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Functional implications of the recognition of factor H-related protein 1 by anti-factor H autoantibodies in autoimmune hemolytic uremic syndrome

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Abstract

The autoimmune form of atypical hemolytic uremic syndrome (aHUS) is characterized by circulating autoantibodies against the complement regulator factor H, and is often associated with deficiency of the factor H-related proteins CFHR1 and CFHR3. We studied whether anti-factor H autoantibodies cross-react with CFHR1, and determined the functional consequence of this cross-reactivity. Autoantibodies from 24 aHUS patients were analyzed for factor H, CFHR1 and CFHR3 binding by ELISA. All 24 anti-factor H IgG bound to SCR20 of factor H, 21 also recognized CFHR1, but none bound to CFHR3. Three patients also had anti-factor H IgA cross-reacting with CFHR1. Analysis of the IgG fractions in CFHR1-deficient patients revealed that CFHR1-IgG complexes were formed during plasma treatment, demonstrating that autoantibodies recognize CFHR1 *in vivo*. The functional relevance of autoantibody binding to CFHR1 was analyzed in hemolysis assays using sheep erythrocytes. Recombinant CFHR1 prevented the lysis caused by patients' plasma containing anti-factor H IgG, but it did not inhibit the lysis caused by a factor H mutation (W1183L) in SCR20. These results demonstrate that CFHR1 protects host cells from complement-mediated damage caused by the factor H-inhibitory autoantibodies. We conclude that exogenous CFHR1 provided during plasma exchange therapy contributes to the neutralization of anti-factor H autoantibodies, thus CFHR1 could be helpful in the treatment of autoimmune aHUS.

Keywords:

autoantibody, complement, factor H, factor H-related proteins, hemolytic uremic syndrome

Introduction

Atypical hemolytic uremic syndrome (aHUS) is a severe kidney disease characterized by hemolytic anemia, thrombocytopenia and acute renal failure, and is associated with dysregulation of complement activation.¹ aHUS-associated mutations and polymorphisms in complement genes,² and genomic rearrangements involving the factor H (*CFH*) and factor H-related (*CFHR*) genes^{3,4} have been identified as disease predisposing factors. In addition, an autoimmune form of aHUS is caused by autoantibodies against the complement regulator factor H (fH).⁵

fH belongs to a protein family which also includes factor H-like protein 1 (alternative splicing product of the *CFH* gene) and five factor H-related (*CFHR*) proteins.⁶ These proteins are composed of four to nine short consensus repeat (SCR) domains, and share various degrees of sequence identity with each other and with fH. In particular, the five *CFHR* proteins have domains homologous to the C-terminal domains SCR19-20 of fH, which are responsible for directing the complement regulatory activity of fH to cell and tissue surfaces.⁷⁻⁹ Autoantibodies of most aHUS patients recognize the C-terminus of fH, and inhibit the protective activity of fH on cells against complement attack.¹⁰⁻¹²

Anti-fH IgG have been identified in ~5-11% of aHUS patients in four cohorts.^{5,10-13} The majority of the patients present with deficiency of *CFHR1* and *CFHR3*, resulting from a homozygous deletion of the genomic region including both genes.¹¹⁻¹⁴ However, anti-fH IgG have also been described in patients having two copies of *CFHR1* and *CFHR3*,^{12,13} and in patients with other genetic alterations, including one patient with homozygous *CFHR1/CFHR4A* deletion,¹³ three patients with combined *CFHR1/CFHR3* and *CFHR1/CFHR4A* deletions,^{12,13} and one patient with combined *CFHR1/CFHR3* deletion and a missense mutation in *CFHR1*.¹³ Furthermore, autoantibodies may occur together with mutations in *CFH*, *CFI*, *CD46* or *C3*.¹² Thus, anti-fH autoantibodies are not associated with a single genetic defect, but in most cases they occur together with a complete deficiency of

CFHR1. Here, we have analyzed whether anti-fH autoantibodies cross-react with CFHR1 and other CFHR proteins, and studied the functional relevance of antibody cross-reactivity.

Results

Anti-fH autoantibodies cross-react with CFHR1

The C-terminal domains of the CFHR1, CFHR3 and CFHR4A proteins, which are affected by genomic rearrangements in most aHUS patients with anti-fH autoantibodies, display 64-100% amino acid sequence identity with SCR19 of fH, and 37-97% identity with SCR20 (**Figure 1a**). We have, therefore, tested whether anti-fH autoantibodies cross-react with these CFHR proteins. Samples from 24 patients with anti-fH IgG from 2 cohorts (German and Spanish) were analyzed; clinical data available have been reported previously.^{11,13,15} All anti-fH IgG bound to SCR19-20 of fH (data not shown).^{10,11,15} The majority (21 out of 24) of the anti-fH IgG analyzed bound to recombinant CFHR1 in ELISA, whereas no significant binding to CFHR3 and CFHR4A was observed (**Figure 1b and Table 1**). Most autoantibodies showed a similar binding intensity to CFHR1 and to fH (e.g. patients P5, P6 and P20), but some had a weaker binding to CFHR1 (e.g. patients P7, P8 and P15). Autoantibodies from three patients did not show significant CFHR1 binding in the assay (e.g. patient P17); interestingly, two of these patients were not CFHR1 deficient (**Table 1**). Binding of two autoantibodies to plasma-derived fH and CFHR1 was also analyzed by Western blot, using patient IgG as the primary antibody. Both native fH and the two CFHR1 isoforms were recognized by the patients' IgG but not by IgG derived from a healthy individual (**Figure 1c**). Recognition of plasma-derived CFHR3 and CFHR4A by the patients' IgG was not observed.

SCR5 of CFHR1 is almost identical to SCR20 of fH, and the previously described mAb C18 recognizes both domains⁹ and blocks binding of native fH and CFHR1 to immobilized C3b and to endothelial cells (**Supplementary Figure S1**). Because mAb C18 has been reported to inhibit autoantibody binding to fH,¹⁰ we tested its effect on CFHR1

cross-reactivity. Complete inhibition of autoantibody binding to CFHR1 was achieved by mAb C18 in all samples analyzed, indicating that there were no additional autoantibodies reacting with the N-terminal SCR1-2 domains of CFHR1 in these samples (**Figure 1d**).

In addition, for the first time, in three of the CFHR1/CFHR3 deficient patients IgA autoantibodies were identified, which also bound to SCR20 of fH and cross-reacted with CFHR1 (**Supplementary Figure S2**).

