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Overall Neutralization of Complement Factor H by Autoantibodies in the Acute Phase of the Autoimmune Form of Atypical Hemolytic Uremic Syndrome

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Complement is a major innate immune surveillance system. One of its most important regulators is the plasma protein factor H (FH). FH inactivation by mutations or by autoantibodies is associated with a thrombotic microangiopathy disease, atypical hemolytic uremic syndrome. In this study, we report the characterization of blood samples from 19 anti-FH Ab-positive atypical hemolytic uremic syndrome patients collected at the acute phase of the disease. Analyses of the functional consequences and epitope mapping, using both fluid phase and solid phase approaches, were performed. The anti-FH Abs perturbed FH-mediated cell protection (100%), inhibited FH interaction with C3 (46%), and caused C3 consumption (47%). The Abs were directed against multiple FH epitopes located at the N and C termini. In all tested patients, high titers of FH-containing circulating immune complexes were detected. The circulating immune complex titers correlated with the disease stage better than did the Ab titers. Our results show that anti-FH autoantibodies induce neutralization of FH at acute phase of the disease, leading to an overall impairment of several functions of FH, extending the role of autoantibodies beyond the impairment of the direct cell surface protection. *The Journal of Immunology*, **2012, 189: 3528–3537.**

The complement system is a part of the innate immune response, acting as a first line of defense against pathogens and participating in immune surveillance and homeostasis. This cascade, which can be activated by the classical, lectin, and alternative pathways, is highly controlled by a number of plasma or membrane regulators to prevent host tissue damage (1). Atypical hemolytic uremic syndrome (aHUS) is considered to be the prototypical disease linked to a dysregulation of the alternative pathway (2). This life-threatening disease associates with acute renal failure, thrombocytopenia, and hemolytic anemia due to microangiopathic lesions occurring without previous infection by shiga toxin-producing bacteria. The main protein implicated in this process is the plasma regulator factor H (FH) (2–4). Genetic abnormalities were found in ~30% of patients, and the presence of anti-FH autoantibodies was detected in 6–10% of patients (5, 6). FH is composed of 20 short consensus repeat (SCR) domains, each consisting of ~60 aa, which have specific ligands and functions. FH acts as a regulator by competing with factor B for the binding to C3b, enhancing the dissociation of the C3bBb complex (decay activity), and acting as a cofactor for factor I (FI)-mediated proteolytic inactivation of C3b into iC3b via the N terminus (7). The central part of the molecule (SCR 6–8) binds to glycosaminoglycans on cell surfaces (8). The C-terminal domains (SCR 19–20) bind to glycosaminoglycans on cell membranes and to C3b and its cleavage fragment C3d (9–11).

The autoimmune form of aHUS (AI-aHUS) was first described in 2005 (5) and occurs mainly in children (12, 13). This form of the disease is highly linked to a homozygous deletion of two complement FH-related (CFHR) genes due to a recombination in the locus called regulator of complement activation in chromosome 1, where *CFH* and its five related genes (encoding CFHR1–5) are located (12, 14). The CFHR proteins share a high degree of sequence homology with several domains of FH. The C-terminal region of

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C.B. and M.-A.D.-D. designed the research; C.B., Y.A., S.H., and M.-A.D.-D. performed the experiments; S.H. and T.S.J. provided vital reagents; S.K.S., B.R., P.N., C.L., A.G., and A.B. are in charge of the patients and provided plasma samples; C.B., L.T.R., W.H.F., C.S.-F., V.F.-B., and M.-A.D.-D. analyzed the data and wrote the paper. All authors read and approved the submission of this manuscript.

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Abbreviations used in this article: aHUS, atypical hemolytic uremic syndrome; AI-aHUS, autoimmune form of atypical hemolytic uremic syndrome; AU, arbitrary unit; CFH, complement factor H; CFHR, complement factor H-related; CIC, circulating immune complex; CIC-FH, circulating immune complexes factor H–anti-factor H IgG; FH, factor H; FI, factor I; RLA, radioligand assay; SCR, short consensus repeat domain.

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CFHR1 is identical to FH except for two amino acids localized in SCR 3–5 and FH SCR 18–20. CFHR1 and CFHR2 share similarities between their N-terminal domains, and the SCR 1–3 of CFHR3 shares common amino acid sequences with the central part of FH. Physiological functions of CFHRs remain incompletely characterized, but CFHR1 and CFHR2 have been shown to play a role in the regulation of C5 and C3 convertases, respectively (15, 16). The *CFHR1/3* homozygous deletion is present in the normal population at a frequency varying between 2 and 8%, depending on the ethnic origin (17). This homozygous deletion is highly suspected to play a role in the disease, but the mechanisms leading to anti-FH autoantibody generation remain unclear. Nevertheless, the characterization of anti-FH autoantibody binding sites and their functional consequences are important to better understand the physiopathological mechanisms of the disease. Using ELISA assays, previous studies mapped the dominant epitope of the autoantibodies in the FH C-terminal domains (13, 18–20). The autoantibodies of three patients were demonstrated to impair FH binding to C3b and resulted in an inefficient FH-dependent cell protection (18, 20).

