Conflict of Interest

Nothing to declare.

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Autoimmune-type atypical hemolytic uremic syndrome treated with eculizumab as first-line therapy

Masataka Hisano,[1] Akira Ashida,[2] Eiji Nakano,[1] Mamiko Suehiro,[1] Yoko Yoshida,[3] Masanori Matsumoto,[3] Toshiyuki Miyata,[4] Yoshihiro Fujimura[3] and Motoshi Hattori[5]

[1]*Department of Nephrology, Chiba Children's Hospital, Chiba,* [2]*Department of Pediatrics, Osaka Medical College, Takatsuki,* [3]*Department of Blood Transfusion Medicine, Nara Medical University, Kashihara,* [4]*Department of Molecular Pathogenesis, Research Institute National Cerebral and Cardiovascular Center, Suita and* [5]*Department of Pediatric Nephrology, Tokyo Women's Medical University, Tokyo, Japan*

Abstract We report a case of atypical hemolytic uremic syndrome (aHUS) in a 4-year-old boy. Although the patient had the typical triad of aHUS (microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney injury), urgent dialysis was not indicated because he had neither oliguria nor severe electrolyte abnormality. He was given eculizumab as first-line therapy, which led to significant clinical improvement, thus avoiding any risk of complications associated with plasma exchange and central venous catheterization. Retrograde functional analysis of the patient's plasma using sheep erythrocytes indicated an increase in hemolysis, suggesting impairment of host cell protection by complement factor H. The use of eculizumab as first-line therapy in place of plasma exchange might be reasonable for pediatric patients with aHUS.

Key words atypical hemolytic uremic syndrome, eculizumab, plasma exchange.

Hemolytic uremic syndrome (HUS) is defined by the typical triad of microangiopathic hemolytic anemia, thrombocytopenia, and acute renal injury. More than 90% of cases in children are secondary to infection with enterohemorrhagic *Escherichia coli* (EHEC) which produces Shiga toxin. The remaining 10% of cases, however, are classified as atypical hemolytic uremic syndrome (aHUS). aHUS has a poor prognosis with a high mortality rate and a high rate of progression to end-stage renal failure.[1] Plasma exchange (PE) has been recommended as first-line rescue therapy for such aHUS episodes, and for prevention of relapse.[2,3] This treatment, however, has some problems in terms of long-term acceptance, and its efficacy is controversial. Also, vascular access carries risk of complications, including bleeding and vascular injury. Eculizumab (Soliris®; Alexion Pharmaceuticals, Cheshire, CT, USA) is a humanized monoclonal anti-C5 antibody that inhibits the terminal complement pathway and hinders the generation of pro-inflammatory C5a and C5b-9 (membrane attack complex: MAC). Recent reports have indicated the efficacy and safety of eculizumab in patients with aHUS.[4,5] In Japan, it was approved for the treatment of aHUS in September 2013.

Here we describe the clinical features of a child with aHUS due to autoantibody against complement factor H (CFH), who was treated successfully with eculizumab as first-line therapy.

Correspondence: Akira Ashida, MD PhD, Department of Pediatrics, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan. Email: ped006@poh.osaka-med.ac.jp

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Case report

The patient was a 4-year-old Japanese boy who was the second child of non-consanguineous parents. He had an elder brother and a younger sister, both of whom were healthy. He had been brought to his family physician with a 2 day history of headache,

nausea, appetite loss, and low-grade fever. Given that anemia, thrombocytopenia, and acute kidney injury were evident, he was tentatively diagnosed as having HUS, and referred to hospital for intensive care. The clinical course is summarized in Figure 1. On admission his complexion was pale and slightly icteric. Other physical data included bodyweight, 14.9 kg; body temperature, 37.5°C; pulse rate, 144 beats/min; and blood pressure, 106/60 mmHg. Neither hepatosplenomegaly nor enlargement of superficial lymph nodes was found. The laboratory findings on admission are summarized in Table 1. Among them, severe anemia, thrombocytopenia, hyperbilirubinemia, elevated lactate dehydrogenase, elevation of serum renal function markers including creatinine, and low C3, were remarkable. Both the direct and indirect Coomb's tests were negative. Hemostatic tests showed that prothrombin time and activated partial thromboplastin time were both within the normal range, but that fibrin/fibrinogen degradation products were elevated. Furthermore, red blood cell (RBC) fragmentation was found in a peripheral blood smear. Stool culture failed to identify Shiga toxin-producing EHEC, or both Shiga toxins 1 and 2. Although the patient had macrohematuria, moderate proteinuria, and elevation of serum renal function markers, he did not fall into the category of oliguria or severe electrolyte abnormality. For this reason, urgent dialysis was not initiated. On the following day (hospital day [HD] 2), fresh frozen plasma (FFP; 23 mL/kg) was infused in order to supply normal complement regulatory factors under a tentative diagnosis of aHUS, given that diarrhea was absent. On the third day (HD 3), however, the patient's clinical symptoms worsened, and RBC concentrates were therefore transfused. Given that plasma a disintegrin-like and metalloproteinase with thrombospondin type I motifs, number 13 (ADAMTS13) activity was 120% on the night of HD 3, the patient was definitively diagnosed as having aHUS, and given eculizumab at a dose of 600 mg. On the second day after eculizumab treatment (HD 5), the macrohematuria dramatically resolved, and thereafter hematology showed gradual improvement. On HD 10, the patient started to receive eculizumab at the maintenance dose (300 mg) by injection every 2 weeks. The patient was vaccinated against *Neisseria meningococcus* and *Streptococcus pneumonia* on HD 29 and HD37, respectively, and received prophylactic antibiotic therapy with cefditoren pivoxil until 2 weeks after vaccination for meningococcus. He was discharged with no sequelae on HD32, and thereafter received an injection of eculizumab at the maintenance dose (300 mg) every 2 weeks. There were no adverse events associated with eculizumab treatment, including infusion reaction or infection, in the whole period of observation, or any further recurrence of aHUS.

Retrograde analysis including hemolytic assay, and Western blotting for detection of anti-CFH antibody and complement factor H-related protein 1/3 (CFHR 1/3) were performed using the patient's plasma, which had been obtained before plasma infusion using the method reported previously.[6,7] Comprehensive gene mutation analysis of CFH, complement factor I (CFI), complement factor B (CFB), C3, membrane cofactor protein (MCP), and thrombomodulin, was also performed as described previously.[6] The patient's plasma enhanced the hemolysis of sheep erythrocytes and this effect was suppressed by addition of purified CFH, indicating impairment of host cell protection by CFH (Fig. 2). Anti-CFH antibody was detected in the patient's plasma, but no deficiency of the protein encoded by CFHR 1/3 was observed (Fig. 2).[8] Additionally, there were no mutations of CFH, CFI, CFB, C3, MCP, or thrombomodulin. Therefore, the patient was diagnosed as having aHUS due to autoantibody against CFH without CFHR 1/3 protein deficiency.

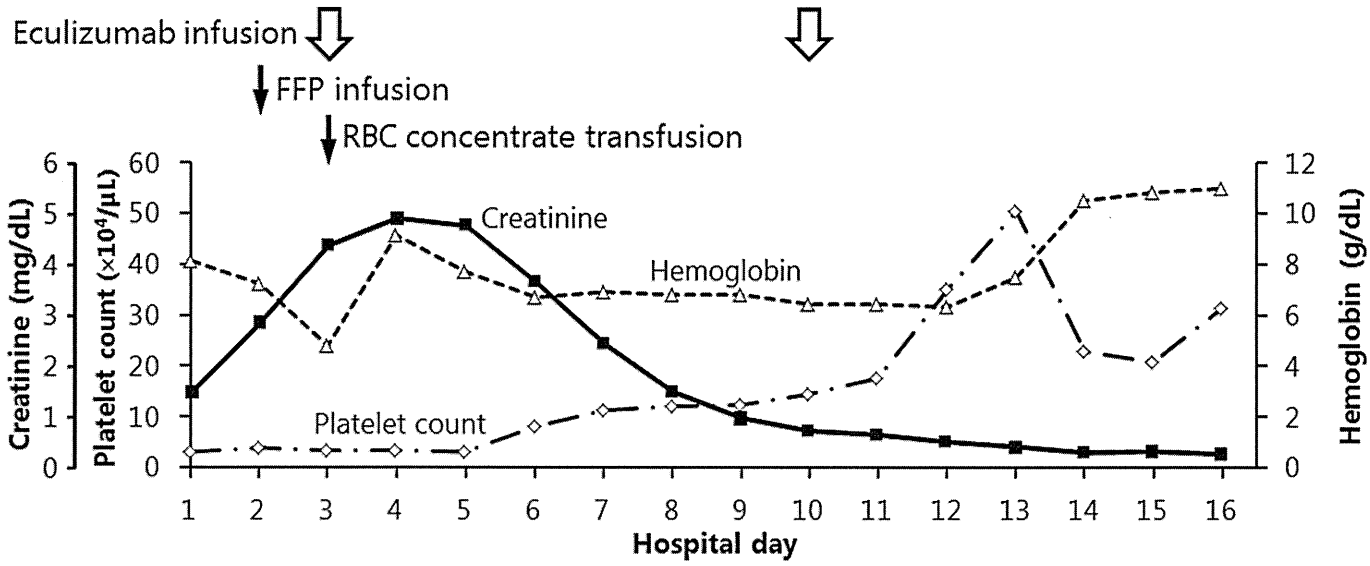

Fig. 1 Clinical course of treatment for atypical hemolytic uremic syndrome. Although fresh frozen plasma (FFP; 23 mL/kg) and red blood cell (RBC) concentrate were transfused on hospital days 2 and 3, laboratory findings including creatinine and platelet count worsened. After initiation of eculizumab, however, all of the laboratory parameters improved.