CFHR1:autoantibody complexes are formed *in vivo*

To study whether anti-fH autoantibodies bind to CFHR1 in plasma, IgG fractions available from four autoantibody positive patients were analyzed by Western blot. As shown in **Figure 2**, fH was present in the IgG fractions of all four aHUS patients, demonstrating the existence of circulating fH:IgG complexes. The IgG fractions from patients P6 and P20 also contained the two CFHR1 isoforms (CFHR1 α and CFHR1 β). Patient P6 was not deficient in CFHR1, and patient P20 was CFHR1-deficient but received plasma treatment. Thus, CFHR1:IgG complexes were formed in CFHR1-containing plasma of the two patients. fH- or CFHR1-immune complexes were not detected in healthy individuals (**Figure 2**).

To further address the *in vivo* generation of CFHR1:autoantibody complexes, we analyzed serum samples from two patients with autoantibodies and homozygous CFHR1/CFHR3 deficiency who had received plasma treatment. Serum samples obtained before or during plasma treatment were subjected to Protein G chromatography. Western blot analyses of the original samples, the flowthrough and the bound fractions (containing IgG and IgG-immune complexes) were performed to detect fH and CFHRs (**Figure 3**). Serum samples from two healthy individuals, one of them with homozygous CFHR1/CFHR3 deficiency, were used as controls; the analysis of these control samples revealed unspecific binding of factor H-like protein 1 to the column (**Figure 3a**). Both free and IgG-bound fH were present in the two samples from patient H108, although in different proportions (**Figure 3b**).

Interestingly, when the patient was under plasma exchange therapy, a significant amount of the exogenous CFHR1 was IgG-bound, although free fH was still available. Similar results were observed upon analysis of the sample from patient H154 obtained during plasma exchange (**Figure 3c**). Here, a high proportion of fH appeared in the flowthrough (suggesting that the autoantibodies were not in excess), but all exogenous CFHR1 was IgG-bound. These data suggest a competition between fH and CFHR1 for the autoantibodies *in vivo*. To directly assay if such a competition occurs, a plasma sample from patient H108 obtained before plasma treatment was incubated with increasing amounts of recombinant CFHR1, and the levels of free and IgG-bound fH were estimated by Western blot upon Protein G chromatography (**Figure 3d**). CFHR1 reduced the bound/free fH ratio in a dose-dependent manner, and some CFHR1 appeared IgG-bound at physiological concentrations. In another set of experiments, the amount of free fH upon addition of CFHR1 was determined by ELISA, with similar results (**Supplementary Figure S3**).

CFHR1 neutralizes anti-fH autoantibodies and thus reverts fH dysfunction

Plasma from aHUS patients with mutations in SCR20 of fH,¹⁶ or with anti-fH autoantibodies,^{10,15} cause complement-mediated lysis of sheep erythrocytes (SRBC). Since SCR20 of fH and SCR5 of CFHR1 differ only in two amino acids, and both proteins are recognized by most anti-fH autoantibodies (**Figure 1**), we studied whether CFHR1 protects SRBC from autoantibody-induced or mutation-induced hemolysis.

Similar to fH, recombinant CFHR1 rescued SRBC from hemolysis when added to autoantibody positive aHUS plasma (**Figure 4a**), whereas the recombinant SCR15-20 fragment of fH had no protective effect. The lysis-inhibitory effect of fH and CFHR1 was dose-dependent, although a higher concentration of exogenous CFHR1 was required to achieve the same degree of inhibition as with fH (**Figure 4b**). This is likely attributed to the potent complement regulatory activities of fH in plasma and on cells, because the affinity of

autoantibodies to fH and CFHR1 was similar. The effect of CFHR1 was also analyzed when the abnormal hemolysis occurred due to a mutation (W1183L) in SCR20 of fH (**Figure 4c**). In this assay, a minor inhibitory effect of CFHR1 was observed at high concentrations, but this effect was very similar to that of recombinant CFHR4B used as control, and thus most probably non-specific. These results suggest that CFHR1 inhibits the autoantibody-induced lysis by binding to the autoantibodies, but not by a direct inhibitory effect on complement-mediated lysis. To elaborate on this finding, we analyzed whether CFHR1 can inhibit the hemolysis induced in normal human plasma by two monoclonal anti-fH antibodies, mAb C18 and mAb OX24, recognizing different SCR domains.

SRBC lysis is caused in normal human plasma by mAb C18, which recognizes SCR20 of fH and mimics the effects of the anti-fH autoantibodies.¹⁵ A dose-dependent inhibition of this lysis was achieved when fH was added, and the same effect was observed with fH SCR15-20 and with CFHR1 (**Figure 5a**). Lysis of SRBC in normal human plasma can also be induced by the mAb OX24, which binds to SCR5 of fH,¹⁷ and likely interferes with the regulatory activities of the adjacent SCR1-4. Addition of fH abrogated the OX24-induced lysis (**Figure 5b**), but the effects of exogenous SCR15-20 or CFHR1 were strikingly different. Increased lysis was observed in the presence of SCR15-20, which is not recognized by mAb OX24 but which competes with fH for cell binding (**Supplementary Figure S4**). On the other hand, the minor effect of CFHR1 was similar to the one observed in the mutation-associated hemolysis assay (**Figure 5c**).

Altogether these data suggest that CFHR1 specifically reverts the fH dysfunction caused by autoantibodies against SCR20.

Discussion

In this report we identify CFHR1 as a second autoantigen of anti-fH autoantibodies in aHUS. We have characterized anti-fH autoantibodies from a total of 24 German and Spanish aHUS patients. All autoantibodies recognized SCR20 of fH. This domain differs only in 2 amino acids from the homologous SCR5 domain in CFHR1; in line with this, 21 of 24 anti-fH IgG and 3 of 3 anti-fH IgA bound CFHR1 *in vitro*. These results agree with and validate similar findings reported for the Newcastle cohort, where autoantibodies from 7 out of 12 aHUS patients recognized SCR19-20 of fH, and those antibodies also bound to the homologous SCR4-5 of CFHR1.¹²

Most of the 24 autoantibody-positive aHUS patients studied here had homozygous CFHR1 deficiency. Nonetheless, anti-fH autoantibodies can also be found in patients expressing CFHR1 (**Table 1**).^{12,14} We observed that autoantibodies from one of the 3 CFHR1-positive patients showed cross-reactivity with recombinant CFHR1 and, importantly, that this interaction occurred *in vivo* (**Figure 2**). CFHR1:IgG immune complexes were also formed in CFHR1-deficient patients undergoing plasma exchange therapy, even when free fH was still available for antibody binding (**Figure 3**). In this context, we considered it relevant to analyze the functional consequences of the CFHR1 cross-reactivity of the anti-fH autoantibodies.

