Table 7. Odds Ratio (OR) for Having Recurrent Wheezing (RW) at 14 Months of Age According to in Univariate Logistic Regression Analysis of Markers in Cord Blood and at 6 Months

	RW 14M (–)		RW 14M (+)		Total				95% CI for OR	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	*P* Value	OR	Lower	Upper
Cord blood										
WBC	12.31	(3.51)	12.11	(4.90)	12.30	(3.58)	.884	0.984	0.793	1.221
Eosinophils	323.6	(183.8)	282.8	(129.0)	321.2	(180.8)	.561	0.999	0.994	1.003
Basophils	75.00	(57.36)	106.5	(77.06)	76.87	(58.77)	.183	1.007	0.997	1.016
IgE	0.57	(0.78)	0.23	(0.12)	0.55	(0.76)	.122	0.082	0.003	1.953
T_H1%	0.25	(0.26)	0.34	(0.38)	0.26	(0.27)	.366	2.752	0.307	24.698
T_H2%	0.19	(0.23)	0.15	(0.20)	0.19	(0.23)	.617	0.376	0.008	17.435
T_C1%	0.58	(0.70)	0.47	(0.61)	0.58	(0.69)	.649	0.751	0.218	2.584
T_C2%	0.02	(0.04)	0.11	(0.25)	0.03	(0.07)	.061	516.318	0.742	359457
CD3%	52.24	(15.30)	53.55	(14.12)	52.33	(15.17)	.801	1.006	0.961	1.052
CD19%	9.33	(5.59)	10.26	(5.15)	9.39	(5.55)	.627	1.028	0.921	1.147
CD4%	40.88	(12.26)	38.84	(9.49)	40.74	(12.08)	.625	0.986	0.931	1.044
$CD8^{bright}$%	11.97	(5.08)	10.74	(4.00)	11.89	(5.00)	.476	0.946	0.813	1.102
$CD56^+CD16^+$%	9.67	(5.54)	10.27	(5.80)	9.72	(5.54)	.755	1.019	0.905	1.148
$CD56^+CD16^-$%	1.66	(1.20)	1.28	(0.59)	1.63	(1.17)	.344	0.662	0.282	1.557
$CD4^+CD25^+$%	11.65	(2.87)	12.39	(3.07)	11.70	(2.88)	.461	1.086	0.872	1.351
IFN-γ (PHA)	579.2	(875.6)	1056.7	(2023.3)	609.5	(980.1)	.204	1.000	1.000	1.001
IL-4 (PHA)	5.62	(4.49)	8.96	(7.19)	5.84	(4.76)	.053	1.117	0.999	1.249
Six months of age										
WBC	10.89	(3.16)	10.47	(2.47)	10.86	(3.11)	.696	0.955	0.759	1.202
Eosinophils	407.9	(554.0)	358.7	(309.8)	404.7	(541.1)	.793	1.000	0.998	1.002
Basophils	57.50	(30.43)	46.51	(20.20)	56.79	(29.94)	.270	0.982	0.951	1.014
IgE	21.07	(36.39)	11.11	(11.79)	20.43	(35.39)	.439	0.984	0.945	1.025
T_H1%	2.06	(1.07)	2.31	(1.42)	2.08	(1.09)	.505	1.215	0.685	2.157
T_H2%	0.34	(0.21)	0.40	(0.18)	0.34	(0.21)	.434	3.192	0.175	58.318
T_C1%	7.06	(8.19)	7.42	(7.58)	7.08	(8.12)	.897	1.005	0.927	1.090
T_C2%	0.05	(0.17)	0.02	(0.03)	0.05	(0.16)	.614	0.033	0.000	19784.957
CD3%	61.60	(13.08)	63.76	(10.09)	61.74	(12.89)	.625	1.014	0.958	1.074
CD19%	14.42	(9.18)	18.13	(9.66)	14.65	(9.22)	.247	1.041	0.972	1.115
CD4%	44.02	(10.53)	43.98	(10.09)	44.02	(10.47)	.992	1.000	0.937	1.067
$CD8^{bright}$%	13.49	(4.93)	14.85	(6.41)	13.58	(5.02)	.433	1.050	0.930	1.186
$CD56^+CD16^+$%	6.95	(4.55)	4.17	(3.45)	6.77	(4.53)	.075	0.779	0.592	1.025
$CD56^+CD16^-$%	0.95	(1.08)	1.05	(0.78)	0.96	(1.06)	.797	1.067	0.651	1.747
$CD4^+CD25^+$%	10.41	(2.21)	10.12	(2.52)	10.39	(2.22)	.712	0.943	0.691	1.287
IFN-γ (PHA)	2981.3	(3381.0)	2536.0	(3368.9)	2951.8	(3369.5)	.701	1.000	1.000	1.000
IL-4 (PHA)	12.73	(10.61)	21.33	(19.46)	13.28	(11.48)	.045	1.043	1.001	1.086

Abbreviations: AD, atopic dermatitis; IFN, interferon; Ig, immunoglobulin; IL, interleukin; PHA, phytohemagglutinin; WBC, white blood cells.

Table 8. Odds Ratio (OR) for Having Recurrent Wheezing (RW) at 14 Months of Age According to Univariate Logistic Regression Analysis of Markers at 6 Months

		RW 14M (–)		RW 14M (+)				95% CI for OR	
			(n/total)		(n/total)	*P* Value	OR	Lower	Upper
Six months of age									
Egg white	≥ class 1	40.46%	(53/131)	55.56%	(5/9)	.380	1.840	0.472	7.170
Cow's milk	≥ class 1	12.98%	(17/131)	0%	(0/9)	.998	0.000	–	–
Wheat	≥ class 1	8.60%	(8/93)	12.50%	(1/8)	.712	1.518	0.165	13.935
Soy	≥ class 1	0.76%	(1/131)	0%	(0/9)	1.000	0.000	–	–
Dog dander	≥ class 1	9.92%	(13/131)	0%	(0/9)	.999	0.000	–	–
Cat dander	≥ class 1	2.29%	(3/131)	0%	(0/9)	.999	0.000	–	–
Df	≥ class 1	2.29%	(3/131)	0%	(0/9)	.999	0.000	–	–

Abbreviations: Df, *Dermatophagoides farinae*.

the 14MRW(–) group as a reference, the risk of becoming 14MRW(+) increased significantly in PHA-induced IL-4 production from PBMCs at 6 months of age (Table 7). Specific IgE analyses at 6 months of age revealed no factor to be significant (Table 8).

Since only 1 factor was significant in the univariate logistic regression, stepwise multivariate logistic regression analysis was not performed.

A ROC curve analysis performed to evaluate the predictability of the PHA-induced IL-4 production from PBMCs at 6 months of age for distinguishing 14MRW(+) from 14MRW(–) revealed the AUC of PHA-induced IL-4 production from PBMCs at 6 months of age to be 0.629 (95% CI, 0.419-0.839; *P*=.196). Hence, higher PHA-induced IL-4 production from PBMCs at 6 months of age is a risk factor—but not a predictive factor—for RW at 14 months of age.

Discussion

We are conducting the GAICS to understand the genetic and environmental factors that affect sensitization to allergens and the development of allergic disorders. We are also investigating which laboratory data are useful for predicting the development of allergic diseases. Although several birth cohort studies have been performed in Europe and United States, the number of birth cohort studies in Asia remains low. Our cohort study is one of the few such studies in an Asian community.

We focused on the relationship between infantile AD/RW and laboratory data (values for IgE, WBC, eosinophils, basophils, and lymphocyte subpopulations, as well as cytokine production in CBMCs or PBMCs). Several new findings in allergic disease have been recorded. The T_H1/T_H2 paradigm provides a useful model for understanding the pathogenesis of allergic diseases, and the immunosuppressive function of the Treg subset is of interest. We also analyzed subpopulations of helper T cells (T_H1/T_H2 or Tregs) in cord blood and peripheral blood at 6 and 14 months of age.

Several lines of research suggest that the prevalence of AD in infancy is about 15% to 20%, although this value varies according to the study. We showed that the prevalence of AD at 6 months and 14 months of age was 24.7% and 19.1%, respectively. Moore et al [18] found the prevalence of AD in first 6 months in the United States to be 17.1%. Dunlop et al [19] reported that the prevalence of AD in 14-year-old Slovak children was 15.6%. Benn et al [20] reported that the prevalence of AD in children aged 18 months in Denmark was 11.5%. Each study was performed in children of different ages and different races in different areas using different diagnostic methods. Our diagnosis was based on physical examination by well-trained pediatric allergists, while other research was based on questionnaires [18,19] or data obtained by a trained interviewer [20]. Our research results are highly representative of the prevalence of AD in Asian infants.

We also investigated the prevalence of RW in infancy. Our study showed that the respective prevalence of RW at 6 months and 14 months of age was 0% and 7.6%. Other reports showed higher values. Henderson et al [21] reported that the prevalence of RW at 6 months and 7-18 months of age in the UK was 8.8% and 15.2%, respectively. Visser et al [22] reported that the prevalence of RW in the first year of life among Dutch infants was 14.5%. However, Chulada et al [23] reported that the prevalence of RW in the USA was 7.6%. Our results were similar to those of Chulada et al and provide important information on the prevalence of RW in Asian infants.

We showed that higher cord blood IgE is significantly associated with AD at 6 months of age (aOR, 1.607). However, ROC curve analysis revealed that AD is not a good predictor of AD at 6 months of age. We previously reported that cord blood IgE level is not a good predictor of infant AD [9]. Other authors [24,25] reported that cord blood IgE cannot be recommended as a screening instrument for primary prevention. The results of our study are consistent with these findings. The predictability of allergic diseases using cord blood IgE has received much attention. However, our latest finding is that cord blood IgE is the only risk factor out of 16 cord blood immunological markers, including relatively new markers such as T_H1, T_H2, T_C1, T_C2, and $CD4^+CD25^+$ cells. These data indicate that cord blood IgE is the best marker of 16 cord blood immunological markers but not an ideal predictor for AD at 6 months of age.