Table 1 Laboratory findings on admission

Peripheral blood		Stool culture	
WBC	9500 /μl	Normal flora	
RBC	300×10^4 /μl	EHEC	(−)
Hb	8.1 g/dL	Shiga-toxin	(−)
Ht	23.2%		
Platelet	1×10^4 /μl	Chemistry	
RBC fragmentation	(+)	TP	6.1 g/dL
		Alb	3.1 g/dL
Hemostatic test		Total bilirubin	2.6 mg/dL
PT	11.2 s	Indirect bilirubin	2.2 mg/dL
PT-INR	1.06	AST	101 IU/L
APTT	27.1 s	ALT	27 IU/L
Fibrinogen	278 mg/dL	LDH	3,570 IU/L
FDP	11.8 μg/mL	BUN	48.7 mg/dL
		UA	9.3 mg/dL
Urinalysis		Cr	1.51 mg/dL
Urine color	Light red	CRP	3.55 mg/dL
Occult blood	(4+)	Na	136 mmol/L
Protein	(2+)	K	3.8 mmol/L
Sediment		Cl	102 mmol/L
RBC	10–15/HPF	CK	403 IU/L
WBC	1–4/HPF	Haptoglobin	<10 mg/dL
Epithelium		Coombs test	
Epithelial cast	1+	Direct	(−)
		Indirect	(−)
Complement activity			
CH50	32.2 IU/L	Serological test	
C3	30.8 mg/dL	Total ANA	<40
C4	21.8 mg/dL	PR3-ANCA	<1.0
		MPO-ANCA	<1.0
ADAMTS13 activity	120%	ss-DNA antibody	<1.0
		ds-DNA antibody	<1.0

ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13; ANCA, anti-neurophil cytoplasmic antibody; APTT, activated partial thromboplastin time; EHEC, enterohemorrhagic *Escherichia coli*; FDP, fibrin/fibrinogen degradation products; INR, international normalized ratio; MPO, myeloperoxidase; PR3, proteinase 3; PT, prothrombin time.

Discussion

Atypical hemolytic uremic syndrome is a rare disease characterized by hemolytic anemia, thrombocytopenia, and acute renal failure secondary to thrombotic microangiopathy. In recent years, aHUS has been found to be associated with dysregulation of the complement alternative pathway. In more than half of patients with aHUS, mutations in genes encoding complement-regulating protein including CFH, CFI, and MCP, have been reported.[1] Additionally, functional CFH deficiency due to autoantibodies against CFH has been reported, and this is highly associated with polymorphic homozygous deletion of genes encoding CFHR proteins 1 and 3.[1] The present patient had had no diarrhea, and neither EHEC nor Shiga toxin had been found in his stools. ADAMTS13 activity was 120%, which was within the normal range. aHUS associated with anti-CFH autoantibody was diagnosed on the basis of additional examinations including gene mutation analysis and Western blot analysis for anti-CFH antibody and proteins encoded by CFHR1/3.

Plasma exchange has been recommended as a first-line therapy for aHUS based on expert opinion rather than clinical trials.[2,3] For management of aHUS associated with anti-CFH autoantibodies, PE with FFP has been done for the purpose of removing anti-CFH autoantibodies and simultaneously supplying the circulating CFH pool. Although combination therapy with immunosuppressants has also been used, the rate of remission in response to short-term PE is 70–80%, and the rate of death or end-stage renal disease as a long-term outcome is 30–40% in patients with anti-CFH autoantibodies.[1] Additionally, it is difficult to determine whether the disease activity is stable and leads to remission, because no international standard for determining anti-CFH antibody and the levels of autoantibodies leading to disease relapse or exacerbation has been established.

In contrast, previous case reports have suggested that eculizumab is effective for treatment of aHUS.[4,5] Additionally, Legendre *et al.* noted the efficacy and safety of long-term eculizumab for thrombotic microangiopathy in aHUS patients, via two prospective phase 2 trials lasting 62–64 weeks.[9] Although reduction of the antibody load plays a very important role in aHUS associated with anti-CFH autoantibodies, eculizumab can effectively block the terminal complement cascade and stop further damage in the presence of anti-CFH autoantibodies. Noone *et al.* reported two cases of CFH autoantibody-positive HUS treated with eculizumab and proposed that eculizumab should be used in the acute phase for arresting the complement-mediated damage.[10]

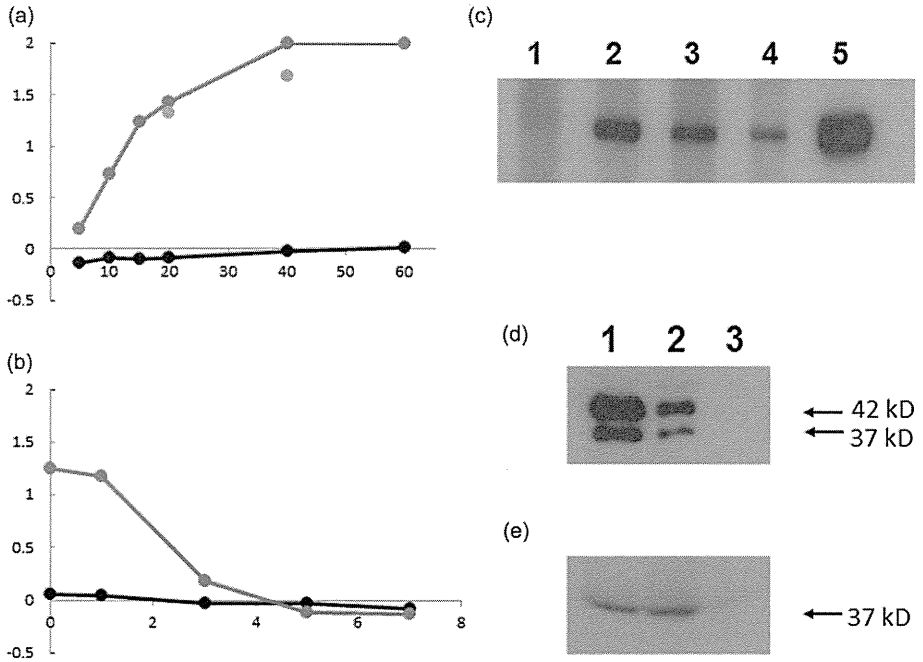

Fig. 2 (a,b) Hemolytic test and (c–e) Western blot analysis for detection of (c) anti-complement factor H (anti-CFH) autoantibodies and (d,e) protein encoded by *complement factor-H related protein CFHR1* and 3. (a) Lysis of sheep erythrocytes by addition of patient plasma. (a) OD_{414} titer sheep erythrocytolysis as a function of patient plasma. Plasma samples ranging from 5 μL to 60 μL were used. (b) Inhibition of enhanced hemolysis with 20 μL of plasma by adding purified CFH in amounts ranging from 0 μg to 7 μg. (—●—) Normal plasma ; (—●—) patient plasma; (●) normal plasma plus purified anti-CFH antibody. (c) Western blot analysis for detection of anti-CFH autoantibody. Lane 1, normal plasma; lane 2, patient plasma in the acute phase (hospital day [HD] 2); lane 3, patient plasma in the chronic phase (HD 31); lane 4, plasma of 3-year-old Japanese boy diagnosed with aHUS associated with anti-CFH autoantibody;[8] lane 5, CFH monoclonal antibody. (d,e) Western blot analysis for (d) CFHR 1 and (e) 3 proteins. (d) Plasma samples from a normal control, the present patient, and a patient who had been previously diagnosed as having deficiency of CFHR plasma proteins and autoantibody-positive HUS (DEAP-HUS)[8] were electrophoresed on a 12.0% SDS-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. After blocking with 5% dried milk, the membrane was incubated for 1.5 h at room temperature with mouse anti-human CFHR1 monoclonal antibody, the concentration of which was adjusted to 1 μg/mL. Then, 10 000-fold-diluted horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody was used as the secondary antibody, and bound mouse monoclonal antibody was visualized using enhanced chemiluminescence substrate (Western Lightning-ECL; Perkin Elmer, Yokohama, Japan). (e) Western blot analysis for detection of CFHR 3 protein was done using the same method as for CFHR 1, with 1500-diluted rabbit anti-human CFHR 3 polyclonal antibody as the first antibody and 20 000-diluted HRP-labeled goat anti-rabbit IgG antibody as the secondary antibody. Lane 1, normal control; lane 2, present patient; lane 3, DEAP-HUS patient.[8]

Given that the present patient had neither oliguria nor electrolyte abnormalities including hyperkalemia, urgent dialysis was not necessary. Therefore, the patient received eculizumab as first-line therapy and was able to avoid the risk of complications associated with these maneuvers. Therapy with eculizumab was very effective, and no adverse events occurred. Zuber *et al.* proposed the use of eculizumab as first-line therapy for all episodes of aHUS in children because of its efficacy and safety, and for avoiding any potential complications of PE.[4]

Conclusion

The present study has demonstrated the efficacy and short-term safety of eculizumab as first-line therapy in the acute phase for aHUS associated with anti-CFH autoantibodies in a pediatric patient.

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Stenosing ureteritis in Henoch–Schönlein purpura: Report of two cases

Katsuaki Kasahara, Osamu Uemura, Takuhito Nagai, Satoshi Yamakawa, Masaru Nakano and Naoyuki Iwata
Department of Pediatric Nephrology, Aichi Children's Health and Medical Center, Aichi-ken, Japan

Abstract Stenosing ureteritis (SU), a rare complication of Henoch–Schönlein purpura (HSP), typically presents with severe symptoms. We report the cases of two HSP patients presenting with gross hematuria, blood clotting, and colicky flank pain, followed by purpura on the lower extremities. Early-stage ultrasonography indicated hydronephrosis, thickened renal pelvic mucous membrane, and ureteral dilatation (UD), suggesting HSP complicated with SU. After early SU treatment with prednisolone, kidney function, thickened renal pelvic mucous membrane, and UD progressively normalized and the pain gradually disappeared. Regular ultrasonography of HSP patients from the onset of gross hematuria can be useful to detect early SU and facilitate conservative therapy with prednisolone. Diagnosis of SU can be easily missed by assuming HSP nephritis, particularly owing to the non-specific symptoms. Common characteristics as well as treatment methods and prognosis of SU are given in the literature review.

