where the study was performed but could easily be applicable to most health care systems in the Western world. In several societies, most medical expenses are covered or reimbursed by the government, whereas in others the same expenses would be handled by private insurance or the individual directly. Our data show that, no matter what the circumstances, there are appreciable economic incentives in favor of home-based SC-Ig treatment.

As stated in our study, the dose of SC-Ig was equivalent to the dose of IV-Ig, as is done in Europe,[11] whereas US recommendations lean toward a 37% higher dose of SC-Ig.[12] If the latter dosage had been used, it would have led to a 37% increase in the cost of immunoglobulins for the SC-Ig group, which in our model would have resulted in the matching of total expenditures for both treatments. The savings from other medical and nonmedical expenses, in turn, would still have offset this, as we have shown. This is especially relevant because the price of the product itself, which constitutes the bulk of the cost, is likely to change in the future. The benefits from the patient's perspective would also remain, facilitating their tolerability of the treatment and easing the burden for families, which in terms of macroeconomics and health care policies are still of the outmost importance.

With the current efforts to improve efficiency in health care delivery and resource management, this type of pharmacoeconomic study could become an example of how to realistically transform a costly therapy for a chronic illness into a more economical and patient-oriented alternative, especially considering the lasting monetary effect on the health care system, which should be substantial.

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Aberrant glycosylation of IgA in Wiskott-Aldrich syndrome and X-linked thrombocytopenia

To the Editor:

Wiskott-Aldrich syndrome (WAS) is a rare X-linked disorder caused by mutations in the *WAS* gene, and it is characterized by the triad of thrombocytopenia, eczema, and susceptibility to infection.[1] WAS exhibits extensive clinical variability, and the genotypes of patients with WAS are also highly variable.[1] Mutations impairing but not abolishing WAS protein expression can cause X-linked thrombocytopenia (XLT), an attenuated form of WAS with minimal or no immunodeficiency.[1] Autoimmune complications are exceedingly common in WAS/XLT and affect 40% to 70% of the patients according to retrospective cohort studies.[1] Glomerulonephritis is a frequent complication of WAS/XLT. It is found in 3.5% to 19% of the cases and can progress to chronic renal failure requiring renal transplantation.[2] IgA nephropathy (IgAN) has been diagnosed in a majority of patients with WAS/XLT.[2,3]

Previous reports revealed that aberrantly *O*-glycosylated IgA, which exhibits galactose deficiency in the *O*-linked glycans in the hinge region of the heavy chain, may play a pivotal role in the pathogenesis of these IgANs.[4] We have previously reported that *Was*-knockout mice develop proliferative glomerulonephritis reminiscent of human IgAN.[5] We measured serum levels of aberrantly glycosylated IgA in *Was*- knockout mice by using lectin-binding assays with elderberry *(Sambucus nigra)* bark and ricinus communis agglutinin I, which recognize terminal sialic acid and galactose residues.[6] These results indicated that aberrant IgA production may be critically involved in the pathogenesis of glomerulonephritis in these mice.[5] Furthermore, aberrant *O*-glycosylation of serum IgA with characteristics similar to those observed in IgAN was detected in a WAS-carrier patient who presented with Henoch-Schönlein purpura.[6] In this report, we evaluated the role of aberrant IgA production in the development of autoimmunity and glomerulonephritis in patients with WAS/XLT.

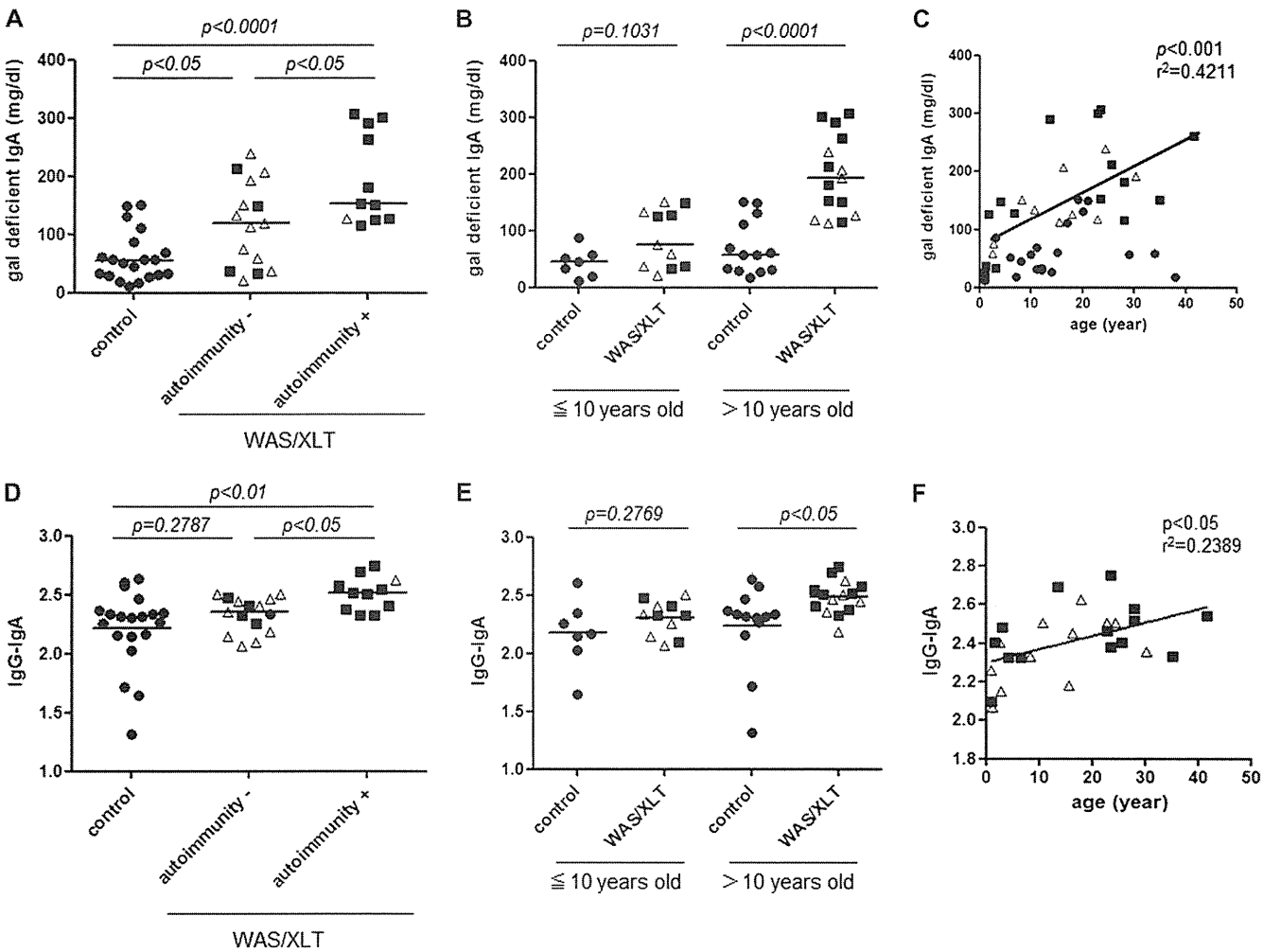

FIG 1. Increased serum galactose-deficient IgA levels and CIC containing IgA and IgG in WAS/XLT. **A,** Serum levels of galactose-deficient IgA. **B** and **C,** Correlation between serum galactose-deficient IgA levels and age. **D,** Serum levels of IgG-IgA CIC. **E** and **F,** The correlation between serum levels of IgG-IgA CIC and age. The *bars* represent the median values. *Open triangles,* XLT; *filled squares,* WAS; *filled circles,* control.

Serum samples were obtained from 26 patients with WAS or XLT and 20 age-matched controls. The clinical characteristics of the patients with WAS/XLT are shown in Table E1 (in this article's Online Repository available at www.jacionline.org). Eleven patients presented with autoimmune complications including IgAN, vasculitis, arthritis, colitis, autoimmune hemolytic anemia, and autoimmune thrombocytopenia.

To examine the terminal galactosylation of IgA molecules, we used lectin-binding assays with *Helix aspersa*, which recognizes terminal *N*-acetylgalactosamine residues[7] (for more information, see the Methods section in this article's Online Repository at www.jacionline.org).

The serum levels of galactose-deficient IgA in patients with WAS/XLT were significantly higher than those in the control group ($P < .001$) (Fig 1, *A*). Interestingly, the levels of galactose-deficient IgA were not significantly higher in patients with WAS than in patients with XLT. However, galactose-deficient IgA levels were significantly higher in patients with WAS/XLT with autoimmune complications than in patients without autoimmune complications ($P < .05$) (Fig 1, *A*). In addition, galactose-deficient IgA levels were significantly higher in patients with WAS/XLT older than 10 years compared with age-matched controls ($P < .0001$) (Fig 1, *B*). In patients with WAS/XLT, we found that galactose-deficient IgA increased in an age-dependent manner (Fig 1, *C*).