fH is a major fluid phase complement regulator that also protects autologous cells from complement-mediated damage.^{7,8} The C-terminus of fH, particularly SCR20, is critical for its cell-protective function, as it interacts with polyanionic molecules on cell surfaces and with C3b deposited during complement activation.^{7-9,18} Mutations in the fH C-terminus or autoantibodies against this region cause reduced fH binding to cells, and thus a reduced protection from complement-mediated damage, which can be measured *in vitro* with a hemolysis assay using SRBC.^{10,15,16,19,20} We used this kind of assay to compare the effect of exogenous fH and CFHR1, and found that CFHR1 could revert the anomalous lysis caused by plasma samples of autoantibody-positive aHUS patients, but not the lysis due to a mutation in

fH SCR20. A similar inhibitory effect of CFHR1 was observed when SRBC lysis was induced in normal human plasma by the mAb C18, which recognizes the C-terminal domains of fH and CFHR1, but CFHR1 could not inhibit the lysis induced by the mAb OX24, which binds to the N-terminus of fH. Therefore, our results indicate that CFHR1 can revert the fH dysfunction caused by anti-fH autoantibodies. Thus, CFHR1 derived from exogenous plasma during therapy could have a beneficial effect by neutralizing the autoantibodies. This also raises the possibility of using recombinant CFHR1 as a decoy for the antibodies.

CFHR1 has been shown to inhibit the terminal pathway when applied at high concentrations.²¹ Nonetheless, in our assays this effect could be excluded because CFHR1 inhibited the autoantibody-mediated lysis already at lower concentrations. The minor effect of CFHR1 on mutation- or mAb OX24-induced lysis was considered unspecific, since CFHR4B had the same effect. In addition, no enhanced hemolysis was observed in CFHR1-deficient normal human plasma¹⁵ or in plasma from 8 aHUS patients lacking both CFHR1 and anti-fH autoantibodies (data not shown).

CFHR1 shares ligand binding capacities with fH due to their very similar C-terminal domains. Thus, CFHR1 binds to C3b and to endothelial cells (Majno et al., *Mol. Immunol.* 2003, 40:174)²¹ (**Supplementary Figure S1**) and has been shown to inhibit the binding and regulatory activity of fH on a bacterial ligand.²² Therefore, it has been suggested that CFHR1 competes with fH for ligand- and cell binding.⁶ Interaction of fH with host cells, however, involves the binding to a complex of cell surface polyanions and deposited C3b,²³ and the affinity of CFHR1 for C3b is lower than that of fH.²¹ Indeed, the two aminoacid differences between SCR20 of fH and SCR5 of CFHR1 have been identified as aHUS-associated fH mutations that cause reduced C3b binding,²⁴ and such CFHR1-like C-terminus is also present in the fH::CFHR1 hybrid protein.⁴ While a wild-type recombinant SCR19-20 fragment competes with fH for cell binding and thus causes SRBC lysis when added to human plasma, a mutant SCR19-20 fragment with the S1191L and V1197A exchanges, corresponding to

L290 and A294 of CFHR1, had an impaired ability to compete with fH and thus to cause hemolysis.²³ In line with these findings, recombinant CFHR1 did not cause SRBC lysis when added to normal plasma at the concentrations tested (up to 4 μ M CFHR1, corresponding to ca. 150 μ g/ml protein). In contrast to this, the fH SCR15-20 fragment enhanced lysis when added at high concentrations due to competition with fH (**Supplementary Figure S4**), and it did not protect SRBC from antibody-induced hemolysis (**Figure 4**), thus rendering this fragment inappropriate for use as an inhibitor of the autoantibodies.

Although there is no consensus treatment for aHUS patients with anti-fH autoantibodies, to apply plasma exchanges combined with immunosuppressive therapy is a logical choice.^{15,25-28} The patients differ, however, in their response and tolerance to these treatments.¹⁵ **A supplementation with purified fH could help restore the complement regulation impaired by the autoantibodies. However, purified fH as a therapeutic agent is currently not available; on the other hand, while it could supply fully active free fH, it does not solve the problem of continuous autoantibody production in the patients. There is also the inherent risk of transmitting infectious agents such as viruses or prion proteins. Thus, a combination of immunosuppression to inhibit autoantibody production, and supplementation with recombinant fH, and/or with CFHR1 that will act as an autoantibody-decoy, could be considered (Supplementary Figure S5). It would be easier to generate recombinant CFHR1 (5 SCR domains) than fH (20 SCR domains). The risk of immunization with recombinant CFHR1 is unlikely to be higher than in the case of plasma exchanges, when exogenous CFHR1 is also provided and CFHR1-immune complexes are generated. Furthermore, CFHR1 is almost a perfect hybrid of SCR1-2 of CFHR2 and SCR18-20 of fH, two closely related proteins present in the patients' plasma. Clinical trials will be necessary to establish the efficacy of these and other treatments (including the administration of fresh frozen plasma, plasmapheresis, plasma exchanges, and immunosuppressive treatments), as well as the effect of complement inhibitors (e.g. anti-C5 antibody) that could be beneficial in the acute phase of**

the disease, when systemic complement activation is often observed.

In conclusion, our results demonstrate that most anti-fH autoantibodies in aHUS patients cross-react with CFHR1 and can recognize this protein when administered exogenously during plasma treatments. These results suggest that plasma exchanges have multiple beneficial effects: in addition to removing autoantibodies and providing free fH, the exogenous CFHR1 prevents the remaining autoantibodies from fH binding. Thus, to include exogenous CFHR1 could be helpful in the treatment of autoimmune aHUS. **Prospective clinical trials will help identify the best therapeutic option for each patient.**

We also report for the first time the presence of anti-fH antibodies of the IgA isotype in aHUS patients. This finding is of potential relevance, because IgAs have been associated with a poor prognosis in thrombotic microangiopathy and in nephropathies.^{29,30} Whether the IgA isotype in autoimmune aHUS is associated with a different disease evolution needs to be addressed in larger patient cohorts.

Materials and methods

Blood samples

Serum or EDTA-plasma samples were obtained from 24 pediatric aHUS patients that had been previously detected to have anti-fH IgG, and from several control individuals. 20 patients (P1 to P20) were from a German aHUS cohort^{10,11,15} and 4 patients (H108, H151, H154 and H177) were of Spanish origin.¹³ The studies were performed with approval of the Research Ethics Committees of the Medical Faculty of the Friedrich Schiller University and of the University Hospital “La Paz”, in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients’ relatives.