Key words gross hematuria, Henoch–Schönlein purpura, Henoch–Schönlein purpura nephritis, hydronephrosis, stenosing ureteritis, ultrasonography.

Henoch–Schönlein purpura (HSP) is the most common childhood systemic small-vessel vasculitis and can cause serious complications such as stenosing ureteritis (SU).[1,2] SU symptoms may be masked by gastrointestinal and renal symptoms and, in some cases, it may be diagnosed incidentally.[2–5] Depending on the course, various reports have stated the necessity of surgical treatment for SU, but the benefits of steroid monotherapy have also been noted.[2,6,7] We report the cases of two HSP patients presenting with ureteral stenosis, gross hematuria, and colicky flank pain that improved with prednisolone (PSL) therapy. Although an extensive literature review found >30 cases reports on the clinical spectrum of the complications, only seven English-language case reports published during 1997–2013 have been included (Table 1).[2–10]

Correspondence: Katsuaki Kasahara, MD, Department of Pediatric Nephrology, Aichi Children's Health and Medical Center, 1-2 Osakata, Morioka-chou, Oobu-shi, Aichi-ken 474-8710, Japan. Email: katsukasa@nifty.com

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Case reports

Patient 1

A 6-year-old boy, diagnosed with gastroenteritis by a local general practitioner, presented with a 3 day history of colicky flank pain and gross hematuria. The patient had a history of severe sinusitis and bilateral vesicoureteral reflux (stage III at 4 years of age), but no history of urinary tract infection (UTI) after 4 years of age. At presentation, he was afebrile and normotensive; on physical examination, tenderness was observed around the navel, with no evidence of purpura or joint pain.

Blood test results were as follows: white blood cell (WBC) count, 19 570/μL; platelet count, 35.0×10^4/μL; creatinine (Cr), 0.39 mg/dL; C-reactive protein (CRP), 7.6 mg/dL (normal, <0.5 mg/dL); complement component 3 (C3), 118 mg/dL (normal, 86–160 mg/dL); and D-dimer, 5.2 μg/mL (normal, <1.0 μg/dL). Urinary test results were as follows: urine protein, 300 mg/dL; urine sediment, 281/μL (normal, <5/μL) and 4865/μL (normal, <5/μL) for WBC and red blood cells (RBC; non-glomerular), respectively. Urine biochemistry was as follows: calcium/Cr ratio (Ca/Cr), 0.1 (normal, <0.31); and β-2 microglobulin (β2MG), 301 μg/L (normal, <230 μg/L). To exclude urolithiasis and colitis, ultrasonography and abdominal

A rapid, fully automated and highly sensitive ADAMTS13 gold particle immunoassay using a routine biochemistry analyser

Thrombotic microangiopathies (TMAs) are pathological conditions, characterized by thrombocytopenia, microangiopathic haemolytic anaemia and organ failure due to platelet thrombi (Moake, 2002). Two typical phenotypes of TMAs are thrombotic thrombocytopenic purpura (TTP) and haemolytic uremic syndrome, both of which are life-threatening generalized diseases. TMAs, however, occur more frequently in association with a variety of underlying clinical conditions, including pregnancy, autoimmune diseases, malignancy and transplantation (Lämmle *et al*, 2005). Therefore, the differential diagnosis of TMAs is critically important and often urged in clinical practice. Among the TMAs, TTP is now well defined by severe deficiency of the activity of the von Willebrand factor (VWF)-cleaving protease, termed ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motifs 13) (Sadler, 2008). However, currently existing assays for ADAMTS13 activity are tedious and time-consuming. To overcome these problems, we have developed a rapid, fully automated and highly sensitive assay for ADAMTS13 activity, termed ADAMTS13 act-GPI.

ADAMTS13 act-GPI is performed using gold particle immuno-agglutination with a biochemistry analyser, such as a Hitachi 7170 automatic clinical analyser (Hitachi, Tokyo, Japan), which is commonly found in routine laboratories. The principle of this novel assay, which consists of two reaction steps, is illustrated in Fig 1A. The first step involves the digestion of the substrate GST-VWF73-His (Kokame *et al*, 2003) by plasma ADAMTS13 for 4·93 min between Points 2 and 3. The second step involves the measurement of particle agglutination generated by immunoreactive lattice formation with the cleaved substrate (GST-VWF10) and two gold particles, coated with anti-GST monoclonal antibody (mAb) (IgG) or anti-N10 mAb (IgG) (Kato *et al*, 2006), respectively. Anti-N10 mAb specifically recognizes the Y1605 residue, which is exposed when the substrate VWF73 is cleaved by ADAMTS13. The degree of particle agglutination is continuously monitored by the change in absorbance (Fig 1B). The decrease in absorbance at 546/660 nm from 335·43 s to 590·04 s is represented as ΔAbs, and a calibration curve was obtained for ΔAbs and ADAMTS13 activity (Fig 1C). This novel assay can be completed in almost 10 min.

Two reagents are essential for ADAMTS13 act-GPI. One is the substrate GST-VWF73-His, which is dissolved at a concentration of 1·0 μg/ml in 10 mmol/l PIPES buffer (pH 5·5) containing 10 mmol/l $CaCl_2$, 5 mmol/l benzamidine, 1 mmol/l transamin, 0·4% argatroban, 0·01% Tween 20, 10% immobilized murine plasma and 3% chondroitin sulfate sodium. The other reagent is a mixture of two kinds of colloidal gold particles, coated with anti-GST mAb (IgG) and anti-N10 mAb (IgG), respectively, prepared according to the method of De Roe *et al* (1987) using gold particles with a mean diameter of 50 nm. The mAb-coated colloidal gold particles are suspended in 5 mmol/l HEPES buffer (pH 7·5) containing 30 g/l mannitol, 2 g/l EDTA-2Na and 0·08% Tween 80.

The detection limit for ADAMTS13 activity with ADAMTS13 act-GPI was determined to be 0·4% of normal, defined as three standard deviations (SDs) above the mean for samples with 0% activity (n = 8). Intra-assay reproducibility with three plasma samples (at 9·1%, 37·2% and 75·9% of plasma ADAMTS13 activity) determined by eight serial measurements was 1·5%, 0·5% and 0·4%, respectively. The inter-assay reproducibility of the same three samples determined on eight different days was 0·9%, 1·0% and 1·0%, respectively. Furthermore, no interference was found with the presence of unconjugated bilirubin (337·0 μmol/l), conjugated bilirubin (359·0 μmol/l), haemoglobin (4·88 g/l), chyle as a turbidity of sample (1550 formazin turbidity units; FTU), or rheumatoid factor (500 IU/ml) in the plasma samples, which were spiked with substances included in Interference Check A and RF plus (Sysmex, Kobe, Japan) (data shown in Fig S1). The specificity of this assay was confirmed by measuring plasma samples that were pre-incubated with two anti-ADAMTS13 mAbs, a neutralizing mAb (A10) and a non-neutralizing mAb (C7) (Yagi *et al*, 2007). Furthermore, EDTA at a final concentration of 10 mmol/l completely abolished ADAMTS13 activity by chelating metal ions (data shown in supporting information, Fig S1).

Comparative studies of ADAMTS13 activity were performed using three methods: ADAMTS13 act-GPI, ADAMTS13 act-enzyme-linked immunosorbent assay (ELISA), and a modified FRETS-VWF73 assay (Kokame *et al*, 2005; Kremer Hovinga *et al*, 2006). For this purpose, the initial determination of ADAMTS13 activity and its inhibitor (neutralizing antibodies) was performed using a chromogenic ADAMTS13 act-ELISA kit (Kainos Laboratories, Tokyo, Japan). Plasma samples used for this study were obtained from the following four groups: 20 genotyped patients with congenital TTP (Upshaw-Schulman syndrome, USS) (Fujimura *et al*, 2011), 30 patients with primary acquired TTP (aTTP) with severe deficient ADAMTS13 activity and