Our study also showed that high total IgE and egg white–specific IgE at 6 months of age were significantly associated with AD at 14 months of age and that the aOR was 1.018 and 23.246, respectively. Moreover, ROC curve analysis showed AD to be a good predictor of AD at 14 months of age. Other authors did not find similar results at this age. Perkin et al [26] reported that total IgE at 12 months was a predictor of eczema at age 5; this finding is similar to ours for total IgE, except for the difference in age. As for sensitization to hen egg, Nickel et al [27] reported that hen egg–specific IgE at 12 months is a valuable marker for subsequent allergic sensitization. Although the outcome of these authors was sensitization to common indoor and outdoor allergens at the age of 3 years, which is different from the outcome we observed, the results are nonetheless similar to ours. We found that high total IgE and sensitization to hen egg are risk factors and predictive factors of AD at 14 months of age. These findings are useful for clinical practice.

In contrast to the importance of IgE in AD, total IgE and specific IgE were not risk factors for RW at 14 months of age. Our results show that high IL-4 production was a risk factor for RW at 14 months of age. IL-4 is a T_H2 cytokine that induces isotype class switching to IgE in human B cells [28,29]. Whether participants with RW at 14 months of age show a subsequent increase in IgE or not will be analyzed in future studies. Although PHA-induced production of IL-4 from PBMCs was a risk factor for RW in our study, the percentage of T_H2 was not, indicating that, although cell count does not change drastically, the ability to produce IL-4 does change in children with RW.

Our cohort is relatively smaller than other birth cohorts. While most studies were based on questionnaires or interviews by nurses, our participants underwent a physical examination by well-trained pediatric allergists, as well as blood tests at 6 and 14 months of age. This makes our study design quite unique. Moreover, we tested new markers of the T_H1/T_H2 paradigm, which includes the T_H1/T_H2 subset, Treg, and $T_H1/$

T_H2 cytokine production. These new markers have not been analyzed in other cohort studies. Our findings provide useful information on allergic disease in infants and on the use of markers in clinical practice.

Total and specific IgE are important markers for AD in infants. In contrast, total and specific IgE are not important markers for RW.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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Norio Kawamoto, MD, PhD

Department of Pediatrics
Graduate School of Medicine, Gifu University
1-1 Yanagido, Gifu 501-1194, Japan
E-mail: noriok-gif@umin.ac.jp

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Characterization of *NLRP3* Variants in Japanese Cryopyrin-Associated Periodic Syndrome Patients

Hidenori Ohnishi · Takahide Teramoto · Hiroaki Iwata · Zenichiro Kato · Takeshi Kimura · Kazuo Kubota · Ryuta Nishikomori · Hideo Kaneko · Mariko Seishima · Naomi Kondo

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Abstract The etiology of cryopyrin-associated periodic syndrome (CAPS) is caused by germline gene mutations in NOD-like receptor family, pryin domain containing 3 (*NLRP3*)/cold-induced autoinflammatory syndrome 1 (*CIAS1*). CAPS includes diseases with various severities. The aim of this study was to characterize patients according to the disease severity of CAPS. Five Japanese patients with four kinds of gene variations in *NLRP3* were found and diagnosed as CAPS or juvenile idiopathic arthritis. Two mutations in *NLRP3*, Y563N and E688K, found in CAPS patients exhibit significant positive activities in the nuclear factor-κB reporter gene assay. Increased serum interleukin (IL)-18 levels were only observed in severe cases of CAPS. In mild cases of CAPS, the serum IL-18 levels were not increased, although lipopolysaccharide- or hypothermia-enhanced IL-1β and IL-18 production levels by their peripheral blood mononuclear cells were detectable. This series of case reports suggests that a combination of in vitro assays could be a useful tool for the diagnosis and characterization of the disease severity of CAPS.

Keywords Autoinflammatory disease · cryopyrin · familial cold autoinflammatory syndrome · interleukin-18 · *NLRP3*

Abbreviations

CAPS	Cryopyrin-associated periodic syndrome
CIAS1	Cold-induced autoinflammatory syndrome 1
CINCA	Chronic infantile neurologic cutaneous and articular
CRP	C-reactive protein
FCAS	Familial cold autoinflammatory syndrome
HEK	Human embryonic kidney
IL	Interleukin
JIA	Juvenile idiopathic arthritis
LPS	Lipopolysaccharide
MWS	Muckle–Wells syndrome
NLRP3	NOD-like receptor family, pryin domain containing 3
NF-κB	Nuclear factor-κB
NOMID	Neonatal-onset multisystem inflammatory disease
PBMCs	Peripheral blood mononuclear cells
TNF	Tumor necrosis factor

Introduction

Cryopyrin-associated periodic syndrome (CAPS) is an autoinflammatory syndrome [1] caused by germline gene mutations in NOD-like receptor family, pryin domain containing 3 (*NLRP3*)/cold-induced autoinflammatory syndrome 1 (*CIAS1*) [2–4]. The diagnosis of CAPS is based on its

H. Ohnishi (✉) · T. Teramoto · Z. Kato · T. Kimura · K. Kubota · H. Kaneko · N. Kondo
Department of Pediatrics, Graduate School of Medicine, Gifu University,
1-1 Yanagido,
Gifu 501-1194, Japan
e-mail: ohnishih@gifu-u.ac.jp

H. Iwata · M. Seishima
Department of Dermatology, Graduate School of Medicine, Gifu University,
Gifu, Japan

R. Nishikomori
Department of Pediatrics, Graduate School of Medicine, Kyoto University,
Kyoto, Japan

H. Kaneko
Department of Clinical Research, Nagara Medical Center,
Gifu, Japan

characteristic clinical phenotypes and examination of gene mutations in *NLRP3*. A hotspot of gene mutations in *NLRP3* is located on exon 3. On the other hand, approximately 40% of cases with the clinically diagnosed severe form of CAPS, chronic infantile neurologic cutaneous and articular (CINCA)/neonatal-onset multisystem inflammatory disease (NOMID) syndrome, have no detectable germline gene mutations in *NLRP3* [5, 6]. Some of these patients have gene mutations in *NLRP3* outside of exon 3, *NLRP12*, or somatic mosaicism of *NLRP3* [5, 7–10]. In some of the remaining typical CAPS patients, the disease-causing mutations cannot be confirmed. Thus, the clinical phenotypes are very important for diagnosing CAPS patients.

Familial cold autoinflammatory syndrome (FCAS) shows the mildest clinical phenotypes in the spectrum of CAPS, such as cold-induced urticaria-like skin rash, while CINCA/NOMID syndrome shows additional severe phenotypes, such as severe arthritis, patella overgrowth, aseptic meningitis, mental retardation, and progressive sensory neural hearing loss [1]. The diagnosis of FCAS is relatively difficult owing to its mild phenotypes compared with the more severe phenotypes of CAPS (CINCA/NOMID syndrome or Muckle–Wells syndrome (MWS)). On the other hand, and similar to other autoinflammatory syndromes such as familial Mediterranean fever, it is important for CAPS treatment to prevent the onset of renal amyloidosis for consideration of the prognosis. Interleukin (IL)-1β inhibitory drugs, such as anakinra, rilonacept, and canakinumab, can prevent the clinical phenotypes of CAPS including renal amyloidosis [11]. However, the usage of IL-1 blockade for the severe form of CAPS may sometimes be an overtreatment for FCAS because the clinical symptoms are relatively mild and the frequency of onset of renal amyloidosis was reported to be low in FCAS patients [11]. Therefore, precise evaluation of the disease severity of CAPS may contribute to a reduction in the usage of IL-1 blockade. Consequently, a convenient objective standard is anticipated for discrimination between the mild and severe forms of CAPS.

In this study, to diagnose CAPS and characterize the differences between the mild and severe forms of CAPS, we evaluated the serum inflammatory cytokine levels, cytokine production levels by peripheral blood mononuclear cells (PBMCs), and cell-based nuclear factor (NF)-κB reporter gene activities of *NLRP3* variants in patients. Our results provide new insights into the characterization of the severity of CAPS.

Methods

Case Reports

The five clinical cases evaluated in this study are described below, and their characteristics are summarized in Table I.

Table I Genotypes and clinical profiles of the patients

	Analyzed age	Onset age	Gender	Diagnosis	Genotype (*NLRP3*)	CNS		Skin	Joint	Others		The inflammatory markers	
						Meningitis	Mental retardation	Urticaria like rash	Arthritis	Hearing loss	Renal amyloidosis	WBC (/μl)	CRP (mg/dl)
Case 1	3 months	3 months	Female	FCAS	Y563N	−	−	+	−	−	−	14,890	0.48
Case 2	34 years	Unknown	Male	FCAS	Y563N	−	−	+	+	−	−	13,120	3.05
Case 3	14 years	11 months	Male	CINCA/NOMID	E688K, G809S	+	+	+	+	−	−	22,500	12.30
Case 4	45 years	Unknown	Female	MWS	E688K	−	−	+	+	+	−	13,640	4.17
Case 5	3 years	3 years	Female	JIA	E378K	−	−	+	+	−	−	15,200	11.69

CNS central nervous system, *FCAS* familiar cold inflammatory syndrome, *CINCA* chronic infantile neurologic cutaneous and articular syndrome, *MWS* Muckle–Wells syndrome, *JIA* juvenile idiopathic arthritis, *WBC* the count of white blood cells, *CRP* the serum C-reactive protein level

All of the patients' family members and healthy control subjects provided informed consent to participate in the study, and the ethical principles of the Declaration of Helsinki were followed.