Microtiter plate assays

Autoantibody binding to fH and to recombinant CFHR proteins was measured by ELISA using MaxiSorp plastic plates (Nunc, Wiesbaden, Germany).¹⁰ Purified fH was purchased from Merck (Schwalbach, Germany). Recombinant CFHR1, CFHR4A and CFHR4B were expressed in the baculovirus-insect cell expression system.³¹ Recombinant CFHR1, CFHR3 and fH SCR15-20 fragment, as well as antibodies and plasma samples, were kindly provided by Peter F. Zipfel (Hans Knöll Institute, Jena). Peroxidase-conjugated anti-human IgA was from Sigma-Aldrich (Taufkirchen, Germany). The mAb 90X and A229 were obtained from Quidel (TECOmedical; Bünde, Germany).

Inhibition experiments were performed with the mAb C18 (Enzo Life Sciences, Lörrach, Germany), which recognizes fH SCR20.⁹ The wells were coated with fH or CFHR1, and the plasma samples were added in the presence or absence of mAb C18.

Hi-Trap Protein G chromatography

EDTA-plasma samples from patients or control individuals were dialyzed against 20 mM sodium phosphate, pH 7.0, and loaded onto 1 ml Hi-Trap Protein G columns (GE

Healthcare, Uppsala, Sweden). Chromatography was performed according to the manufacturer's instructions using an ÄKTA-Prime system (GE Healthcare). IgG and IgG-immune complexes were eluted with 0.1 M Glycine-HCl, pH 2.7, and immediately neutralized with appropriate volumes of 1.5 M Tris-HCl, pH 8.8. After dialysis against PBS, IgG concentration was determined by reading A_{280} ($E^{0.1\%} = 1.35$), and the samples stored at -20°C .

The flowthrough fraction obtained from a control sample (containing all plasma proteins except for IgG) was further processed to obtain a preparation of plasma-derived fH and CFHR proteins by affinity chromatography on Heparin-Sepharose as described.¹³ Briefly, fH and CFHR proteins were retained in the Heparin-Sepharose column at low ionic strength (50 mM NaCl) and further eluted at 500 mM NaCl. This protein preparation was free from IgG and albumin, among other plasma proteins.

Western blot assays

Western blot analyses of fH and CFHR proteins in plasma samples, and in the flowthrough and IgG fractions obtained after Hi-Trap Protein G chromatography, were performed using polyclonal antibodies as described.¹³

Western blots to determine if the IgG fractions of aHUS patients with anti-fH autoantibodies recognize plasma-derived fH and CFHRs were also performed. 25 μg of the protein preparation was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Purified IgG from patients H154 and H177, or from a control individual, were added as primary antibodies, and HRP-conjugated goat anti-human IgG (Biosource, Invitrogen Corporation, USA) as secondary antibody. The membranes were developed using a chemiluminescent substrate (ECL Advance kit; GE Healthcare) as described.¹³

Hemolysis assays

The ability of purified fH, recombinant CFHR1 and fH SCR15-20 to inhibit lysis of sheep erythrocytes (SRBC) was compared using three kinds of assays: 1) Lysis induced by anti-fH autoantibodies. fH, CFHR1 or SCR15-20 were added to 20% autoantibody-positive plasma for 5 min at 20°C, then incubated with SRBC for 30 min at 37°C as described.¹⁰ 2) Lysis induced by mAbs. fH, CFHR1 or SCR15-20 were added to 10% normal human plasma in the presence of the mAb OX24 (binding to SCR5 of fH)¹⁷ or mAb C18, and then incubated with SRBC. 3) Lysis induced by a fH mutation. fH, CFHR1 or CFHR4B were added to 10% plasma from an aHUS patient carrying the mutation W1183L in fH SCR20,¹⁶ and then incubated with SRBC. Hemoglobin release was determined by reading the absorbance at 414 nm.

Disclosure

The authors declare no conflict of interest.

Supplementary material

Figure S1. Binding of CFHR1 to surface bound C3b and to endothelial cells is inhibited by mAb C18.

Figure S2. Anti-factor H IgA autoantibodies bind to the factor H C-terminus and to CFHR1.

Figure S3. Analysis of free and total factor H in patient's plasma upon addition of recombinant CFHR1.

Figure S4. The factor H SCR15-20 fragment, in comparison with CFHR1, efficiently competes with factor H for binding to cells and thus causes sheep erythrocyte lysis.

Figure S5. Influence of treatment options on the formation of factor H- and CFHR1-immune complexes.

Supplementary information is available at *Kidney International's* website.

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Authorship Contributions

P.S.C. and M.J. designed the study; S.S., C.A.G., E.F.R., H.S., P.S.C. and M.J. performed the experiments; S.S., P.S.C. and M.J. wrote the paper.

Titles and legends

Table 1. CFHR1/CFHR3 phenotype and autoantibody cross-reactivity with CFHR1

Figure legends:

Figure 1. aHUS-associated anti-fH autoantibodies cross-react with CFHR1.

(a) Alignment of the factor H-related proteins CFHR1, CFHR3 and CFHR4A with factor H (fH) based on amino acid sequence similarity. The individual SCR domains of the CFHR proteins are vertically aligned to the most similar domains of fH. The percentage of amino acid identity between the C-terminal domains of CFHR proteins and homologous domains in fH is shown. Binding sites of two anti-fH monoclonal antibodies used in this study (OX24 and C18) are also shown. (b) The binding of 24 anti-fH autoantibodies to recombinant human CFHR proteins was analyzed by ELISA. Eight representative cases are shown to illustrate differential binding to CFHR1 and lack of binding to CFHR3 and CFHR4A. (c) Western blots of purified fH, and of a plasma preparation containing fH and CFHR proteins. Rabbit polyclonal anti-fH antibody (rb), IgG from patient H154 (H154), IgG from patient H177 (H177) or IgG from a control individual (C) were used as primary antibodies. Plasma-derived fH and the two CFHR1 isoforms (α and β) were recognized by IgG from the two patients, but not by the control's IgG. (d) The mAb C18, which recognizes SCR20 of fH,⁹ inhibits autoantibody binding to fH,¹⁰ and to CFHR1, as shown here for three representative cases.

Figure 2. In vivo generation of CFHR1:autoantibody immune complexes.

