Table 3 Total biotin distributions in various drinks available in Tokyo*

Drink	Total biotin (μg/mL)**	
	Median	Range
Vinegar[1] (n=3)	1.40	(2.33–0.977)
Edible oil[2] (n=4)	1.14	(1.58–0.422)
Wine[3] (n=3)	0.428	(1.36–0.285)
Sake[4] (rice wine; n=13)	0.406	(1.07–0.129)
Bovine milk[5] (n=3)**	0.364	(0.624–0.164)
Beer[6] (n=10)	0.318	(1.99–0.076)
Milk powder (ordinary)[7] (n=3)	0.301	(0.730–0.233)
Peptide milk for allergy[8] (n=3)	0.532	(0.743–0.492)

*Median, distribution, and top product name and producer are indicated. Milk powder and milk for people with allergies were dissolved in hot water in accordance with the procedures of the suppliers. **Presence of seasonal difference: i.e., the summer product value is higher than winter product value. [1]Pure Rice Vinegar (Japan Livelihood Cooperative Association, Tokyo, Japan). [2]Oil for salad (soybean and rapeseed) (Nisshin Oillio Group, Ltd., Tokyo, Japan). [3]Red wine (Shinshu Concord Co., Shijiri, Nagano, Japan). [4]Seishu Senkoma (Senkoma Brewery Co. Ltd., Shirakawa, Fukushima, Japan). [5]Hokkaido Tokachi low-fat milk (Japan Livelihood Cooperative Association), [6]Kirin Ichiban Shibori (Kirin Holdings Co. Ltd, Tokyo, Japan), [7]Hagukumi for 0–9 months (Morinaga Milk Industry Co. Ltd., Tokyo, Japan), [8]Morinaga MA-mi (Morinaga Milk Industry Co. Ltd., Tokyo, Japan).

previous study (1), we confirmed the seasonal differences in biotin content of bovine milk (first reported by Dr. Umetaro Suzuki); i.e., milk in summer contains a biotin concentration that is approximately 3 times that in winter. A similar range in total biotin content was also observed for ordinary milk powder (Table 3). This may due to the seasons in which the raw materials (bovine milk) were obtained by the producers.

Free biotin is the important nutrition, since free biotin should be liberated and obtained from the usual bound-form biotin via hydrolysis by the amidase (biotinidase) in animals and in some bacteria and fungi, which are not able to synthesize biotin. Typical foodstuffs that contain high ratio of free biotin are summarized in Table 4. Sera from healthy humans, microbes and plant vegetable cells usually contain less than 10% free biotn (lower part of Table 4). Foodstuffs containing high amounts of free biotin included good nutritional materials such as natto, chicken egg-yolk and milk. These foodstuffs seem to have their own mechanisms against invasion of microbes (upper part of Table 4).

Milk and milk powder are very important foodstuffs for infants, who receive nutrition for several months after birth from only milk. The biotin contents of milk and milk powders are summarized in Table 5. We found that all the tested milk and milk powders, except for GSD (glycogen storage disease) formulas, contained sufficient amounts of biotin (Table 5). Milk powder C (follow-up milk) contains relatively lower amounts of total and free biotin; however,

Table 4 Typical examples of high free-biotin containing foodstuffs and possible protection mechanisms against potential pathogens (bacteria, fungi, etc)*

Foodstuff	Total biotin (μg/g)	Free biotin (μg/g)	Ratio of free biotin (%)	Protection method (Potential)
Hikiwari Natto (Okame; dried)	558	514	92.1	poly(gamma-glutamic acid) (pH 6.5)
Chicken egg-yolk	35.2	31.7	90.1	Avidin, lysozyme (egg-white) and pH 5.3 (egg-yolk)
Sake (rice wine)** (Tatsuizumi)	0.334	0.251	75.0	Ethanol (12%)
Bee pollen	10.7	6.41	59.9	Bacteriocin peptide***
Milk powders (ordinary; n=3)	0.301	0.172	57.1	Dried powder
Pollen (Anemone)	23.9	10.5	43.9	Cell wall of cellulose
Bovine milk** (product of February)	0.164	0.0669	40.8	Immunoglobulin A Lactoferrin
Beer** (Kirin Ichiban Shibori)	1.99	0.736	37.0	Ethanol (6%)
Royal jelly A	1120	376	33.6	10-Hydroxydecanoic acid
Royal jelly B	180	37.6	20.9	
Royal jelly C	20.6	6.95	33.7	
Rice bran	30.4	6.02	19.8	Cell wall of cellulose
Human serum**	1.80	0.122	6.8	
Mozuku (dried)	11.7	0.411	3.5	
Dried yeast (Ebios)	15.5	1.29	8.3	
Dried Bacillus natto	49.7	3.42	6.9	

*Total and free biotin were measured as described in the Materials and Mehthods section. **Expressed as μg/mL. ***Refer to (8).

infants who are 9 mo of age or older can consume nutrients from ordinary foodstuffs. Although soy milk is a foodstuff intended for adults, both soy milk and soy baby formula contained a high concentrations of total biotin (Table 5). Furthermore, soy formula also contained a high concentration of free biotin (Table 5). Soy formula may be a good milk for babies.

Biotin deficiency may occur in babies consuming milks with low levels of free biotin and low free biotin ratios. In fact, two babies receiving Milfy (Meiji) and one baby receiving New MA-1 (Morinaga) were found to be biotin deficient at our hospital (6). Elemental formula (Meiji) may also cause biotin deficiency in babies with normal biotinidase (7). One 3-yr-old female GSD patient (apparently normal biotinidase with heat labile *Km*; Michaelis constant) receiving GSD formulas D and N produced by Meiji has also been found to be biotin deficient (Dr. Kenji Ihara, personal communication). Babies and adults exhibiting unstable (heat labile) biotinidase may have a tendency to become biotin deficient (Table 6). The two biotin deficient patients showed increased *Km* values (decreased affinity) compared with fresh controls after treatment at 37°C for 4 h. The biotin-deficient baby received Milfy (Meiji) and was biotin deficient. This baby was then administerd 10 mg/day of biotin for 13 wk, and his biotin status improved (serum total biotin 3.89 μg/mL, free biotin 0.052 μg/mL) and dermatitis disappeared. The biotin-deficient adult in Table 6 took Ebios (dried yeast) and Yakult (*Lactobacillus casei, Shirota*) for 4 mo, and her biotin status improved slightly (serum total biotin 2.04 μg/mL, free biotin 0.077 μg/mL). This patient seems to have improved slightly; however, the precise neurological tests may be necessary to be studied on this patient.

In conclusion, precise knowledge of the biotin

Table 5 Summary of the biotin contents of milk, milk powders and special milk powders for milk-related conditions*

Milk	Total biotin (μg/mL)	Free biotin (μg/mL)	Ratio of free biotin (%)	Appearance of dermatitis patient**
Human breast milk[1]	0.376	**0.209**	55.6	
Bovine milk[2]	0.364	0.248	68.1	
Ordinary milk powders				
Milk powder A[3]	0.730	0.338	46.3	–
Milk powder B[4]	0.301	**0.172**	57.1	–
Milk powder C[5]	**0.233**	**0.162**	69.5	–
Special milk powders (only milk peptides are present; for milk allergies)				
MA-mi (Morinaga)	0.743	0.494	66.5	–
New MA-1 (Morinaga)	0.532	**0.164**	**30.8**	+ (1 case)
Milfy (Meiji)	0.492	**0.056**	**11.4**	++ (2 cases)
Pepdiet[6] (Beanstalk-Snow)	1.18	0.792	67.1	–
MCT formula[7] (Meiji)	1.15	0.909	79.0	–
MCT formula[8] (Meiji)	1.25	0.754	60.3	–
Elemental formula[9] (Meiji)	1.73	**0.251**	**14.5**	?
Lactoless[10] (Meiji)	1.18	**0.094**	**7.97**	?
GSD formula Day[11] (Meiji)	**0**	**0**	+	
GSD formula Night[11] (Meiji)	**0**	**0**	+	
Soy milk[12]	2.98	1.99	66.8	
Soy formula[13]	1.36	0.899	66.1	–

*Total and free biotin were measured as described in the Materials and Methods section. Milk powders were dissolved as indicated by the suppliers. [1] 9-mo-old baby, [2] product of October; Glyco Co., Yoji-yuryo-gyunyu, [3] for 0–9 mo; Morinaga Hagukumi, [4] for 0–9 mo; Yukijirushi Pure, [5] for 9 mo – 3 y; follow-up milk; Yukijirushi Tacchi, [6] plus a small amount of milk proteins, lecithin and edible oils; for lactose intolerance, [7] defatted milk and lecithin, [8] defatted milk, lecithin and added essential fatty acids, [9] amino acids+ edible oils; for lactose intolerance and galactosemia, [10] Casein and edible oils; for lactose intolerance and galactosemia, [11] Casein milk, for liver-type glycogen storage disease; GSD, [12] Marusan-Ai Co. Ltd., [13] Bonlacto I; Wakodo.

contents of foodstuffs is expected to be useful in improving the health and development of babies and adults.