Next, levels of circulating immune complexes (CIC) containing IgA and IgG (IgG-IgA CIC) in serum were determined by using sandwich ELISA. These levels were found to be significantly higher in patients with WAS/XLT with autoimmune disorders than in the control group ($P < .01$) (Fig 1, *D*). Notably, IgG-IgA CIC levels were significantly higher in patients with WAS/XLT than in patients without such complications ($P < .05$) (Fig 1, *D*). In addition, IgG-IgA CIC levels were significantly higher in patients with WAS/XLT older than 10 years of age than in age-matched controls ($P < .05$) (Fig 1, *E*). Similar to galactose-deficient IgA levels, the elevation in IgG-IgA CIC levels was age dependent (Fig 1, *F*). Interestingly, IgG-IgA CIC levels were positively correlated with the serum levels of galactose-deficient IgAs (see Fig E1 in this article's Online Repository at www.jacionline.org).

Patient 16 underwent bone marrow transplantation (BMT).[3] After BMT, he achieved full donor chimerism and his clinical

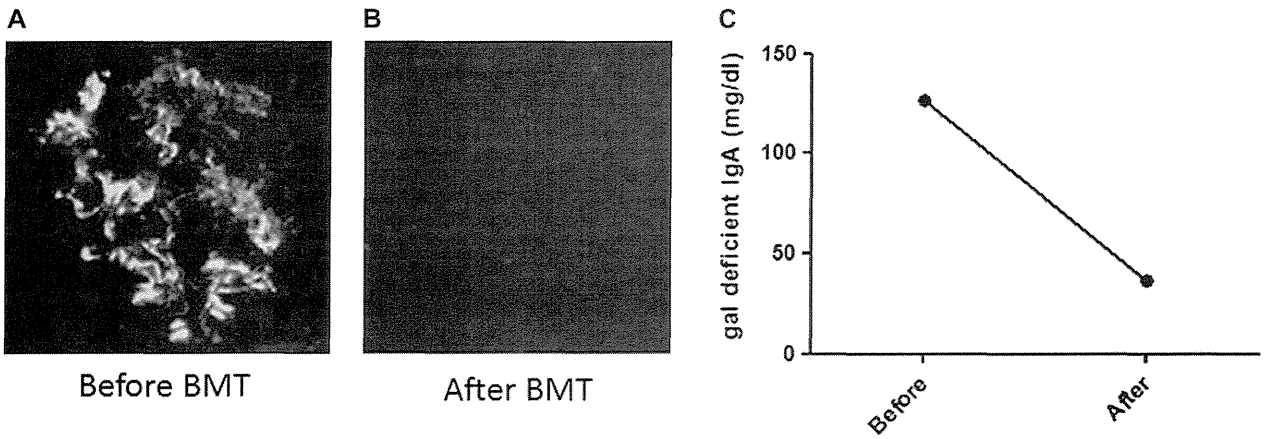

FIG 2. Immunofluorescence study of kidney biopsy specimens and changes in the levels of galactose-deficient IgA in patient 16. **A** and **B,** IgA deposition in mesangial area disappeared after BMT. **C,** The production of galactose-deficient IgA decreased after BMT.

symptoms were improved. Laboratory findings 24 months after BMT were as follows: platelets increased from 8.7×10^9/L to 23.2×10^9/L; serum IgA levels decreased from 264 mg/dL to 131 mg/dL; urinalysis results changed from 3+ proteinuria and sediment containing more than 100 red blood cells/high-power field with casts to normal findings. The IgA-dominant immune deposits in the renal mesangium and glomerular injury significantly improved after BMT (Fig 2, *A*), and the serum levels of galactose-deficient IgA also markedly decreased (Fig 2, *B*).

In this study, we demonstrated that serum galactose-deficient IgA levels and the levels of IgG-IgA CIC increased in patients with WAS/XLT in an age-dependent manner. Interestingly, these levels significantly increased in patients with WAS/XLT with autoimmune manifestations. These findings suggest that increased levels of galactose-deficient IgA with age might be one of the factors contributing to the development of autoimmunity in WAS/XLT. Furthermore, we observed that IgA-dominant immune deposits in the renal mesangium and glomerular injury in a patient with XLT (patient 16) were significantly reduced after BMT. Galactose-deficient IgA levels were also markedly decreased after BMT. These findings indicate that aberrant IgA production in WAS/XLT may result from the immune dysregulation associated with the syndrome and lead to the development of IgAN as one of the several autoimmune complications of WAS that can be corrected with immune reconstitution by BMT.

Some of our patients with WAS/XLT without autoimmune complications showed increased serum levels of galactose-deficient IgA and IgG-IgA CIC. It is possible that increase in IgA and IgG-IgA CIC serum levels precede the clinical presentation of IgAN. Therefore, careful monitoring is considered for these patients.

Altered oligosaccharide biosynthesis in lymphocytes from patients with WAS has been extensively studied[8]; however, the specific mechanisms responsible for the aberrant glycosylation of IgA remain unclear. Previous research suggested the possibility of age-dependent increases in the B-cell populations that produce aberrant IgA.[5] Interestingly, our results showed that aberrant glycosylation of IgA and production of IgG-IgA CIC in patients with WAS/XLT were also increased in an age-dependent fashion. Another possibility is that IgA molecules may be influenced by other factors induced by WAS protein deficiency, including TH_2-dominant changes in cytokine balance.[5,9] Further studies, however, are necessary to validate this hypothesis and clarify the mechanisms responsible for the increased aberrant IgA production in patients with WAS.

Overall, our observations suggest that aberrant IgA production plays a role in the pathogenesis of the renal disease observed in patients with WAS, perhaps through the accumulation of IgA-containing CIC and consequent glomerular injury.

In conclusion, our findings indicate that aberrant IgA production due to mutations in the *WAS* gene may be critically involved in the development of autoimmune-mediated glomerulonephritis. Further studies are required to clarify the specific mechanisms responsible for the aberrant glycosylation of IgA in WAS.

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Antigen-specific T-cell responses in patients with non–IgE-mediated gastrointestinal food allergy are predominantly skewed to T_H2

To the Editor:

IgE-mediated allergy is triggered by cross-linking of antigen-specific IgE antibodies on the cell surfaces of mast cells and basophils, followed by local accumulation and activation of inflammatory cells, including eosinophils and T_H2 cells. T_H2 cells produce such cytokines as IL-4, IL-5, and IL-13, which promote IgE production and eosinophilopoiesis and play central roles in the development of chronic allergic inflammation. On the other hand, non–IgE-mediated allergies, such as hypersensitivity pneumonitis, are considered mediated by cellular immunity, which has not been thought to involve antigen-specific T_H2 cells because IgE antibody would be detected if T_H2 cells were activated. Non–IgE-mediated gastrointestinal food allergies include food protein–induced enterocolitis syndrome (FPIES), food protein–induced proctocolitis, and food protein–induced enteropathy. The precise underlying mechanisms are almost unknown, except for a fundamental role of TNF-α,[1] presumably because this disease entity is relatively rare in incidence and is encountered during infancy in human subjects but not seen in experimental animals. Here, for the first time, we were able to detect antigen-specific T_H2 cell responses in infants with non–IgE-mediated gastrointestinal food allergies by analyzing 89 blood samples collected from all over Japan.

The antigen-specific lymphocyte stimulation test is a classic method for investigating antigen-specific T-cell proliferation and theoretically should be applicable to the study of gastrointestinal food allergies. However, a couple of previous studies demonstrated that the antigen-specific lymphocyte stimulation test was useful, whereas another study found no such usefulness.[2] We hypothesized that this controversy was due to contamination of the antigen preparations with LPS and tested this hypothesis. The limulus amebocyte lysate assay detected high concentrations of LPS in commercially available milk protein preparations, as previously reported (see Table E1 in this article's Online Repository at www.jacionline.org).[3] In addition, significant lymphoproliferative responses were found in the presence of as little as 10 pg/mL LPS (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org), and PBMCs from younger children showed more pronounced lymphoproliferation in response to LPS (see Fig E1, *B*). Therefore we attempted to remove contaminating LPS from milk protein preparations by passing them through a prepacked endotoxin affinity column. However, a high LPS concentration was detected even after that treatment (see Table E1), and therefore we obtained a special β-lactoglobulin preparation with very low contaminating LPS levels (kindly provided by Bean Stalk Snow, Tokyo, Japan). Further studies were performed by using these milk protein preparations, which contained LPS at a final concentration of less than 5 pg/mL.