IgG fractions isolated from plasma of four autoantibody-positive aHUS patients and from two healthy individuals were analyzed for the presence of fH and CFHR proteins by Western blot using a fH antiserum. Patient P6 had normal levels of CFHR1 and CFHR3, whereas the other patients presented homozygous CFHR1/CFHR3 deficiency. fH was detected in the IgG fractions from the four patients (lanes 1-4), but not from the healthy individuals (lanes 5-6).

The two glycosylated forms of CFHR1 (CFHR1 α and CFHR1 β) were identified in patient P6 (lane 1), who had normal levels of CFHR1, and in a sample from patient P20 obtained after plasma therapy (lane 4). CFHR1 was not detected in the samples from patients P3 and P10, who were CFHR1-deficient (lanes 2-3), and in the control IgG samples (lanes 5-6).

Figure 3. Characterization of IgG-immune complexes before and during plasma treatment.

Western blot analysis of serum samples from two control individuals (Control 1 and Control 2) and two aHUS patients (H108 and H154) upon Hi-Trap Protein G chromatography. The original (OR), flowthrough (FT) and bound fractions (B) were analyzed for the presence of fH and CFHR proteins using rabbit polyclonal antibodies (also recognizing factor H-like protein 1 [CFHL1], an alternative splicing product of the *CFH* gene). The two aHUS patients and the control individual 1 have homozygous deficiency of CFHR1 and CFHR3 (DefR1R3), while the control individual 2 has normal expression of these two proteins.

(a) Analysis of the control samples revealed unspecific binding of CFHL1 to the Protein G column, but there was no binding of fH or CFHR1. (b) In the untreated sample from patient H108 both free and IgG-bound fH were detected. In the sample obtained during plasma exchange treatment, most of fH and of the exogenous CFHR1 appeared IgG-bound, indicating the presence of fH- and CFHR1-immune complexes. (c) In patient H154 all the exogenous CFHR1 was detected in the IgG-fraction, while a great proportion of fH was still autoantibody-free. CFHR3, CFHR4A and CFHR5 were not detected in the IgG fractions obtained from controls or patients (not shown). (d) A plasma sample from patient H108 was incubated with increasing concentrations (0-1 μ M) of recombinant CFHR1 at room temperature for 1 hour, submitted to Hi-Trap Protein G chromatography and analyzed by Western blot. The ratio of free fH (FT lanes)/IgG-bound fH (B lanes) band intensity increases with the CFHR1 concentration, demonstrating a competition between fH and CFHR1 for

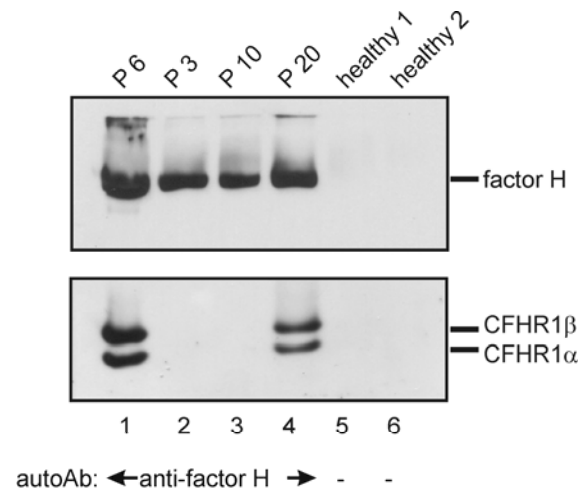
autoantibody binding.

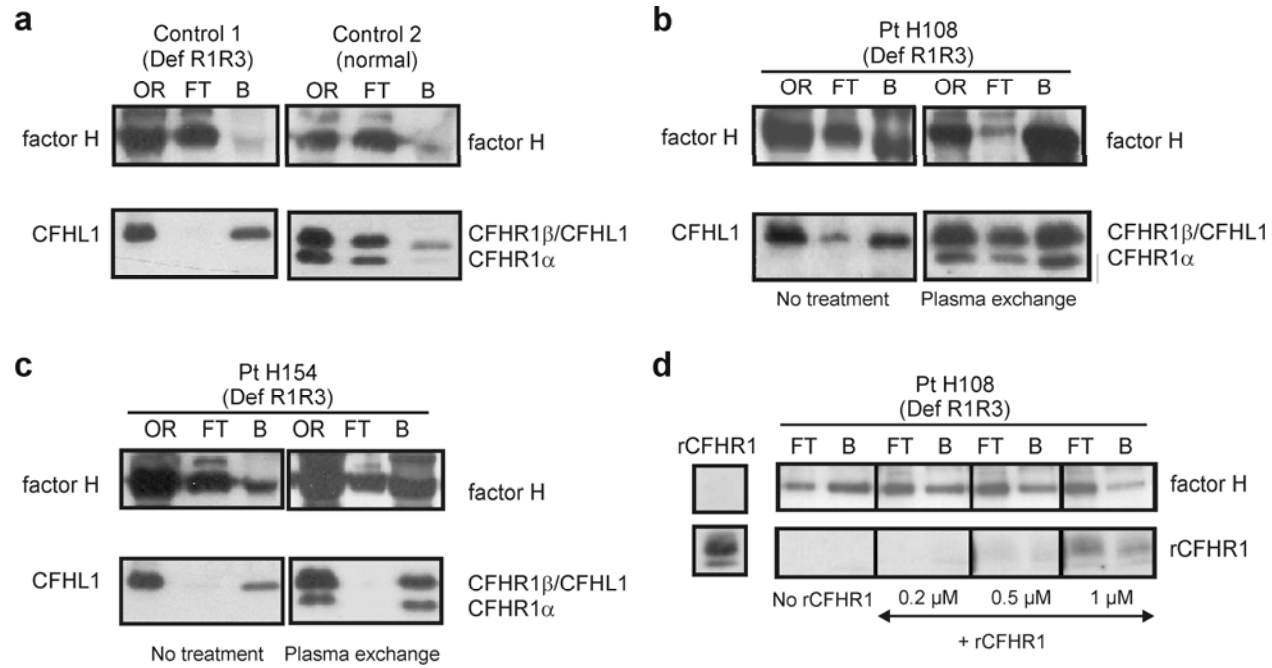
Figure 4. CFHR1 reverts the anomalous lysis caused by anti-fH autoantibodies but not by a fH mutation.