Case 1 The onset of disease (FCAS) in this patient occurred at 3 months of age. She exhibited a recurrent generalized urticaria-like skin rash upon exposure to cold temperatures (Fig. 1a). Progressive sensory neural hearing loss and renal amyloidosis were not seen. Her serum C-reactive protein (CRP) levels were continuously and slightly increased (0.24–2.1 mg/dl).

Case 2 Case 2 was the father of case 1. He was a 34-year-old male with a recurrent urticaria-like skin rash, fever, conjunctivitis, and arthralgia that developed following fatigue or exposure to cold temperatures. The precise time of his disease onset was unknown. Progressive sensory neural hearing loss and renal amyloidosis were not seen [12]. His CRP levels were continuously increased (1.52–3.98 mg/dl).

Case 3 The onset of disease (CINCA/NOMID) in this patient occurred at 11 months of age. Continuous aseptic meningitis, urticaria-like skin rash, arthritis at the end of the fingers, and Raynaud's symptoms were observed. Arteriosclerosis of the ophthalmic artery was found. However, severe patella overgrowth was not seen. At 14 years of age, he had heart failure with myocarditis, which was considered to be a rheumatic characteristic. The patient died suddenly at 19 years of age (the detailed clinical case will be described elsewhere by Teramoto et al.).

Case 4 Case 4 was the mother of case 3. The precise time of her disease (MWS) onset was unknown. Initially, she was diagnosed with rheumatic arthritis and received oral prednisolone therapy. She suffered progressive sensory neural hearing loss at 30 years of age and underwent artificial cochlea replacement therapy at 48 years of age. This was greatly effective in improving her hearing ability. Meningitis and renal amyloidosis were not seen.

Case 5 The onset of disease in this patient occurred at 3 years of age. Fever that continued for more than 2 weeks, severe polyarthritis (serum matrix metalloproteinase-3 of >800 ng/ml), and recurrent urticaria-like non-itchy skin rash (Fig. 1b) were observed. Lymphadenopathy, hepatosplenomegaly, and serositis were not seen. Patella overgrowth, aseptic meningitis, progressive sensory neural hearing loss, and renal amyloidosis were not seen. Rheumatoid factor was negative. Other autoantibodies, including anticyclic citrullinated peptide antibody, were not detected. Her serum CRP and ferritin levels were increased (11.69 mg/dl and 255.1 ng/ml, respectively). Based on the below-mentioned hereditary traits and the results of in vitro functional assays, we diagnosed this patient as juvenile idiopathic arthritis (JIA), according to the criteria for JIA from the International League of Associations for Rheumatology [13]. A combination therapy with steroid and tocilizumab was effective.

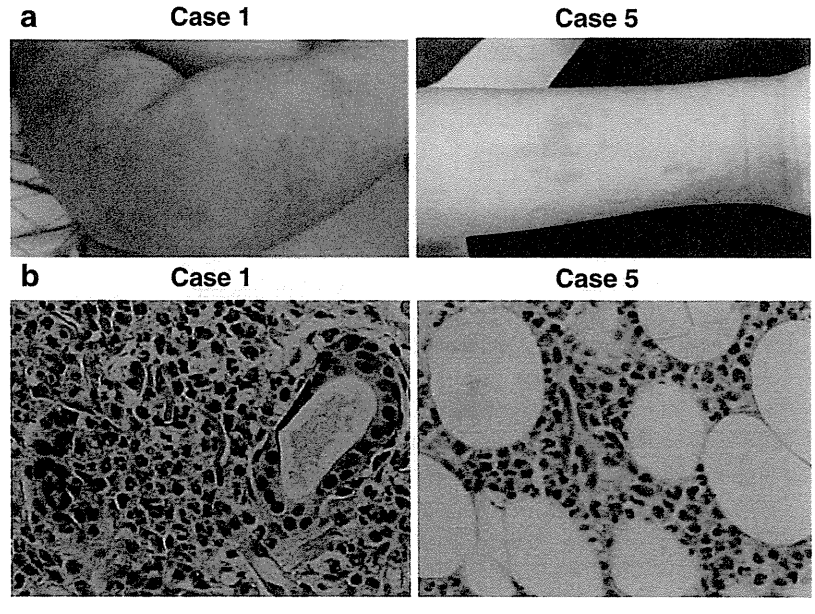

Fig. 1 Urticaria-like skin rash of cases 1 and 5. **a** Clinical appearances of the urticaria-like rash of cases 1 and 5. **b** Histopathological examinations of biopsy specimens from the skin rash of cases 1 and 5. Both skin biopsies show a recurrent cold-induced non-itchy urticaria-like skin rash and also show neutrophil infiltration

DNA Sequencing

Genomic DNA was extracted from leukocytes using SepaGene (Eidia, Tokyo, Japan). A DNA fragment of the *NLRP3* gene was amplified by PCR and analyzed using Big Dye Terminator Bidirectional Sequencing (Applied Biosystems, Foster City, CA, USA).

Cell Culture

PBMCs were isolated from heparinized blood from control donors and patients by gradient centrifugation in Ficoll-Paque (GE Healthcare, Uppsala, Sweden). The PBMCs were cultured in medium consisting of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 pg/ml). Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (high glucose-containing DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Vector Preparations

A cDNA encoding *NLRP3* tagged at the C terminus with a FLAG epitope (NLRP3-FLAG) was cloned into the plasmid vector pcDNA3.1+ (Invitrogen). Mutants of *NLRP3* (E378K, Y563N, E688K, and G809S) were generated using a GeneEditor In Vitro Site-Directed Mutagenesis System (Promega, Madison, WI, USA). An ASC variant 1 tagged at the C terminus with a myc epitope (ASC1-myc) was also cloned into pcDNA3.1+. An NF-κB luciferase reporter vector (pGL4.32-luc2P/NF-kappaB-RE/Hygro) and a *Renilla* luciferase reporter vector (pGL4.74-hRluc/TK) were purchased from Promega.

NF-κB Reporter Gene Activity

HEK293T cells in 96-well plates were transfected with 16 ng/well of pcDNA3.1+ control vector or pcDNA3.1+ NLRP3-FLAG vector (wild-type or mutant-type) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The pcDNA3.1+ ASC1-myc vector, NF-κB luciferase reporter vector, and *Renilla* luciferase reporter vector were cotransfected. After transfection, the cells were cultured for 24 h. The luciferase reporter gene activities were analyzed using a Dual-Luciferase Reporter Assay System (Promega). The statistical significance of differences in the luciferase activities between the wild-type and mutant genes in the NF-κB gene reporter assays was analyzed by the Kruskal–Wallis test, and further analysis was performed by the Bonferroni/Dunn test. Statistical significance was assumed for values of $P<0.05$.

Lipopolysaccharide- or Hypothermia-Induced Assays

PBMCs were suspended at 1×10^6 cells/ml in culture medium and cultured in the presence or absence of 10 or 100 ng/ml of LPSO127 (Sigma) for 24 h in six-well plates at 30°C or 37°C in a humidified atmosphere containing 5% CO_2.

Measurements of Tumor Necrosis Factor-α, IL-6, IL-1β, IL-1ra, and IL-18

Sera from the patients and healthy control subjects ($n=10$; age range, 1–35 years) were stored at −80°C until analysis. The sera of cases 1 and 2 were collected when they had the cold-induced rash, but not fever. The sera of cases 3, 4, and 5 were collected during a fever episode as an autoinflammatory symptom. Culture supernatants in test tubes or microtest plates were centrifuged to remove the cells and then stored at −80°C until analysis. The tumor necrosis factor (TNF)-α, IL-6, IL-1β, IL-1ra, and IL-18 concentrations were measured using a Human TNF-α Immunoassay Kit (BioSource, Camarillo, CA, USA), Human IL-6 Immunoassay Kit (BioSource), Human IL-1β Immunoassay Kit (BioSource), Quantikine Human IL-1ra/IL-1F3 ELISA Kit (R&D Systems, Minneapolis, MN, USA), and Human IL-18 ELISA Kit (MBL, Nagoya, Japan), respectively. The detection limits of the cytokine measurement kits were as follows: TNF-α, 1.7 pg/ml; IL-6, 2.0 pg/ml; IL-1β, 1.0 pg/ml; IL-1ra, 6.26 pg/ml; IL-18, 12.5 pg/ml. Values under the detection limits were shown as not detected. The serum cytokine levels were measured at two points at least, and the average values were calculated. The cytokine production levels by PBMCs were measured in duplicate and the average values were calculated. We defined cytokine levels of more than the mean+2 SD as increasing.

Results

Detection of Gene Variations in *NLRP3*

In the five patients, four heterozygous missense variations (E378K, Y563N, E688K, and G809S) of the *NLRP3* gene were identified (Table I). Interestingly, case 3 showed compound heterozygous gene variations, E688K and G809S, while his mother (case 4) had only one mutation, E688K, of *NLRP3*. The G809S allele was inherited from his asymptomatic father. In case 5, a novel missense variation, E378K, in *NLRP3* was identified. In addition, a heterozygous mutation, E148Q, in *MEFV* was identified. Gene mutations in *TNFRSF1A*, *MVK*, *NLRP12*, and *NOD2* were not found.

The genotypes of *NLRP3* and *MEFV* in her asymptomatic mother were the same. It should be noted that E378K and G809S were not present in the *INFEVERS* database (http://fmf.igh.cnrs.fr/ISSAID/infevers/) [14] and were confirmed as rare variants that were not identified in the 100 ethnically matched control subjects.

