COMPLEMENT FACTOR H FUNCTIONAL ASSAY MAY HELP TO MONITOR ATYPICAL HAEMOLYTIC URAEMIC SYNDROME: A PILOT STUDY

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ABSTRACT

Background: Atypical haemolytic uraemic syndrome (aHUS) results from uncontrolled complement system activation. Complement factor H gene mutations are common causes of aHUS. Plasmatherapy, including plasma infusions and/or plasma exchanges, has been tried in this setting with various successes. At present, we lack a specific marker to monitor functional factor H deficiency-related aHUS.

Methods: We report the use of factor H functional assay in three patients with atypical haemolytic uraemic syndrome. This assay is based on the requirement of soluble complement regulators that bind sheep red cells to prevent haemolysis. As factor H is highly abundant in the plasma, its defect results in haemolysis. Factor H activity was also measured among plasma donors.

Results: One patient suffered from a plasma-dependent form of atypical haemolytic uraemic syndrome. Plasma exchanges restored higher factor H activity and were associated with a 15-months disease-free period. In the two other patients, one with a failing renal graft and the other on chronic dialysis, a bout of thrombotic microangiopathy was preceded by a drop of haemolytic activity below normal values. Plasma from healthy donors (N = 65) showed only minimal variations of Factor H activity (mean activity: 98.3%, SD = 4.0).

Conclusion: These preliminary data suggest that factor H activity could be of interest in both the diagnosis and the treatment by plasmatherapy of factor H-related aHUS.

Key words: Atypical Haemolytic Uraemic Syndrome, Complement Factor H, Haemolytic Assay, Plasmatherapy, Transplantation

BACKGROUND

Haemolytic uraemic syndrome (HUS) is a rare disease characterised by the triad of non-immune (Coombs negative) haemolytic anaemia, thrombocytopenia and renal impairment (1, 2). Thrombocytopenia and acute kidney injury result from diffuse platelet microthrombi formation mainly into the renal microcirculation, while anaemia is related to a microangiopathic process and is due to erythrocytes hitting the walls of narrowed vessels, resulting in schistocytes formation, low serum haemoglobin and elevated LDH blood levels.

Two different forms of HUS are described. The most frequent, called “Typical” HUS, occurs in 2/100,000 individuals per year with a peak incidence under 6 years of age. This is a bacterial disease usually due to Shiga-toxin producing E. coli strains (ie E. coli 0157:H7), this toxin being directly responsible of the microthrombi formation and glomerular lesions. Infected subjects will develop haemorrhagic diarrhea and for 5 to 20% of them, HUS. This disease is often epidemic and has a seasonal predilection for the warm months. The prognosis is usually good with complete recovery of renal function in 70% of cases, however in some series one bemoans until 5% mortality. In case of terminal kidney disease, typical HUS does not relapse on the kidney transplant. Some authors include a particular form of pneumococci associated HUS (neuraminidase associated HUS) into this category.
The second form of HUS; “atypical HUS” (aHUS), accounts for only 10% of all HUS. Its incidence rate is about 2-3 cases/year/million inhabitants. aHUS can occur as a sporadic or a familial condition, from neonatal period to adulthood. Globally its prognosis is very poor with a 25% mortality rate during the acute phase and more than 50% developing end-stage renal disease (ESRD). In addition, some patients have a chronic, relapsing form of the disease. For those with ESRD, the risk of a recurrence on the kidney transplant is high. Generally, relapses occur within the first year following the transplantation and lead to the loss of the graft.

aHUS can be associated with heterogeneous conditions such as drugs, neoplasia, pregnancy, HIV infection or auto-immune disorders. Actually, more and more, aHUS appears to be related to a deficiency in the regulation of the complement alternative pathway following various triggers that promote complement activation and/or endothelial dysfunction. Currently, more than 60% of aHUS are explained by one or several mutations in genes coding for proteins in the complement alternative pathway (3). This proportion increases each year. Most abnormalities are consecutive to the absence or defects of regulatory proteins (4) (factor I, membrane cofactor protein, thrombomodulin (5) and complement factor H (CFH)) while others are related to gain-of-function mutations resulting in an excessive complement activation (factor B and C3 mutations) (6, 7).