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Table 6 Thermal instability of serum biotinidase in biotin-deficient patients*

Serum	*Amo* (L/mol × 1/s)	*V* (pmol per min per mg)	*Km* (μmol/L)	*Kip* (μmol/L)	*Rep* (mol/L × 1/s × 10^{-3})	*Cap* (1/s)
Healthy baby (1 yr, female)						
(Biotin status: Total biotin 3.21 μg/mL, free biotin 0.180 μg/mL)						
Fresh	22.2	156	8.91	494	97.6	46.5
Treated	18.1#	131#	9.03#	485#	80.6#	38.5#
Biotin-deficient baby (4 mo, male; dermatitis and alopecia)**						
(Biotin status: Total biotin 2.31 μg/mL, free biotin 0.535 μg/mL)						
Fresh	25.5	182	9.02	365	83.9	46.3
Treated	8.63	122	**17.9**	271#	41.8	19.0
Healthy adult (male)						
(Biotin status: Total biotin 1.80 μg/mL, free biotin 0.122 μg/mL)						
Fresh	27.9	131	5.94	359	59.3	40.7
Treated	20.6#	109#	6.71#	501	69.3#	37.8#
Biotin-deficient adult (32 yr, female; optical atropy, polyneuropathy [sensory dominant])						
(Biotin status: Total biotin 1.14 μg/mL, free biotin 0.041 μg/mL)						
Fresh	18.4	63.6	4.45	342	27.7	22.4
Treated	6.11	89.3	**18.5**	530	59.9	19.1#

*The thermal instability test was performed as follows: serum was diluted 11-fold with serum dilution buffer containing 1 mM EDTA and 10% glycerol (5). The diluted serum was then incubated at 37°C for 4 h. After 10-fold dilution of this treated serum, the biotinidase activity was measured as described previously (5). *Amo*; affinity for substrate. *V*; specific activity. *Kip*; competitive inhibition constant by the product biotin. *Rep*; repulsion. *Cap*; enzyme capacity. Fresh refers to is non-heat treated controls. #: Parameters that fluctuated by no more than 30%, were considered to be stable. **This patient received Milfy (Meiji; Table 5) and experienced biotin deficiency.

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BRIEF REPORT

Mizoribine for renal sarcoidosis: effective steroid tapering and prevention of recurrence

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Abstract Sarcoidosis is a systemic disease of unknown etiology that is characterized by chronic non-caseating granulomatous inflammation with tissue destruction. It is an uncommon disease in children, and renal sarcoidosis in particular is very rare in adults and children. A 17-year-old boy with renal sarcoidosis was referred to our hospital with an initial diagnosis of pyelonephritis. Prior treatment with various antibiotics had not been effective, but tentative oral daily prednisolone (PSL) had partially ameliorated his symptoms, although the symptoms recurred during steroid tapering. We detected non-caseating granulomatous interstitial nephritis and numerous sclerotic glomeruli in a second biopsy specimen, compatible with the diagnosis of renal sarcoidosis. The patient was treated with pulsed methyl-prednisolone and oral daily doses of PSL and mizoribine (MZR). During the treatment with MZR, the PSL was successfully tapered, and the patient has since presented no signs of recurrence. Our treatment of this patient shows that treatment with MZR can allow steroid sparing and prevent recurrence in a patient with sarcoidosis.

Keywords Children · Granulomatous interstitial nephritis · Mizoribine · Prednisolone · Renal · Sarcoidosis

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Introduction

The lungs, eyes, joints, skin, and thoracic lymph nodes are the organs and tissues primarily affected in sarcoidosis. The most common renal signs of sarcoidosis are nephrocalcinosis and nephrolithiasis related to hypercalciuria and hypercalcemia [1]. Granulomatous interstitial nephritis (GIN) is another type of renal abnormality. Based on autopsy findings, 15 40% of adult patients with sarcoidosis can be considered to have GIN [1 3], but most of the cases are clinically silent. Sarcoidosis rarely develops in children, and reports of childhood isolated GIN are quite limited [4]. While corticosteroids are generally effective against GIN [2, 3], the disease occasionally recurs with the reduction or discontinuation of corticosteroids, which is a serious issue [2, 3]. Immunosuppressive agents are used in such refractory cases [5 7]. We report here our clinical experience with an adolescent male with GIN who was successfully treated with mizoribine (MZR) and corticosteroid.

Case report

A 17-year-old boy was referred to our hospital because of chronic kidney disease and chronic diarrhea for 3 years. He had had an episode of intermittent high fever every 2 3

weeks at the age of 14 and had been admitted for investigation and treatment in another hospital. At the previous hospital, a urinary culture initially revealed *Pseudomonas aeruginosa* (1×10^3 CFU) and *Escherichia coli* (1×10^4 CFU). The urinary white blood cell (WBC) count was 31~50/high power field (hfp), and the urinary β2-microglobulin (β2MG) level was between 1500 and approximately 4000 ng/mL (normal <250). The patient was diagnosed with pyelonephritis and treated with antibiotics. However, the symptoms did not ameliorate after the disappearance of these bacteria. The C-reactive protein (CRP) level and erythrocyte sedimentation ratio (ESR) were also elevated. Contrast abdominal computed tomography (CT) revealed patchy defects of enhancement on bilateral kidneys, consistent with pyelonephritis or acute focal bacterial nephritis. Vesicoureteral reflux was ruled out by voiding cystourography. The 99mTc dimercaptosuccinic acid scan showed a defect of bilateral upper pole. Therapy with various antibiotics was tried, but none elicited a response in the patient. The histological findings on the specimen obtained during the first ultrasound-guided renal biopsy were normal, suggesting the biopsy specimen was from normal renal tissue. An additional open biopsy was refused by the patient. After ruling out the possibility of an infectious disease, neoplasm and rheumatic diseases, the patient was tentatively treated with 20 mg per day of prednisolone (PSL) for 6 months from the onset. Surprisingly, his fever immediately subsided, but the diarrhea and abdominal pain persisted (Fig. 1). Hypertension was controlled with enalapril. However, during steroid tapering to 5 mg, the patient developed fever and high levels of CRP and creatinine (Cr). At this point the patient was transferred to our hospital.

The chest, abdominal, and neurological examinations carried out upon admission to our hospital revealed no abnormalities. His height was 162.5 cm (25th percentile), body weight was 43 kg (3rd percentile), blood pressure was 98/56 mmHg, and body temperature was 36.5°C. The results of the urinalysis were: protein, negative; red blood cells (RBC) <1/hpf; WBC 6–10/hpf. Laboratory analysis of blood parameters revealed: a hemoglobin concentration of 11.3 g/dL, leukocyte count 6860/μL, platelet count 367×10^3 /μL, total protein 8.7 g/dL, serum albumin 4.9 g/dL, blood urea nitrogen 24 mg/dL, serum Cr 3.03 mg/dL, urate 7.6 mg/dL, sodium 136 mEq/L, potassium 4.1 mEq/L, chloride 102 mEq/L, calcium 10.1 mg/dL, phosphate 2.9 mg/dL, CRP 6.1 mg/dL (normal 0.0–0.2), ESR 53 mm/1 h, immunoglobulin (Ig)G 1666 mg/dL, soluble interleukin (IL)-2R 2260 U/mL, and angiotensin converting enzyme 12.8 IU/L (normal 8.3~21.4). The serum electrolyte was normal. Collagen diseases were also ruled out. Urinary chemistries were as follows: β2MG 18,160 ng/mL (normal <250), N-acetyl-beta-d glucosaminidase 3.1 U/L (normal 0.97–4.17), calcium 3.6 mg/dL, and Cr 37.9 mg/dL. Urinary and throat bacteria cultures were negative. A second renal biopsy was performed, and the specimen showed severe interstitial nephritis, globally sclerosing glomeruli, and non-caseating granuloma characteristic of sarcoidosis (Fig. 2). Chest and abdominal CT and X-ray scans showed no abnormal findings nor lymphadenopathy. No ophthalmologic abnormality was detected. A nonspecific intestinal mucosal inflammation without non-caseating granuloma was found

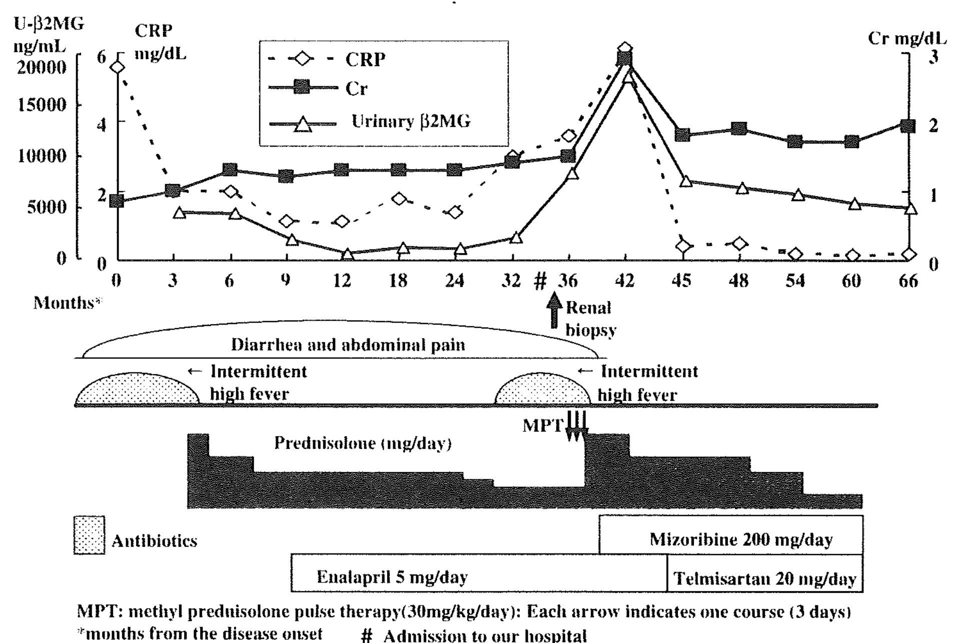

Fig. 1 Clinical course of the patient. *CRP* C-reactive protein, *Cr* creatinine, *β2MG* β2-microglobulin