Next, to elucidate what types of antigen-specific immune responses are induced in patients with gastrointestinal food allergies, we cultured PBMCs from patients and control subjects in the presence and absence of LPS-depleted milk component proteins. The study enrolled 65 patients with gastrointestinal food allergies, 12 patients with IgE-mediated cow's milk allergy (CMA) who showed only nongastrointestinal symptoms on ingestion of milk, and 12 control subjects who showed absolutely no symptoms on ingestion of milk. Table I[4] summarizes the clinical symptoms, clinical diagnosis, and demographic data for the 2 patient groups. None of the patients with gastrointestinal food allergies had detectable levels of IgE against milk proteins in sera. We were unable to recruit infants with IgE-mediated CMA who were age matched with the infants with non–IgE-mediated

TABLE I. Demographic characteristics of the patients

	IgE-mediated CMA		Gastrointestinal food allergies	
	No.		No.	
Age (mo)	12	38.0 (26.5-60.0)	65	2.0 (1.0-4.0)
Male/female sex	12	7/5	65	40/25
Day of onset	12	—	65	32.5 (7.0-115.5)
Symptoms at onset				
Vomiting	12	0% (0/12)	65	53.8% (35/65)
Bloody stool	12	0% (0/12)	65	47.7% (31/65)
Diarrhea	12	0% (0/12)	65	47.7% (31/65)
Failure to thrive	12	0% (0/12)	65	38.4% (22/65)
Lethargy	12	0% (0/12)	65	38.4% (22/65)
Fever	12	0% (0/12)	65	18.5% (12/65)
Eczema	12	100% (12/12)	65	7.7% (5/65)
Wheeze	12	33.3% (3/12)	65	0% (0/65)
Laboratory data				
Milk-specific IgE (IU/mL)	12	56.95 (11.74-90.8)	65	<0.34 (<0.34)
Peripheral blood eosinophils (%)		Not examined	53	7.7 (3.6-13.5)

Data are expressed as medians (interquartile ranges). The inclusion criteria were as follows: (1) gastrointestinal symptoms were present more than 2 hours after ingestion of milk and (2) 3 of Powell's criteria were fulfilled,[4] including (a) switch to therapeutic milk leading to resolution of symptoms, (b) differential diagnosis from other disorders, and (c) verified body weight gain. A definitive diagnosis based on the results of oral food challenge tests that were performed after complete resolution of the initial symptoms was achieved in 19 patients. Patients with gastrointestinal symptoms within 2 hours after ingestion of milk were excluded. On the basis of such symptoms as vomiting, diarrhea, and failure to thrive, the patient group (n = 65) consists of 34 patients with FPIES, 4 patients with food protein–induced enteropathy syndrome (enteropathy), and 27 patients with food protein–induced proctocolitis syndrome (proctocolitis). A definitive diagnosis based on the results of oral food challenge tests was achieved in 13 and 6 patients with FPIES and proctocolitis, respectively. None of the patients underwent endoscopic biopsy.

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METHODS

Patients

Serum samples were obtained from 26 patients with WAS or XLT and 20 age-matched controls (WAS/XLT, 15.5 ± 12.0 years; controls: 14.6 ± 10.0 years). The clinical characteristics of the patients with WAS/XLT are shown in Table E1. Eleven patients presented with autoimmune complications including IgAN, vasculitis, arthritis, colitis, autoimmune hemolytic anemia, and autoimmune thrombocytopenia. This study was approved by the institutional review boards at Kanazawa University and the National Human Genome Research Institute, National Institutes of Health, Bethesda, Md. All specimens were used after the receipt of informed consent.

Detection of IgA-containing CIC

Levels of CIC containing IgA and IgG in serum were determined by using ELISA. Microtiter plate wells were coated with 15 μg/mL F(ab')2 fragment of goat antihuman IgG (Pierce Biotechnology, Rockford, Ill), washed, and blocked with BSA (Sigma Chemical Company, St Louis, Mo) in PBS (pH 7.4) containing 0.05% Tween-20 (BSA-PBS-Tween). Serum samples diluted 100-fold with the same buffer were then used. After 3 hours of incubation at room temperature, test samples were washed with BSA-PBS-Tween and incubated for 1 hour at room temperature with horseradish peroxidase–labeled goat antihuman IgA (Bethyl Laboratories, Inc, Montgomery, Tex) diluted 1:10,000. Samples were incubated with TMB substrate (Bethyl Laboratories, Inc) after washing with the same buffer, and the optical density was then measured at 450 nm.

Measurement of lectin-binding serum IgA levels

To examine the terminal galactosylation of IgA molecules, lectin-binding assays were designed by using *Helix aspersa* (HAA) (Vector Laboratories, Burlingame, Calif), which recognizes terminal *N*-acetylgalactosamine residues (24,25). Microtiter plates coated with 15 μg/mL F(ab')2 fragment of goat antihuman IgA (Jackson ImmunoResearch Labs, West Grove, Pa) were incubated with 100 μL of diluted (100 μg/mL) serum samples. In order that the HAA can recognize *N*-acetylgalactosamine, the terminal neuraminic acid was removed by incubation with 100 μL of 20 mU/mL neuraminidase (Roche Diagnostic Corp, Indianapolis, Ind) in acetate buffer (pH 5) for 3 hours at 37°C. After washing, 100 μL of biotin-labeled *N*-acetylgalactosamine-specific lectin HAA (2 μg/mL, Sigma) was added to each well and incubated for 3 hours at 37°C. Peroxidase-conjugated streptavidin (0.1 μg/mL; Pierce, Rockford, Ill) was added, and the amount of lectin bound was measured. Total IgA levels were also measured by using horseradish peroxidase–labeled goat antihuman IgA diluted 1:10,000. The ratio of the absorbance at 450 nm between IgA bound to HAA and total IgA (HAA/IgA ratio) was calculated. The amount of galactose-deficient IgA in serum at the time of blood drawing was estimated by using the following formula: serum IgA concentration × HAA/IgA ratio.

Statistical analysis

The results are expressed as mean ± SD. Statistical significance was determined by using the Mann-Whitney test. Differences were considered significant if the *P* value was less than .05.

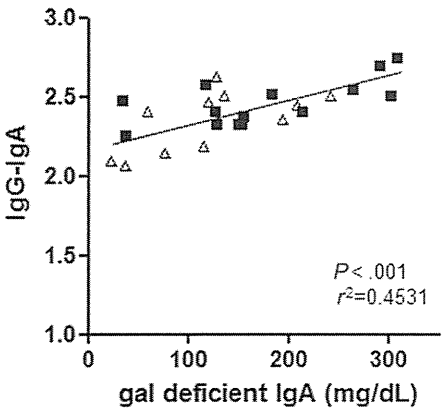

FIG E1. Positive correlation between serum levels of IgG-IgA CIC and galactose-deficient IgAs.

TABLE E1. Clinical characteristics of the patients with WAS/XLT

Patient	Age (y)	Sex	Diagnosis	Autoimmune diseases
1	1	Male	XLT	No
2	2	Male	XLT	No
3	24	Male	XLT	No
4	10	Male	XLT	No
5	22	Male	XLT	No
6	16	Male	XLT	No
7	30	Male	XLT	No
8	2	Male	XLT	No
9	8	Male	XLT	No
10	1	Male	XLT	No
11	15	Male	XLT	No
12	25	Male	WAS	No
13	3	Male	WAS	No
14	4	Male	WAS	No
15	1	Male	WAS	No
16	18	Male	XLT	IgA nephropathy
17	23	Male	WAS	Autoimmune hemolytic anemia
18	6	Male	WAS	Autoimmune thrombocytopenia
19	22	Male	WAS	Systemic vasculitis
20	28	Male	WAS	IgA nephropathy
21	13	Male	WAS	Colitis
22	23	Male	WAS	IgA nephropathy
23	1	Male	WAS	Vasculitis
24	41	Male	WAS	Arthritis
25	28	Male	WAS	IgA nephropathy
26	35	Male	WAS	IgA nephropathy

Pediatr Blood Cancer 2013;60:836–841

Wiskott–Aldrich Syndrome Presenting With a Clinical Picture Mimicking Juvenile Myelomonocytic Leukaemia

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Background. Wiskott–Aldrich syndrome (WAS) is a rare X-linked immunodeficiency caused by defects of the WAS protein (*WASP*) gene. Patients with WAS typically demonstrate micro-thrombocytopenia. ***Procedures.*** The report describes seven male infants with WAS that initially presented with leukocytosis, monocytosis, and myeloid and erythroid precursors in the peripheral blood (PB) and dysplasia in the bone marrow (BM), which was initially indistinguishable from juvenile myelomonocytic leukaemia (JMML). ***Results.*** The median age of affected patients was 1 month (range, 1–4 months). Splenomegaly was absent in four of these patients, which was unusual for JMML. A mutation analysis of genes in the RAS-signalling pathway did not support a diagnosis of JMML. Non-haematological features, such as eczema (n = 7) and bloody stools (n = 6), ultimately led to the diagnosis of WAS at a median age of 4 months (range, 3–8 months), which was confirmed by absent (n = 6) or reduced (n = 1) WASP expression in lymphocytes by flow cytometry (FCM) and a *WASP* gene mutation. Interestingly, mean platelet volume (MPV) was normal in three of five patients and six of seven patients demonstrated occasional giant platelets, which was not compatible with WAS. ***Conclusions.*** These data suggest that WAS should be considered in male infants presenting with JMML-like features if no molecular markers of JMML can be detected. Pediatr Blood Cancer 2013;60:836–841.