NF-κB Reporter Gene Activities of the *NLRP3* Variants

Figure 2 shows the ASC-dependent NF-κB activities of the *NLRP3* variants in vitro. The NF-κB reporter gene activities were increased by the Y563N and E688K mutations in *NLRP3*. The activities were higher for D303N (as a positive control *NLRP3* mutation that was previously identified in a CINCA/NOMID patient [5]) and E688K than for the FCAS mutation, Y563N. E378K and G809S did not cause any significant increases in the activities. Initially, we suspected that case 5 had CAPS. However, based on these results, we were able to confirm the diagnosis of case 5 as JIA, rather than CAPS.

Cytokine Profiles of the Patients

The serum IL-1β, IL-6, and TNF-α levels were not detected in the sera of the healthy control subjects. Although we were unable to detect IL-1β in the patients' sera, we clearly detected the serum IL-18 and IL-1ra levels in all cases (Fig. 3a, b). The serum IL-18 levels were extremely high in the CINCA/NOMID (case 3), MWS (case 4), and JIA (case 5) patients compared with the control subjects. The serum IL-1ra and IL-6 levels were increased in cases 2, 3, 4, and 5 (Fig. 3b, c). The serum TNF-α levels were increased in cases 1, 2, and 3 (Fig. 3d).

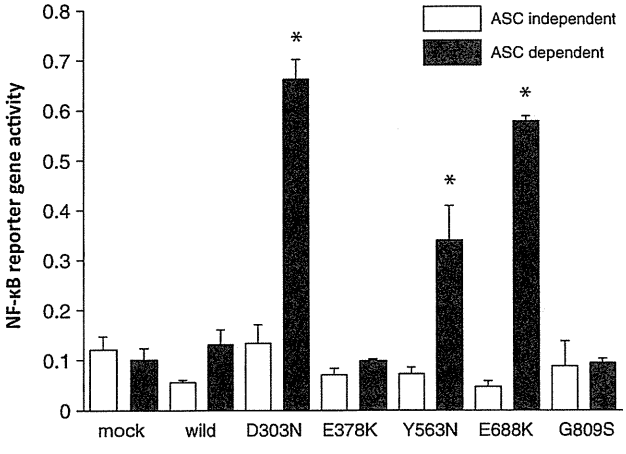

Fig. 2 NF-κB reporter gene activities of the *NLRP3* variants. The *white bars* indicate the NF-κB reporter gene activities of the *NLRP3* variants without cotransfection of ASC, while the *black bars* indicate these activities with cotransfection of ASC. The data shown are the means±SD of triplicate assays. The ASC-dependent NF-κB reporter gene activities are increased for the variants with D303N, Y563N, and E688K. The activities for the CINCA/NOMID mutations, D303N and E688K, are higher than those for the FCAS mutation, Y563N. The variants with E378K and G809S do not show any significant increases in the activities. *$P<0.05$

Interestingly, the serum IL-18 levels in the FCAS patients (cases 1 and 2) did not show any increases compared with the control subjects (Fig. 3a). Furthermore, the levels of spontaneous IL-1β production by PBMCs from the CINCA/NOMID (case 3) and MWS (case 4) patients were increased, whereas those of the control subjects, FCAS patients, and JIA patient (cases 1, 2, and 5) did not show any increases (Fig. 4a).

The lipopolysaccharide (LPS)-induced cytokine production levels by PBMCs from the FCAS and JIA patients are shown in Fig. 4b–d. The IL-1β and IL-18 production levels were increased in the FCAS patients compared with the control subjects. However, TNF-α did not show any significant changes. Comparisons of the cytokine production levels by the PBMCs cultured at 30°C and 37°C are shown in Fig. 5. The PBMCs from the FCAS patients showed obvious increases in the IL-1β and IL-18 production levels after culture at the lower temperature with no stimulation.

Discussion

The diagnosis of CAPS is still based on the clinical symptoms and recognition of a syndrome. Detection of a pathogenic *NLRP3* mutation can confirm the CAPS diagnosis. However, to confirm the diagnosis of CAPS patients with novel identified *NLRP3* variations, some functional experiments regarding the effects of the *NLRP3* mutations, such as the NF-κB luciferase reporter gene assay used in this study, are necessary because of the existence of nonfunctional missense variations of *NLRP3* [7]. Furthermore, although there are many previously reported missense mutations of *NLRP3* associated with CAPS in the *INFEVERS* database [14], the mutations with confirmed functional evidence are limited. In this study, we identified *NLRP3* gene mutations in five patients who were suspected of having autoinflammatory syndromes. Two mutations of *NLRP3*, Y563N and E688K, were previously reported to be disease-causing mutations [15, 16], although in vitro functional assays were not performed. Y563N was first identified in FCAS patients who were diagnosed based on the clinical criteria of FCAS [16, 17]. Our FCAS patients (cases 1 and 2) showed a skin rash, occasional fever, and mild arthritis and did not show any severe symptoms, such as neurological disorders, hearing loss, and renal amyloidosis. On the other hand, E688K was first identified in an Italian male CINCA/NOMID patient [15] who was described as having a skin rash, hearing loss, fever, and transient arthritis without persistent deformities of the involved joints. Our patients with E688K

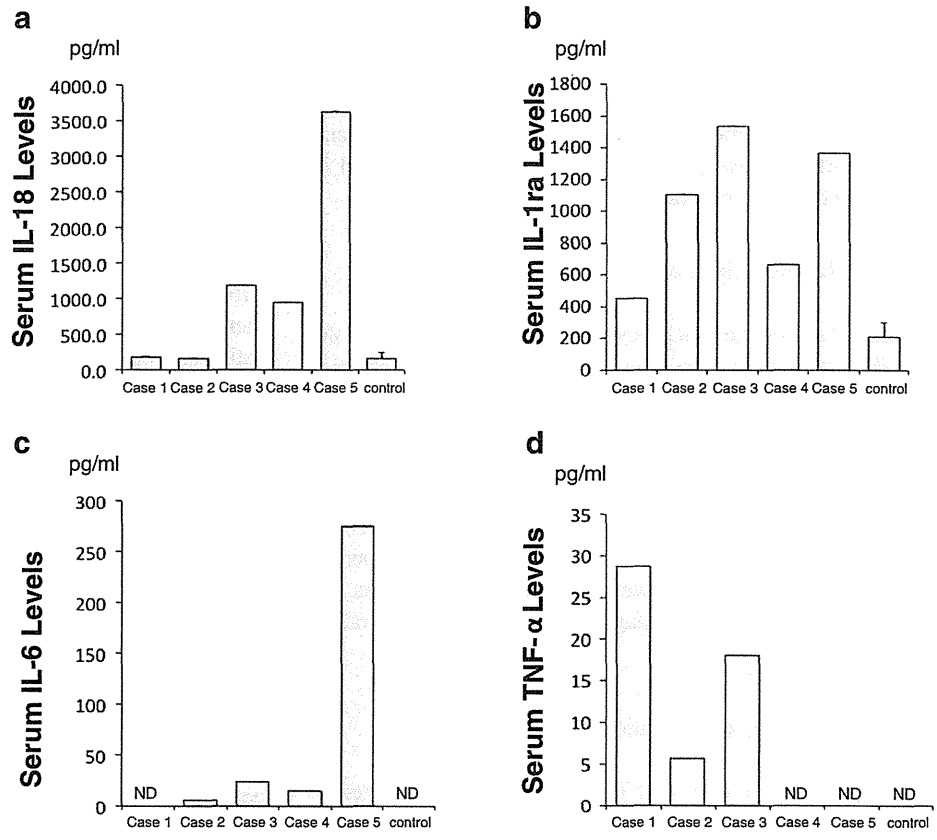

Fig. 3 Serum inflammatory cytokines in the four CAPS cases. IL-1β, IL-6, and TNF-α were not detected in the sera of the control subjects. The means ±D of the serum IL-18 and IL-1ra levels of the healthy control subjects were 169.2±85.7 and 213.4±87.1 pg/ml, respectively (n=10)

(cases 3 and 4) also had no strong deformities of the joints, but had obviously more severe phenotypes than FCAS, such as aseptic meningitis and hearing loss. In the present study, the E688K mutation in the MWS and CINCA/NOMID syndrome patients showed significantly stronger NF-κB activities than the Y563N mutation identified in the FCAS patients. Our findings indicate that the clinical phenotypes and values of the ASC-dependent NF-κB activity assay are well correlated with the genetic mutations, consistent with a previous report [18]. However, the artificial reporter gene assay system used may have little to do with the function of the CAPS pathophysiology, and limited numbers of *NLRP3* variants have been assessed using the assay in the present and previous studies, thereby making it difficult to prove this hypothesis at the present time. Consequently, further experiments including large amounts of pathogenic mutations and accumulation of detailed clinical information about the disease severity of CAPS are necessary to confirm this hypothesis. It should be noted that low-penetrance mutation, G809S, did not show positive activity with this in vitro assay system. But the clinical phenotype of case 3 was obviously more severe than case 4, although the father of case 3, who also was found to have G809S, was as symptomatic. Because of the discrepancy between the patient and the father, it remains unclear whether G809S is a pathogenic mutation or, alternatively, if there is an alternative genetic explanation for disease in the patient not detected by genomic DNA sequencing.

On the other hand, it requires time to build the above-mentioned in vitro experimental system. For the rapid diagnosis and characterization of CAPS, a simple screening system is necessary. In this study, we measured several serum inflammatory cytokine levels in our patients (Fig. 3). The serum IL-6 level is usually used for evaluating the disease severity of rheumatoid arthritis [19]. Moreover, the serum IL-18 level was recently reported to reflect the disease severity of not only JIA but also other diseases such as allergic diseases [20, 21]. In our CAPS patients, the serum levels of IL-18, but not IL-1β, seemed to be correlated with the disease phenotypes. Although the precise reason for this dissociation between the IL-18 and IL-1β levels in the sera is unknown, IL-1β may be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. In fact, serum IL-1ra, which is the counter-regulator of IL-1, was increased in our CAPS patients. Thus, the serum IL-18 levels may be used as an appropriate marker for the evaluation of treatments, although it is unlikely that serum IL-18 can contribute to the differential diagnosis between CAPS and other diseases.