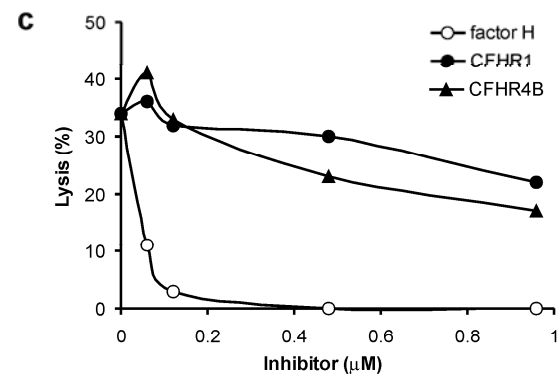
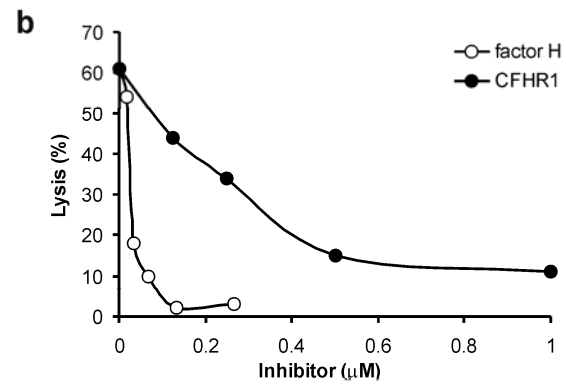
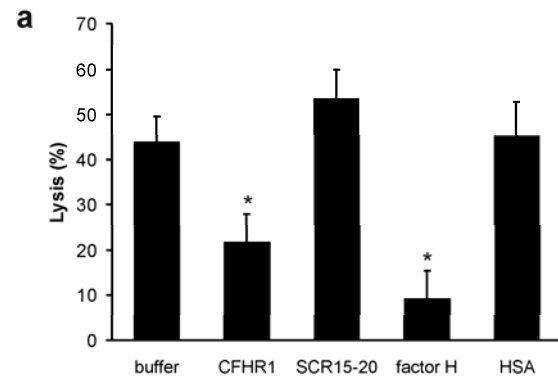
(a) An anti-fH autoantibody-positive plasma (P16) causes enhanced lysis of SRBC (buffer). The effect of fH, recombinant fH fragment SCR15-20, recombinant CFHR1, and human serum albumin (HSA) on the hemolysis was tested. Proteins were added at 1 μ M concentration, and hemoglobin release was measured in the supernatant after 30 min incubation at 37°C. Data represent mean + SD from three independent experiments. Asterisks indicate significant differences ($p < 0.01$; Student's *t*-test) from the control, HSA-treated plasma. (b) fH and recombinant CFHR1 inhibited the anomalous SRBC lysis observed in 10% plasma from the autoantibody-positive patient H154. Complete inhibition was achieved at 1 μ M CFHR1. (c) The anomalous lysis observed in the plasma from an aHUS patient with the mutation W1183L in SCR20 of fH is fully inhibited by addition of fH, as previously described.¹⁶ By contrast, the effect of recombinant CFHR1 was similar to that of recombinant CFHR4B, included as a control, and therefore considered to be unspecific. The experiments in panels b and c were performed in parallel twice, with similar results.

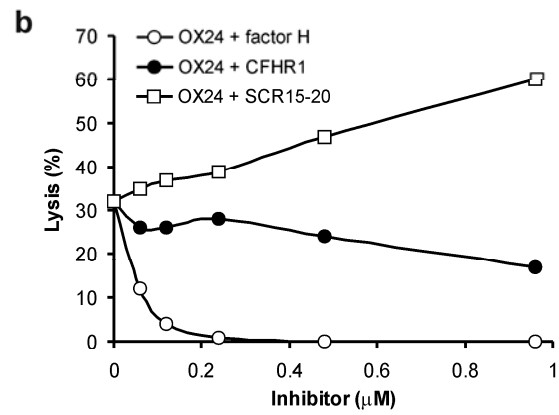
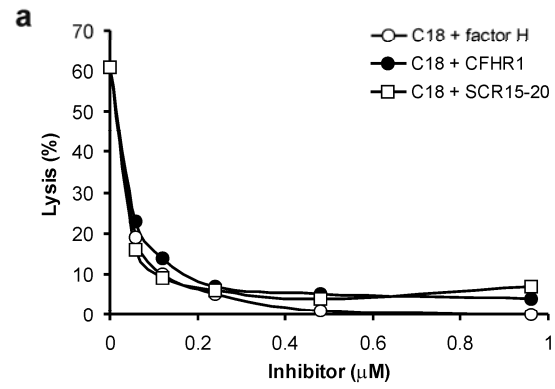
Figure 5. CFHR1 reverts the lysis caused by mAb C18 but not by mAb OX24.

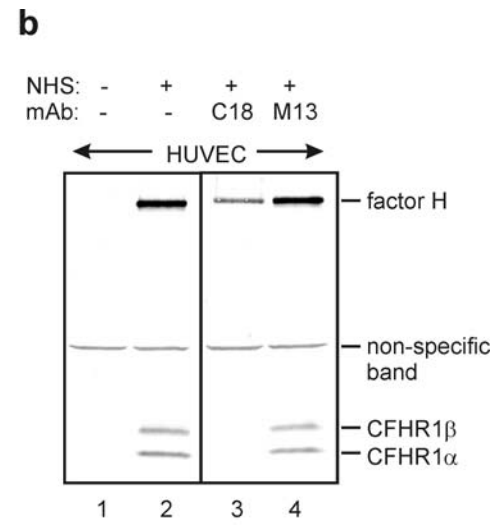
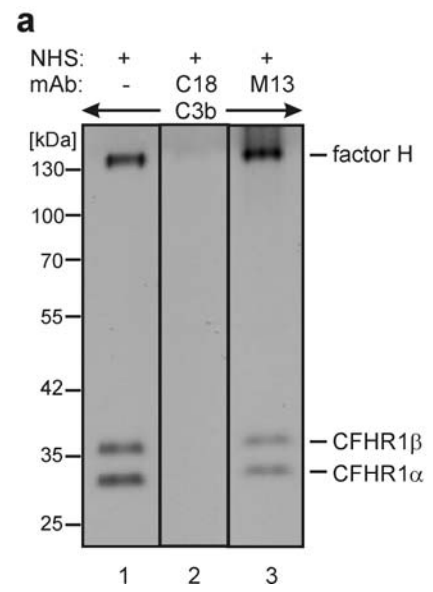
(a) Addition of 1.5 μ g mAb C18, which recognizes SCR20 of fH and SCR5 of CFHR1, to 10% normal human plasma induces SRBC lysis. This lysis was inhibited by exogenous fH, CFHR1 and the fH SCR15-20 fragment, added in the indicated concentrations. (b) The mAb OX24, which recognizes fH but not SCR15-20 or CFHR1, also induces SRBC lysis in normal human plasma. Accordingly, the OX24-induced lysis is inhibited by exogenous fH, but not by SCR15-20 or by CFHR1. The experiments in panels A and B were performed in parallel twice with similar results.