Key words: children; juvenile myelomonocytic leukaemia; Wiskott–Aldrich syndrome

INTRODUCTION

Wiskott–Aldrich syndrome (WAS) is a rare X-linked recessive disorder, characterized by micro-thrombocytopenia, eczematous skin disease, and recurrent infections. The incidence of WAS is 1–10 in 1 million male new-borns. Affected patients have a predisposition to autoimmune diseases and lymphoid malignancies [1,2]. The responsible gene is *WASP*, which encodes the 502 amino acid WASP protein [3]. WASP is expressed selectively in hematopoietic cells and is involved in cell signalling and cytoskeleton reorganization [3]. Specific types of defects in WASP are often but not invariably associated with the severity of disease and clinical phenotype. Lack of WASP expression causes the most severe phenotype (i.e., classic WAS), whereas inactivating *WASP* missense mutations allow residual protein expression and can cause less severe X-linked thrombocytopenia (XLT) [4,5]. Gain-of-function mutations generate X-linked neutropenia (XLN) [6,7].

Juvenile myelomonocytic leukaemia (JMML) is a rare disease in children that occurs with an estimated incidence of 1–2 cases per million [8]. JMML has characteristics of both myelodysplastic syndrome (MDS) and myeloproliferative disorders (MPD) and is categorized in the MDS/MPD category in the World Health Organization (WHO) classification [9–11]. Clinical and haematological manifestations of JMML include hepatosplenomegaly, skin rash, lymphadenopathy, leukoerythroblastosis, monocytosis, and thrombocytopenia. Recent studies show that deregulated activation of the RAS/MAPK signalling pathway plays a central role in the pathogenesis of JMML. Gene mutations in either the *RAS*, *PTPN11*, *NF1*, or *CBL* genes involved in this pathway are detected in about 80% of JMML patients [12–18].

Micro-thrombocytopenia is the key haematological finding in patients with WAS. However, myelopoiesis and erythropoiesis are usually not affected, despite the fact that WASP is expressed in various hematopoietic cells [19]. The present report describes seven cases of male infants with classical WAS who demonstrated haematological abnormalities mimicking JMML. Importantly, patients can present with JMML-like features before the full clinical manifestations of WAS become apparent. Moreover, nor-

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mal mean platelet volume (MPV) and the presence of the giant platelets complicated the diagnostic evaluation in some of our patients.

PATIENTS AND METHODS

Patients

In 2007, we described a case of a male patient (patient #1) with WAS who demonstrated JMML-like clinical features [20]. Briefly, thrombocytopenia was detected shortly after birth. He suffered from bloody diarrhoea from the age of 9 days. At the age of 42 days, leukocytosis with myeloid/erythroid precursors and monocytosis was detected. Bone marrow (BM) aspirates showed hypercellularity with significant predominance of myelopoiesis and dysplastic features. The morphological features were compatible with JMML. Subsequently, the white blood cell (WBC) count increased to 52.0×10^9/L with the appearance of peripheral blasts (3%) and persistent fever. Intravenous administration of various antibiotics had no effect on fever and leukocytosis. Oral 6-mercaptopurine (6-MP) was administered, which resulted in disappearance of leukocytosis. Positive results of cytomegalovirus (CMV)-IgM/IgG and a low level pp65 CMV-antigen (Ag) cells were transitionally noted without CMV-related symptoms. Intravenous administration of ganciclovir (GCV) led to the elimination of CMV-Ag but not to any improvement of JMML-like features. At the age of 7 months, mild atopic dermatitis-like eczema was recognized, which finally led to the clinical and molecular diagnosis of WAS.

The MDS committee of the Japanese Society of Paediatric Hematology/Oncology (JSPHO) study coordinating center of the European Working Group of MDS in Childhood (EWOG-MDS) perform the morphological review of peripheral blood (PB) and BM smears and laboratory examinations for the diagnosis of JMML in Japan and Germany, respectively. By January 2011, WAS was diagnosed in six Japanese males (including patient #1) and one German male who were initially referred with a suspected diagnosis of JMML. Patient #4 was recently reported [21]. Approval for the study was obtained from the institutional review board of Nagoya University, Nagoya, Japan, and University of Freiburg, Freiburg, Germany. Informed consent was provided by parents according to the Declaration of Helsinki.

Diagnostic Tests for Wiskott–Aldrich Syndrome

Intracellular WASP expression in lymphocytes was analysed by flow cytometry (FCM) by the standard method described previously [4,22]. DNA purification and sequencing of genomic DNA, RNA isolation, reverse transcription-polymerase chain reaction, and sequencing of cDNA for the mutational analysis of *WASP* gene was performed as reported previously [23].

Diagnostic Tests for Juvenile Myelomonocytic Leukemia

Mutational screening for *PTPN11*, *NRAS*, and *KRAS* genes was performed in six patients, as previously reported [24–27]. In patients #6 and #7, the c-CBL gene, which has been recently found in about 10% of JMML patients, was also screened as described previously [16,18]. None of the patients had clinical signs of neurofibromatosis type 1 (NF1). *In vitro* colony assay for granulocyte–macrophage colony stimulating factor (GM-CSF) hypersensitivity assay was performed as a supportive diagnostic tool for JMML as previously reported [28,29].

RESULTS

Clinical Characteristics and Laboratory Findings

The clinical characteristics of these patients are summarized in Table I. Thrombocytopenia and bloody diarrhoea were observed soon after birth in all patients except for patient #6. JMML-like clinical manifestations occurred within the first few months of life. Eczema developed between 0 and 3 months after birth in all patients. Splenomegaly was seen in three of seven patients and massive splenomegaly was present in two patients. At the presentation of JMML-like features, episodes of recurrent infections, which suggest an immunodeficiency, were not observed in any patients. However, in three patients, recurrent bacterial, or viral infections (cases #5, #6, and #7) were documented during the clinical course.

The laboratory findings at the presentation of JMML-like disease are summarized in Table II. The WBC count was increased in all patients except for in patient #7. Monocytosis and myeloid/erythroid precursors were seen in PB in all patients. All patients had anaemia. The MPV before platelet transfusions ranged between 6.9 and 7.9 fl (normal, 7.2–11.7 fl) in the five patients that were evaluated. Hb F levels were normal in three patients examined. The platelet morphology demonstrated anisocytosis in all patients. Occasional giant platelets, which are defined as platelets bigger than red cells, were observed in six patients. These features were unusual for WAS. Full BM with significant predominance of myelopoiesis and a marked left shift of the myeloid lineage was seen in all patients. The number of megakaryocytes was normal or increased. Dysplasia in megakaryopoiesis, myelopoiesis, and erythropoiesis was observed in seven, four, and four patients, respectively. The common dysplasia in the megakaryopoiesis included hypolobulations of nuclei and small megakaryocytes with single or double round nuclei. In the myelopoiesis, nuclear abnormalities such as double nuclei, ring nuclei, or pseudo-Pelger–Huet anomaly nuclei were often seen. The dysplasia of erythropoiesis was mild, if observed, and included nuclear lobulation and double nuclei. The karyotype was normal in all patients. The serum levels of immunoglobulin were variable (Table II). Evaluation of T cell function revealed normal responses to phytohemagglutinin and concanavalin A in the four patients that were examined. The numbers of peripheral T and B cells and the CD4/8 ratio were normal in four patients. Patient #7 demonstrated B-lymphocytopenia and an elevated CD4/8 ratio.

Diagnostic Tests for Juvenile Myelomonocytic Leukemia

Molecular analysis of *PTPN11*, *N-RAS*, and *K-RAS* genes (n = 7) and the c-*CBL* gene (n = 2) documented no mutations in any of the examined patients. *In vitro* GM-CSF hypersensitivity was performed in all patients but patient #1 and was positive only in patient #4.