CFH is a very abundant plasma glycoprotein (140-650 mg/L), produced mainly by the liver (8). The corresponding gene (located on human chromosome 1q32) is in fact a cluster of six genes: the CFH gene itself and five CFH-related genes (CFHR). All the derived plasma glycoproteins, CFH itself, CFH-like protein 1 and many CFH-related proteins are made of short consensus repeat (SCR) domains sharing a high sequence identity with each other.

CFH itself is made of 20 SCR that allow the CFH to bind, on one hand to cell surface polyanions such as sialic acid and heparin, and on the other to the complement factors C3b, C3c, C3d and C3. The N-terminal (SCR 1-4) of this protein is crucial for the regulatory activity of the protein whereas the C-terminal domain (SCR 18-20) is responsible for the fixation to the cell surface. About 80 CFH mutations contribute to nearly one third of aHUS (1). In turn, anti-CFH autoantibodies, that generally occur in association with complement mutations - typically CFHR1 and CFHR3 deletions- explain approximately 10% of aHUS (9). Most of the CFH mutations affect the C-terminal domain of the protein (10). However, the inheritance of these mutations is variable and their penetrance is limited. Often, a second hit, consisting in an endothelial injury, is necessary to trigger the disease (11).

Most patients are heterozygous and have normal CFH levels (12). However, CFH is only effective as an oligomer (13), and the abnormal CFH molecules will prevent the formation of this functional oligomer, leading to low CFH activity (“dominant negative effect”) (1). On the contrary, the homozygous (and some compound heterozygous) CFH mutations lead to quantitative CFH deficiency and are characterised by very low C3 levels due to the uncontrolled and continuous complement activation. Besides, the former lead more frequently to membranoproliferative glomerulonephritides (14).

CFH mutations-related aHUS are particularly severe with 70% of patients developing ESRD or death (3). Early disease recurrence occurs in 60% of kidney allografts (15).

While new therapeutics, such as the anti-complement monoclonal antibody eculizumab or CFH concentrates, are currently under investigation for controlling aHUS bouts recurrence (16, 17), clinicians are still using plasmapheresis in case of CFH-associated aHUS flare, with variable success (18, 19, 20, 21, 22, 23, 24, 25, 26) In the absence of any randomised control trials assessing plasmapheresis in the context of aHUS, we find the expert recommendations published by the European Paediatric Study Group for HUS in 2009 helpful (27).

The aim of this pilot study was to measure CFH activity by using an existing haemolytic assay. The plasmapheresis and the disease evolution in 3 patients with aHUS were monitored.

METHODS

Immunochemical assays

Complement factor H activity assay in serum

Complement factor H activity assay in serum was first described by Pangburn et al (28) in a research setting, and has been subsequently developed by Sánchez-Corral et al for clinical samples (29). It is based on the propensity of sheep erythrocytes to undergo lysis in contact with deficient CFH containing serum. As do most human cells, sheep erythrocytes are covered by sialic acid containing polysaccharides that can bind CFH, preventing in this way the activation of the complement system. But in contrast with human erythrocytes, sheep erythrocytes lack membrane complement regulators (CD35, CD46, CD59) and strictly rely on fluid phase complement regulators - such as CFH- to prevent haemolysis. Therefore, when normal serum is added to sheep red blood cells, no or only a weak haemolysis occurs. On the opposite, addition of CFH deficient serum leads to a substantial haemolysis, consecutive to an uncontrolled activation of the complement alternative pathway.