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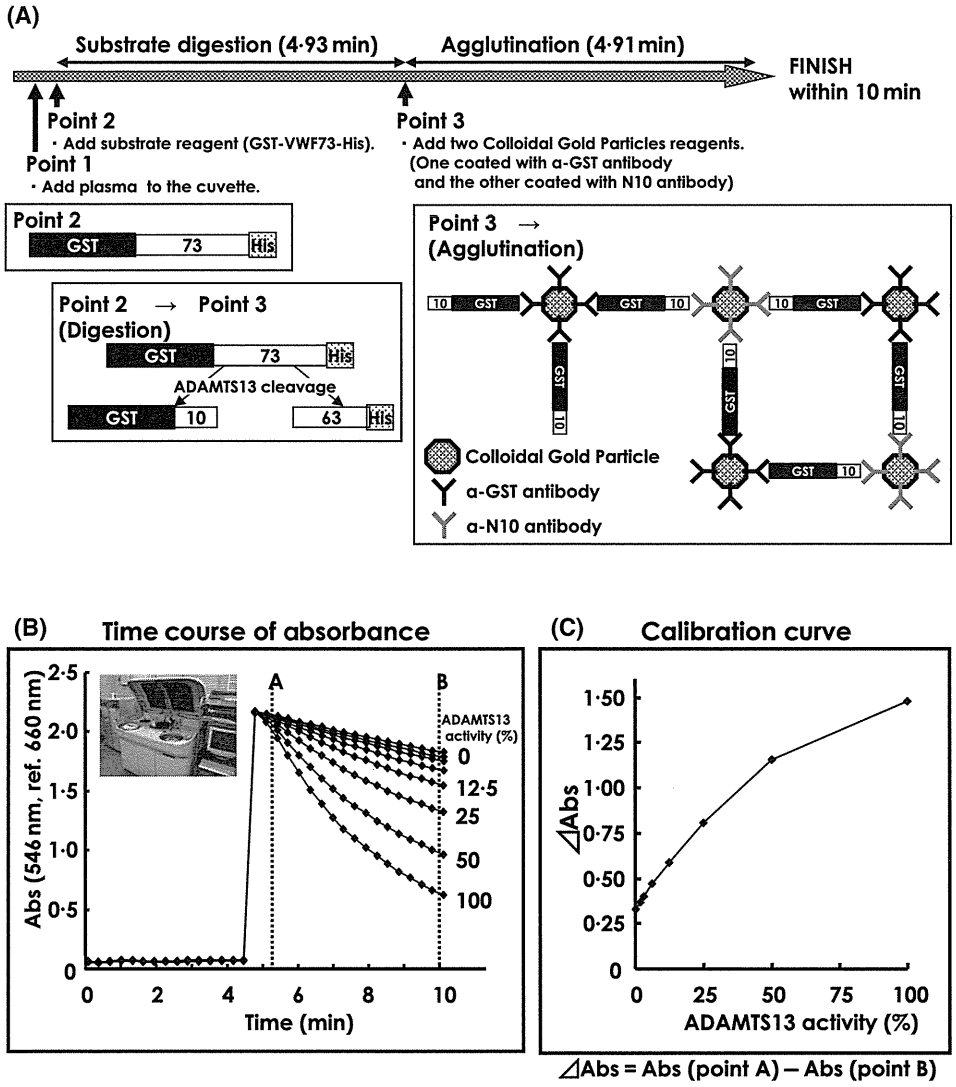

Fig 1. Determination of ADAMTS13 activity using novel ADAMTS13 act-GPI. (A) Schema illustrating the principle of the assay. There is only one manual step, putting a centrifuged tube of blood in the same manner that plasma is usually prepared as a sample into the analyser. The time-table and reactions in ADAMTS13 act-GPI are as follows. At Point 1, 5 μl of the sample is transferred to an assay cuvette. At Point 2, 90 μl of the substrate reagent, containing GST-VWF73-His, is added and stirred. Subsequently, the reaction mixture is incubated for 4·93 min at 37°C for the enzymatic reaction. After enzymatic digestion, at Point 3, 90 μl of the reagent containing colloidal gold particles coated with anti-GST and anti-N10 are added and stirred. The reaction between the particles and GST-VWF10, generated by ADAMTS13 activity in the sample, results in agglutination. This assay can be completed within approximately 10 min. (B) The time course of changes in absorbance. The time course with calibrants that have 100%, 50%, 25%, 12·5%, 6·25%, 3·125%, 1·5625% and 0% ADAMTS13 activity are depicted. The change in the ratio of absorbance values for 660 and 546 nm (secondary and primary wavelengths, respectively) from 335·43 s (dotted line A) to 590·04 s (dotted line B) was calculated and expressed as ΔAbs. The calibrants were prepared with pooled normal plasma sequentially diluted by pooled normal plasma that was heat inactivated. Inset: a photograph of the analyser. (C) A representative standard curve of ADAMTS13 activity.

positive ADAMTS13 inhibitors (>1·0 Bethesda U/ml), 120 patients with a variety of TMAs based on clinical and laboratory data, with more than 3% ADAMTS13 activity by ADAMTS13 act-ELISA, and 30 normal individuals aged 20–40 years (15 females and 15 males). The calibrants were prepared by pooled normal plasma (prepared from 30 males and 30 females aged 20–40 years) sequentially diluted with heat inactivated pooled normal plasma.

Plasma levels of ADAMTS13 activity determined by ADAMTS13 act-GPI in the aforementioned four categories are shown in Fig 2A. The correlation in plasma ADAMTS13 activity levels between ADAMTS13 act-ELISA (x-axis) and ADAMTS13 act-GPI (y-axis) is shown in Fig 2B, with the regression line ($y = 1{\cdot}1488x - 3{\cdot}3263$) and the correlation coefficient ($r = 0{\cdot}941$). Further, the correlation in plasma ADAMTS13 activity levels between FRETS-VWF73 (x-axis)

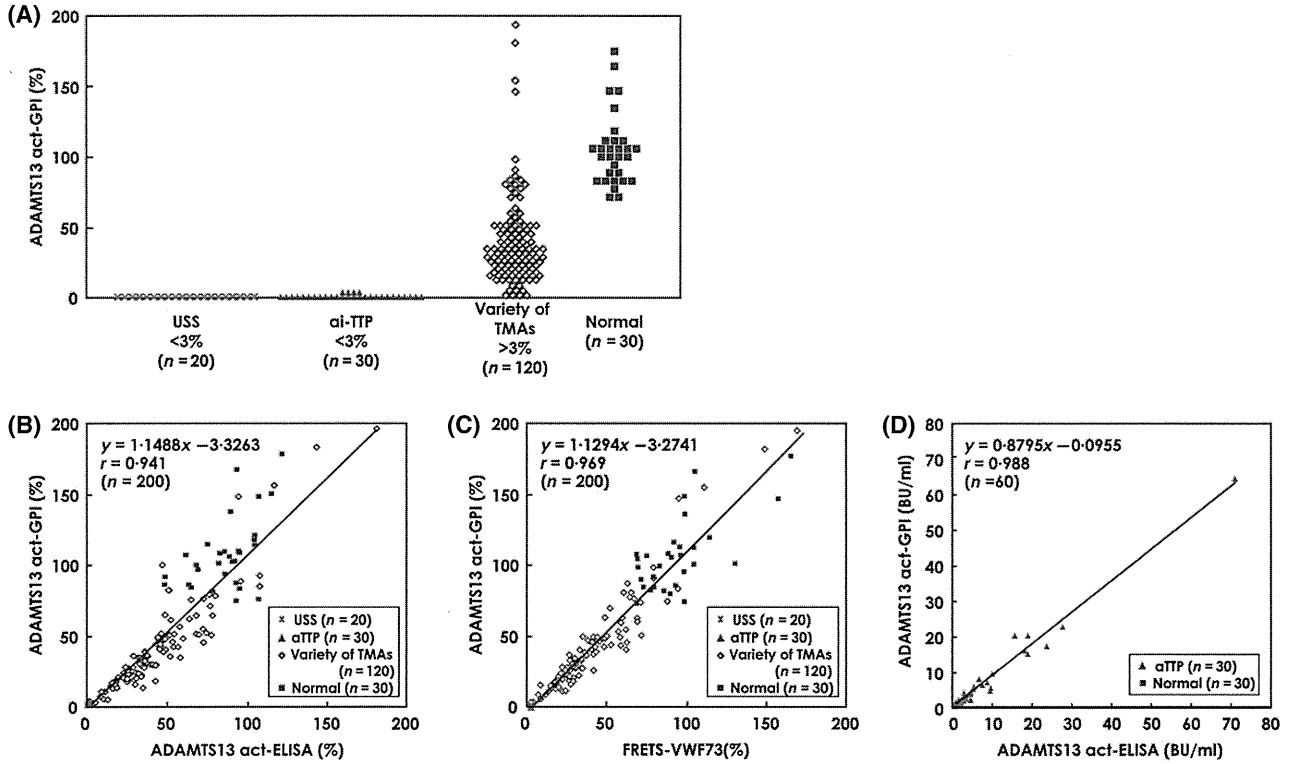

Fig 2. (A) Plasma ADAMTS13 activity levels determined by ADAMTS13 act-GPI. All the samples used in this study were 0·32% citrated plasma stored at −80°C until use. Plasma was obtained from the following four groups: 20 genotyped patients with congenital thrombotic thrombocytopenic purpura (TTP) (Upshaw-Schulman syndrome, USS), 30 patients with primary acquired TTP (aTTP) with severe deficiency of ADAMTS13 activity and positive for ADAMTS13 inhibitors (>1·0 Bethesda U/ml), 120 patients with a variety of suspected TMAs, based on clinical and laboratory information, with more than 3% ADAMTS13 activity by ADAMTS13 act-ELISA, and 30 normal individuals aged 20–40 years (15 females and 15 males). (B) The correlation in plasma ADAMTS13 activity levels between ADAMTS13 act-ELISA and ADAMTS13 act-GPI. (C) The correlation in plasma ADAMTS13 activity levels between FRETS-VWF73 and ADAMTS13 act-GPI. (D) The correlation in titres of plasma ADAMTS13 inhibitor determined using ADMTS13 act-ELISA and ADAMTS13 act-GTI in 30 aTTP patients and 30 healthy individuals (see text for details).

and ADAMTS13 act-GPI (y-axis) is shown in Fig 2C, with the regression line (y = 1·129x − 3·2741) and r (0·969). The plasma ADAMT13 activity levels, determined by three methods in four groups, are shown in Table S1. In Fig 2D, the correlation in plasma ADAMTS13 inhibitor titres determined by ADMTS13 act-ELISA (x-axis) and ADAMTS13 act-GTI (y-axis) is shown in 30 aTTP patients and 30 healthy individuals, with the regression line (y = 0·8795x − 0·0955) and r (0·988).

Although these three ADAMTS13 activity assays operate on totally different principles, the values obtained using these assays were highly correlated. We propose that ADAMTS13 act-GPI could be useful in clinical practice because automation has the advantages of rapidity, high-throughput performance and decreasing human contact with infectious materials.

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Author contribution

SK, ST and YF designed the research study; SK, MT and AI performed experiments; MM collected plasma samples; SK analysed data; MT supported construction of assay procedure; SK and YF wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

The authors have some conflicts of interest relevant to this manuscript submitted to *British Journal of Haematology.*

Employment: Seiji Kato and Mutsumi Tanaka are employees of Alfresa Pharma Corporation. Tomohiro Samori is an advisor of Japan Clinical Laboratories, Inc.