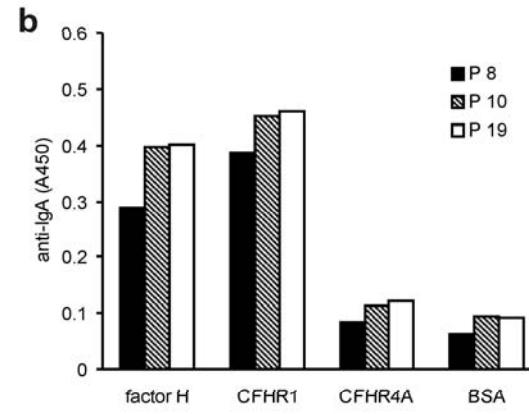
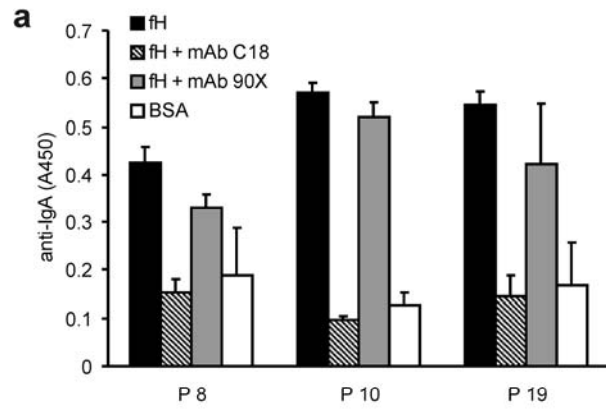


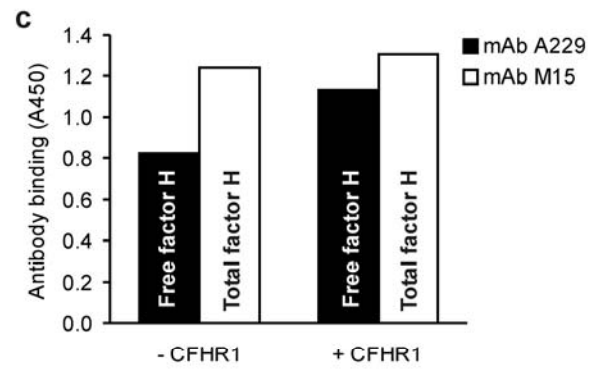
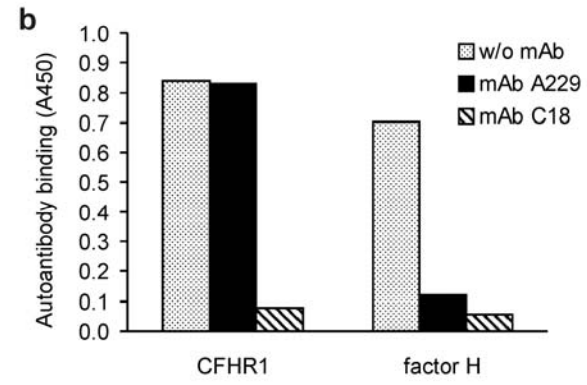
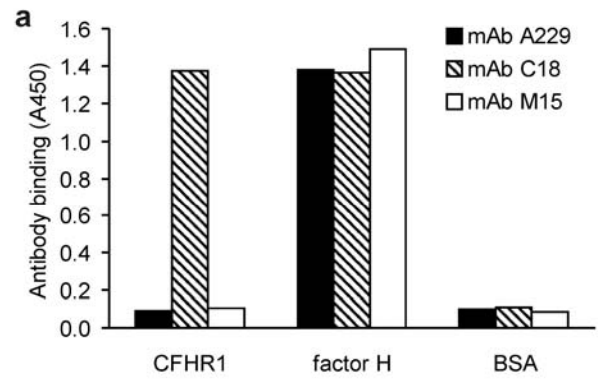


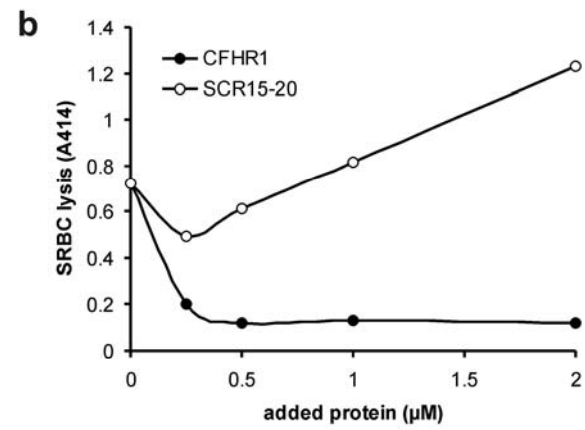
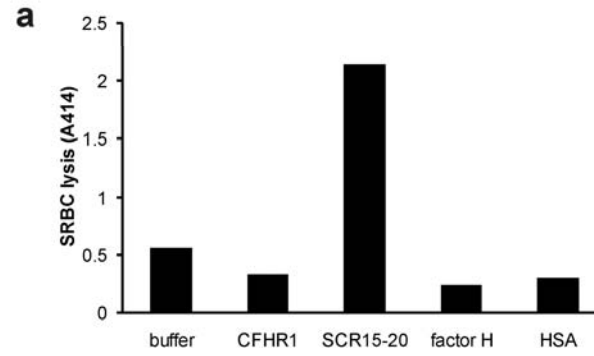