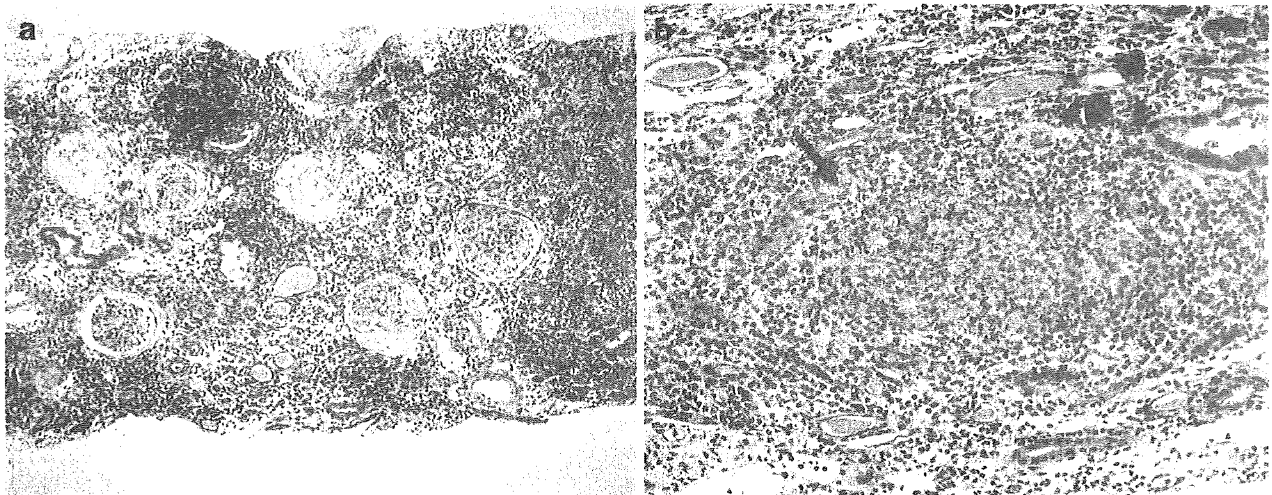

Fig. 2 Renal pathological findings. **a** Diffuse interstitial nephritis, sclerosing glomeruli, and severe tubular atrophy. Hematoxylin eosin stain, magnification ×40. **b** Non-caseating epithelioid granulomas (*arrow*). Hematoxylin eosin stain, magnification ×400

by endoscopy. Tuberculosis was ruled out by a PCR analysis and bacterial culture of renal and intestinal samples. The final diagnosis was renal sarcoidosis. He was treated with three courses of methyl-prednisolone pulse therapy (MPT: 30 mg/kg per day, 3 days) followed by 20 mg per day of oral PSL and 200 mg per day of MZR. Following the treatment with MPT, the intermittent high fever, pyuria, and elevated CRP and ESR resolved. It is possible that the intermittent high fever and chronic diarrhea were due to the sarcoidosis. Serum Cr levels dropped down to 1.9 mg/dL. The PSL was then successfully tapered to 5 mg per day, and there has been no elevation of CRP or ESR and no recurrence of clinical manifestations for 2 years (Fig. 1). Enalapril was discontinued because of cough, and telmisartan was started. However, chorioretinitis developed with tapering of the PSL to 10 mg per day; this condition subsequently responded to steroid eye-drops. Ophthalmologic findings were compatible with ocular sarcoidosis, which further confirmed his diagnosis.

In our patient, MZR contributed to successful steroid tapering and the prevention of renal sarcoidosis recurrence. The peak concentration of MZR was 4.5 μg/mL. No adverse event of MZR was experienced.

Discussion

The diagnosis of sarcoidosis could not be determined sooner because of the absence of typical respiratory, ocular, or cutaneous symptoms. The absence of an extra-renal lesion frequently delays the diagnosis [8–10]. Therefore, most patients show renal insufficiency at the time of diagnosis, which results in persistent renal insufficiency even if the patients initially respond well to steroid therapy. O'Riordan et al. [8] and Robson et al. [9] reported a total of 12 adult patients with isolated GIN or sarcoidosis. All of these showed renal insufficiency at the time of the initial presentation. With the exception of one patient, who rapidly progressed to end-stage renal failure, all of the remaining 11 patients responded to the steroid therapy, but renal function never normalized in any of them.

The only approach to diagnose GIN is renal biopsy. However, GIN lesions are usually scattered, as was the case in our patient. In this setting, precise biopsy of a lesion is not easy. Our first biopsy site was decided upon based on the findings of contrast abdominal CT and gallium scintigraphy, but the results were negative. Ultrasound-guided needle biopsy had a number of limitations in our case in terms of sampling diseased tissue. Unfortunately, a second biopsy was refused by the patient; open or CT-guided biopsy should have been considered at the initial admission.

Apart from sarcoidosis, tubulointerstitial nephritis and uveitis syndrome (TINU syndrome), tuberculosis, and drug-induced interstitial nephritis should be considered for the differential diagnosis of GIN. We ruled out these diseases based on the results of laboratory tests, ophthalmological examination, and clinical history. In addition, chorioretinitis, which is compatible with the diagnosis of ocular sarcoidosis, further confirmed this diagnosis.

In general, sarcoidosis shows a good response to steroids but tends to relapse with the tapering or discontinuity of steroid therapy if the duration or amount of steroid is insufficient [3, 4, 9]. In 20% of adults [2] and 22% of children [3], GIN has been found to recur during withdrawal or discontinuity of the steroid. Immunosuppres-

sive agents, such as cyclosporine [5], chloroquine [6, 7], methotrexate [6, 11], mycophenolate mofetil (MMF) [12–14], cyclophosphamide [15], and tumor necrosis factor blockade [10, 16], have been reported to be effective in preventing the recurrence during the tapering of steroids; MZR has been shown to be effective in this regard.

To our knowledge, this is the first report of the clinical benefit of MZR in renal sarcoidosis. Mizoribine selectively inhibits inosine monophosphate dehydrogenase and guanosine monophosphate synthetase; consequently, it inhibits T cell and B cell proliferation, macrophage activation, and inflammation [17]. The immunosuppressive mechanism of MZR is similar to that of MMF. There have been some reports of the clinical benefit of MMF against sarcoidosis [12–14], suggesting that these two drugs may have the same mechanism.

The therapeutic benefit of MZR has been reported in nephrotic syndrome, lupus nephritis, and IgA nephropathy in children [18–21]. According to several recent studies, the peak blood concentration of MZR should be maintained between 3.0 and 6.0 μg/mL in order to inhibit human mixed-lymphocyte reaction [17]. In our patient, the peak concentration was 4.5 μg/mL. The MZR blood concentration should be carefully monitored in patients with renal insufficiency because MZR is eliminated into the urine. Compared to other immunosuppressive agents, MZR has few severe adverse effects, such as nephrotoxicity, gonadotoxicity, and myelosuppression [18–21].

This is the first report showing the therapeutic benefit provided by MZR in a patient with renal sarcoidosis, but more cases will be needed to establish its clinical benefit. Nevertheless, early diagnosis is crucial to prevent persistent renal insufficiency.

Conflict of interest The authors report that there is no conflict of interest.