Buffers used were: VBS, 2.5 mM veronal, 1.5 mM Na veronal, 144 mM NaCl, 7 mM MgCl2, pH 7.2-7.4; VBS containing 0.1% gelatin (GVB); GVB containing 10 mM EGTA (GVB/EGTA-10); GVB containing 10 mM EGTA and 50 mM EDTA (GVB/EGTA-10/EDTA-50); VBS containing 2 mM EDTA (VBS/EDTA-2). Haemolytic assay: 40 µL of serum or plasma were diluted in 40 µL of GVB/EGTA-10. A duplicate of each sample dilution was prepared in the same buffer plus 50mM EDTA (GVB/EGTA/EDTA-50/EDTA-50), in order to check a possible complement-independent lysis of the cells. Twenty µL of sheep erythrocytes (1 x 10^8 cells/µL in GVB/EGTA-10) were then added and the mix was immediately transferred at 37°C in a water bath and incubated for 30 min. The following controls were conducted in parallel, in duplicates: 20 µL of sheep erythrocytes plus 80 µL of GVB/EGTA-10 (spontaneous haemolysis in GVB/EGTA-10) or 80 µL of GVB/EGTA-10/EDTA-50 (spontaneous haemolysis in GVB/EGTA-10/EDTA-50) or 80 µL of water (total haemolysis). The reaction was stopped by adding 500 µL of ice-cold VBS/EDTA-2 in all tubes, excepted 500 µL of water in the control “total haemolysis”. The samples were centrifuged, and the OD of supernatant was determined at 405 nm. For each sample, a corresponding blank was performed by measuring the A405 of 40 µL of serum or plasma diluted in 560 µL of VBS/EDTA-2 (A405 blank). The percent lysis was determined as follows. For each sample, a corrected A405

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Fresh frozen plasma units containing citrate phosphate dextrose

We assessed the CFH concentration and activity in samples obtained from the blood bank. Samples were obtained at time of transfusion for various indications and then frozen at –20°C until measurement with the same method as described above in serum.

Practical aspects

CFH activity and concentration assays must be performed on freshly collected blood samples without anticoagulation and sent to the laboratory within four hours following blood withdrawal, or alternatively, serum can be separated and be sent frozen, on dry ice, in aliquots to the laboratory. MCP expression is analysed on blood collected on EDTA, kept and sent to the laboratory at room temperature (for more information please contact Dr Patrick Stordeur at patrick.stordeur@ulb.ac.be).

RESULTS

Case description

Patient 1 is a 5-years-old girl, born from consanguineous Caucasian parents with no history of renal disease. She presented a first non-diarrhoeal HUS episode that recovered with daily plasma infusions (10 ml/kg). Eight days later, the HUS relapsed and plasma exchanges were started with a volume of 40 ml/kg BW, 5 exchanges a week for 2 weeks. Complete clinical and biological remission was reached after 10 plasma exchanges. However a second recurrence occurred 15 days later and it was then decided to maintain the patient on chronic plasma exchanges (40 ml/kg BW, once a week). After 20 months, the patient stays free of any clinical or biological relapse: platelet counts and LDH levels remain within the normal range, although haptoglobin level remains persistently low. Renal function recovered fully.

Initial evaluation revealed that ADAMTS 13 activity, C3 and C4 levels were in the normal range. The expression of MCP on leukocytes was normal, as well as complement factor I concentration. Although the concentration of factor H was also normal, (52 mg/dl), normal range 37-73 mg/dl), the CFH functional activity was reduced to 9% (normal range 86-103%). We could not obtain any genetic analysis in this patient. After initial biochemical assessments we started to measure CFH activity and concentration before each plasma exchange. (Figure 1).

We observed that after the initiation of plasma exchanges, the CFH activity increased from 9 to 54%. This increase was sustained during the whole observation period. Nevertheless, despite the fact that the treatment was given on a regular basis, the CFH activity varied greatly over time with the lowest level of activity being 37% and the highest 96%. No clinical or biological recurrence of HUS was observed within the same period.

Patient 2 is a 36-years-old man with an affected mother who died from HUS. He is suffering from a familial form of HUS with functional CFH deficiency with autosomal inheritance. He bears 2 complement mutations. First, two AA changes (S1191L and V1197A – on the same allele) in the ADAMTS 13 enzyme. He bears 2 complement mutations. First, two AA changes (S1191L and V1197A – on the same allele) in the ADAMTS 13 enzyme. He bears 2 complement mutations. First, two AA changes (S1191L and V1197A – on the same allele) in the ADAMTS 13 enzyme.
SCR 20 of the CFH protein (9). This well-known mutation predisposes to aHUS (30) and is thought to be the result of a gene conversion event from CFH (SCR20) to CFHR1 (SCR5). Indeed, mutations inside the C-terminal domain of the CFH were shown to be associated with both C3b/C3d and heparin abnormal binding on host surfaces (31). Of note, this patient had normal levels of CFH at any time and only a slightly decreased C3 level during non-crisis periods. Second, one AA change (I231G) in the CD55 gene – also called DAF for decay accelerating factor – was found in the patient and his father. DAF is another complement regulatory protein acting by disassociating the alternative and classical pathway convertases. However neither the familial history of our patient, nor experimental evidences argue to date for any role for DAF mutations in aHUS pathology (29). The expression of DAF was not assessed at that time.