Patent: Alfresa Pharma corporation holds a patent (WO2006085441) for the ADAMTS13 activity assay. Seiji Kato, Masanori Matsumoto and Yoshihiro Fujimura are the inventors of the patent.

Correspondence

Seiji Kato[1]
Mutsumi Tanaka[1]
Ayami Isonishi[2]
Masanori Matsumoto[2]
Tomohiro Samori[3]
Yoshihiro Fujimura[2]
[1]*Division of Diagnostic R&D, Alfresa Pharma Corporation, Osaka,* [2]*Department of Blood Transfusion Medicine, Nara Medical University, Nara, and* [3]*Research Division, Japan Clinical Laboratories, Inc., Kyoto, Japan. E-mail: seiji-katoh@alfresa-pharma.co.jp*

Keywords: ADAMTS13, thrombotic thrombocytopenic purpura, thrombotic microangiopathy, assay

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Effect of interfering substances, anti-ADAMTS13 monoclonal antibody and EDTA on the ADAMTS13 act-GPI.

Table S1. Plasma ADAMTS13 levels activity determined by three methods in four groups.

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RESEARCH ARTICLE

A Novel Quantitative Hemolytic Assay Coupled with Restriction Fragment Length Polymorphisms Analysis Enabled Early Diagnosis of Atypical Hemolytic Uremic Syndrome and Identified Unique Predisposing Mutations in Japan

Yoko Yoshida[1], Toshiyuki Miyata[2], Masanori Matsumoto[1], Hiroko Shirotani-Ikejima[2], Yumiko Uchida[2], Yoshifumi Ohyama[3], Tetsuro Kokubo[3], Yoshihiro Fujimura[1]*

1 Department of Blood Transfusion Medicine, Nara Medical University, Kashihara, Japan, **2** Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Suita, Japan, **3** Molecular and Cellular Biology Laboratory, Graduate School of Medical Life Science, Yokohama City University, Yokohama, Japan

* yoshifuji325@naramed-u.ac.jp

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Abstract

For thrombotic microangiopathies (TMAs), the diagnosis of atypical hemolytic uremic syndrome (aHUS) is made by ruling out Shiga toxin-producing *Escherichia coli* (STEC)-associated HUS and ADAMTS13 activity-deficient thrombotic thrombocytopenic purpura (TTP), often using the exclusion criteria for secondary TMAs. Nowadays, assays for ADAMTS13 activity and evaluation for STEC infection can be performed within a few hours. However, a confident diagnosis of aHUS often requires comprehensive gene analysis of the alternative complement activation pathway, which usually takes at least several weeks. However, predisposing genetic abnormalities are only identified in approximately 70% of aHUS. To facilitate the diagnosis of complement-mediated aHUS, we describe a quantitative hemolytic assay using sheep red blood cells (RBCs) and human citrated plasma, spiked with or without a novel inhibitory anti-complement factor H (CFH) monoclonal antibody. Among 45 aHUS patients in Japan, 24% (11/45) had moderate-to-severe (≥50%) hemolysis, whereas the remaining 76% (34/45) patients had mild or no hemolysis (<50%). The former group is largely attributed to CFH-related abnormalities, and the latter group has C3-p.I1157T mutations (16/34), which were identified by restriction fragment length polymorphism (RFLP) analysis. Thus, a quantitative hemolytic assay coupled with RFLP analysis enabled the early diagnosis of complement-mediated aHUS in 60% (27/45) of patients in Japan within a week of presentation. We hypothesize that this novel quantitative hemolytic assay would be more useful in a Caucasian population, who may have a higher proportion of CFH mutations than Japanese patients.

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Competing Interests: YF received a research grant from Alexion Pharmaceuticals and the patent royalty for the ADAMTS13 assay from Alfresa Pharma Corporation. MM received the patent royalty for the ADAMTS13 assay from Alfresa Pharma Corporation. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

Introduction

Thrombotic thrombocytopenic purpura (TTP) with predominantly neurological involvement and hemolytic uremic syndrome (HUS) with predominately renal failure are both life-threatening systemic diseases that are often clinically indistinguishable. They are categorized as thrombotic microangiopathies (TMAs) [1, 2]. It is now well documented that TTP is caused by deficiency of ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13) activity, either because of genetic abnormalities or acquired autoantibodies [3, 4]. On the other hand, more than 90% of HUS cases are associated with Shiga toxin-producing *Escherichia coli* (STEC) infection, termed STEC-HUS or typical HUS. The remaining 10% or so, which does not involve STEC infection, is called atypical HUS (aHUS) [5].

Most cases of aHUS are caused by uncontrolled complement activation due to genetic abnormalities in the alternative pathway, including complement factor H (CFH), complement factor I (CFI), membrane cofactor protein (MCP), thrombomodulin (THBD), complement component C3 (C3), and complement factor B (CFB) [6]. Acquired autoantibodies against CFH can also mediate aHUS; they are frequently associated with homozygous gene deletion of CFH-related (CFHR) proteins 1 and 3 [7–9]. More recently, recessive mutations in diacylglycerol kinase ε, a protein kinase C inhibitor, were also shown to cause aHUS. Diacylglycerol kinase ε normally blocks signaling of arachidonic acid-containing diacylglycerols involved in platelet activation [10]. However, unlike ADAMTS13 deficiency in TTP and STEC infection in typical HUS, making a diagnosis of aHUS is not easy. In fact, comprehensive gene analysis takes at least several weeks, and can only detect genetic abnormalities in approximately 70% of the patients with aHUS [11].

In 2004, Sanchez-Corral et al. [12] introduced a qualitative hemolytic assay using sheep red blood cells (RBCs). This assay is based on the principle that in normal individuals, exogenous human CFH, via its glycosaminoglycan-binding domains in the C-terminal portion, binds to the sialic acid-rich surface of sheep RBCs, to which C3b binds and is then proteolytically inactivated by CFI. Ultimately, this results in inhibition of hemolysis. In contrast, mutant CFH may not readily bind to the surface of sheep RBCs, which results in an inability to block hemolysis associated with C3b generated from spontaneous hydrolysis, followed by the formation of membrane attack complex. In 2014, Roumenina et al. [13] reported on a modified hemolytic assay using serum or EDTA plasma. However, depending on how they were prepared and preserved, serum specimens often give inconsistent results in the hemolytic assays. In addition, it is not possible to measure ADAMTS13 activity using EDTA plasma because the enzyme is a divalent cation-dependent metalloproteinase [4]. Thus, existing hemolytic assays are qualitative in nature.

In this study, we have developed a quantitative hemolytic assay using sheep RBCs with human citrated plasma spiked with or without the novel inhibitory anti-CFH murine monoclonal antibody (mAb) O72. This was followed by genetic analysis of 45 aHUS patients in Japan. We found that this novel quantitative hemolytic assay plus RFLP analysis can be used for the early diagnosis of aHUS patients in Japan. Subsequent gene analysis identified a unique predisposing mutation accumulated in the Kansai district of Japan. Thus, although the number of tested patients is still small, the genetic abnormalities of aHUS patients in Japan appear to be different from those in Western countries.

Materials and Methods

Ethics statement

The study protocol was approved by the Ethics Committees of Nara Medical University Hospital and National Cerebral and Cardiovascular Center, and complied with the principles expressed in the Declaration of Helsinki. All patients were given written informed consent to participate in this study. All animal studies were approved by the Institutional Review Board of Nara Medical University. To sacrifice animals we used cervical dislocation, and all efforts were made to minimize suffering.

Patients

Since 1998, our laboratory at Nara Medical University has enrolled patients with suspected TMA based on clinical characteristics across Japan [14]. As of the end of 2013, we established a registry of 1,214 patients with TMA. Patient plasmas were sent to our laboratory, and firstly ADAMTS13 activity was measured. Of these 1,214 patients, severe deficiency of ADAMTS13 activity (<5% of normal) was found in 467 patients, of which 52 had congenital TTP and 415 had acquired TTP. The primary diagnosis of aHUS was made based on Japanese criteria for aHUS [15]: microangiopathic hemolytic anemia (hemoglobin <10 g/dl), thrombocytopenia (platelet count of $<150\times10^9$/l), and acute renal failure (pediatric patients: serum creatinine 1.5-fold higher than the age- and gender-specific Japanese Society for Pediatric Nephrology reference values [16]; adult patients: meeting diagnostic criteria for acute kidney injury (AKI)) with no severe ADAMTS13 activity deficiency or STEC infection. In this study, however, the diagnosis of aHUS was also made according to the exclusion criteria used in the UK [17] and Spain [18], where patients with organ or hematopoietic stem cell transplantation, systemic lupus erythematosus and pregnancy-associated are excluded. As a result, there were 77 aHUS patients in our cohort as of the end of 2013, of whom 45 patients belonged to 40 families that were analyzed by the novel hemolytic assay, and then followed by genetic analysis. Among them, 43 patients were tested using freshly prepared citrated plasmas, but in two patients (2P1 and 3G1) the sera stored at -80°C were only available for analysis. Plasma or serum levels of CFH and C3 in 43 of 45 patients were described in S1 Table. C3 level was determined by immune-nephelometry (SRL, Inc., Japan). CFH level was measured by Laurell's immunoelectrophoresis using rabbit anti-CFH serum prepared in our laboratory. A value of 100% of plasma CFH antigen level was defined by the amount in the pooled normal plasmas, prepared from a total of 20 healthy individuals (10 males and 10 females).