Diagnostic Tests for Wiskott–Aldrich Syndrome

FCM analysis showed absent (n = 6) or reduced (n = 1) WASP expression in the lymphocytes, which led to the confirmation of a diagnosis of WAS (Table III). Mutations of *WASP* genes

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TABLE I. Clinical Features of the Patients

Patient	1	2	3	4	5	6	7
Age at the detection of thrombocytopenia	At birth	At birth	At birth	At birth	1 month	4 months	2 months
Age at the onset of JMML like haematological features	1 month	3 months	1 month	1 month	1 month	4 months	2 months
Age at the onset of eczema	1 month	3 months	Soon after birth	3 months	1 month	3 months	2 months
Age at the onset of bloody diarrhoea	At birth	20 days	At birth	1 week	1 month	No	1 month
Hepatomegaly/splenomegaly (cm under the costal margin)	Yes (3)/no	Yes (3)/yes#	No/no	No/no	No/no	Yes (5)/yes (7.5)	Yes (6)/yes (6)
Infectious episodes before the diagnosis of WAS	CMV antigenemia	No episode	No episode	No episode	Fever of unknown origin	Otitis media	Adenovirus and Rotavirus in stool
Infectious episodes between the diagnosis of WAS and HSCT	No episode	No episode	No episode	No episode	Bacterial and RSV pneumonia Rotavirus gastroenteritis	Otitis media Anal abscess	CMV pneumonia
HSCT (age)	10 months	10 months	17 months	4 months	18 months	13 months	7 months
Donor/stem cell source	U-CBT	MSD-BMT	U-CBT	MSD-BMT	1 antigen MMUD-BMT	MUD-BMT	MUD-BMT
Survival (age at the time of the last follow-up)	Alive (6 years 5 months)	Alive (5 years 4 months)	Alive (4 years 8 months)	Alive (12 months)	Alive (1 year 9 months)	Alive (1 year 6 months)	Alive (1 year 7 months)

JMML, juvenile myelomonocytic leukaemia; WAS, Wiskott–Aldrich syndrome; RSV, respiratory syncytial virus; CMV, cytomegalovirus; # splenomegaly was noted only by ultrasound; HSCT, hematopoietic stem cell transplantation; U-CBT, unrelated cord blood transplantation; MSD-BMT, bone marrow transplantation from an HLA matched sibling donor; MUD-BMT, BMT from an HLA matched unrelated donor; MMUD-BMT, BMT from an HLA-mismatched unrelated donor.

TABLE II. Laboratory Findings Accompanying the Juvenile Myelomonocytic Leukaemia-Like Haematological Features

Patient	1	2	3	4	5	6	7
Peripheral blood							
WBC count ($\times 10^9$/L)	35.5–50.0	12.0–18.0	13.5–22.1	15.0	35.0–50.0	6.0–12.0	7.5
Monocyte count ($\times 10^9$/L)	8.9	1.0–1.5	8	2.3	1.1	1.0–1.5	1.3
Blasts (%)	3	2	2	4	2	0	1
Immature myeloid/erythroid cells	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes	Yes/Yes
Eosinophils (%)	3	12	4	7	2	5	2
Platelet count ($\times 10^9$/L)	44	40–90	31	24	53	11	26
MPV (fl)[a]	7.0	7.4	NE	6.9	7.5	NE	7.9
Platelet anisocytosis/giant platelets	Yes/Yes	Yes/Yes	Yes/Yes	Yes/No	Yes/Yes	Yes/Yes	Yes/Yes
Hb (g/dl)	8.9	8.0	9.2	6.1	11.6	9.5	8.0
Bone marrow							
Cellularity	Full[b]	Full	Full	Full	Full	Full	Full
M/E ratio	33	4	7	5.4	11	2	2
Blasts (%)	3.5	0.5	1	0	2	3.5	2
Karyotype	46,XY	46,XY	46,XY	46, XY	46, XY	46,XY	46,XY
Immunological examination							
Age at examination (months)	8	5	2	2	10	4	2/3/5
IgG (mg/dl)	2,554	468	638	102	792	3,780	1,170/2,120/2,070
IgM (mg/dl)	156	64	37	<5	33	353	122/244/156
IgA (mg/dl)	49	52	38	39	129	124	25/45.4/58.2
IgE (mg/dl)	494	368	89	8	16	1,330 (10 months)	258/693/7,995
LBT (PHA, ConA)	Normal	Normal	NE	NE	NE	Normal	Normal
CD4/8 ratio	Normal	Normal	NE	Normal	NE	Normal	Increased (7.0/22.2/1.1)

WBC, white blood cell; MPV, mean platelet volume; M/E myeloid-/erythroid-cells; LBT, lymphoblastic test; PHA, phytohemagglutinin; conA, concanavalin A; NE, not evaluated. [a]Normal range (7.2–11.7 fl). [b]The cellularity was high (full bone marrow), which was normal for infants.

varied between patients. In patient #1, sequencing of *WASP* cDNA identified five nucleotides (CCGGG) inserted at position c.387 in exon 4, causing a frameshift at codon 140 that gave rise to a premature stop signal at codon 262, as reported previously [20]. Patients #2 and #3 had previously known nonsense mutations in exon 1 and exon 4, which led to the absence of WASP expression and a moderate to severe clinical phenotype of WAS [4,30–32]. Patient #4 had a known deletion in intron 8, which cause a frameshift and absence of WASP expression [4,5]. Patient #5 had a known splice anomaly in intron 6, which reduced expression of WASP and led to a clinical phenotype of either XLP or WAS [4,32]. Patient #6 had known deletion in exon 1, which was associated with a classic WAS phenotype [33]. Patient #7 had a nonsense mutation in exon 1, which has not been previously described.

Clinical Course of Patients

Patient #1 received 6-MP to control leukocytosis. In other patients, the JMML-like features were stable until allogeneic hematopoietic stem cell transplantation (HSCT), which was performed at the age of 4–18 months. All patients are alive after HSCT at the time of the last follow-up (Table I). Graft failure was observed in patient #7, and a second HSCT is currently planned for this patient.

DISCUSSION

Although WASP is expressed ubiquitously in hematopoietic cells and although *in vitro* results suggest that WASP is involved in the proliferation and differentiation of all hematopoietic progenitors, overt defects are restricted to micro-thrombocytopenia and immune-dysfunction in classical WAS. We previously described a case of a male presenting with a clinical picture of JMML, in whom WAS was ultimately diagnosed (patient #1) [20]. These haematological abnormalities had not been previously reported in patients with WAS. Since then, we have encountered six additional patients with WAS who presented with similar clinical characteristics. Morphological features were not distinguishable from JMML. Moreover, normal MPV and the presence

TABLE III. Results of the Diagnostic Tests for Wiskott–Aldrich Syndrome

Patient	1	2	3	4	5	6	7
Age at examinations	8 months	4 months	4 months	3 months	8 months	4 months	3 months
WASP protein expression	Absence	Absence	Absence	Absence	Reduced	Absence	Absence
WASP mutation	Exon 4 c.387–421 ins 5nt	Exon 1 c.37C>T	Exon 4 c.424C>T	Intron 8 c.777+1_+4 delGTGA	Intron 6 c.559+5G>A	Exon1 c.31delG	Exon 1 c.C55>T
Mutation type	Insertion	Nonsense	Nonsense	Deletion	Splice anomaly	Deletion	Nonsense
Predicted protein change	Frameshift stop aa 262	R13X	Q142X	Frameshift stop aa 246	Frameshift stop aa 190	Frameshift stop aa 37	Q19X

of giant platelets in three and six patients, respectively, initially argued against a diagnosis of WAS, because micro-thrombocytes are known as a key diagnostic feature of WAS and XLP. The JMML-like features developed shortly after birth in all patients, before the full clinical picture of WAS become apparent. In our patients with JMML-like features, signs of immune defects were not present. Without recent advances in molecular diagnostic tests for WAS and JMML, it might otherwise be impossible to establish a diagnosis of WAS in these patients. Absent or reduced WASP expression by FCM–WASP and detection of *WASP* mutation ultimately led to a diagnosis of WAS. The mutations were distributed in different exons and introns, and there was no clustering. Thrombocytopenia since birth and some of the observed clinical features (e.g., atopic dermatitis-like eczema, persistent bloody stool, lack of splenomegaly) were unusual for JMML but were compatible with WAS.

The deregulated RAS signalling pathway plays a central role in the pathogenesis of JMML, and mutational analyses of *PTPN11*, *RAS*, and *c-CBL* genes located in the RAS signalling pathway have become important diagnostic tests. Mutations of one of these genes and a clinical diagnosis of NF1 can be found in more than 80% of patients with JMML. However, in up to 20% of patients without any molecular markers, a diagnosis of JMML relies on unspecific clinical and laboratory observations. We suggest that WAS should be considered within the differential diagnosis in male infants with clinical features of JMML if no mutations of the RAS signalling pathway can be detected. Importantly, clinicians should not exclude a diagnosis of WAS if the MPV is normal or if giant platelets are present. Rarely, patients with WAS can present with normal or large platelets [34,35].

The pathogenesis of JMML-like feature in these patients is unknown. There is no evidence that WASP is related to the RAS signalling pathway. The activation of this pathway does not seem to be a major cause of JMML-like features in our patients, because GM-CSF hypersensitivity was demonstrated only in one of six patients examined. Patients with WAS have an increased risk of viral infections. CMV, Epstein–Barr virus (EBV) and human herpes virus-6 (HHV-6) infections can mimic JMML in infants [36,37]. However, extensive screening failed to detect viral infections at the time, at which these patients presented with JMML-like features, except for patient #1, in whom CMV antigen was detected.