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LETTER TO THE EDITORS

Efficacy of mycophenolate mofetil for steroid and cyclosporine resistant membranoproliferative glomerulonephritis type I

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Sirs,
We read with great interest the article entitled "Remission of resistant MPGN type I with mycophenolate mofetil and steroids" by De et al. [1]. We encountered a similar case and would like to comment on the efficacy of mycophenolate mofetil (MMF) for refractory membranoproliferative glomerulonephritis (MPGN) type I. A 13-year-old boy was transferred to our hospital because of steroid-resistant nephrotic syndrome with hypocomplementemia. He had been treated with 2 mg/kg of prednisolone (PSL) for 4 weeks, but systemic edema, massive proteinuria and hypertension had not improved. When he was admitted to our hospital, the results of urinalysis were: protein 2,498 mg/dl, creatinine 399 mg/dl, β2 microglobulin 5,691 μg/dl (normal <250 μg/dl), and red blood cell (RBC) count over 50 per high-power field (HPF). Those of blood biochemistry were: 4.4 g/dl of total protein, albumin 2.0 g/dl, serum creatinine 0.83 mg/dl, blood urea nitrogen 33 mg/dl, uric acid 7.3 mg/dl, total cholesterol 404 mg/dl, triglycerides 296 mg/dl, complement (C)3 21 mg/dl (86~160 mg/dl), C4 21 mg/dl (17~45 mg/dl), CH50 20 U/ml (30~40 U/ml), anti-nuclear antibody (negative), and anti-double stranded DNA antibody (negative). Renal biopsy showed diffuse and severe MPGN type I. All the glomeruli showed marked lobulation, intracapillary hypercellularity, thickening of the capillary walls and double contour. Immunofluorescence showed intense staining of all capillary walls and mesangial cells with C3 and immunoglobulin (Ig)G. An electron micrograph showed electron-dense deposits only in the subendothelial space. The patient was initially treated with three courses of methyl prednisolone pulse therapy (MPT, one course; 1 g of methyl prednisolone for 3 days) followed by PSL orally. Lisinopril and losartan were used for hypertension and proteinuria. Proteinuria was dramatically reduced after three courses of MPT, but it was still in the nephrotic range (Fig. 1). Additional cyclosporin was also insufficient to induce remission of proteinuria. In the end, he was started on MMF 3 months from the initiation of treatment. Proteinuria decreased promptly soon after he had started on MMF. Two months later the proteinuria had completely resolved, even though PSL had been withdrawn. Under treatment with PSL, cyclosporin A (CsA) and MMF, he has not had proteinuria for 8 months. Hematuria has also

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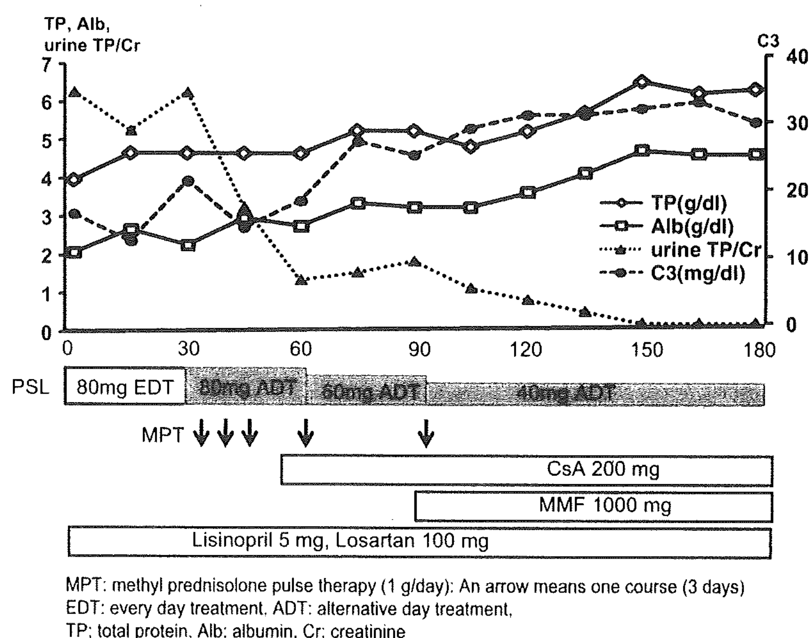

Fig. 1 Clinical course of the patient (*MPT* methyl prednisolone pulse therapy, 1g per day. An *arrow* indicates one course (3 days). *EDT* every day treatment, *ADT* alternate day treatment, *TP* total protein, *Alb* albumin, *Cr* creatinine)

improved gradually. Serum C3 level is still low, but is slowly reaching around 40 mg/dl. Further studies are needed to conclude whether MMF is really effective against MPGN, but, in the light of our clinical experience, MMF emerges as a new therapeutic option against refractory MPGN.

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ORIGINAL ARTICLE

Single dose of rituximab for refractory steroid-dependent nephrotic syndrome in children

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Abstract We conducted a multicenter prospective trial to evaluate the efficacy, safety and pharmacokinetics of a single dose of rituximab (375 mg/m^2 body surface area) for the treatment of children with refractory steroid-dependent nephrotic syndrome (SDNS). All patients (n=12) were able to discontinue steroids at a median of 74 days after treatment. The frequency of relapses per 6 months was significantly reduced and the steroid-free period per 6 months was significantly increased after treatment compared with those before treatment. The condition in nine of the patients (75%) relapsed at a median of 129 days after treatment, and seven patients were given additional rituximab due to steroid dependency. Most of the relapses developed simultaneously with recovery of B-cells. However, three patients (25%) did not have a relapse with B-cell recovery and the disease was kept in remission for more than 1 year. None of the patients developed life-threatening adverse events. This is the first report of a prospective study of a single dose of rituximab for refractory SDNS. Treatment with a single dose of rituximab may be effective for refractory SDNS, but its efficacy to prevent relapses was transient in most of the patients.

Keywords Refractory steroid-dependent nephrotic syndrome · Children · Clinical trial · Rituximab · Pharmacokinetics

Introduction

Idiopathic nephrotic syndrome is the most frequent glomerular disease of childhood. Most cases respond to steroid treatment, but approximately 40% of the children develop

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frequent-relapsing nephrotic syndrome or steroid-dependent nephrotic syndrome (SDNS). In Japan these patients are usually treated with immunosuppressants such as cyclosporine (CyA), cyclophosphamide (CPA), mizoribine (MZR) and mycophenolate mofetil (MMF). However, some patients continue to have relapses, and the condition remains steroid-dependent despite these treatments. Such patients often suffer from severe steroid toxicity, such as cataracts, obesity, osteonecrosis and growth failure. Moreover, these immunosuppressants also have significant adverse effects. Chronic nephrotoxicity is a well-known side effect of CyA, and long-term use of this drug is known to carry a high risk for chronic CyA nephrotoxicity [1], although Kranz et al. reported that long-term use of cyclosporine for SDNS was relatively safe [2]. Because of the possibility of gonadotoxicity (azoospermia), it is recommended that CPA be used within limited cumulative doses. MZR and MMF are still off label for SDNS in Japan. Therefore, even if these immunosuppressants are effective, it is difficult for these drugs to be used for a long period.

It has recently been reported that rituximab (anti-CD20 monoclonal antibody) was successfully used to treat patients with refractory nephrotic syndrome [3–14]. To date, there have been eight reports of a total of 34 patients with SDNS who received rituximab treatment [3–10]. In those reports, rituximab treatment for SDNS that was resistant to immunosuppressants prevented relapses and resulted in the patients' discontinuation of steroids. Thus, it is likely that rituximab is very effective for SDNS. In most of the previous studies, rituximab was given at a dose of 375 mg/m^2 body surface area (BSA) once weekly for 4 weeks, because this dosage is recommended for patients with B-cell lymphoma. However, Smith reported successful treatment of a SDNS patient with a single dose of rituximab [6]. We also successfully treated two patients with refractory steroid-resistant nephrotic syndrome with a single dose of rituximab [12]. Thus, the optimal dose of rituximab for childhood refractory SDNS has not been established.

Therefore, we conducted a multicenter prospective study to examine the efficacy and safety of a single dose of rituximab for refractory SDNS in children. Since there have been no previous reports of rituximab pharmacokinetics in children, we also examined the pharmacokinetics of rituximab and time courses of peripheral B-cell counts after rituximab administration in children with refractory SDNS.

Materials and methods

Patient population

Patients included in this trial met the following criteria: (1) they were younger than 20 years old, (2) they had idiopathic nephrotic syndrome, (3) the disease was steroid-dependent, and conventional immunosuppressants such as CyA, CPA, MZR and MMF were being taken, and (4) they had no history of rituximab treatment. Approval of the off-label use of rituximab and of the study protocol was obtained from the institutional review boards of the National Center for Child Health and Development, Kobe University Graduate School of Medicine, Yokohama City University Medical Center and Saitama Children's Medical Center. All patients' parents gave their written informed consent.

Treatment

Before rituximab infusions, complete blood counts, biochemical parameters (total protein, albumin, blood urea nitrogen, creatinine, uric acid, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, electrolytes, etc.), peripheral B-cell counts, and titers of hepatitis B virus and hepatitis C virus were examined. Chest radiographs and electrocardiograms were obtained, and echocardiography was also carried out. We administered rituximab intravenously, in a single dose of 375 mg/m^2 BSA (maximum 500 mg) after obtaining remission with prednisolone (PSL). In order to minimize infusion reactions, we gave the patients acetaminophen and cyproheptadine hydrochloride 30 min prior to rituximab infusions. Steroids were not infused prior to rituximab infusions. Patients were observed in the hospital for at least 24 h after rituximab infusion.

Follow-up

For all patients, clinical and laboratory parameters, including complete blood counts, biochemical parameters, serum immunoglobulin levels and CD19+ B-cell counts by flow cytometry were monitored once a month for 6 months. Steroid dosages were tapered and discontinued by 6 months after the rituximab infusions, although the detailed protocol for tapering steroid dosage was not restricted. The protocols for discontinuation of immunosuppressants and PSL for relapses were not restricted in this trial. Patients were followed-up for at least 1 year after they had been given the rituximab infusion. Frequency of relapses, steroid-free periods, and dosages of steroids were evaluated before and after rituximab treatment. B-cell depletion was defined as a CD19+ countof fewer than five cells per cubic millimeter at any time, and B-cell recovery was defined as a CD19+ cell count of more than15 cells per cubic millimeter.