Purification of CFH from human plasma

Anti-CFH rabbit polyclonal antibody (pAb) was a gift from Professor Emeritus Teizo Fujita of Fukushima Medical University. Twenty milligrams of anti-CFH IgG pAb were coupled to 2 g of CNBr-activated Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. Ten milliliters of fresh frozen plasma were diluted three times with starting buffer consisting of 50 mM Tris-buffered saline (pH 7.4) containing 20 mM ε-aminocaproic acid, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine-HCl (BZ), 5 mM EDTA, and 0.02% sodium azide (NaN_3), and then applied to the immunoadsorbent column. The bound proteins were eluted with 0.1 M glycine-HCl (pH 2.7) containing 2 mM BZ and 0.02% NaN_3. The eluted protein fractions were immediately neutralized and dialyzed overnight at 4°C against 20 mM Tris-HCl buffer (pH 8) containing 2 mM BZ and 0.05% NaN_3. After dialysis, the samples were applied to a Mono Q 5/50 GL column (GE Healthcare Bio-Sciences AB) and the bound proteins were eluted with a linear salt gradient of 0 to 1 M NaCl.

Purified plasma CFH was eluted as a single major peak at 0.3 M NaCl. These fractions were pooled, concentrated with Aquacide II (Calbiochem, San Diego, CA, USA), dialyzed against Tris-buffered saline, and then stored in aliquots at −80°C until use.

Preparation of anti-CFH monoclonal antibodies (mAbs)

BALB/c mice were injected intraperitoneally with 50 μg of purified human CFH mixed with Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA) every two weeks for a total of four times. After immunization, the procedure for anti-CFH mAb production, which consisted of cell fusion, culture, and cloning, was performed according to a well-established method [19]. Six different clones that produce anti-CFH IgG mAbs were obtained, and their specificities were confirmed by Western blot analysis. The immunoglobulin subclass of the anti-CFH mAbs was determined using the Mouse/Rat Monoclonal Antibody Isotyping Test Kit (AbD Serotec, Kidlington, UK).

Construction of expression plasmids for recombinant CFH and CFH domains

To produce recombinant human CFH in budding yeast, various lengths of *CFH* fragments with a C-terminal Pk (V5) tag were inserted into a p416GAL1 vector [20] using the Sequence and Ligation Independent Cloning method unless otherwise noted [21]. In addition to full-length CFH, four truncated forms of CFH were generated as follows: short consensus repeat (SCR) 1–5 (plasmid pM7315), SCR6–10 (pM7316), SCR11–17 (pM7317) and SCR18–20 (pM7318). In addition, SCR18–20 was divided into three peptides: SCR18 and 19 (pM7336), SCR 19 and 20 (pM7340), and SCR 18 and 20 (pM7341). Full-length *CFH* (3693 bp) or these truncated forms were PCR-amplified from sequence-verified human *CFH* cDNA from the Mammalian Gene Collection (Clone ID: 40148771) with the corresponding primers, as well as a Pk (V5) tag fragment (132 bp) that was amplified by PCR from pM4376 [22]. As for the construction of pM7336, the fragment was prepared from pM7335 using the restriction enzyme SacI and XhoI, and was inserted in the p426GAL1 vector [20]. In pM7341, the expression plasmid for SCR18 and 20 was obtained in a two-step PCR reaction. The first PCR reaction was performed with two primer pairs, after which the fragments were connected by the second PCR (primer TK12150-TK12151). Detailed information on each recombinant CFH and its domain expression plasmid and primer are shown in S2 Table.

Expression of recombinant CFH and its fragments and Western blot analysis

Saccharomyces cerevisiae BY4741 (Euroscarf, Frankfurt, Germany) was transformed with each of the expression plasmids for recombinant CFH and its fragments. Transformed *Saccharomyces cerevisiae* cells were cultured in 20 ml of SD medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose) or SG medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 4% galactose) at 30°C until the mid-logarithmic growth phase. The treatment of cell extracts and immunoblot analysis was performed as previously described [23]. Anti-CFH mAb O72 (1:1000) and horseradish peroxidase (HRP)-labeled anti-mouse IgG (1:2000; #sc-2005, Santa Cruz Biotechnology, Dallas, TX, USA) were used for the detection of recombinant CFH and its fragments. Samples were developed using enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific, Waltham, MA, USA, and Merck Millipore, Darmstadt,

Germany) and detected on an LAS 4000 image analyzer (GE Healthcare, Buckinghamshire, England).

Hemolytic assay using citrated human plasma and sheep RBCs

Sheep RBCs were purchased from Japan Ram Co (Fukuyama, Japan). Hemolytic assays using sheep RBCs were performed according to the method of Sanchez-Correal et al and Roumenina et al [12, 13], except that throughout our study we used citrated plasma unless otherwise noted.

Briefly, we diluted 5–60 μl of normal or tested citrated plasma or serum into 100 μl with AP-CFTD buffer (2.5 mM barbital, 1.5 mM sodium barbital, 144 mM NaCl, 7 mM $MgCl_2$, and 10 mM EGTA, pH 7.2–7.4). Each sample of diluted plasma (100 μl) was then mixed with 100 μl of sheep RBC suspensions prepared with AP-CFTD buffer to a final concentration of 2.5×10^6 cells/μl. The mixture was further incubated at 37°C for 30 min. After incubation, the reaction was quenched by the addition of 1 ml of VBS-EDTA buffer (2.5 mM barbital, 1.5 mM sodium barbital and 144 mM NaCl, and 50 mM EDTA, pH 7.4). The mixtures were then centrifuged at 800 g at 4°C for 10 min, and the absorbance of the supernatant was measured at 414 nm. Plasma, diluted with AP-CFTD buffer containing 2 mM EDTA was treated in the same manner and used as a blank. In this study, 100% hemolysis was defined as the absorbance at 414 nm obtained with 20 μl of normal citrated plasma spiked with mAb O72 (200 μg/ml). The percentage of hemolysis in the patients was calculated as follows: the absorbance of the 20 μl plasma samples with the corresponding blank subtracted was divided by the absorbance of 20 μl normal plasma spiked with mAb O72. The hemolytic assay was performed in duplicate, and the mean value was used for calculation or shown as the result of the hemolytic assay. To determine the normal ranges of hemolysis, we tested hemolysis on freshly prepared citrated plasmas from 20 healthy individuals (10 males and 10 females).

In some experiments, to assess the inhibitory effect of anti-CFH IgG mAbs, we incubated 20 μl of normal plasma mixed with each anti-CFH mAb or control mouse IgG at a final concentration of 50, 100, 200, 300, or 400 μg IgG/ml at room temperature for 30 min. Subsequently, these mixtures were diluted to 100 μl with AP-CFTD buffer. The diluted samples were mixed with 100 μl of sheep RBC suspensions (final concentration, 1×10^6 cells/μl) and these mixtures were assayed as described above.

Determination of anti-CFH autoantibody status with Western blot

The presence of anti-CFH autoantibodies in each patient's plasma or serum was evaluated using Western blot analysis, as previously described [24]. Briefly, purified CFH was loaded on a 5% SDS-PAGE gel under non-reducing conditions, and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membrane was cut into strips of 0.5 cm in width. Each strip was incubated with a 1:100 dilution of a patient plasma or serum sample, followed by HRP-labeled goat anti-human IgG antibody (1:1000; #074–1006, KPL, Gaithersburg, MD, USA). Bound HRP-labeled antibodies were visualized using an enhanced ECL substrate (Western Lightning Plus ECL, PerkinElmer, Waltham, MA, USA).

Determination of anti-CFH autoantibody titer with ELISA

Plasma or serum samples of five patients with anti-CFH autoantibodies detected by Western blot were further analyzed by a CFH IgG ELISA kit (Abnova, Taipei, Taiwan). All procedures were performed according to the manufacturer's instructions. Samples of patient specimens were analyzed in duplicate and diluted if needed. Various concentrations of CFH IgG (0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250 Arbitrary Unit (AU)/ml) were prepared by supplied 10,000 AU/ml

stock solution. Antibody titers were calculated by the standard curve composed of above concentrations of CFH IgG (3.9–250 AU/ml).

Determination of CFH-related proteins using Western blot

Tested plasmas or sera were collected before plasma therapy. Two microliters of plasmas or sera were electrophoresed on a 12% SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and blocked with 5% skim milk. Blotted proteins were incubated with a monoclonal mouse anti-human CFHR1 antibody (1 μg/ml, #MAB4247, R&D Systems, Minneapolis, MN, USA) or a polyclonal rabbit anti-human CFHR3 antibody (1:1500, #16583-1-AP, Proteintech, Chicago, IL, USA). After three washes, the blots were incubated with a HRP-labeled anti-mouse or rabbit IgG antibody (1:10000 and 1:20000, respectively; #074–1806, #074–1506, KPL). Bound antibodies were visualized using the aforementioned methods.

Genetic analysis and structure model

Genetic analysis was performed at the Department of Molecular Pathogenesis, the National Cerebral and Cardiovascular Center as previously described [24]. Genomic DNA was extracted from peripheral blood leukocytes of patients and their family members. The coding exons and the intronic flanking regions of *CFH* (NM 000186.3), *C3* (NM 000064.2), *MCP* (NM 002389.4), *CFI* (NM 000204.3), *CFB* (NM 001710.5), and *THBD* (NM 000361.2) were amplified using PCR and sequenced. The adenine of the ATG translation initiation start site was designated as the +1 position and the initial Met was denoted as +1. Of the 45 patients, W1 was sequenced at other institution [25].

The mutations, which have previously been reported as the cause of aHUS, were described as 'predisposing mutation'. The rare mutations were described as 'potentially predisposing mutation'. RFLP analysis was performed to detect C3-p.I1157T mutation as previously described [24]. Amplified DNA fragments were digested with the restriction enzyme SspI (New England Biolabs, Ipswich, MA, USA) and the products were electrophoresed to determine the genotype based on the cleaved bands.