Leukocyte adhesion deficiency (LAD)-1 is a rare immunodeficiency caused by a mutation in the beta-2 integrin gene. The firm adhesion of leukocyte to the blood vessel wall is defective in LAD-1, which results in leukocytosis, mimicking JMML [38]. A defect of leukocyte adhesion due to abnormal integrin beta clustering has been described in the context of WAS [39]. A mechanism similar to that seen in LAD1 may be present in WAS with JMML-like features.

A recent report showed that WASP localizes to not only the cytoplasm but also to the nucleus and has a role in the transcriptional regulation at the chromatin level in lymphocytes [40]. Active *WASP* mutations, which cluster within the GTP-ase binding domain of WASP (L270P, S272P, and I294T), cause XLN and myelodysplasia [6,7]. Further, increased apoptosis associated with increased genomic instability in myeloid cells and lymphocytes has been described in the context of active *WASP* mutations [41,42]. Further research may identify new roles of WASP in transcriptional regulation and genomic stability in haematopoiesis, which may explain the JMML-like features, seen in WAS patients.

In conclusion, WAS should be considered in the differential diagnosis in male infants presenting with JMML-like features if no molecular markers of JMML can be demonstrated. A normal MPV and the presence of giant platelets do not exclude a diagnosis of WAS. Clinical information, such as bloody stool and eczema, may be helpful in pursuing a diagnosis of WAS in an infant with JMML like features.

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CASE REPORT

A novel Wiskott–Aldrich syndrome protein mutation in an infant with thrombotic thrombocytopenic purpura

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Abstract

Thrombotic thrombocytopenic purpura (TTP) has not yet been reported to be associated with mutations in the Wiskott–Aldrich syndrome (WAS) gene. WAS is an X-linked recessive disorder characterized by thrombocytopenia, small platelet size, eczema, recurrent infections, and increased risk of autoimmune disorders and malignancies. A broad spectrum of mutations in the WAS protein (WASP) gene have been identified as causing the disease. In this study, we report on a 2-month-old Japanese boy who presented with cytomegalovirus (CMV) infection and TTP. The activity of von Willebrand factor cleaving metalloproteinase, ADAMTS13 was low and the antibody against ADAMTS13 was positive (3.6 Bethesda U/mL). Although TTP was improved by plasma exchange and steroid pulse therapy, thrombocytopenia persisted and regular transfusions of irradiated platelets were needed. Tiny platelets were found on a peripheral blood smear. CMV genome was positive in peripheral blood by polymerase chain reaction and the CMV viremia continued to persist despite intravenous gancyclovir therapy. Through direct sequencing of genomic DNA of the WASP gene in the patient, we identified a novel mutation of WASP gene: the seventh nucleotide in exon 11 (G) had been deleted (1345delG). This mutation causes a frameshift and a stop codon at amino acid 470. Western blotting demonstrated a truncated WAS protein. To our knowledge, this is the first report describing TTP in WAS patients with novel mutation in the WASP gene.

Key words Wiskott-Aldrich syndrome; thrombotic thrombocytopenic purpura; autoimmunity

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Wiskott–Aldrich syndrome (WAS) is a rare X-linked disorder with variable clinical phenotypes that correlate with the type of mutations in the WAS protein (WASP) gene (1). The WASP gene is composed of 12 exons containing 1823 base pairs and encodes a 502-amino acid protein that appears to be of central importance for the function of hematopoietic stem cells (2). Mutations of WASP gene are located throughout the gene, although some hot spots have been identified (3). The type of mutation strongly influences the clinical severity of WAS (3). Mutations that abolish WASP expression are mainly associated with a severe clinical phenotype (full blown WAS) and a life expectancy below 20 yr of age (4). Mutations, on the other hand, result in residual expression of a full-length point-mutated WASP, are often associated with X-linked thrombocytopenia (XLT) (5), corresponding to a longer life expectancy (6). A scoring system based on clinical symptoms has been developed to differentiate these distinct clinical phenotypes caused by WASP gene mutations (2, 3, 7). Autoimmune complications are frequently observed in WAS and patients who develop autoimmune diseases are assigned to a high-risk group with poor prognosis (1). The incidence of autoimmunity in WAS is high in the US and European populations (40–72%), whereas a lower incidence was reported in Japan (22%)

(1, 6). The most common autoimmune manifestation in WAS is hemolytic anemia (36%), followed by vasculitis (including cerebral vasculitis; 29%), arthritis (29%), neutropenia (25%), inflammatory bowel disease (9%), and IgA nephropathy (3%) (8). Henoch–Schönlein purpura, dermatomyositis, recurrent angioedema, and uveitis have also been reported in some patients (6, 9). Moreover, in some cases, multiple autoimmune manifestations are observed.

Autoimmune hematological diseases are characterized by the production of antibodies against blood proteins and cells, and comprise immune thrombocytopenia, autoimmune hemolytic anemia, acquired hemophilia, and thrombotic thrombocytopenic purpura (TTP). TTP is a rare but severe disease characterized by mechanical hemolytic anemia and consumptive thrombocytopenia leading to disseminated microvascular thrombosis that causes signs and symptoms of organ ischemia and functional damage. von Willebrand factor (vWF) is synthesised in endothelial cells and assembled in larger multimers that are present in normal plasma. The larger multimers, called unusually large vWF (ULvWF), are rapidly degraded in the circulation into the normal size range vWF multimers by a specific vWF-cleaving protease, ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motif 13) (10). ADAMTS13 deficiency leads sequentially to the accumulation of ULvWF multimers, platelet aggregation and platelet clumping, which is characteristic of the disease. ULvWF multimer accumulation in TTP is associated with absent or markedly diminished ADAMTS13 activity due to an inherited or acquired deficiency (11). An inhibitory autoantibody to the ADAMTS13 metalloproteinase has been found in patients with acquired TTP (11).

Here, we report a male infant who presented with cytomegalovirus (CMV) infection and acquired TTP which led to the diagnosis of WAS. A novel mutation, one nucleotide deletion at position 1345 (1345delG) in exon 11 was identified. To our knowledge, this is the first report regarding WAS with TTP as an autoimmune disease.

Materials and methods

Flow cytometric analysis of WASP expression

Intracellular staining with anti-WASP mAb was performed as described by Kawai *et al.* (12) In brief, peripheral blood mononuclear cells (PBMCs) from both a healthy control and the patient were first fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and stained with phycoerythrin (PE)-labeled CD3 (PharMingen, San Diego, CA, USA), CD19 (Beckman Coulter, Fullerton, CA, USA), or CD56 (PharMingen) mAb. Then cells were permeabilized in 0.1% Triton X-100 in Tris-buffered saline (pH 7.4) with 1% fetal calf serum (FCS) and 0.1% NaN_3 for 5 min. Subsequently, these cells were reacted with 10 mg/mL of anti-WASP (5A5) (12) or isotype-matched control mouse IgG2a mAb (PharMingen) for 20 min on ice, washed, and then incubated with 10 mg/mL of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG2a antibody (Southern Biotechnology Associates, Birmingham, AL, USA). The stained cells were immediately analyzed on an EPICS XL (Beckman Coulter).

Anti-WASP antisera and Western blot analysis

B-Lymphoblatoid cell lines (B-LCLs) were established by inoculating PBMCs from healthy controls and the patient with Epstein–Barr virus (EBV) – containing supernatant (6). B-LCLs from healthy control and the patient were suspended at 1.0×10^7/mL in lysis buffer containing 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5% aprotinin, and 10 μg/mL leupeptin at pH 7.5 and were kept on ice for 30 min. From each sample, 40 μg total protein was loaded onto a sodium dodecyl sulfate polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membranes were incubated with rabbit anti-WASP antibody (Ab 503) against a synthetic peptide (aa's 209–226 of WASP) (6) at 1 : 5000 dilutions. The membranes were incubated with alkaline phosphatase-conjugated goat antirabbit immunoglobulin (Promega, Madison, WI, USA). Results were visualized by incubation with AP buffer (100 mM Tris–HCl, pH 9.5; 100 mM NaCl; and 5 mM $MgCl_2$).

DNA purification and sequencing of genomic DNA

Genomic DNA was extracted from the patient's PBMCs using Sepa-Gene (Seikagaku kogyo, Tokyo, Japan). Purified genomic DNA samples were amplified with primer pairs designed to span each exon and exon/intron junction, and the specific causative mutation was identified by direct sequencing as described previously (6). For gene sequencing, informed consent by the patient's family and approval by institutional review boards was obtained.