Our patient experienced a first HUS episode at age of 24 resulting in ESRD. He received a first cadaveric kidney graft at age of 26 but lost it from recurrence less than 2 years later. At the age of 35, he received a second cadaver kidney graft (Figure 2a). His peak and current panel reactive anti-HLA antibodies were 86% and 7%. He had 2/1/1 HLA A/B/DR incompatibilities with his donor. He was given thymoglobulin, tacrolimus, methylprednisolone and mycophenolate mofetil. A plasma exchange was performed just before transplantation and intensive plasma infusions were started thereafter. He developed 2 biopsy-proven humoral rejections: the first on day 8, which was treated by high doses methylprednisolone, intravenous immunoglobulins (IVIG, total dose 1.2 g/kg) and intensive plasma infusions (100 ml/kg the first week, 80 ml/kg the second) and a second one at day 24 treated by 3 plasma exchanges, followed by four injections of 500 mg of Rituximab over three weeks and then 0.2 mg/kg injection of IVIG per month. At that time, plasma infusions were started at 20 ml/kg every two weeks. The serum creatinine stabilised at 1.7–1.9 mg/dl after 3 months. Of note, LDH, thrombocytes, haptoglobin and schizocytes levels were normal. Progressive loss of graft function associated with haemolytic markers was noted and a waitlist biopsy was performed, which was reduced below 50% of normal value at 3 months. It remained stable two weeks after transplantation (86%– dotted line), CFH antigenic level (mg/dL), its normal lower limit (86%– dotted line), CFH antigenic level (mg/dL), its normal interval (from 37 to 83 – dotted area), plasma exchanges (grey circles) and creatininemia (mg/dl– grey triangles). Of note, LDH, thrombocytes, haptoglobin and schizocytes levels were normal. Progressive loss of graft function associated with haemolytic markers developed since month 5. Haemodialysis had to be resumed at month 7.

All the CFH concentration assessments performed throughout the second transplantation period remained within normal limits (Figures 2b). In contrast (Figures 2b and 2c), the regulatory activity of complement factor H decreased during the same period. The CFH activity value was almost normal at 81% before transplantation (normal 86–103%). It remained stable two weeks after transplantation (78%), under intensive plasma infusions, in the context of rejection. However, less frequent infusions resulted in a drop of factor H activity, which was reduced below 50% of normal value at 3 months. At that time, LDH, thrombocytes, haptoglobin and schizocytes levels were still normal. We began to observe biological signs of HUS at the start of the 5th month (platelets < 150.000/mm3, LDH > 225 UI/l and haptoglobin < 30 mg/dL). We also noticed a mirror image between creatinine, that increased steadily from the 4th month onwards, and the regulatory activity of factor H that continued to decline. A basal rate of 25% activity of factor H coincided with the need to resume dialysis (urea 240 mg/dL, creatinine 7.9 mg/dL, potassium 5.6 mEq/L).

Patient 3 is a 20-years-old girl who experienced a first HUS episode following Shiga-Toxin associated diarrhoea one year ago, that, despite intensive plasma therapy, resulted in ESRD, chronic epilepsy and severe relapsing pancreatitis. She is also bearing a heterozygous deletion of four nucleotides within the SCR20 of the CFH gene. She is presently treated by peritoneal dialysis. After she developed a first haemolytic flare, we decided to monitor prospectively the CFH serum activity and concentration on a monthly basis (Figure 3). CFH concentrations were low, around 30 mg/dL (normal range 37–73 mg/dL), and remained stable over time. By contrast, her CFH activity varied widely over time, ranging from 11 to 100% (normal: 86–103%). When we began the CFH activity monitoring, it was very low and concomitant to a bout of
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DISCUSSION

This paper reports on the use of a functional assay to assess the activity of CFH. Despite considered as classical (1), this test has yet to be used in clinical practice. We made several pilot observations suggesting that the quantification of CFH activity is of potential interest for diagnosis, prognosis and monitoring of aHUS patients, although further prospective studies are needed to validate these observations and to assess both the sensitivity and the specificity of this laboratory assay.