The crystal structures of the complexes of C3b/CFH-SCR1-4 (ID: 2WII) and C3d/CFH-SCR19-20 (ID: 3OXU) were retrieved from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) [26, 27]. Molecular graphic imaging was generated by using the PyMOL molecular visualization system (Schrödinger, Portland, OR).

Results

Characterization of the six anti-CFH mAbs

Six murine anti-CFH mAbs, termed O37, O52, O72, Q34, R27, and R35, reacted with CFH purified from plasma with Western blot under both non-reducing and reducing conditions (Fig 1A). Immunoglobulin isotyping revealed that five mAbs (O37, O72, Q34, R27, and R35) were IgG1κ and one mAb (O52) was IgG2aκ. Then, we evaluated the ability of these six anti-CFH IgG mAbs to induce hemolysis through inhibition of CFH function, as originally reported by Józsi et al and Strobel et al [28, 29].

As shown in Fig 1B, two anti-CFH IgG mAbs (O52 and O72) induced strong hemolysis, almost indistinguishably from each other. Three other mAbs (R27, R35, and O37) induced slightly enhanced hemolysis, but the mAb Q34 did not induce any appreciable hemolysis.

Next, we determined the epitope of the anti-CFH mAb O72 using recombinant CFH fragments expressed by yeast with Western blot. As shown in Fig 1C, the anti-CFH mAb O72 reacted not only to full-length recombinant human CFH, but also to SCR18–20. However, mAb

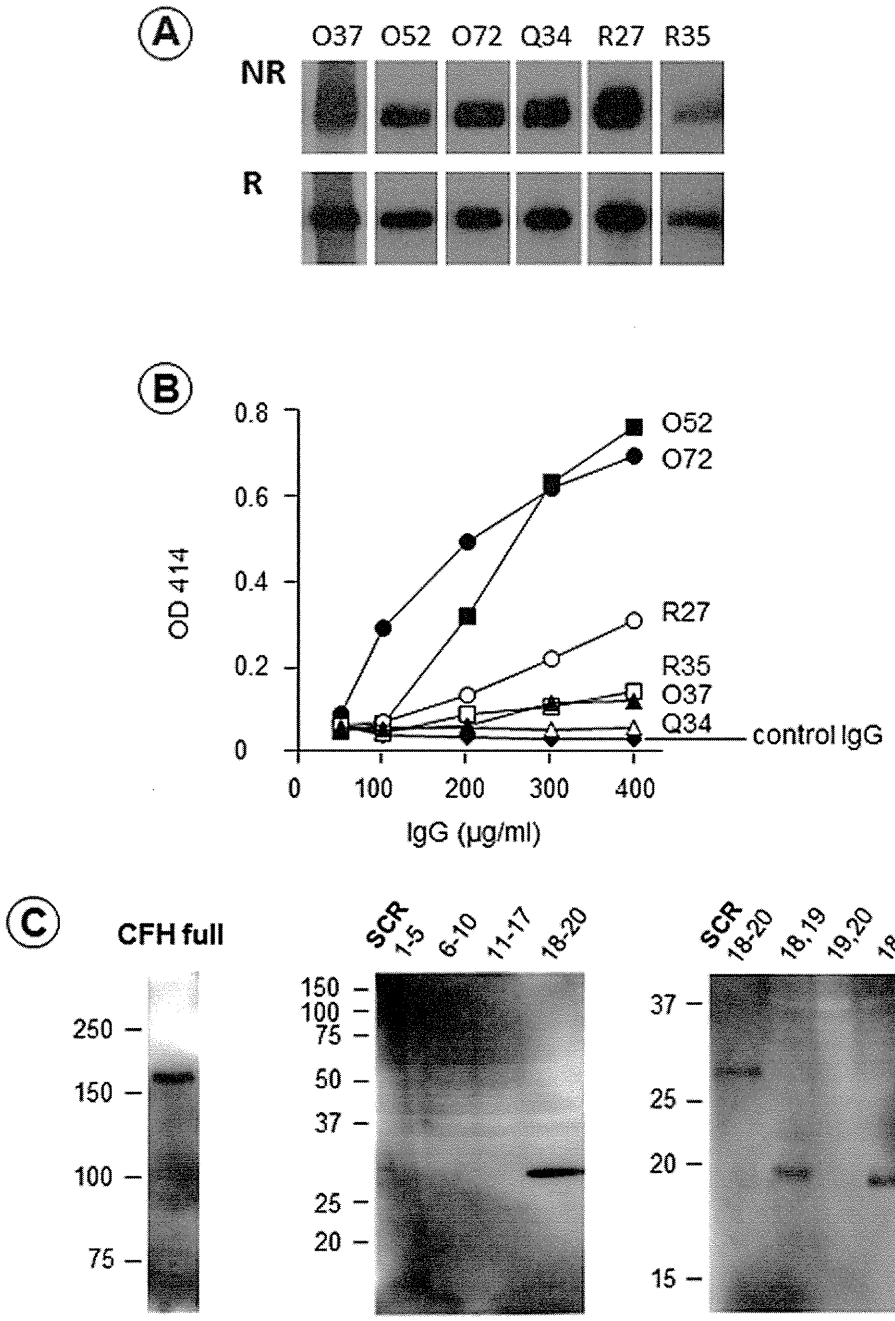

Fig 1. Production of six anti-complement factor H (CFH) murine monoclonal antibodies (mAbs), and their effects on lysis of sheep red blood cells (RBCs). (A) Western blot analysis. All six anti-CFH mAbs reacted with purified human CFH on Western blot under both non-reducing (NR) and reducing (R) conditions. **(B) Effects of six anti-CFH mAbs on lysis of sheep RBCs.** Twenty microliters of normal plasmas spiked with anti-CFH mAbs were incubated with sheep RBCs at a final concentration of 50 μg/ml to 400 μg/ml. Two plasma samples, treated with anti-CFH mAb O52 and O72, induced strong hemolysis in a dose-dependent manner. Three mAbs (R27, R35, and O37) resulted in slightly enhanced hemolysis. As for mAb Q34, appreciable hemolysis was not detected, similar to control IgG. **(C) Epitope analysis of mAb O72 using recombinant CFH expressed by yeast.** The mAb O72 reacted with full length CFH (left) and short consensus repeat (SCR) 18–20 (middle). Moreover, the right panel shows that mAb O72 reacted with peptide of SCR18–19, and SCR18 and 20, but not SCR19–20, indicating that the epitope of mAb O72 resided in SCR18.

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O72 did not react with SCR1–5, SCR6–10, or SCR11–17. To narrow down mAb O72 epitope, we prepared three short peptides within SCR18–19, SCR19–20, and SCR18 and 20. The Western blot clearly indicated that mAb O72 reacted with both peptide of SCR18–19, and SCR18 and 20, but not with SCR19–20, indicating that mAb O72 epitope resided in SCR18. Similarly, we determined the mAb O52 epitope to be in SCR16 (data not shown).

Optimal quantitative hemolytic assay

Since normal plasma spiked with anti-CFH mAb O72 consistently induced enhanced hemolysis in the sheep RBCs assay through blocking the C-terminus region of CFH from binding to sialic acid on the RBCs surface, spiked normal plasma was used as a positive control in the hemolytic assays throughout this study. Two groups of investigators have previously indicated that the hemolytic assays could be performed using serum, EDTA plasma, or citrated plasma [12, 13]. A majority of patients with TMA often need an assay of ADAMTS13 activity concurrently for ruling out TTP, which can only be stably measured using citrated plasma. Thus, we evaluated conditions for a quantitative hemolytic assay using citrated plasma as follows.

First, the optimal sheep RBC count was evaluated as follows: 20 μl of normal plasma spiked with anti-CFH mAb O72 (final concentration, 200 μg IgG/ml) or control mouse IgG was incubated for 30 min at room temperature, and then diluted to 100 μl with AP-CFTD buffer. To this mixture, we added 100 μl of sheep RBC suspensions at various cell counts ranging from 0.1×10^6 to 2.5×10^6 cells/μl, which was then further incubated at 37°C for 30 min. After incubation, the reaction was quenched by adding 1 ml of VBS-EDTA buffer. As shown in Fig 2A, the maximum difference in hemolysis between normal plasma samples spiked with mAb O72 and control IgG mAb was observed at the sheep RBC count of 2.5×10^6 /μl.

Second, the degree of hemolysis induced by normal plasma samples spiked with mAb O72 was compared to that of plasma samples from a patient with a CFH-p.R1215Q mutation (described below) by increasing the volume of plasma added. As shown in Fig 2B, normal plasma alone at the volume of 5–60 μl did not induce appreciable hemolysis, but plasma samples spiked with mAb O72 or plasma samples with a CFH-p.R1215Q mutation showed enhanced hemolysis in a dose-dependent manner, which was almost indistinguishable up to a volume of 40 μl.

Third, hemolytic assay performed using three different blood specimens from a healthy individual were compared: 1) serum prepared from a blood collection tube, 2) EDTA plasma, and 3) citrated plasma. As shown in Fig 2C, appreciable hemolysis was not noted up to 20 μl in all specimens, while normal citrated plasma spiked with mAb O72 showed enhanced hemolysis at that volume. On the other hand, normal citrated plasma alone showed minimal hemolysis at 40 μl, but both serum and EDTA plasma at this volume showed enhanced hemolysis without any additives.

Fourth, we evaluated the coagulation time interval for generating serum after blood collection on the hemolytic assay, since thrombin or other proteases generated during coagulation may affect hemolysis via complement activation. For this purpose, 50 ml of whole blood was drawn from a healthy individual, which was separated into 5 ml aliquots in sterile glass test tubes. The blood was kept at room temperature for 2, 4, 24, and 48 hours, and 7 days. At each time interval, blood was centrifuged at 800 g for 15 min at 4°C to separate serum, which was stored at −80°C until use. The hemolytic assay was performed using these freezing-thawed serum samples. As shown in Fig 2D, serum samples obtained at the interval of 2 and 4 hours showed slightly enhanced hemolysis at a volume of 40 μl. However, serum obtained at 7 days no longer showed enhanced hemolysis, even in the presence of mAb O72.

