress associated with SHH signaling.

Results.

Medulloblastoma in DSBR deficient mice.

While germline inactivation of *DNA ligase IV (Lig4)*, *Xrcc4* or *Xrcc2* is lethal at mid or early gestation, coincident inactivation of *p53* rescues lethality, and promotes a spectrum of tumors by ten weeks of age including lymphoma and medulloblastoma (9, 14, 35, 36). In order to carefully evaluate medulloblastoma exclusive of lymphoma, we generated mice carrying