Patient and results

The patient was the first son of healthy and non-consanguineous Japanese parents, born at term following an uncomplicated pregnancy, and his body weight at birth was 2888 g. His past medical history was unremarkable. At the age of 2 months, he presented with fever, intermittent tachypnea, and general petechiae. On examination, he looked pale and icteric. He had hepatosplenomegaly, but did not have lymphoadenopathy or eczema. Peripheral blood analysis disclosed severe anemia and thrombocytopenia with hemoglobin (Hb) of 3.9 g/dL (normocytic), reticulocytes of 37.8% and platelet count of 11×10^9/L. The mean platelet volume was 5.8–8.1 fL (normal range, 9.0–10.7 fL) and morphology

showed small platelets. White blood cell count (WBC) was 12.3 × 10^9/L. Laboratory investigations revealed the following: serum total bilirubin (T-bil) 3.5 mg/dL (indirect 2.4 mg/dL), lactate dehydrogenase (LDH) 3264 IU/L, aspartate aminotransferase (AST) 210 IU/L, alanine aminotransferase (ALT) 73 IU/L, gamma-glutamyltranspeptidase (γGTP) 257 IU/L, blood urea nitrogen (BUN) 12 mg/dL and creatinine (Cre) 0.22 mg/dL. His prothrombin time, activated partial thromboplastin time and fibrinogen were normal. D-dimer was 7.8 μg/mL (normal range, 0–0.5 μg/mL) and haptoglobin was 8.9 mg/dL with a negative Coombs' test. Furthermore, peripheral blood smears showed fragmented red blood cells. Urinalysis revealed microscopic hematuria.

The patient was diagnosed as having TTP and treated with steroid pulse and plasma exchange (PE) therapy (40 mL/kg/d) for six consecutive days. The patient responded with elevations in the Hb to 8.0 g/dL. LDH decreased to 600 IU/L. Further serum analysis on admission showed a noticeable decrease in ADAMTS13 activity to <0.5% (normal, 70–120%), with the existence of anti-ADAMTS13 IgG autoantibody. Anti-ADAMTS13 IgG autoantibody was evaluated with the chromogenic ACT enzyme-linked immunosorbent assay (ELISA) with the Bethesda method in the Department of Blood Transfusion, Nara Medical University. One Bethesda unit is defined as the amount of inhibitor that reduces the enzymatic activity by 50% of the control value, and values >0.5 U/mL are considered significant (13, 14). Our patient showed markedly decreased ADAMTS13 activity (<0.5%) and tested positive for anti-ADAMTS13 IgG autoantibody (3.6 Bethesda U/mL) at the onset of TTP.

Viral serology study showed a positive result for CMV IgM. CMV was subsequently identified by a urine shell vial culture method and a plasma polymerase chain reaction test for CMV (PCR-CMV) demonstrated significant viremia with 7.0 × 10^5 copies/mL. Administration of intravenous ganciclovir (10 mg/kg/d) was initiated. Gancyclovir therapy was continued until viral loads were stable at around 1000 copies/mL and did not seem to further decline. His platelet counts, however, did not rise and the child required repeated platelet transfusions. A trial of intravenous immunoglobulin (IVIG) as well as a trial of systemic prednisone failed to induce a rise in platelet counts. Antiplatelet antibodies were negative. He also developed several episodes of gastroenteritis due to norovirus and methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia secondary to soft tissue infection or pneumonia, despite the monthly administration of prophylactic treatment with intravenous immunoglobulin. The presence of thrombocytopenia, small sized platelets, frequent potentially life-threatening infections and autoimmune disease led to the consideration of WAS. WASP expression was examined by flow cytometric analysis of intracellular WASP expression and a reduced expression level was detected (Fig. 1A). Western blot analysis of lysates from the normal control showed that WASP was normally expressed (66 kDa), but a truncated WASP was expressed in the patient (Fig. 1B). Sequencing of WASP genomic DNA identified a one-nucleotide (G) deletion at the position of exon 11, that cause a frameshift, resulting in the generation of a premature stop signal at codon 470 (Fig. 1C and 1D). This mutation has not been previously described. Immunological analysis of peripheral blood revealed normal percentages and numbers of $CD3^+$ T cells (1.35 × 10^9 cells/L), $CD19^+$ B cells (0.85 × 10^9 cells/L) and $CD16^+CD56^+$ NK cells (0.78 × 10^9 cells/L). Analysis of cytolytic activity against K562 target cells demonstrated a normal functional activity of the patient's NK cells compared with that from control.

Discussion

The 502-amino acid protein, WASP, consists of five functional domains: an N-terminal *Drosophila*-enabled/vasodilator-stimulated phosphoprotein homology 1 (EVH1) domain, a basic region (BR), a GTPase-binding domain (GBD), a polyproline-rich region (PRR) and a C-terminal verpolin cofilin homology domains/acidic region (VCA) domain (3) (Fig. 1D). Since the causative gene was first isolated and cloned in 1994(15), various unique mutations have been reported in the WASP gene, spanning all 12 exons. Here, we report a novel WASP gene mutation identified in a Japanese boy, that is, deletion of one nucleotide (G) in exon 11 (1345delG), which leads to a frameshift, resulting in a stop codon at amino acid 470. Most missense mutations are localized to the EVH1 domain, and a mutated WASP often cannot bind to WASP-interacting protein (WIP), leading to defective WASP expression (16). However, since 1345delG mutation causes the partial deletion of WASP in VCA domain, but still maintains an intact EVH1 domain for WIP binding, we can assume that the mutant WASP can bind to WIP and is relatively stable, which protects the truncated WASP from being degraded. But, due to the lack of the VCA area, the truncated WASP cannot combine with the actin-related protein (ARP) 2/3 complex, which plays a key role in cytoskeletal remodeling. WASP, in the active form, binds the ARP 2/3 complex, which gives rise to nucleation of actin filaments at the side of pre-existing filaments, thus creating a branching network of actin at the plasma membrane (8). The activity of the ARP2/3 complex was shown to contribute to a variety of cellular functions, including change of cell shape, motility, endocytosis, and phagocytosis (17).

While many thought that autoimmunity was more common in patients with complete WASP deficiency, recent reports show that autoimmunity can occur in both severe and attenuated cases of the disease (6). Antibody-mediated cytopenias are the most frequent manifestation of autoimmune reactions but various vascular and organ-based autoimmune processes have also been reported (18). Although 22–72% of reported WAS cases suffered from autoimmune disorders,

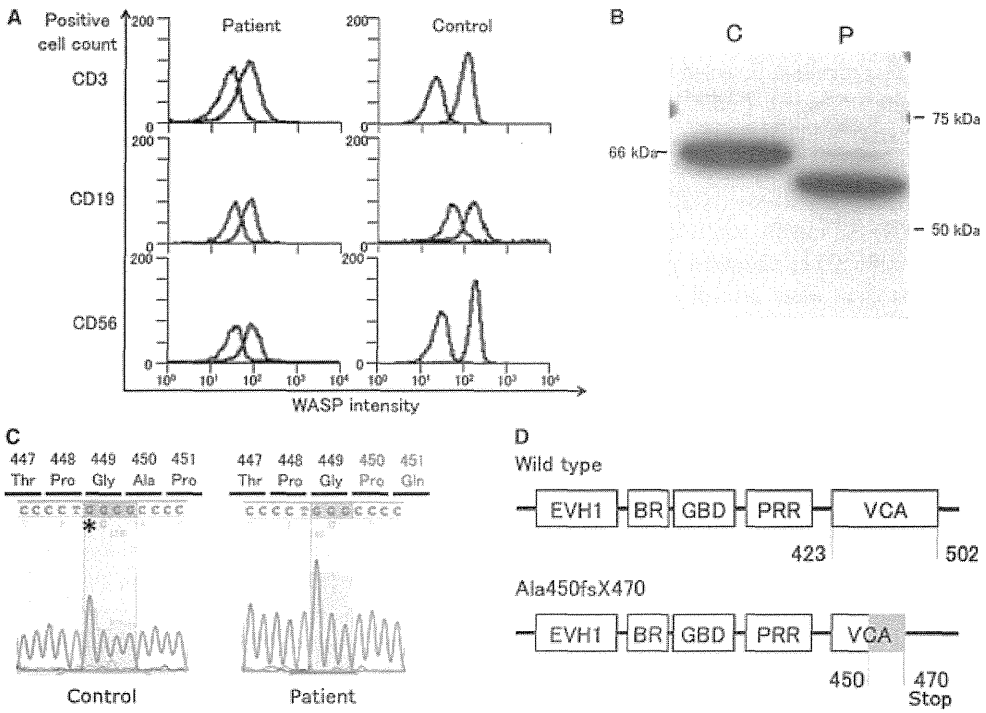

Figure 1 (A) Intracytoplasmic Wiskott–Aldrich syndrome (WAS) protein (WASP) expression analysis by flow cytometry. Histograms represent anti-WASP staining compared with isotype control in different lymphocyte subsets as indicated. (B) Anti-WASP Western blot analysis from peripheral blood mononuclear cells (PBMCs). The lysate from normal individual expressed WASP at a normal size (66 kDa), and a truncated WASP was expressed in the patient's PBMCs. C: normal control, P: patient. (C) Mutation analysis of the WASP gene. Electropherogram shows the deletion in exon 11 of the WASP gene. The position of the deletion is indicated by the asterisk on the wild-type sequence, and the changes of amino acids in the patient are shown. (D) Wild type and 1345delG-mutated WASP. EVH1, Ena/VASP homology 1 domain; BR, basic region; GBD, GTPase-binding domain; PRR, proline-rich region; VCA, verpolin cofilin homology domains/acidic region.

none of them developed TTP (8, 19). Why the present case developed TTP as an autoimmune disorder is not clear. Thrombotic microangiopathy (TMA) including TTP has been shown to occur in the setting of bacterial infections, viral infections, autoimmune diseases, malignancies, pregnancy related complications, and certain medications such as ticlopidine, cyclosporine, and tacrolimus (20). To date, there are several case reports of active CMV infection associated with TMA in both immunocompetent and immunosuppressed individuals. Although the exact pathogenesis by which CMV infection results in TMA is unknown, CMV has been shown to injure endothelial cells either by direct infection or indirectly by initiating an abnormal immune response (20, 21).