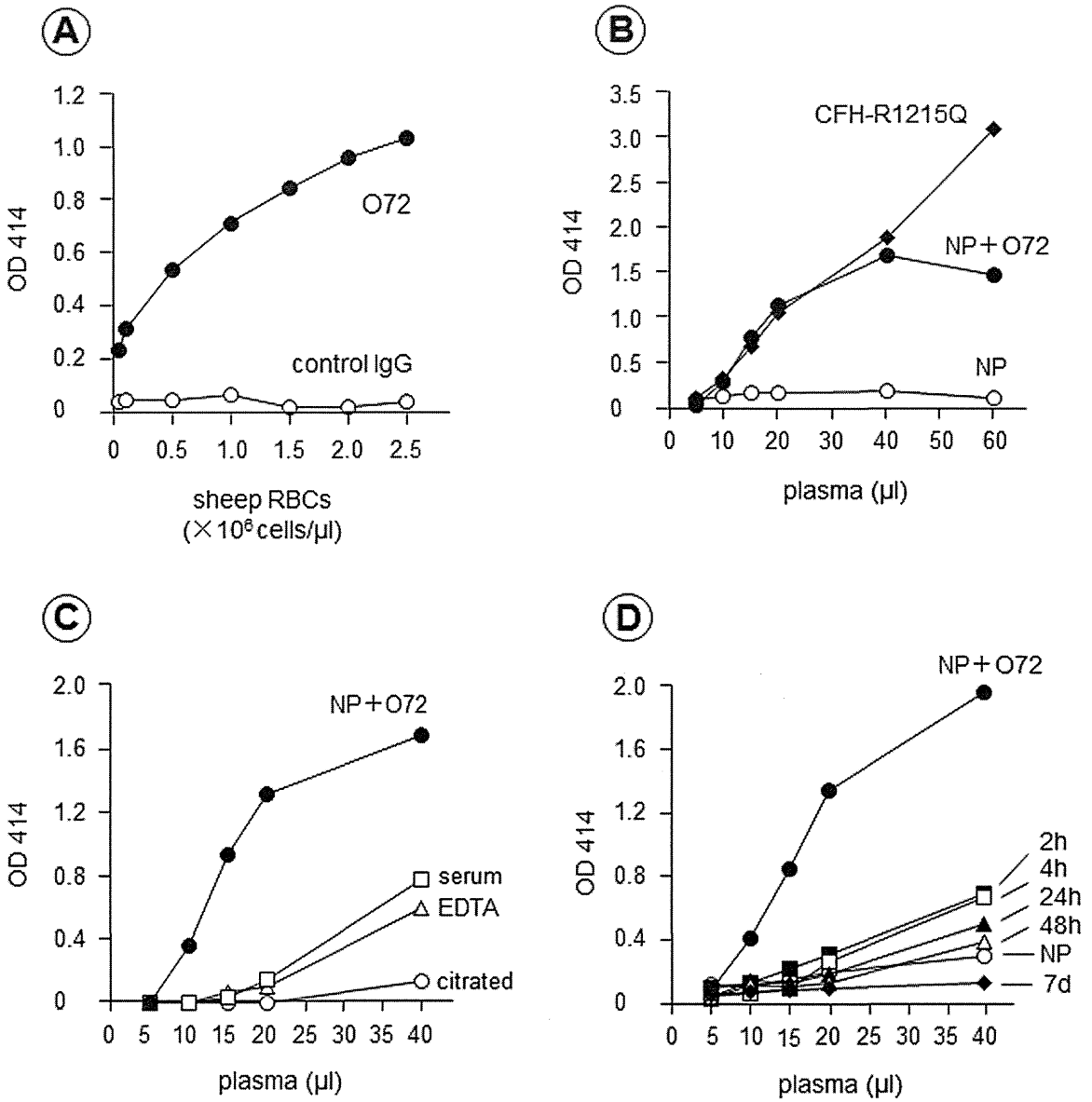

Fig 2. Optimization of the hemolytic assay: sheep red blood cells (RBCs) concentration, anti-complement factor H (CFH) monoclonal antibody (mAb) O72, and blood specimen type. (A) Evaluation of the optimal sheep RBC count. Various counts of sheep RBCs were incubated with 20 μl of normal plasma spiked with anti-CFH mAb O72 (200 μg IgG/ml, NP + O72) or control IgG. Maximum hemolysis was observed at the sheep RBC count of 2.5×10^6/μl; thus, all subsequent experiments were performed using this sheep RBC count. **(B) Comparison of hemolysis induced by mAb O72 and CFH mutant protein.** The degree of hemolysis caused by normal plasma spiked with mAb O72 or plasma from a patient with a CFH-p.R1215Q mutation was quite comparable up to 40 μl. **(C) Screening of optimal blood specimen type.** Three different specimens (serum, EDTA plasma, and citrated plasma) from one healthy donor were analyzed using the hemolytic assay. Citrated normal plasma spiked with mAb O72 was used as a positive control. In serum and EDTA plasma, mild hemolysis of sheep RBCs was detected at 40μl. No appreciable hemolysis was observed at any volume of citrated plasma. **(D) Evaluation of the influence of the coagulation time interval on hemolytic reaction.** Serum obtained 2 and 4 hours after blood collection showed mildly enhanced hemolysis at 40 μl. This mild hemolysis was lower in serum obtained 24 and 48 hours prior. No appreciable hemolysis was detected in serum obtained 7 days prior.

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Taken together, sheep RBCs at a final concentration of 2.5×10^6 cells/μl and citrated plasma were chosen for the assay. Furthermore, 100% hemolysis was defined as the absorbance at optimal density (OD) 414 nm obtained with 20 μl of normal citrated plasma spiked with mAb O72 (200 μg/ml). Under these conditions, freshly prepared citrated plasmas from 20 healthy

individuals were tested using the hemolytic assay, and showed the degree of hemolysis as 5.4±1.8% (mean ± standard deviation) (Fig 3).

Quantitative hemolytic assay plus genetic analysis for aHUS

In total, 45 aHUS patients underwent genetic analysis for six candidate genes (*CFH*, *C3*, *MCP*, *THBD*, *CFB*, *CFI*). The results of the hemolytic assay and complement abnormalities are shown in Fig 3.

The degree of hemolysis was classified into four categories based on the percentage of hemolysis as follows: severe hemolysis: >75% hemolysis (9 patients), moderate hemolysis: 50–75% hemolysis (2 patients), mild hemolysis: 25–50% (6 patients), and no apparent hemolysis: <25% (28 patients).

1.Severe hemolysis: >75%. There were nine unrelated patients (2I1, 2M1, X1, 3T1, W1, 2Y1, 2P1, 3O1, and 3A1) with more than 75% hemolysis, of whom seven patients (excluding 3O1 and 3A1) had CFH-related abnormalities (Fig 3). Among them, three patients (2I1, 2M1 and X1) carried a predisposing mutation, either p.R1215Q or p.R1215G in the SCR20 domain of CFH. Moreover, anti-CFH autoantibodies were detected in four patients (3T1, W1, 2Y1 and 2P1). The antibody titers in three patients except for 2P1 were 46,784, 13,700, and 360 AU/mL, respectively (S3 Table). Patient 2P1 showed deficiency of CFHR1 protein in serum [30], and 2Y1 displayed low but detectable levels of CFHR1 according to Western blot. Both 3T1 and W1 did not have any deletions in CFHR1 and CFHR3. Of the two remaining patients, Patient 3A1 had no mutations in the six candidate genes, but the degree of hemolysis decreased from 100% to 30% during plasma exchange treatment and to 7% after initiation of eculizumab therapy.

In a female Patient 3O1, a heterozygous mutation at p.K1105Q within the thioester-containing domain (TED) of C3 was identified, but no mutations in CFH were identified. As shown in Fig 4A, her father and brother, who have the same mutation but were asymptomatic, showed similar severe hemolytic reactions (Fig 4B). Hemolysis observed in these three family members was corrected by the addition of purified CFH (Fig 4C). The patient's mother did not have an enhanced hemolytic reaction or the mutation. Anti-CFH autoantibodies were negative in all members of this family tested.

A potentially important and interesting finding from the hemolytic assay was observed in the family of Patient 2I1, who had a heterozygous p.R1215Q mutation in CFH (Fig 5A). As shown in Fig 5B, strongly enhanced hemolysis was detected in this patient, his mother, and his older brother (brother 1) in the hemolytic assay. Dose-dependent inhibition of enhanced hemolysis was seen when purified CFH was added to the reaction mixtures for these three individuals (Fig 5C), who all carried the same p.R1215Q mutation in CFH. One potentially predisposing mutation, THBD-p.T500M, was also identified in the patient and his mother (Fig 5A). Interestingly, the patient's mother (now 61 years old) and brother 1 (now 34 years old) have never had any episodes of TMA. We also identified that unaffected X1's father with the CFH-p.R1215Q mutation had hemolysis greater than 50% in the hemolytic assay.

2.Moderate hemolysis: 50–75%. Two aHUS patients, 2G1 and 2K1, had 50–75% hemolysis, but we were unable to identify any predisposing mutations through the analysis of the six candidate genes (Fig 3). In Patient 2G1, anti-CFH autoantibodies were detected by both Western blot and CFH IgG ELISA (4,813 AU/mL) (S3 Table), although she had no deficiency in CFHR1 and CFHR3 proteins.

On the other hand, no autoantibodies were identified in Patient 2K1 (Fig 6A). Samples from her parents and two children were also analyzed using the hemolytic assay. As shown in Fig 6B, her father and both children showed severely enhanced hemolysis, but her mother showed