The diagnosis of FCAS seems to be relatively difficult because of its mild phenotypes compared with the other more severe phenotypes of CAPS. The serum inflammatory

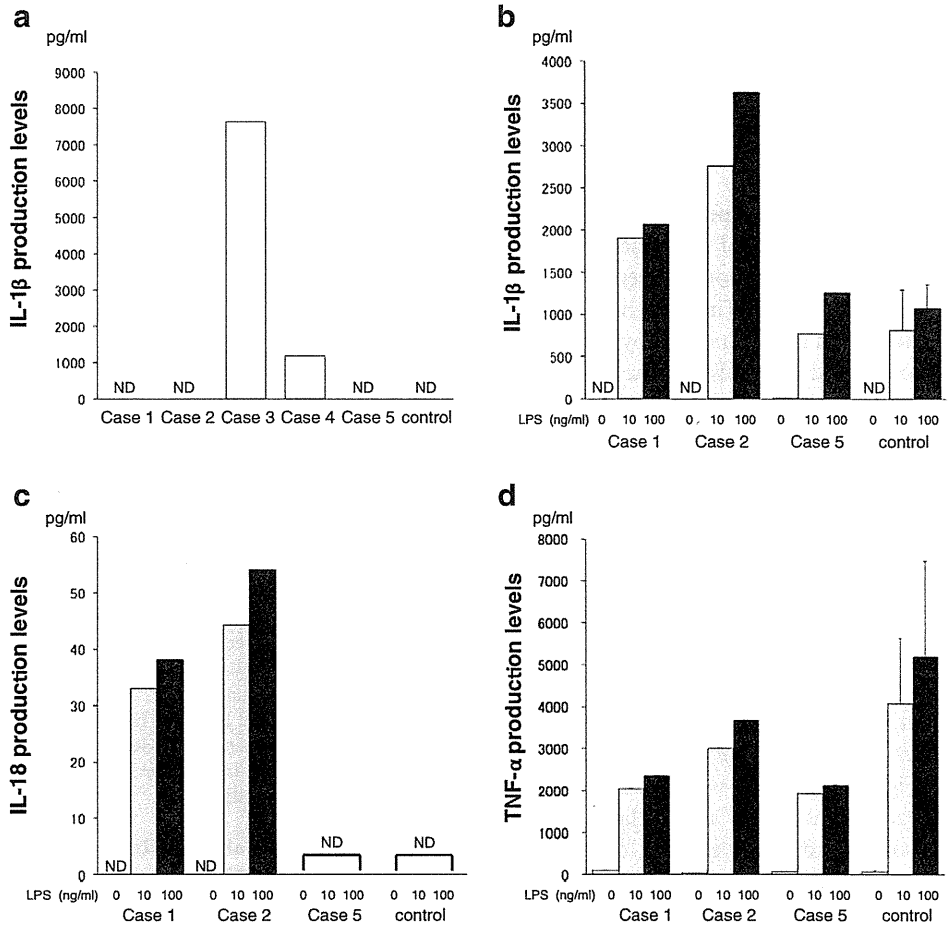

Fig. 4 LPS-induced cytokine production levels in the patients. **a** The *white bars* indicate the spontaneous IL-1β production levels by PBMCs. Increased IL-1β production by PBMCs from case 3 (CINCA/NOMID syndrome) and case 4 (MWS) is detected, whereas no increases are observed for the PBMCs from the control subjects and cases 1, 2 (FCAS), and 5 (JIA). **b**, **c** The LPS-induced IL-1β and IL-18 production levels by PBMCs from the FCAS patients are increased compared with PBMCs from the control subjects. **d** The TNF-α production levels by PBMCs from the FCAS and JIA patients do not show any significant changes. In **b**–**d**, the *white bars* indicate the cytokine production levels without stimulation and the *gray* and *black bars* indicate the cytokine production levels after stimulation by 10 and 100 ng/ml LPS, respectively

cytokine levels in our FCAS patients did not show any typical increases, unlike the case for the CINCA/NOMID patient (Fig. 3), indicating that the establishment of an effective and easy screening method is important for the diagnosis of FCAS. Therefore, we focused on the cytokine production levels in these patients' blood cells. First, IL-1β production by nonstimulated PBMCs was observed in our CINCA/NOMID and MWS patients (cases 3 and 4, respectively), as reported previously [5]. However, no enhancement of spontaneous IL-1β production was observed in our FCAS patients (cases 1 and 2) (Fig. 4a), suggesting that this method may not be suitable for screening of FCAS.

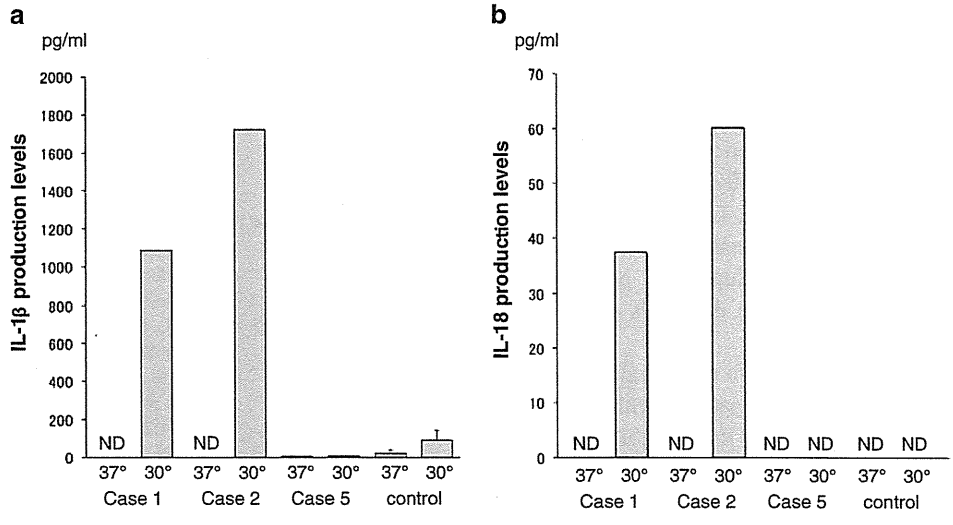

Fig. 5 Hypothermia-induced cytokine production levels by PBMCs from the FCAS and JIA patients. **a**, **b** Comparisons of the cytokine production levels by PBMCs cultured at 30°C and 37°C. The PBMCs from the FCAS patients (cases 1 and 2) show obvious increases in the IL-1β and IL-18 production levels after culture at lower temperature with no stimulation

Furthermore, the LPS- or hypothermia-induced cytokine production levels by the PBMCs showed marked elevation of IL-1β or IL-18 (Figs. 4a–c and 5b), as reported previously [16, 22]. The phenomena for hypothermic culture were similar to the findings in our recent report that NF-κB activity induced by LPS stimulation through TLR4 is enhanced in low-temperature cultures [23], although the precise mechanism of the association between the *NLRP3* variations and the low-temperature stimulation requires further clarification. These findings suggest that the cytokine production assays induced by LPS or hypothermia stimulation should be helpful for the diagnosis of FCAS. It should be noted that the serum IL-18 levels could be detected in all of the non-CAPS subjects, although the production levels of IL-18 from their PBMCs were lower than the detection limit. This might be dependent on the long half-life of IL-18 in human blood compared with the above-mentioned half-life of IL-1β.

The discrimination between CAPS and JIA cases is sometimes difficult because of their similar clinical characteristics. Interestingly, although case 5 had a rare missense variation in *NLRP3* (E378K) and some of her clinical symptoms were similar to those of CAPS (Table I), the E378K variant did not show enhancement of NK-κB activity (Fig. 2). This gene variation was inherited from her mother who did not show any inflammatory symptoms. Case 5 showed strong polyarthritis, continuous fever, and a recurrent generalized urticaria-like erythema as well as symptoms of CAPS. In particular, histopathological examination of a biopsy specimen from her skin rash revealed infiltration of neutrophils and mononuclear cells, representing similar findings to case 1 (Fig. 1). Thus, it was difficult to discriminate CAPS by the clinical symptoms alone in this case.

Therefore, to discriminate between CAPS and JIA in this case, we focused on her cytokine profiles. Her serum IL-6 and IL-18 levels were extremely high compared with not only the healthy controls but also the other CAPS patients (Fig. 3a, c). These observations resembled the serum cytokine pattern of systemic-onset JIA [21, 24]. Furthermore, the LPS-induced and hypothermia-induced IL-1β and IL-18 production levels by PBMCs from case 5 showed no increases compared with the control subjects (Figs. 4b, c and 5a, b). Recently, Saito et al. [5] reported that another screening method, LPS-induced monocyte cell death, was effective for diagnosing CAPS. The monocytes in case 5 did not show LPS-induced cell death. These objective results also supported the diagnosis of case 5 as JIA, rather than CAPS.

In this study, we evaluated several methods for the limited genotypes of patients with *NLRP3* variants. According to comparisons of the clinical phenotypes of previous case reports and our cases, the disease severity seems to be correlated with the serum cytokine levels and the ex vivo and in vitro responses and is almost completely determined by the specific mutations, which appear to suggest that other genetic or epigenetic determinants or environmental factors do not play a significant role.

Conclusions

A precise and easy method for the diagnosis of CAPS has not yet been established. The characteristics of the clinical phenotypes and the identification of proven gene variations of *NLRP3*, as the etiology of CAPS, are very important for diagnosing CAPS. In addition, the serum IL-18 levels and NF-κB activities of patients with the *NLRP3* variants reflect the phenotypes of disease severity. Evaluation of the cytokine profile is also a useful tool for diagnosing and discriminating the severity of CAPS.