Thrombocytopenic purpura concurrently occurs in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome, scleroderma, Still's disease, polymyositis, and myasthenia gravis (22). While the present case has no autoimmune disorders other than TTP, Monteferrante *et al* (23). presented a patients with WAS who developed SLE at the age of 12 yr. The definitive phenotype in patients with mutations in the WAS gene may manifest only late in life and never reach the medical literature (6). Nikolov *et al.* and Humblet-Baron *et al.* (24, 25) have found that older WASP deficient mice develop anti-nuclear and anti-dsDNA antibodies at much higher rates than isogenic controls with titers approaching those of other autoimmune-prone mouse strains. In WASP deficient mice over 6 months of age, Nikolov *et al.* (24) found circulating immune complexes, immune complex deposition in the kidney, and mild nephritis resembling the IgA nephropathy seen in some patients with WAS. As infants with WAS may not yet have developed the final clinical phenotype, careful observation for unexpected clinical phenotypes is warranted.

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ORIGINAL RESEARCH

Chronic Granulomatous Disease: Two Decades of Experience From a Tertiary Care Centre in North West India

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Abstract Chronic granulomatous disease (CGD) results from an inherited defect in the phagocytic cells of the immune system. It is a genetically heterogenous disease caused by defects in one of the five major subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. There is a paucity of data from India on CGD. We herein describe the clinical features in 17 children with CGD from a single tertiary referral center in India. A detailed analysis of the clinical features, laboratory investigations and outcome of 17 children 7 with X-linked (XL) and 10 with autosomal recessive (AR) form was performed. Diagnosis of CGD was based on an abnormal granulocyte oxidative burst evaluated by either Nitroblue Tetrazolium (NBT) test or flow cytometry based Dihyrorhodamine 123 assay or both. The molecular diagnosis was confirmed by genetic mutation analysis in 13 cases. The mean age at diagnosis and the age at onset of symptoms was significantly lower in children diagnosed with XL- CGD compared those with AR disease. Mutations were detected in *CYBB* gene in 6 patients with XL-CGD and *NCF-1* gene mutations were observed in 7 cases of AR- CGD. The course and outcome of the disease was much worse in children diagnosed with X-linked form of disease compared to AR forms of the disease; 4/7 (57 %) children with X-CGD were dead at the time of data analysis. This is one of the largest series on chronic granulomatous disease from any developing country.

Keywords Chronic granulomatous disease · NADPH oxidase · CYBB gene · NCF-1 gene · dihydrorhodamine

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Introduction

Chronic granulomatous disease (CGD) is an inherited and genetically heterogenous immunodeficiency disorder resulting from defects of one of the subunits of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex in phagocytic cells. It is a rare disease affecting between 1in 2,00,000 and 1 in 2,50,000 live births [1]. The actual incidence is likely to be higher due to underdiagnosis of patients presenting with milder disease phenotype. CGD was initially described in 1954 [2] and 1957 [3], but it was not well characterized as a distinct clinical entity until 1959 [4].

NADPH oxidase complex is composed of five major subunits. Two of these *gp91phox* (cytochrome b-245 β

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polypeptide) and *p22phox* (cytochrome b-245 α polypeptide) are membrane bound components encoded by the *CYBB* gene and the *CYBA* gene respectively. The remaining three components of the complex include *p47phox, p67phox and p40phox* encoded by the corresponding genes namely, *NCF1* (neutrophil cytosolic factor 1), *NCF2* (neutrophil cytosolic factor 2) and *NCF4* (neutrophil cytosolic factor 4) [5]. All the components of the NADPH oxidase except for the gp91phox are not phagocyte specific and expressed in other tissues as well [6]. Therefore defects in the components other than gp91phox may have subtle effects on other tissues.

The NADPH oxidase complex catalyzes the conversion of molecular oxygen O_2 to superoxide anion (O_2^-) and other reactive oxygen intermediates. Therefore, defects in any of the components of the NADPH oxidase complex results in impaired killing of intracellular microorganisms and renders patients with CGD susceptible to recurrent and often life threatening infections with bacteria and fungi. X-linked recessive form of the disease due to mutations in the *CYBB* gene encoding for gp91phox accounts for approximately 65 % of patients with CGD. Mutations in the *NCF1* gene encoding for the *p47phox* account for 30 % of the cases whereas *CYBA* and *NCF2* mutations are detected in <5 % patients each. Only one patient with mutation in *NCF4* has been reported thus far [7]. The risk of mortality in CGD is estimated to be 1–5 % annually and is likely dependent on the mode of inheritance i.e. X-linked or AR.

There is a paucity of data on chronic granulomatous disease from developing countries although large series have been published from Europe and USA. The clinical spectrum of disease including the type of infections, frequency of breakthrough infections, morbidity and mortality are likely to be different in the context of a developing country. Hence we embarked to perform a retrospective analysis of our cohort of chronic granulomatous disease diagnosed and managed over the last 2 decades.

Patients and methods

A detailed data analysis of 17 children diagnosed with chronic granulomatous disease from August 1993 to April 2013 was performed. The study was conducted in the Pediatric Allergy and Immunology, Unit, Advanced Pediatrics Centre and the Department of Immunopatholgy, Postgraduate Institute of Medical education and Research (PGIMER), Chandigarh. Our Institute serves as tertiary level referral centre for North West India . The study was approved by the Department Review Board in consonance with the existing practice at our institution. Data were retrieved from the case records and files of the Pediatric Immunodeficiency Clinic at the Advanced Pediatrics Centre, Postgraduate Institute of Medical Education and Research (PGIMER). Evaluation of the clinical manifestations included the age at presentation and diagnosis, presenting complaints and detailed laboratory parameters.

Diagnosis of CGD was based on an abnormal granulocyte oxidative burst evaluated by either Nitroblue tetrazolium test [8, 9] (NBT, $n=10$) or flow cytometry based Dihydrorhodmaine (DHR)123 assay [10, 11] ($n=1$) or both ($n=6$). These tests were also performed on the parents and siblings where available ($n=9$) to determine the mode of inheritance. The NBT test was performed using leucocyte rich plasma whereas heparin anticoagulated whole blood was used for the DHR assay . Phorbol Myristate acetate (PMA) was used for stimulation of neutrophils in the DHR assay and yeast cells and/or PMA where used for stimulation in the NBT dye reduction test.

Complete blood count including a total and differential leucocyte count, mean platelet volume, absolute lymphocyte count, eosinophil and neutrophil counts were determined in each case using a five part automated hematology analyzer.

Serum Immunoglobulins IgG, A and M were estimated by end-point nephelometry using a semi-automated nephelometer MININeph (The Binding Site, Birmingham, UK). Lymphocyte subset analysis was done using BD Tritest™ CD45PerCP, CD3-FITC and CD19- PE antibody cocktail from BD Biosciences (San Jose, USA) following a lyse no wash protocol. Briefly 50 µl of EDTA anticoagulated blood was added to 20 µl of the antibody cocktail in a FACS tube, mixed, vortexed and solution. After 10 min the tubes were vortexed and acquisition was performed on a BD FACSCalibur flow cytometer.

Investigations also included X-rays and CT scan when indicated.

Genetic mutation analysis results was performed in 13 cases. The mutation analysis was not done in four cases because some of the children were diagnosed when facilities for mutation studies were not available or the children had died before these studies could be performed. The mutation analysis was conducted at the National Defense Medical College, Saitama, Japan and at the Department of Pediatrics and Adolescent Medicine, The University of Hong Kong, Hong Kong after obtaining written informed consent from the parents.

Results

Seventeen patients (15 males and 2 females) from 15 families with a diagnosis of CGD were included in the analysis. 7 children (41 %) children were diagnosed with X-linked form of CGD and 10 (59 %) were found to have an AR form of disease (Table I). Definite mutation analysis was available in 13/17 patients. Mutations were detected in CYBB gene was detected in 6 patients classified as definite X-CGD. Similarly mutations in NCF-1 gene were detected in 8 patients (definite