The present study, performed with samples collected from 19 patients at the acute phase of the disease, reports the extensive characterization of anti-FH autoantibodies. A study of the functional consequences was performed by eight different assays and epitope mapping by three approaches. Anti-FH autoantibodies impaired FH-mediated cell protection, weakened FH interaction with C3 fragments, and in some cases disturbed the FI cofactor activity. Thus, our results demonstrate that in AI-aHUS, the Abs bind multiple epitopes localized in the N- and C-terminal and central domains of FH and form stable immune complexes. The autoantibodies also bind to CFHR1 and CFHR2 proteins. Collectively, our results suggest that the autoimmune form of aHUS is due to an impairment of several functions of FH, not only its ability to restrict complement activation on self surfaces.

Materials and Methods

Cohorts/patients

Nineteen patients presenting clinical and biological criteria of aHUS were studied. The selection was based on the presence of anti-FH IgG in samples collected at the acute phase of the disease before any blood-derived products or immunosuppressive treatment was administered. Informed consent was obtained from each patient or parents of children, and the study was approved by the Ethics Committee (Comité de Protection des Personnes Ile de France V, IDRCB2008-A00144-51). The anti-FH IgG titer was determined by ELISA as previously described (14). The clinical data of these patients were previously reported (6). For 14 patients, samples were also collected during remission, that is, at least 1 y after disease onset and at a time when no evidence of hemolysis or platelet consumption was observed.

Samples collected from aHUS patients with a homozygous *CFHR1/3* deletion without anti-FH IgG were used as controls. All of the blood specimens used were EDTA plasma samples.

Genetic analysis

All patients were screened for mutations and polymorphisms in the *CFH*, *CFI*, and *CD46* genes. The number of *CFHR1* and *CFHR3* genes was determined by multiplex ligation-dependent probe amplification for each patient as previously described (14).

Anti-FH IgG subtypes determination

The IgG subtypes were determined by an anti-FH ELISA as previously described with some modifications. After plasma incubation and washing, isotype-specific mouse Abs directed against IgG1, IgG2, IgG3, and IgG4, diluted 1:1000 in Tween 20/PBS, were incubated for 1 h at room temperature, followed by an additional incubation with an anti-mouse IgG Ab labeled with HRP (Santa Cruz Biotechnology, Yvelines, France).

Nonsensitized sheep erythrocytes hemolytic assay

Samples from six patients with anti-FH Abs were collected at the acute phase and in remission. Plasma from aHUS patients with complete CFHR1/3 deficiency but without anti-FH Abs ($n = 7$) and healthy donor plasma ($n = 20$) were used as controls. Diluted plasma (25%) was added to sheep erythrocytes (10^8) and incubated for 30 min at 37°C in buffer (7 mM $MgCl_2$, 10 mM EGTA [pH 7.2–7.4], 2.5 mM barbital, 1.5 mM sodium barbital, and 144 mM NaCl). After the addition of 1 ml 0.9 M NaCl, sheep erythrocyte lysis was measured by absorbance at 414 nm, as previously described (21).

ELISA for FH binding to C3(H_2O), C3c, and C3d

Patient IgG samples were purified using Melon Gel IgG purification kits (Thermo Scientific, Courtaboeuf, France) according to the manufacturer's instructions and used immediately to avoid IgG precipitation. Microtiter plates (Nunc microtiter microplates; Thermo Scientific) were coated with 2 μg/ml C3(H_2O) (Calbiochem, Lyon, France) or C3c or C3d (CompTech, Tyler, TX) overnight at 4°C. Purified human FH (0.01 mg/ml) was preincubated with purified patient IgG (0–0.5 mg/ml) overnight at 4°C. After blocking with 0.1% BSA in PBS, the preformed FH–anti-FH IgG complexes were added in duplicate to the coated plates. FH binding was revealed using the anti-FH mAb (OX23; Santa Cruz Biotechnology) diluted at 1:250, incubated for 1 h at 37°C, followed by an incubation with an HRP-labeled anti-mouse IgG. OX24 and L20/3 mAbs (Santa Cruz Biotechnology) directed against the N- and C-terminal domains of FH, respectively, were used as controls at the concentration of 0.002 mg/ml. Each experiment was performed at least three times.

FI cofactor activity test

IgG purified from each patient (20 μg/ml) was incubated with purified FH (CompTech; 20 ng) at 37°C for 30 min. Purified FI (CompTech; 20 ng) and C3(H_2O) (Calbiochem; 100 ng) were then added to the mixture and incubated for 0, 3, 6, or 8 min at 37°C. The reaction was stopped, and Western blot was then performed to reveal the generated C3 fragments using a goat anti-C3 Ab (Calbiochem) followed by incubation with a labeled secondary Ab (rabbit anti-goat HRP; Santa Cruz Biotechnology).