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Conflicts of Interest The authors have declared no conflicts of interest.

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Structural property of soybean protein P34 and specific IgE response to recombinant P34 in patients with soybean allergy

HIDEYUKI MORITA[1,2], HIDEO KANEKO[1,2], HIDENORI OHNISHI[1], ZENICHIRO KATO[1], KAZUO KUBOTA[1], TAKAHIRO YAMAMOTO[1], EIKO MATSUI[1], TAKAHIDE TERAMOTO[1], TOSHIYUKI FUKAO[1], KIMIKO KASAHARA[1] and NAOMI KONDO[1]

[1]Department of Pediatrics, Graduate School of Medicine, Gifu University; [2]Department of Pediatrics, Nagara Medical Center, Gifu, Japan

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Abstract. Soybean allergy is one of the important food allergies because soybean is widely used in processed foods. P34 has been identified as the main allergen in soybeans. The main objective was to analyze the structural property of recombinant P34 and the P34 antigen-specific IgE response in soybean allergy using recombinant P34. Recombinant P34 was expressed by the BL21 (DE3) strain of *Escherichia coli*. Purified recombinant P34 showed oligomerization and binding to endotoxin. The binding of recombinant P34 to endotoxin was confirmed by LPS pull-down assay. High-density SDS treatment dissociated oligomeric recombinant P34 and removed endotoxin. Both native P34 and purified recombinant P34 showed almost identical structural properties as determined by circular dichroism analysis. We analyzed recombinant-P34-specific IgE antibodies by the ImmunoCAP System. In ImmunoCAP using recombinant P34, all sera from healthy controls were classified as negative. A correlation was found between the specific IgE antibodies to whole soybean and recombinant P34 ($r=0.526$, $P<0.05$). The sera from 3 of 9 (33%) patients with outgrown soybean allergy and 6 of 9 (66%) patients with soybean allergy were classified as positive. SDS-treated recombinant P34 retained its structure and biological activity. Recombinant P34 is a useful tool for the analysis of antigen-specific response in soybean allergy. It may be possible to develop a modified form of recombinant P34 for the diagnosis or treatment of soybean allergy using specific immunotherapy techniques.

Correspondence to: Dr Hideo Kaneko, Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu 501-1194, Japan
E-mail: hideo@gifu-u.ac.jp

Abbreviations: CD, circular dichroism; DLS, dynamic light scattering; LST, lymphocyte stimulation test; rP34, recombinant P34

Key words: endotoxin, ImmunoCAP, recombinant P34, SDS, soybean allergy, specific IgE

Introduction

Soybean is one of the main sources of protein in human nutrition (1,2), and soybean is used in an increasing number of products because of its health benefits (3,4). However, in the United States, approximately 0.4% of children are allergic to soybean and many infants must not be fed soybean-based formula and baby foods (5). Soybean-induced allergic symptoms may range from skin, gastrointestinal, or respiratory reactions to anaphylaxis (5,6). Although the primary treatment for food allergies is to avoid the causative agent, it is difficult to avoid soy protein because of its extensive use in prepared and processed foods.

There are more than 20 soybean proteins that cause allergies (7). Recently, it has been reported that Gly m 5 (β-conglycinin) and Gly m 6 cause severe allergic reactions in Europe (8). In other reports, three proteins, Gly m Bd 60k, Gly m Bd 30k (P34), and Gly m Bd 28k represent the main seed allergens in soybean-sensitive patients (9,10). P34 is the allergen most strongly and frequently recognized by the IgE antibodies in the sera of soybean-sensitive patients with atopic dermatitis (3,11). In several IgE binding studies, more than 65% of soybean allergic patients with atopic dermatitis exhibited an allergic response to P34 (11-14). P34 is regarded as the major, or immunodominant, soybean allergen and is a target allergen for producing low-allergen-content hypoallergenic soybean products (15-17). Moreover, P34 shares high sequence homologies with the main peanut allergen Ara h 1, the dust mite allergen Der p 1, cow's milk caseins, and papain (18-20). Thus, peanuts and soybeans contain common allergenic components and, for this reason, IgE antibodies to peanut proteins may cross-react with soybean proteins.

First, we tried to express recombinant P34 (rP34) in *Escherichia coli* using a previously reported method (21). However, recombinant proteins expressed in *E. coli* carry the risk of endotoxin contamination. Endotoxin causes false-positive results in biological-cell-based assays, such as the cell proliferation assay and must be removed particularly when evaluating allergen-stimulated T cell proliferation (22). The methods for removing endotoxin vary greatly and depend on the structure and characteristics of particular allergens (13,22,23).

On the other hand, for serological diagnosis of soybean allergy, specific IgE antibodies to soybean proteins can be detected using commercially available tests. However, these tests are unsatisfactory for the identification of patients with soybean allergy. Specific IgE antibodies to major allergens in other foods, such as omega-5 gliadin, are highly useful for the diagnosis of food allergies (24). The measurement of specific IgE antibodies to rP34 may be useful. Furthermore, for a precise serological diagnosis, a tertiary folded soluble recombinant allergen protein is necessary.

In this study, we expressed rP34 using an *E. coli* expression system for the evaluation of clinical manifestation. Our purified rP34 had almost identical structural properties to native P34. Furthermore, ImmunoCAP System using rP34 was carried out to analyze the specific IgE antibodies to P34 in patients with and without soybean allergy.

Materials and methods

Soybean extracts. Soybean (Fukuyutaka) flakes from commercial sources were ground to a fine powder using pestle and mortar and extracted by incubation with 20 mmol sodium phosphate and 1 mmol sodium chloride (1:20 wt/vol, pH 7.2) overnight at 4°C. Soybean mRNA was obtained using an RNeasy Plant Mini kit (Qiagen). Reverse transcription was carried out using an RNasin ribonuclease inhibitor (Promega) and M-MLV reverse transcriptase (Invitrogen). The sense and antisense primers used for PCR were designed on the basis of the cDNA sequences: 5'-GATTCGATCGAAGGTCGTAAGAAA ATGAAGAAGGAAC-3' for the sense primer and 5'-GCGGCC GCAAGAGGAGAGTGATCAACTCTTC-3' for the antisense primer. The resulting DNA fragment was purified using a Gene Clean kit, ligated to a pUC118 plasmid, and transformed into the *E. coli* strain JM109. The resulting plasmid, pUC118/P34, was digested with *Eco*RI and *Not*I, and the fragment containing the P34 gene was inserted into pET24b and expressed in *E. coli* BL21 (DE3). The initial expression, purification and refolding of rP34 were performed in accordance with the method of Babiker *et al* (21).

Purification of histidine-tagged recombinant P34 using Ni Sepharose column. rP34 was purified using a Ni Sepharose column (Ni Sepharose 6 Fast Flow, 5 ml, GE Healthcare). The bound protein was eluted with 20 ml of 100 mM phosphate buffer containing 500 mM imidazole, pH 7.4.

Conformational change of recombinant P34. To produce the monomeric form of P34, we used a previously reported method with modification (14). The homogeneity of rP34 was determined by dynamic light scattering (Dyna Pro-99, Proterion). The solution containing the oligomeric form of rP34 was treated with an equivalent volume of 4% SDS solution containing 10% 2-ME and heated at 98°C for 10 min. The solution was then loaded onto a Sephacryl S-200 column pre-equilibrated with 0.1 M sodium phosphate buffer (pH 7.6) containing 1% SDS and 10 mM 2-ME. The column was run at 20°C using the same buffer and the fractions containing P34 were collected.

Endotoxin removal from recombinant P34. Detoxi-Gel (Pierce) was used to remove contaminating endotoxin in accordance with the manufacturer's instructions. The collected samples were dialyzed against 50 mM sodium phosphate buffer (pH 7.6) overnight at 4°C. The level of endotoxin was measured by a chromogenic limulus amebocyte lysate assay (endotoxin single test; Wako Pure Chemical Industries). The collected sample was dialyzed against 50 mM sodium phosphate buffer (pH 7.6) overnight at 4°C to remove any remaining SDS and stored at -20°C.

Endotoxin was also removed by Triton X-114 phase separation, as described by Liu *et al* (25). Triton X-114 was added to rP34 to a final concentration of 1% and incubated for 30 min at 4°C with constant stirring, followed by a 10-min incubation at 37°C and centrifugation at 20,000 x g at 25°C for 10 min.

N-terminal sequencing of recombinant P34. After separation by SDS-PAGE for 1 h at 200 mA in 25 mM Tris, 192 mM glycine, and 10% methanol, the proteins were electroblotted onto a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was briefly stained with CBB R250 (Wako, Japan) and destained extensively in 45 and 90% methanol solutions containing 7% acetic acid. Amino acid sequence analysis of rP34 was carried out using an Edman degradation technique using a pulse liquid automatic sequencer (Model 491HT, Applied Biosystems).

Circular dichroism (CD). For SDS-treated proteins, SDS concentrations of 0, 1 and 4% (0, 3.5×10^{-2} and 1.4×10^{-1} M) were used. Optically clear solutions in phosphate buffer were used to record CD spectra (195-250 nm) in a 1 mm rectangular quartz cell (JASCO) using a CD spectrometer (JASCO). The secondary structure was determined by visual assessment of the spectra and using the computer program CDPro. Native P34 was provided by Professor S. Nagaoka of Gifu University.

Dynamic light scattering (DLS). DLS was carried out using a DynaPro-99 molecular-sizing instrument equipped with a microsampler (Protein Solutions). rP34 samples containing 0, 1 and 4% SDS were used in the experiment. The DynaPro-99 instrument was operated in accordance with the DLS machine protocol to estimate the molecular weight of rP34. Data were analyzed using the Dynamics 5.0 software (Protein Solutions).