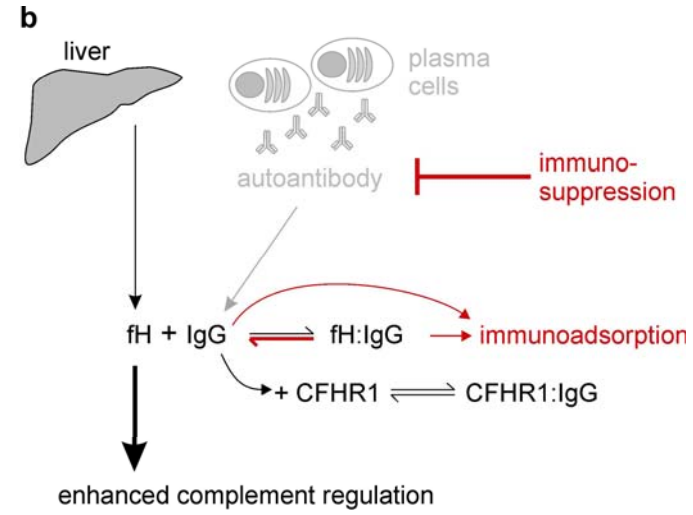
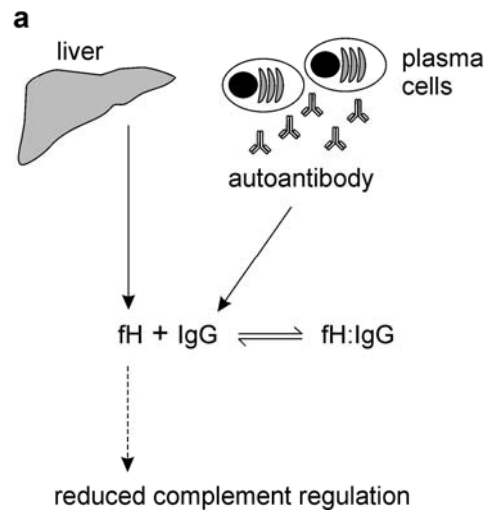












Supplementary figure legends:

Supplementary Figure S1. Binding of factor H and CFHR1 to surface bound C3b and to endothelial cells is inhibited by mAb C18.

(a) To study the binding of plasma factor H and CFHR1 to solid phase C3b, microtiter plate wells were coated with 500 ng C3b. Human plasma samples (diluted to 20%) were incubated on the C3b-coated wells in the presence or absence of the anti-factor H mAbs C18 (recognizing SCR20) and M13 (binding within SCRs 15-18).⁹ C3b-bound proteins were then eluted with sample buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose membrane, and analyzed by Western blot using a factor H antiserum. Binding of both factor H and CFHR1 to immobilized C3b was inhibited by mAb C18 (lane 2), but not by mAb M13 (lane 3). A representative experiment is shown.

(b) Binding of plasma factor H and CFHR1 to endothelial cells was analyzed as previously described for factor H.⁹ Human umbilical vein endothelial cells (HUVEC) were incubated with or without 20% human plasma in the presence or the absence of anti-factor H mAbs C18 or M13. After extensive washing, the cells were lysed and the cell extracts were analyzed by Western blot using a factor H antiserum. Binding of both factor H and CFHR1 to the cells was inhibited by mAb C18 (lane 3), but not by mAb M13 (lane 4). A representative experiment is shown.

Supplementary Figure S2. Analysis of anti-factor H IgA autoantibodies.

(a) IgA autoantibodies that bind to factor H (fH) were detected in plasma samples from 3 patients by ELISA (black bars). As indicated by inhibition experiments, their binding site is also located in SCR20. The mAb C18 that binds to SCR20 inhibited IgA autoantibody binding (patterned bars) to the same level as the background binding to BSA (empty bars). The mAb 90X that binds to SCR1 had no inhibitory effect (grey bars). Data are means + SD

from three experiments. **(b)** IgA autoantibodies from the three patients bind to factor H and to recombinant CFHR1, but not to CFHR4A or to BSA.

Supplementary Figure S3. Detection of free factor H by ELISA.

The amounts of free and total factor H were determined by ELISA using mAb A229 and M15,¹⁶ respectively. **(a)** The mAb A229 binds to the factor H C-terminus but – in contrast to mAb C18 – it does not recognize recombinant CFHR1. The mAb M15 binds within SCR11-18 of factor H,¹⁶ and does not bind to CFHR1. **(b)** The mAb A229 inhibits the binding of autoantibodies to factor H, but not to CFHR1; thus mAb A229 recognizes only free factor H. The mAb M15 does not inhibit autoantibody binding to factor H.^{10,16} **(c)** When 4 μ M recombinant CFHR1 was added to the serum of patient 6, more free factor H was detected (black bars, mAb A229) in relation to the total factor H (white bars, mAb M15).

Supplementary Figure S4. The factor H SCR15-20 fragment, in comparison with CFHR1, efficiently competes with factor H for binding to cells and thus causes lysis of sheep erythrocytes.

(a) Addition of factor H or CFHR1 to 20% normal human plasma does not induce anomalous lysis of sheep erythrocytes (SRBC). However, the factor H SCR15-20 fragment caused hemolysis of SRBC, suggesting a competition between this fragment and factor H for cell binding. All proteins were added at 2 μ M concentration. A representative experiment out of two is shown. **(b)** The SCR15-20 fragment has a biphasic effect on the SRBC lysis induced by mAb C18. At lower concentrations it inhibited the lysis because it binds to the mAb C18 and neutralizes this mAb. At higher concentrations, most likely due to competition with factor H for cell binding, the SCR15-20 fragment further enhanced the lysis. This biphasic effect was not observed for CFHR1. A representative experiment out of three is shown.

Supplementary Figure S5. Immune complex formation and treatment possibilities in autoimmune aHUS.

(a) Factor H (fH) is continuously produced in the liver and is released into the circulation. Autoantibodies are produced by plasma cells. The concentrations of free factor H and free autoantibodies, and the affinity of the autoantibodies to factor H, will determine the concentration of the fH:IgG complexes, which will differ among patients. The equilibrium of this binding reaction can be shifted by influencing these concentrations; e.g. a reduction of IgG concentration will shift the reaction towards dissociation of the complexes until a new equilibrium is reached. (b) Plasma therapy applied in the acute phase of the disease can increase the concentration of free factor H, and remove some of the circulating autoantibodies and immune complexes. CFHR1 in the infused plasma will also complex some autoantibodies. Immunoabsorption only removes the free and complexed autoantibodies. Exogenous factor H and CFHR1 added as plasma proteins or as purified/recombinant proteins would complex a part of free autoantibodies and increase the concentration of free factor H. All these measures are useful to acutely reduce the amount of autoantibodies and/or to increase the amount of free, fully functional factor H. However, to stop the production of autoantibodies, immunosuppression and elimination of plasma cells need to be attempted. A late attempt with rituximab may fail as it does not target long-lived plasma cells due to the lack of the CD20 antigen on these cells.