Firstly, our observations confirm that the haemolytic assay is altered in some patients with aHUS. This was the case for the 3 patients reported here. Two of them (Patients 2 and 3) bore a CFH mutation, confirming that these ones could be associated with low factor H activity. Importantly, we observed that CFH activity could be altered even in the absence of CFH antigenic deficiency (Patients 1 and 2). These observations are in accordance with previously published data (30, 31, 32) demonstrating some cases of mutations in the C-terminal domain of CFH associated with normal CFH antigenic levels and low activity of the haemolytic test. Moreover, Jozsi, Strobel et al (32, 33) showed that the result of this test can be modified by anti-CFH auto-antibodies as well. However, Johnson et al (34) have noticed that some CFH mutations were not associated with a defective haemolytic assay. Obviously, further studies are needed to investigate if other complement abnormalities could also lead to a positive haemolytic assay. Since CFH activity assay reflects the capacity of human serum to prevent complement mediated haemolysis of sheep red cells, we hypothesise that a significant mutation into other important fluid phase complement regulatory proteins (CF1, C3, CFH related proteins, C4BP, CFB, clusterin (35), soluble thrombomodulin form (5), other unknown polymorphisms impacting the serum resistance to haemolysis) could also result in a positive haemolytic assay (36). Of note, relevant membrane-bound protein mutations will not be detected by this test. This fact together with the incomplete penetrance of the atypical HUS argues for a combination of genetic and functional testing to better predict clinically relevant complement abnormality. In support of both the sensitivity and the specificity of this assay, and of interest in the context of plasmatherapy for atypical CFH-associated HUS, we observed that the inter-individual variation of CFH activity and concentration among normal plasma donors was quite negligible. Therefore, it does not seem useful to select plasma donors on the basis of CFH activity.

Secondly, we observed an association between low CFH activity levels and haemolysis flares. In patient 1, the drop in CFH activity was concomitant with the haemolytic bout, and increased again during effective plasma exchanges. This is not a formal demonstration that plasma exchanges restore CFH activity but it is highly suggestive since our patient was clinically plasma-dependent. Plasma exchanges as well as increased CFH activity were associated with clinical remission in this patient. In patient 2 a very low CFH of 25% activity was again associated with a haemolytic flare and with graft loss. CFH activity began to decrease even before any traditional haemolytic parameters did. In patient 3, low CFH activity was detected at the time of a haemolytic flare and prospective testing revealed a new drop in CFH activity one month before a new aHUS flare. Our results therefore suggest that CFH activity could be a sensitive/predictive marker of HUS flares or treatment response.

Thirdly, we observed that CFH activity levels vary greatly over time in patients affected by HUS. Although our experience is limited to three patients, we noticed that none developed HUS relapse as long as the CFH functional activity remained above 40%. Therefore, these observations are consistent with the fact that CFH activity must reach a certain threshold in order to prevent complement-mediated haemolysis. Corollary with this hypothesis, Nathanson et al (19) noticed that, in the context of complete CFH deficiency, HUS progressed to terminal renal failure despite the fact that the circulating CFH level was increased to 22-24% by intensive plasma exchanges (normal values given by the author: between 65 and 140%).

In summary, we found that the assay of CFH activity is easy and inexpensive, and, if confirmed in larger series, might help in diagnosing as well as in treating CFH deficiencies. With regard to therapy monitoring, further studies are needed to establish the cut-off CFH value above which complement activation and atypical HUS is unlikely to occur.

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Figure 3: represents patient 3's haptoglobin concentration (mg/dL-grey squares), CFH activity (%) black diamonds) and concentration (mg/dL-white triangles) and finally, C3 concentration (mg/dL-black circles, dotted line) during monthly follow-up.
CONFLICT OF INTEREST: None.

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