LPS pull-down assay. A pull-down assay of the recombinant protein using labeled LPS was carried out as previously described (26,27). Briefly, 10 μg of biotinylated LPS (Alexis Biochemicals) was absorbed onto 20 μl of streptavidin agarose (Vector Laboratories) and incubated with 10 μg of LPS-free rP34 at room temperature for 1 h. After washing with PBS containing 0.1% Triton X-100, the precipitated protein was detected by silver staining.

Homology modeling of P34. The 31 kDa cysteine protease SPE31 (PDB code, 2b1 m) was selected from a protein databank (www.rcsb.org/pdb) as the most homologous template for P34. The structural modeling of P34 was performed using the MOE software (Chemical Computing Group, Inc.).

Subjects. Patients with a history of soybean allergy were selected as subjects of this study (Table I), and grouped on the basis of the results of the open challenge test or the accidental

episodes of ingestion as follows: the outgrown group (n=9) had a history of soybean allergy and produced soybean-specific IgE, but was currently tolerant to soybean; the allergic group (n=9) was reactive to soybean; and the healthy control group (n=13) was negative for all allergens in ImmunoCAP System (Phadia AB, Uppsala, Sweden) and had no history of food allergies. Five patients (Patients 11, 13, 15, 16 and 18) had allergic reactions (urticaria, cough and wheeze) within 2 h after soybean ingestion, and six patients (Patients 10, 12-15 and 17) had skin symptoms after more than two hours. Informed consent was obtained from the families of all the subjects.

Western blot analysis. Western blotting using an anti-P34 monoclonal antibody was carried out in accordance with the method of Tsuji *et al* (28). Samples were electrophoresed on 15-25% gradient gels (XV PANTERA GEL). Separated proteins were then transferred onto membranes using an iBlot system (Invitrogen) at 30 mA for 14 min. The membranes were blocked with blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 3% BSA) for 2 h at room temperature. After two 5-min washes in TBS-T, they were reacted with the patients' sera for 2 h. After washing four times, the blots were incubated with goat anti-human IgE conjugated to HRP (1:5,000) for 2 h. Blots were washed four times as described above. The P34 monoclonal antibody was a kind gift from Professor T. Ogawa of Kyoto University.

Specific IgE antibody. The level of specific IgE antibody to our rP34 in these patients was measured by ImmunoCAP System. In the case of using an undiluted reagent, ImmunoCAP enables the quantification of specific serum IgE antibodies in the range of 0.35 to 100 kUA/l. However, for comparative purposes, the above-mentioned range is often converted into 7 scores, in accordance with the internal calibrator system, as follows: class 0, negative; class 1, 0.35-0.7 kUA/l; class 2, 0.7-3.5 kUA/l; class 3, 3.5-17.5 kUA/l; class 4, 17.5-50 kUA/l; class 5, 50-100 kUA/l; and class 6, >100 kUA/l. IgE levels >0.35 kUA/l in serum are considered to indicate positivity for IgE by the manufacturer.

Statistical analysis. Pearson's correlation coefficient was used to estimate correlations between two numerical variables. P-values <0.05 are considered statistically significant.

Results

High-density SDS-treatment was useful for removal of endotoxin from rP34. The purified rP34 was detected using an anti-P34 monoclonal antibody (Fig. 1A). The N-terminal amino acid sequence of the recombinant protein (KKMKKEQTS) was identical to that of soybean P34 (amino acid number, 123-131). As mentioned above, recombinant proteins expressed in *E. coli* carry the risk of endotoxin contamination. To remove endotoxin from this protein, rP34 was applied to the endotoxin removal column. However, all of the proteins absorbed onto the column. To further investigate the binding ability of P34 to LPS, we performed pull-down assay. LPS was clearly pulled down with rP34 (Fig. 2). We found that high-density SDS treatment, but not Triton X-114 treatment, dissociated oligomeric recombinant P34 and

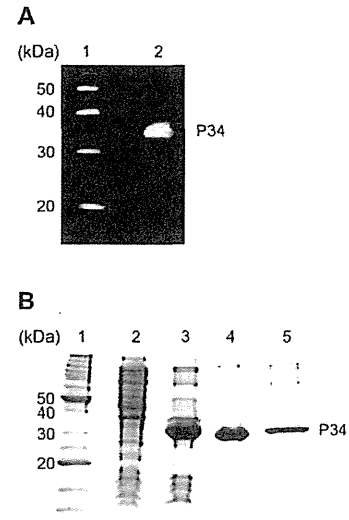

Figure 1. Purification of recombinant P34. (A) Immunoblot of recombinant P34 with an anti-P34 monoclonal antibody (1:3,000). Lane 1, molecular weight marker; lane 2, recombinant P34. (B) SDS-PAGE using a 15% gel. Polypeptides were visualized by Coomassie blue staining. Lane 1, molecular weight marker; lane 2, soluble fraction; lane 3, insoluble fraction; lane 4, refolded protein; lane 5, recombinant P34 without endotoxin.

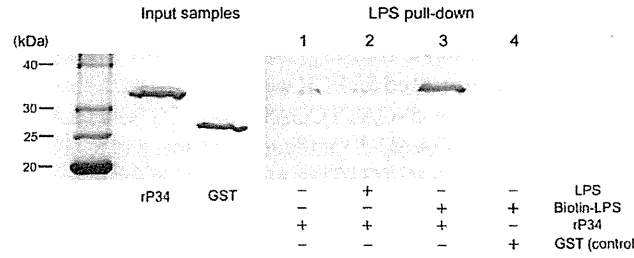

Figure 2. LPS pull-down assay. Lane 1, 10 μg of recombinant P34 was incubated with 30 μl of packed avidin beads. Lanes 2-3, 10 μg of recombinant P34 was incubated with 10 μg of non-biotinylated (lane 2) or biotinylated LPS (lane 3) and 30 μl of packed avidin beads. Lane 4, GST protein was added as a control. After washing, recombinant P34 bound to LPS was detected by silver staining.

removed endotoxin. The final yield of purified rP34 decreased to ~40% of that in the sample prior to endotoxin removal (Fig. 1B). The final yield after purification was about 11.6 mg/l of LB medium culture.

Both native P34 and SDS-treated endotoxin-free rP34 showed almost identical structural properties. SDS-induced structural conformation changes were determined from the appearance of a minimum peak at 205 nm and a weak shoulder at 222 nm in the CD spectra. No significant differences were observed between native P34 and SDS-treated rP34 in terms of the shape of the CD spectra and molar ellipticity values at 222 nm, which reflect the α helix content of the folded protein (Fig. 3). It should be noted that there was actually a minor difference between native and rP34, but this may be attributable to the C-terminal hexa-histidine tag.

Structurally, P34 belongs to the papain family, whose individual members share a common mechanism of catalysis. The model of the P34 structure was constructed on the basis of the structure of the papain-like protein family protein SPE31

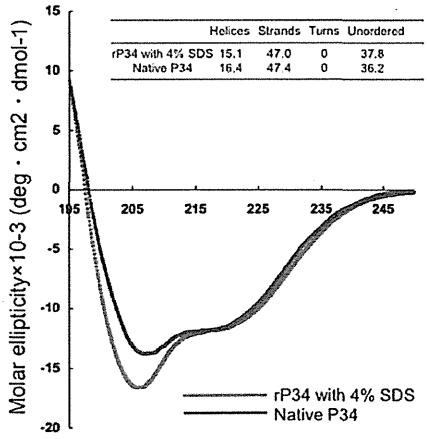

Figure 3. Circular dichroism spectra of native P34 and recombinant P34 exposed to SDS. The secondary structures of both native and recombinant P34 after SDS treatment were estimated using a computer program (CDPro).

(Fig. 4). Analysis of the sequence alignments for P34 identified SPE31 as sharing 78% sequence identity (29). The overall structure of P34 contains two independent domains, namely, the L domain and R domain, which form a cleft. The major secondary structural part of the L domain forms the β-sheet and that of the R domain forms the α-helix.

Reactivity of IgE in sera from soybean allergic patients to rP34 examined by ImmunoCAP System. A total of 31 sera (13 controls, 9 outgrown, and 9 patients with soybean allergy) were available for the present study. The levels of rP34-specific IgE antibodies in the sera from outgrown and allergic patients measured by ImmunoCAP System are shown in Table I. Among the 31 sera investigated, 9 were positive for the specific IgE antibodies to rP34 and 22 were negative. A correlation was found between specific IgE antibodies to soybean and rP34. The correlation value was 0.526 and $P<0.05$. All sera from healthy controls were negative for the antibodies. Six of the 9 (66%) outgrown and 3 of the 9 (33%) allergic patients were also negative for the antibodies. The positive sera belonged to class 1 (4 sera), class 2 (3 sera) and class 3 (2 sera). No serum belonged to classes 4 to 6. Four of 5 patients with sera belonging to class 2 or 3 were not tolerant to soybean. The median concentrations of specific IgE antibodies to rP34 were 1.63 kUA/l (range, 0.35-5.00) in the allergic group and 0.50 kUA/l (range, 0.35-1.26) in the outgrown group.

Discussion

Recently, recombinant allergens have become available for diagnostic and therapeutic purposes (23,30,31). It is particularly important to evaluate T cell activation for diagnosis and specific immunotherapy using allergens. However, the use of recombinant antigens contaminated with endotoxin leads to nonspecific cytokine production and affects T cell proliferation, thereby masking any antigen-specific responses. In our previous study, we showed that endotoxin-free rP34 might be a useful tool for evaluation of soybean allergy (32). It is essential to remove endotoxin from recombinant proteins before using them in the analysis of antigen-specific responses.