Measurement of serum rituximab levels

Serum rituximab levels were quantified by a proprietary enzyme-linked immunosorbent assay (ELISA) at Covance

Laboratories, Inc. (Chantilly, VA, USA). The ELISA-0145 method is a sandwich ELISA format for the quantitative determination of rituximab in human serum. The 1/100 diluted calibrators, controls, and samples were pipetted on to a microtiter plate precoated with polyclonal goat anti-rituximab, incubated, and then washed. A goat anti-mouse IgG F(ab')^2 horseradish peroxidase (HRP) conjugate was then incubated, followed by another plate washing. Bound HRP conjugate was detected with a tetramethyl benzidine (TMB) substrate, which was read colorimetrically on a plate reader.

Rituximab pharmacokinetic analysis

We obtained serum samples to examine serum rituximab levels before and just after infusion, after 24 hours, and after 1 week, and then once a month until 6 months after treatment. The maximum serum concentration (Cmax) of rituximab was an actual measured value. The area under the serum concentration time curve (AUC), total clearance (CL), elimination half-life (T1/2) and volume of distribution (Vdss) were calculated by non-compartmental methods using the computer program MOMENT [15].

Statistical analysis

We used the Wilcoxon signed-rank test to compare the number of relapses and steroid-free periods before and after rituximab infusion, and Student's *t*-test and analysis of variance (ANOVA) to analyze PSL dosages, B-cell counts, and serum immunoglobulin levels. The Kaplan–Meier method was used for the analysis of the probabilities of relapse-free survival and B-cell depletion. Statistical significance was established at $P<0.05$.

Results

Patients' characteristics

Between February and September 2007, 12 patients (eight boys and four girls) with SDNS were enrolled in our trial at four institutions. The clinical characteristics of the patients are shown in Table 1. Renal biopsies revealed minor glomerular abnormalities (minimal change disease) in 11 patients and focal segmental glomerulosclerosis in one patient. All patients had a history of CyA treatment. All patients suffered from severe steroid-dependent disease and could not discontinue steroid usage under one or two immunosuppressants at the time of rituximab infusion. Mean duration of steroid therapy before rituximab treatment was 6.1±3.2 years. The effects of severe steroid toxicity, such as short stature, obesity, osteoporosis, hypertension and cataract, were observed in almost all patients.

Clinical courses after rituximab treatment

Clinical courses after rituximab infusion are shown in Table 2. All patients were able to discontinue PSL usage at a median of 74 days after the infusion. For eight patients, immunosuppressants were also able to be discontinued. Complete B-cell depletion was achieved in ten of 12 patients (88%). In

Table 1 Patients' characteristics (*IS* immunosuppressant, *Rtx* rituximab, *M* male, *F* female, *FSGS* focal segmental glomerulosclerosis, *CyA* cyclosporine A, *CPA* cyclophosphamide, *MZR* mizoribine, *PSL* prednisolone, *MGA* minor glomerular abnormalities, *CHL* chlorambucil, *FK* tacrolimus, *MMF* mycophenolate mofetil, *SD* standard deviation)

Patient no.	Gender	Age at onset (years)	Duration of steroid treatment (years)	Age at Rtx treatment (years)	Renal histology	History of previous IS	Treatments at the time of Rtx therapy
1	M	5	9.3	14	FSGS	CyA, CPA, MZR	PSL, CyA, MZR
2	M	8	7.7	16	MGA	CyA, MZR	PSL, MZR
3	M	15	1.5	18	MGA	CyA	PSL, CyA
4	M	4	8.3	12	MGA	CyA, CPA, CHL, MZR	PSL, CyA, MZR
5	M	2	2.7	5	MGA	CyA, CPA, MZR	PSL, CyA
6	M	10	10.6	19	MGA	CyA, MZR	PSL, MZR
7	F	8	1.9	10	MGA	CyA, CPA, MZR	PSL, CyA
8	M	2	7.9	10	MGA	CyA, FK, CPA, MZR	PSL, FK, MZR
9	M	2	9.0	15	MGA	CyA, CPA, MMF	PSL, CyA, MMF
10	F	9	2.5	12	MGA	CyA, CPA, MZR, MMF	PSL, CyA, MMF
11	F	1	4.5	11	MGA	CyA, CPA	PSL, CyA
12	F	3	6.7	10	MGA	CyA, MMF	PSL, MMF
Mean		5.8	6.1	12.7			
SD		4.3	3.2	3.9			

Table 2 Clinical courses after rituximab infusion (*IS* immunosuppressant, *Rtx* rituximab, *D/C* discontinued, *MZR* mizoribine, *CyA* cyclosporine A, *FK* tacrolimus, *MMF* mycophenolate mofetil)

Patient no.	Cessation of PSL after Rtx infusions (days)	IS treatment after Rtx infusions	B-cell recovery after Rtx infusions (days)	First relapse after Rtx infusions (days)	Summary of the clinical courses
1	63	D/C MZR on day 0 D/C CyA on day 175	119	129	Relapses on days 129 and 190, additional Rtx on day 190
2	83	D/C MZR on day 116	146	161	Relapse on day 161, additional Rtx on day 175, relapse on day 316, four doses of additional Rtx from day 328
3	68	D/C CyA on day 89	245	Not relapsed	Maintaining remission on day 365
4	73	D/C MZR on day 0 D/C CyA on day 122	118	104	Relapses on days 104 and 128, additional Rtx on day 141, relapses on day 253, four doses of additional Rtx from day 273
5	57	D/C CyA on day 112	Not depleted	199	Relapses on days 199 and 221, restarted CyA on day 233
6	75	D/C MZR on day 0	152	124	Relapses on days 124 and 194, four doses of additional Rtx from day 203, relapse on day 217
7	58	D/C CyA on day 140	147	128	Relapses on days 128 and 156, additional Rtx on day 167, relapse on day 213, restarted CyA on day 272
8	76	D/C MZR on day 0	104	8	Relapses on days 8, 139 and 173, additional Rtx on day 196,
		FK→CyA on day 8			Relapses on days 197, 253 and 302, two doses of additional Rtx from day 343
9	123	D/C CyA on day 74 Continued MMF	151	353	Relapse on day 353
10	120	D/C CyA on day 78 Continued MMF	Not depleted	Not relapsed	Maintaining remission on day 365
11	55	D/C CyA on day 112	84	125	Relapses on days 125 and 146, additional Rtx on day 153
12	172	Continued MMF	171	Not relapsed	Maintaining remission on day 365
Median	74.0		146.5	129.0	
Range	55–172		84–245	8–353	

two patients (patients 5 and 10), B-cells decreased but were not depleted (fewer than five per cubic millimeter). Minimum CD19+ B-cell counts were 61 per cubic millimeter and 44 per cubic millimeter, and the reduction in CD19+ B-cells was 87% and 91%, respectively.

Nine patients had relapses of nephrotic syndrome (NS) at a median of 129 days after the rituximab infusion. Most of the relapses developed simultaneously with the recovery of B-cells. Seven of these patients (patients 1, 2, 4, 6, 7, 8, and 11) were given additional rituximab infusions at an average of 175 days after the first rituximab infusion, due to steroid dependency. Patient 8 suffered relapse 8 days after the first rituximab infusion, although complete B-cell depletion was achieved, but his steroids were discontinued on day 76. After two steroid-free months, he suffered from two more relapses. Although he was given an additional single-dose rituximab infusion on day 196, the condition relapsed again, which led to two additional doses of rituximab infusions from day 343. Two patients (patients 5 and 7) were restarted on CyA. The disease in three patients (patients 3, 10, and 12) remained in remission for 12 months after infusions.

Comparison of clinical parameters before and after rituximab treatment

The frequency of relapses per 6 months was significantly reduced, and the steroid-free period per 6 months was significantly increased after treatment, compared with those before treatment (Table 3). Mean PSL dosages

Table 3 Frequency of relapses and steroid-free periods before and after rituximab infusion (*Rtx* rituximab)

Patient no.	Number of relapses		Steroid-free period (days)	
	During the 6 months before Rtx infusions	During the 6 months after Rtx infusions	During the 6 months before Rtx infusions	During the 6 months before Rtx infusions
1	3	2	12	64
2	1	1	0	77
3	3	0	47	114
4	2	2	0	34
5	4	0	5	126
6	2	1	0	64
7	4	2	13	72
8	2	3	0	63
9	4	0	0	60
10	5	0	7	64
11	2	2	0	69
12	2	0	0	9
Mean	2.83*	1.08*	7.0**	68.0**
S.D.	1.19	1.08	13.5	30.7

*P=0.016; **P=0.0005

(before infusion 0.82±0.36 mg/kg per day) were significantly reduced between 2 months and 5 months after rituximab infusion (2 months 0.32±0.17 mg/kg per day; 3 months 0.08±0.09 mg/kg per day; 4 months 0.07±0.15 mg/kg per day; 5 months 0.39±0.44 mg/kg per day), but they increased again at 6 months (0.45±0.63 mg/kg per day) (Fig. 1a).

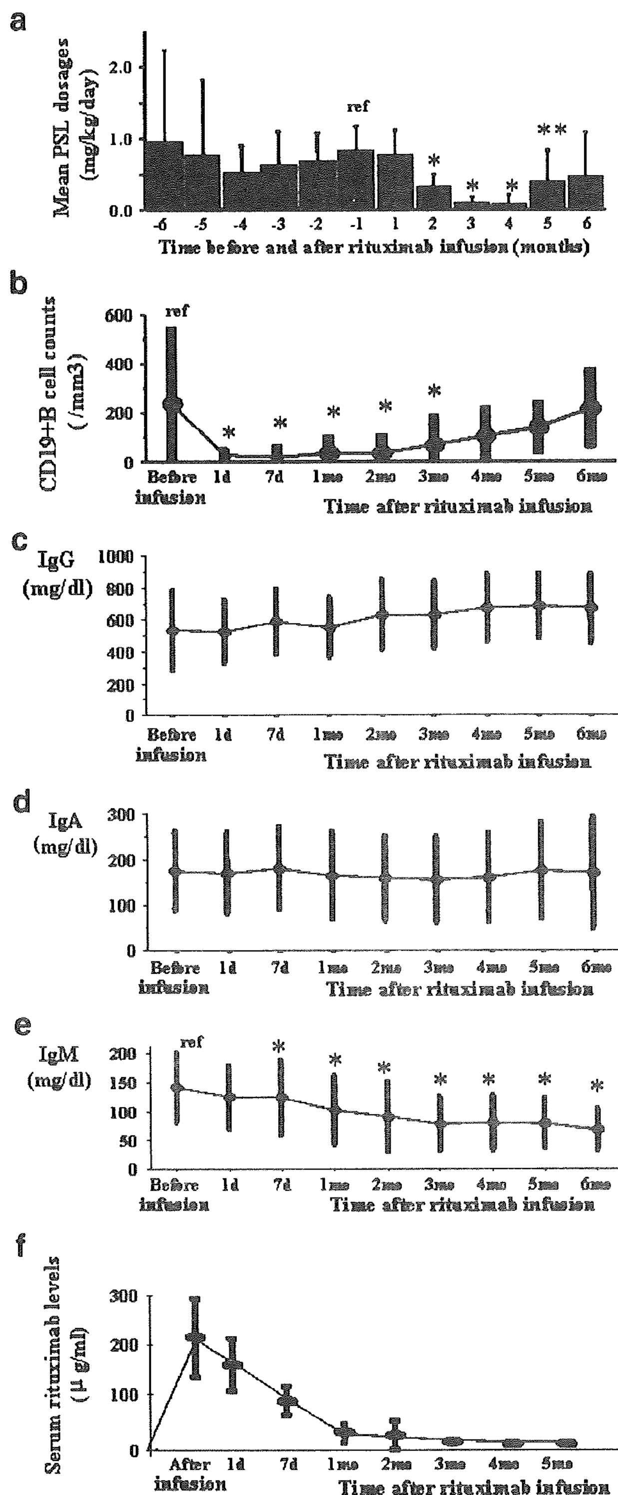
B-cell counts and serum immunoglobulin levels

B-cells were depleted in ten of 12 patients. Peripheral B-cell counts (before infusion 238.7±314.7 per cubic millimeter) rapidly decreased immediately after rituximab infusion (24 hours 28.3±29.2 per cubic millimeter). B-cells gradually increased at 4 months (105.2±120.6 per cubic millimeter) and returned to baseline levels at 6 months (218.0±166.2 per cubic millimeter) (Fig. 1b). Serum immunoglobulin (Ig)G and IgA levels did not change during the clinical course, but serum IgM levels gradually decreased (before infusion 135.7±58.4 mg/dl; 1 month 94.1±58.3 mg/dl; 6 months 61.2±25.4 mg/dl) after rituximab treatment (Fig. 1c–e).

Fig. 1 Clinical parameters after rituximab treatment. Values are means±standard deviations (SDs). **a** Mean PSL dosages before and after rituximab treatment. *P<0.01, **P<0.05 compared with before infusion (reference). **b** CD19+B-cell counts after rituximab treatment. *P<0.01 compared with before infusion (reference). **c–e** Serum immunoglobulin (Ig)G, IgA and IgM levels after rituximab treatment. *P<0.01 compared with before infusion (reference). **f** Serum rituximab levels after infusion. *d* days, *mo* months

Pharmacokinetics

Figure 1f shows serum rituximab levels after the drug infusion. Cmax obtained just after the drug infusion was 220.0±78.9 μg/ml. Serum rituximab levels at 24 hours,



1 week, 1 month, 2 months and 3 months after the infusion were 166.2±56.2 μg/ml, 92.5±29.8 μg/ml, 27.4±18.4 μg/ml, 18.5±29.8 μg/ml and 3.0±4.4 μg/ml, respectively. They were undetectable (<0.5 μg/ml) in all patients at 5 months. A detailed pharmacokinetics study was carried out in five patients. T1/2 was 14.6±5.2 days, AUC was 83.2±53.1 mg·h/ml, Vdss was 2.2±0.37 l/m^2 and CL was 5.83±2.97 ml/h per square millimeter.

Probability of relapse-free survival and B-cell depletion

Figure 2a shows the probability of relapse-free survival by the Kaplan–Meier method. The median time to first relapse (50% survival time) in our patients was 129 days. Figure 2b shows the probability of CD19+ B-cell depletion by the Kaplan–Meier method. The median time to B-cell recovery in our patients was 119 days (50% survival time).

Adverse events

Mild reactions to the infusions were observed in five of 12 patients (42%). These reactions included fever and hypotension (one patient), tachycardia (one patient), hypertension (one patient), facial flushing (one patient), and mild respiratory distress (one patient). None of the patients developed serious adverse events that required discontinuation of the trial. One patient (patient 5) developed a fever of unknown etiology between days 45 and 98 but recovered spontaneously.

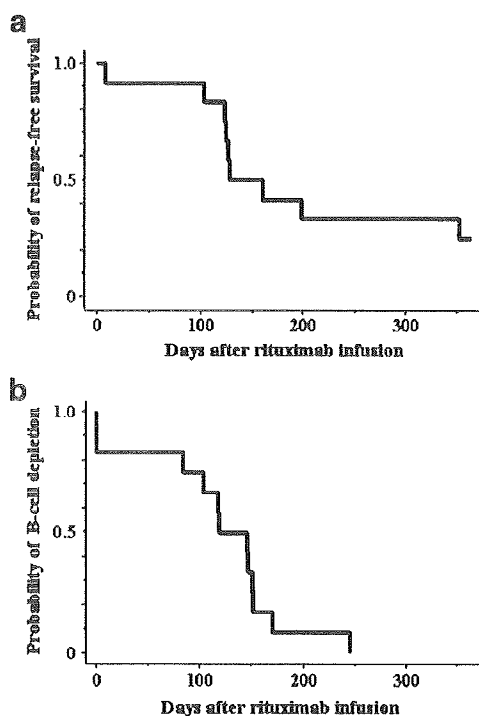


Fig. 2 Probability of relapse-free survival (**a**) and of B-cell depletion (**b**)

Discussion

This is the first report of a prospective study of single-dose therapy with rituximab for refractory SDNS. All patients were able to discontinue steroids after rituximab treatment. We found that the frequency of relapses was significantly reduced, the steroid-free period was significantly increased and mean steroid dosages were significantly reduced after rituximab treatment, suggesting that rituximab is effective for patients with SDNS. However, the efficacy of the treatment was transient in most patients. Nine of 12 patients (75%) had relapses within 1 year, and most of the relapses developed simultaneously with the recovery of B-cells. Seven of the patients needed additional rituximab treatment due to steroid dependency. It is of note that two out of the three patients who did not have a relapse and one of the patients who did not suffer relapse until day 353 did not stop their PSL until day 120 or later. Therefore, it is possible that the speed of PSL withdrawal was related to relapses, although further studies are required.

There have been no previous reports on rituximab pharmacokinetics in children. Table 4 shows previous reports of rituximab pharmacokinetics [16–22]. Most of the previous pharmacokinetic studies were carried out on adult patients with B-cell lymphoma, and most of them were given four doses of rituximab. The only large rituximab pharmacokinetics study of patients that did not have lymphoma was reported by Breedveld et al. [22]; however, they treated their patients with two doses of 1,000 mg rituximab. Vieira et al. reported pharmacokinetics of single-dose therapy with rituximab in kidney transplanted adults [16]. In that study, nine patients were treated with a single dose of rituximab (n= 3 per group) at 50 mg/m^2, 150 mg/m^2 or 375 mg/m^2 and the data for Cmax per dose (micrograms per milliliter per milligram) were almost the same among the three groups (0.30, 0.35, 0.30 each). Our data (Cmax 220.0 μg/ml; T1/2 14.6 days; AUC 83.2 mg·h/ml) were comparable with those of the patients who were treated with a dose of 375 mg/m^2 in the study by Vieira and colleagues, although the disease was different, suggesting that the rituximab pharmacokinetics profile in children is similar to that in adults.