During the processes of protein purification, we noted a strong affinity of rP34 to endotoxin derived from the host strain. In this study, we clearly showed the LPS binding ability of P34 by the LPS pull-down assay (Fig. 2). It is reported that P34 contains many hydrophobic residues and is, thus, insoluble in aqueous solutions (16). The structural model of rP34 (Fig. 4C) indicated that many of the hydrophobic residues (shown in yellow) are located superficially, and thus may contribute to the strong affinity of rP34 to endotoxin. On the other hand, DLS analysis showed that rP34 treated with 4% SDS had a molecular weight of 34.9 kDa, whereas non-SDS-treated rP34 had a molecular weight of 790 kDa, indicating that SDS treatment disrupted the rP34 oligomers. This finding was consistent with the finding of a previous study, in which Ogawa *et al* (14) isolated native P34 in its oligomeric form (>300 kDa). In our recent study and in this study, it was also possible that these SDS-induced conformational changes enabled the separation of endotoxin from rP34 (32). From this findings, we speculated that the oligomerization property of P34 may influence its affinity to endotoxin.

It was reported that P34 is a receptor of the glycolipid elicitor syringolide produced by *Pseudomonas syringae*

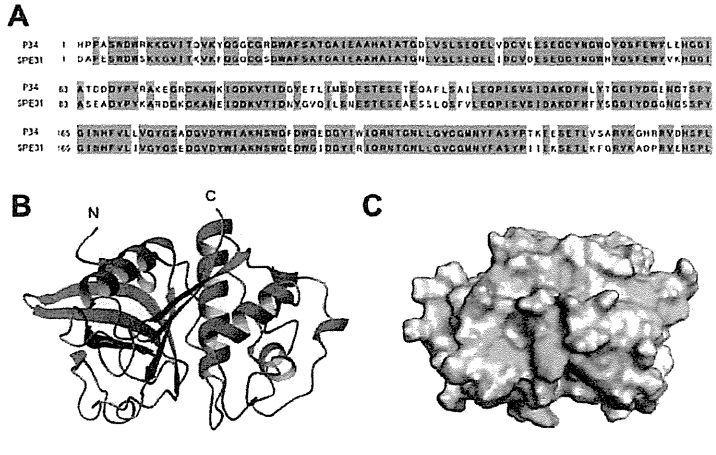

Figure 4. Structural model of recombinant P34. (A) Alignment of P34 and SPE31 amino acid sequences. P34 shares 78% amino acid sequence identity with SPE31. (B) Structural model of mature P34. (C) Distribution of hydrophobic residues on P34. The hydrophobic residues are shown in yellow.

Table I. Characteristics of outgrown subjects and soybean allergic subjects.

					ImmunoCAP values			
					Soybean		rP34	
Patient	Gender	Age (years)	Symptoms caused by soybean intake	Total IgE (IU/ml)	Class	kUA/l	Class	kUA/l
1	M	6	Outgrown	3,540	2	4.14	2	1.26
2	F	1	Outgrown	133	1	1.40	1	0.56
3	M	4	Outgrown	156	0	<0.35	0	<0.35
4	M	5	Outgrown	233	2	0.84	0	<0.35
5	M	4	Outgrown	108	2	1.07	0	<0.35
6	M	3	Outgrown	377	2	1.94	0	<0.35
7	F	2	Outgrown	426	2	2.14	0	<0.35
8	F	3	Outgrown	374	2	3.60	1	0.61
9	M	9	Outgrown	977	2	1.02	0	<0.35
10	M	1	Urticaria	>16,000	5	88.2	3	5.00
11	M	4	Wheeze, urticaria	1,960	4	20.9	2	1.13
12	M	2	Erythema	31	1	0.59	0	<0.35
13	F	1	Cough, urticaria	626	2	1.63	0	<0.35
14	F	1	Urticaria	23.2	0	<0.35	0	<0.35
15	M	6	Cough, urticaria	1,490	5	76.4	1	0.41
16	M	1	Wheeze, urticaria	2,460	3	9.31	3	3.61
17	M	2	Urticaria	1,850	6	>100	1	0.45
18	F	6	Wheeze, urticaria	>16,000	5	57.1	2	2.93

(29,33). This suggests that P34 plays an important role in plant defenses against bacteria. Endotoxin is also recognized as a glycolipid elicitor and can induce defense responses in rice cells, including reactive oxygen generation and defense-gene expression. Both endotoxin and syringolide have considerable similarities in their immunological roles as pathogen-associated molecular patterns (PAMPs) (34). This suggests that P34 also recognizes the endotoxin in soybean in a manner similar to that of the Toll-like receptor 4 (TLR-4), MD-2, and CD14 in the mammalian innate immune systems (34-36). It is of particular interest that a recent report showed that the LPS-binding protein Der p 2 which is one of the major allergens of house dust mites, modulates mouse TLR-4 signaling and is associated with atopic dermatitis (37). Trompette *et al* (37) suggested that lipid-binding proteins possess the intrinsic adjuvant activity that underlies the phenomenon of allergenicity. The endotoxin binding ability of P34 may also be associated with allergic reactions in humans through a similar mechanism.

A previous report has described the linear B cell epitope on P34 (13). However, the conformational B cell epitopes on P34 remain to be fully elucidated. It is desirable to maintain the three-dimensional structure of P34, both for accurate evaluation of T cell responses and for the elucidation of conformational epitopes. High-density SDS may cause conformational changes in proteins. In a previous report, CD analysis of soybean glycinin (11S) and β-conglycinin showed that SDS treatment increased both the α-helical and unordered structures of both proteins at the expense of the β-sheet structure (38). In this case, the relatively low-density SDS treatment ($2x10^{-3}$ M) may have changed the conformation of these proteins. However, in the present study, no significant changes in the secondary structure of P34 occurred after high-density SDS treatment. Estimation of the secondary structure from the recorded CD spectra indicated no significant differences between non-SDS-treated native P34 and SDS-treated rP34 (Fig. 3). This suggests that both of the proteins form a similar folding pattern. Both CD and DLS analyses indicated that rP34 retained its secondary structure even after high-density SDS treatment. Our purified rP34 is useful for the analysis of linear or tertiary epitopes because it retains the tertiary structure of its protein.

We established the method using the ImmunoCAP system with purified rP34 for the first time. Measurement of the concentration of specific IgE antibodies to rP34 is more useful than IgE Western blotting (data not shown). A correlation was found between specific IgE antibodies to soybean and rP34. Patient 16, whose serum with specific IgE antibody to soybean had a relatively low score of 3, showed severe allergic reactions within two hours after soybean ingestion. It is assumed that the allergic reaction in this patient was caused mainly by P34 in soybean. On the other hand, the finding that patients 15 and 17 showed a high score for their serum with specific IgE antibodies to soybean but a low score of 1 for specific IgE antibodies to rP34 may indicate an allergic reaction to soy protein other than P34. These results showed that the rP34 ImmunoCAP system we established could play a role as a soy allergic diagnostic tool or could indicate outgrown allergy.

In conclusion, our proposed new purification method for rP34 did not affect the tertiary protein structure. The strong affinity of P34 for endotoxin/LPS suggests that P34 may modulate the immunological responses underlying allergenicity.

Furthermore, the ImmunoCAP system using our purified rP34 that retains its tertiary structure may be useful for the identification of patients with soybean allergy or for the indication of outgrown allergy.

Acknowledgements

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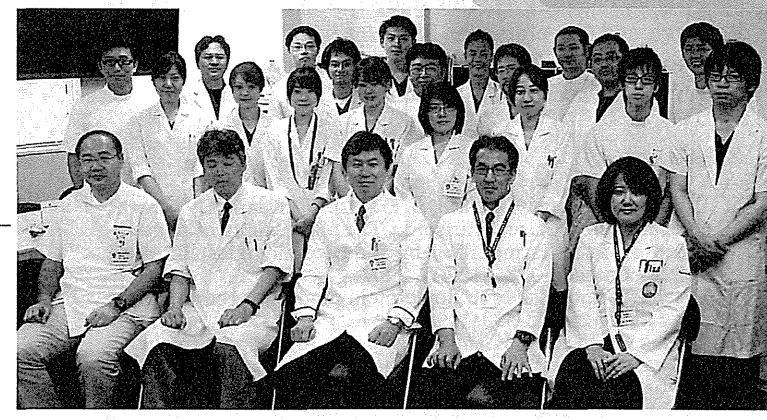
研究会／施設紹介
インタビュー

The Front Line

鹿児島大学大学院
医歯学総合研究科呼吸器内科学
講師 東元 一晃先生
教授 井上 博雅先生



前列左から2人目より順に寒川卓哉医局長，井上博雅教授，東元一晃講師，水野圭子病棟医長．

「喘息死ゼロ作戦」における取り組み ～鹿児島大学呼吸器内科学～

はじめに

鹿児島県はこれまで喘息死（人口10万人当たりの死亡者数）が全国平均の2倍以上と極めて多く，都道府県別でもほぼこの10年常にワースト3位以内という順位で推移していることが知られてきました．われわれはこの状況を打開すべく，2007年から鹿児島大学呼吸器内科系グループを中心に地域医療における喘息診療のネットワーク構築を目指して活動を開始．これらの活動は約5年を経てようやくある一定の成果を上げつつあるようにみえます．

そういった中，2010年6月には鹿児島大学呼吸器内科学教室が開講，それまで3つの内科系講座に分散していた力が結集し，診療，研究，教育などさまざまな問題を解決していく環境が整ってきています．

本稿では鹿児島県における「喘息死ゼロ作戦」の概要を紹介しながら，喘息診療のなかで医療連携や大学の果たす役割について考えてみたいと思います．

鹿児島県の喘息死の状況とその背景

ガイドラインの認知や吸入ステロイド薬を中心とした標準治療の普及により，わが国の年間喘息死亡者数は1995年に7,253人とピークを示した後，順調に減少し，特に2000年以降の約10年間でほぼ半減，近い将来2,000人を下回ることも予想されています（図1）．しかしなが