Cmax of rituximab in our study (220.0 μg/ml) were comparable to serum levels after initial infusion of rituximab in previous reports on B-cell lymphoma (range 188.5–245.3 μg/ml) (Table 4). The peak level after four doses of rituximab was almost twice as high as that of a single dose in previous reports. Apart from in this study, we treated children with refractory SDNS with four doses of rituximab (n=4). In those patients treated with four doses of rituximab, the maximum serum levels of rituximab (403.4±122.9 μg/ml; range 318.7–584.8 μg/ml) were similar to those in the previous studies (Table 4) and were almost twice as high as those in this study. Berinstein et al. showed that the mean T1/2 was longer and the mean CL was smaller after four doses of

Table 4 Previous reports on rituximab pharmacokinetics data (*T1/2* elimination half-life, *AUC* area under the concentration–time curve, *Vss* volume of distribution at steady state, *CL* clearance)

Author	Disease	Number of patients	Age (years)	Dose of rituximab	Serum levels after the initial infusion (μg/ml)	Serum levels after the last infusion (μg/ml)	T1/2 (days)	AUC (mg·h/ml)	Vss	CL
Berinstein et al. [17]	B-cell lymphoma	166 (14)[a]	22–79	375 mg/m² ×4	239.1	460.7	8.6[d,e]	–	–	9.2 ml/h[c]
Davis et al. [18]	B-cell lymphoma	38 (10)[b]	31–80	375 mg/m² ×4	245.3	426.4	12.7[c]	–	–	–
Tobinai et al. [19]	B-cell lymphoma	4	31–66	250 mg/m² ×4	–	64.3	23.4[d,e]	91.3[c]	10.76 l [d,e]	168 ml/h[d,e]
	B-cell lymphoma	8	36–75	375 mg/m² ×4	–	92.1	16.2[d,e]	118.2[c]	11.16 l [d,e]	44 ml/h[d,e]
Iacona et al. [20]	B-cell lymphoma	7	Adults	375 mg/m² ×4	188.5	347.2	19.9[e,f]	–	–	5.4 ml/h per square meter[e]
Regazzi et al. [21]	B-cell lymphoma	22	Adults	375 mg/m² ×4	203.3	377.7	21.4[e,f]	–	3.52 l/m² [e]	5.1 ml/h per square meter[e]
	Autoimmune disease	14	Adults	375 mg/m² ×4	192.9	323.6	20.2[e,l]	–	4.45 l/m² [e]	6.6 ml/h per square meter[e]
	AL amyloidosis	4	Adults	375 mg/m² ×8	203.7	493.5	–	–	–	–
	Refractory lymphoma	8	Adults	375 mg/m² ×6	188.5	248.0	22.4[e,f]	–	4.03 l/m² [e]	5.2 ml/h per square meter[e]
Breedveld et al. [22]	Rheumatoid arthritis	111	Adults	1,000 mg ×2	–	453	20.7[c]	233.0[c]	4.56 l [c]	9.6 ml/h[c]
Vieira et al. [16]	Transplanted kidney	3	30–44	50 mg/m² ×1	25.90	–	9.88[c]	5.19[c]	–	–
	Transplanted kidney	3	34–55	100 mg/m² ×1	88.57	–	14.14[c]	21.3[c]	–	–
	Transplanted kidney	3	46–52	375 mg/m² ×1	232.67	–	12.36[c]	47.8[c]	–	–

[a] Pharmacokinetics data were analyzed in 14 patients

[b] Pharmacokinetics data were analyzed in ten patients

[c] Non-compartment model

[d] One-compartment model

[e] Two-compartment model

[f] T1/2β

rituximab than those of the initial dose, and that this phenomenon was probably due to circulating B-cells, which played the role as antibodies of rituximab at the time of the initial infusion [17]. Collectively, four doses of rituximab may provide higher maximum serum levels, a longer T1/2 and smaller CL, even in childhood refractory SDNS. In addition, only two of six patients had a first relapse within 1 year after the treatment (261 days and 270 days after the treatment, respectively) in previously reported studies using a dose of 375 mg/m^2 BSA once weekly for 4 weeks [3–5, 7, 9], whereas the median time to first relapse was 129 days in our patients. Also, the time to B-cell recovery was 270 days and 360 days after the treatment, respectively, in a previously reported two patients on a dose of 375 mg/m^2 BSA once weekly for 4 weeks [4, 5], while the median time to B-cell recovery was 119 days in our patients. Therefore, a multicenter, randomized, double-blind, placebo-controlled trial for patients with childhood refractory SDNS is in progress in Japan to evaluate the efficacy and safety of four doses of rituximab. Alternatively, a single dose of rituximab every several months could be effective for childhood refractory SDNS, for which further studies are needed to examine the optimal protocol of this drug.

Mechanisms by which rituximab can prevent relapses in patients with refractory SDNS remain unclear. For more than 30 years, nephrotic syndrome was thought to be primarily a disorder of T-cell function. However, recently, a number of clinical observations provided evidence of an important role for B-cells in the development of nephrotic syndrome. Kemper et al. found increased levels of both sCD23 (a marker of B-cell activation) and sCD25 (a marker of T-cell activation) during relapses of SDNS [23]. Cho et al. also observed a significantly higher expression of CD23 in fresh B-cells from patients with active minimal change nephrotic syndrome [24]. Several studies have shown increased production of interleukin (IL)-13 and elevated expression of IL-13 mRNA in patients with minimal change nephrotic syndrome [24, 25]. IL-13 is one of the cytokines associated with type 2 T helper (Th2) cells, leading to antibody production and allergic reactions, which are caused by immune responses involving B-cells.

As well as being the source of plasma cells, B-cells are also involved in the presentation of antigens to T-lymphocytes, and they secrete co-stimulatory signals required for CD4 T-cell activation [26]. Therefore, B-cell depletion by rituximab may block T-cell activation induced by B-cells or B-cell-derived factors. Tokunaga et al. showed that rituximab decreases CD40- and CD80-expressing cells among activated B-cells in patients with systemic lupus erythematosus (SLE), and it also down-regulates CD40L and CD69 on CD4-positive cells [27]. Those results imply that rituximab can inhibit the interaction between these B-cells and activated T-cells. However, the roles of CD40L in patients with active SDNS are not yet known and will need to be investigated in the future.

In our study adverse events associated with rituximab were acceptable. Mild reactions to the infusion were observed in five of 12 patients (42%). Steroids were not infused before rituximab treatment in our protocol. There might have been fewer infusion reactions if steroids had been given immediately before the rituximab treatment. There were no serious adverse events during the patients' clinical courses.

In conclusion, a single dose of rituximab may be effective for patients with childhood refractory SDNS. However, its efficacy to prevent relapses was transient in most of the patients.

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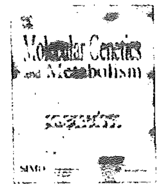

High frequency of acid α-glucosidase pseudodeficiency complicates newborn screening for glycogen storage disease type II in the Japanese population

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ABSTRACT

To investigate the feasibility of newborn screening for glycogen storage disease type II (GSDII; Pompe disease or acid maltase deficiency) in the Japanese population, we assayed the acid α-glucosidase activity in dried blood spots from 715 Japanese newborns and 18 previously diagnosed patients using a fluorometric procedure. The enzyme activity of apparently healthy newborns showed a bimodal distribution. The median activity of the minor group (31 individuals, 4.3% of the samples) was 6.5 times lower than that of the major group. Four of the 715 control samples (0.56%) fell in the patient range. We then analyzed genomic DNA, extracted from the same blood spots, for the occurrence of two sequence variants, c.1726G>A and c.2065G>A, known to cause "pseudodeficiency". This analysis revealed that 27 of 28 individuals homozygous for c.[1726A; 2065A] belonged to the minor group. One c.[1726A; 2065A] homozygote had just slightly higher activity. Twelve of the 18 patients with GSDII either had one (9 cases) or two (3 cases) c.[1726A; 2065A] alleles. The frequency of this allele was double in the patient compared to the control group (0.42 *vs* 0.19) at the expense of a lower frequency of the c.[1726G; 2065G] and c.[1726G; 2065A] alleles (0.58 *vs* 0.71 and 0 *vs* 0.1). These findings illustrate that c.[1726A; 2065A] homozygosity among apparently healthy individuals (3.9 per 100) complicates newborn screening for GSDII in Japan, and further that one or more pathogenic mutations are associated with the c.[1726A; 2065A] allele.
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Enzyme replacement therapy for lysosomal storage diseases has focused attention on the need for early diagnosis in order to optimize the therapeutic outcome. Along this line several initiatives have been taken to develop methods for newborn screening. Most methods are based on the direct measurement of lysosomal enzyme activities in dried blood spots (DBSs) [1–5]. Other procedures include antibodies to increase the specificity of the assay, or to determine the amount of enzyme protein rather than activity, or to probe lysosomal disease markers [6–9]. Multiplex assays with the measurement of several lysosomal enzyme activities are aimed to improve the cost effectiveness of newborn screening [10–13].

From several clinical trials since 1999, the picture emerges that patients with glycogen storage disease type II (GSDII) can benefit from enzyme replacement therapy [14–18]. GSDII, also known as Pompe disease or acid maltase deficiency (OMIM No. 232300) is an autosomal recessive disorder of glycogen metabolism resulting from a generalized deficiency of the lysosomal enzyme acid α-glucosidase (AαGlu[1]; EC 3.2.1.20/3). The enzyme deficiency causes intralysosomal glycogen storage in numerous tissues, but predominantly in muscle. The disorder exhibits a broad clinical spectrum with regard to age of onset, cardiac involvement and progression of skeletal muscle dysfunction. The effect of therapy in severely affected infants is readily recognized by regression of the cardiomegaly, prolonged survival and acquirement of motor skills. Beneficial effects of enzyme replacement therapy in children, adolescents and adults with GSDII also have been reported and are promising, but the crucial outcomes of larger clinical trials is still to be awaited as well as the long term effects [17–20]. Further, it appears that infants with rather well preserved muscle morphology respond better to therapy than those who are diagnosed late and have severe muscle damage at start of treatment. Early diagnosis seems a must in GSDII to optimize any form of therapeutic intervention [21].

Recently, we have established an assay procedure for the reliable diagnosis of GSDII in mixed leukocytes whereby acarbose is used to inhibit the interfering α-glucosidase activity of

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[1] *Abbreviations used:* AαGlu, acid α-glucosidase; GSDII, glycogen storage disease type II; 4MU-αGlc, 4-methylumbelliferyl α-D-glucopyranoside; DBS, dried blood spot.

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maltase-glucoamylase [22]. Of note, acarbose was introduced earlier to eliminate the interfering maltase-glucoamylase activity in DBS assays [10] and is more suitable for that purpose than maltose [3]. Given the recent interest in newborn screening and the awareness that the diagnosis of GSDII in Japan and in other Asian populations might be complicated by the existence of a "pseudodeficiency" allele [23,24], we performed a pilot experiment investigating the feasibility of newborn screening in Japan. "Pseudodeficiency" of AαGlu is associated with two SNPs, c.1726G>A (p.G576S) and c.2065G>A (p.E689K) that have a different distribution in Asian compared with Caucasian populations [23–25]. Substitution p.E689K caused by c.2065G>A characterizes the "GAA4" allozyme, which is found in Chinese and Japanese populations with frequencies of 0.27–0.28 and 0.27–0.31, respectively, and reduces the AαGlu activity by 50% at most [26–28; JSNP, http://snp.ims.u-tokyo.ac.jp/map/cgi-bin/aa_XM.cgi?NM_000152.2]. On the contrary, substitution p.G576S caused by c.1726G>A reduces the activity to such extent that it falls into the patient range [24]. Recently, it was shown that the structural changes brought about by each of the two substitutions are small and do not affect the active site of AαGlu [23].

Here, we present the results of the experiment in which we measured the AαGlu activity in DBSs with a fluorometric procedure while we performed in parallel haplotype analysis on DNA extracted from the same spot. Based on our findings we conclude that the high frequency of the "pseudodeficiency" allele in the Japanese population complicates the finding of an enzymatic screening procedure that is both sensitive and specific.

Subjects, materials and methods

Subjects and DBS collection

Seven hundred and fifteen Japanese newborns (second to fifth day postpartum) and 18 Japanese patients with GSDII were enrolled in this study. The patient group included one patient with classic infantile form, 6 with juvenile form, 10 with adult form and one with unknown phenotype. The DBSs on filter paper were obtained with the standard heel-stick for collecting newborn screening samples, or prepared by drop-wise addition of EDTA-blood samples on the filter paper (filter paper #510AD01, Advantec, Tokyo, Japan) that is routinely used for newborn screening in Japan. DBSs were dried at room temperature for at least 3 h but no more than 16 h, and were subsequently stored at −20 °C in sealed plastic bags until use. Written informed consent was obtained from all subjects, and all samples from these subjects were prepared and analyzed in accordance with the protocols approved by the institutional responsible committee.

Chemicals and reagents

4-Methylumbelliferyl α-D-glucopyranoside (4MU-αGlc) was purchased from Sigma-Aldrich (St. Louis, MO). Acarbose, 4-methylumbelliferone and Proteinase K were from Toronto Research Chemicals (North York, Canada), Nacalai Tesque (Osaka, Japan) and Roche (Basel, Switzerland), respectively. Ampdirect™ Plus with NovaTaq™ Hot Start DNA polymerase was obtained from Simadzu (Kyoto, Japan). Other chemicals were of reagent grade and from Sigma-Aldrich or Nacalai Tesque.

Enzymatic assay

A 3.2-mm diameter disk was punched out from the DBS on the filter paper and incubated in a well of a 96-well clear microwell-plate (Corning, New York, NY) with 100 μL distilled water by mixing gently for 1 h at room temperature. The water extract was used for the enzymatic assay. The disk was recovered for DNA extraction and genotype analysis. The AαGlu activity was measured fluorometrically with 4MU-αGlc as substrate according to our previous report with minor changes [22]. Briefly, 20 μL of the extract was added to 40 μL of the substrate solution containing 2.0 mmol/L 4MU-αGlc in 0.2 mol/L citrate/0.4 mol/L sodium-phosphate buffer, at pH 4.0 with 4.5 μmol/L acarbose (3.0 μmol/L in final concentration), in a 96-well black microwell-plate (PerkinElmer, Boston, MA). The reaction mixture was incubated at 37 °C for 24 h, and the reaction was stopped by addition of 190 μL of 0.2 mol/L glycine-NaOH buffer at pH 10.7 containing 0.1% Triton X100. The fluorescence intensity was measured with the CORONA spectrofluorometer (MTP-600F, Colona Electric, Hitachinaka, Japan) at excitation and emission wave-lengths of 360 nm and 450 nm, respectively, and corrected for substrate blank. We used a stock solution of 100 μmol/L 4-methylumbelliferone in 20 mmol/L sodium-phosphate buffer (pH 7.0) to calibrate the measurement of liberated 4-methylumbelliferone. The enzyme activity was expressed as pico moles 4-methylumbelliferone released per hour per 3.2 mm diameter disk (pmol/h/disk). Each assay was performed in duplicate. The measured values per group are expressed as means ± SD unless otherwise indicated.

Disk clean-up and genotype analysis

After extraction with distilled water for assay of enzyme activity, the disk was recovered, washed with a 100 μL solution of 0.1% Triton X100 in water and incubated in 100 μL digestion buffer containing 0.2 mg/ml Proteinase K, 0.5% sodium dodecyl sulfate, 5 mM EDTA, 400 mM NaCl and 20 mM Tris–HCl (pH 8.0), in a 1.5 ml reaction tube, at 55 °C for 1 h. The reaction was terminated by heating for 10 min on a heat block at 95 °C. The disk was rinsed twice with 500 μL of 10 mM Tris–HCl containing 1 mM EDTA (pH 8.0), once with 400 μL isopropanol, and then dried on a heat block at 70 °C for 60 min. The cleaned-up disk was stored at 4 °C until use for genotype analysis. Genotype analysis was performed by PCR-based detection (amplification refractory mutation system; ARMS). Each cleaned-up disk was cut into 4 pieces with scissors or with a scalpel, and the pieces were placed into 4 PCR tubes each containing 10 μL Ampdirect™ Plus (including PCR buffer and dNTPs), 0.5 units NovaTaq™ Hot Start DNA polymerase, and a set of specific primers (each 0.5 μmol/L), in a total volume of 20 μL reaction mixture. We designed 4 different oligonucleotide primers for 4 PCR sets including either, 5′-TACAACCTGCACAACCTCAACG-3′ (F1) or 5′-TACAACCTGCACAACCTCAACA-3′ (F2) as forward primer and either, 5′-GGCCTGCTGGGCCGACTC-3′ (R1) or 5′-GGCCTGCTGGGCCGACTT-3′ (R2) as reverse primer for the amplification of 4 different *GAA* alleles characterized by different SNPs: the combination F1 + R1 for c.[1726G; 2065G], F1 + R2 for c.[1726G; 2065A], F2 + R1 for c.[1726A; 2065G], and F2 + R2 for c.[1726A; 2065A]. Each oligonucleotide primer was designed to have a one-base mismatch nucleotide at the −4 base position from the 3′ terminal end to improve the selectivity for allele detection. PCR was performed under the following conditions; an initial denaturation at 96 °C for 10 min; 40 cycles amplification with denaturation at 96 °C for 20 s, annealing at 64 °C for 20 s and extension at 72 °C for 90 s; and extra extension at 72 °C for 7 min. The PCR products (1209 bp fragments) were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. This method allows determining 10 different diplotypes, which were constructed from the combination of the 4 haplotypes (Tables 1 and 2). To confirm the reliability of the present method, DNA sequencing analysis was performed according to the procedure described elsewhere [24] for all diplotypes from 18 individuals.