ELISA to detect CFHR1 binding to C5

This assay was performed according to Heinen et al. (15). Serial dilutions of purified CFHR1 (Abnova, Le-Perray-en-Yvelines, France; 0–15 μg/ml in buffer [10 nM Na_2HPO_4, 27 mM KCl, 1.4 M NaCl, and 2% BSA]) were incubated overnight in microtiter plates at 4°C. After blocking with 1% BSA in PBS, human purified C5 (25 μg/ml; Calbiochem) diluted in 0.1% gelatin–veronal-buffered saline was added to the plate and incubated for 1 h at 37°C. After washing, biotinylated anti-C5 Ab (Abcam, Paris, France) diluted 1:500 was added, followed by an incubation with HRP-streptavidin (Dako, Trappes, France) diluted 1:1000.

Alternatively, microtiter plates (Nunc microtiter microplates) were coated with 0.01 mg/ml CFHR1 overnight at 4°C. After blocking, purified IgG (0.5 mg/ml) from two CFHR1-sufficient patients were added in duplicate and incubated for 1 h at 37°C. After washing, C5 (25 μg/ml) was added to the plate and incubated for 1 h at 37°C. After washing, C5 binding was revealed as described above.

Production of SCR 19–20 and SCR 1–4 fragments

SCR 19–20 was expressed in yeast as previously described (22). SCR 1–4 was produced largely according to Pechtl et al. (23), except for the following main modifications. First, DNA encoding human FH domains 1–4 (residues 19–264) was amplified from a human liver cDNA library (Stratagene) with specific primers, and the product was inserted into the yeast expression vector pPicZαB (Invitrogen, Courtaboeuf, France). A His-tag was added to the C terminus of FH 1–4 using the QuickChange site-directed mutagenesis kit (Stratagene) with the following primer: 5′-GGATGGCGTCCGTTG-CCTTCATGTGAACATCATCATCATCATCATTGATCTAGAACA-3′. The product was transformed into yeast and expressed as the FH 19–20 fragment (22). For purification, the pH of the culture supernatant was adjusted to 7, and after centrifugation, the supernatant was applied to an Ni-NTA agarose column (Qiagen, Courtaboeuf, France). Elution was performed using a series of 75, 100, 150, and 250 mM imidazole in 50 mM phosphate and 300 mM NaCl (pH 8.0). The best fractions (i.e., those containing the main peak of FH 1–4) were combined and the buffer was changed with a concentrator (Amicon Ultra, Ultracel, 3000 m.w. cut-off) to 20 mM sodium carbonate (pH 9.0) containing 1 mM EDTA. The protein was bound to a Resource Q ion exchange column (1 ml; GE Healthcare, Buc, France) and eluted with a gradient up to 1 M NaCl in the same buffer. As a final step, the protein was purified with Superdex TM 75 10/300 GL gel filtration using PBS as the running buffer.

ELISA for patient IgG binding to FH, SCR 19–2O, and SCR 1–4 constructs

Microtiter plates (Nunc microtiter microplates) were coated overnight at 4°C at equal molarity (67 nM) with FH (Calbiochem) or FH fragments

(0.01 mg/ml FH, 0.001 mg/ml SCR 19–20, and 0.002 mg/ml SCR 1–4). After blocking with 1% BSA in PBS, the wells were incubated with plasma from 14 aHUS patients diluted 1:50 in 0.1% Tween 20/PBS for 1 h at 37°C. After washing with 0.1% Tween 20/PBS, IgG binding was measured using anti-human IgG labeled with HRP and diluted 1:2000 (SouthernBiotech, Montrouge, France). Each sample was tested in duplicate.

Generation of recombinant fragments of FH, CFHR1, CFHR2, and CFHR3

Total RNA from human liver tissue was isolated using TRIzol (Life Technologies, Cergy Pontoise, France). After DNase treatment, cDNA was synthesized using AMV reverse transcriptase (Roche Diagnostics, Mannheim, Germany). Human FH and CFHR cDNA were amplified with specific primers for each construct using the Expand Long Template PCR System (Roche Diagnostics), controlled and extracted with the QIAquick gel extraction kit (Qiagen). FH and CFHR cDNA constructs were cloned into the expression vector pcDNA3.1/v5-His TOPO (Invitrogen) and used to transform *TOP10* bacteria (Invitrogen) according to the manufacturer's instructions. The transformed bacteria colonies were selected in medium containing 100 μg/ml ampicillin and expanded for plasmid DNA preparation. Plasmid DNA was extracted and purified using QIAprep plasmid spin columns (Qiagen). The sequence of the cloned cDNA was confirmed using Applied Biosystems automated sequencers.

Radioligand assay

[^{35}S]cysteine-labeled recombinant proteins (Amersham Biosciences, Pantin, France) were produced by an in vitro transcription/translation assay using a TNT coupled transcription/translation System (Promega, Charbonnières, France) according to the manufacturer's instructions. The products were applied to a NAP-5 column (GE Healthcare) with reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 1% Tween 20 [pH 7.2]) to remove free [^{35}S]cysteine. After control of the radiolabeled constructions on SDS-PAGE, a defined amount of each labeled recombinant protein providing 15 kcpm was incubated in presence of 10 μl plasma containing anti-FH IgG in a 96-well deep plate overnight at 4°C. Fifty microliters of protein G-Sepharose (GE Healthcare) was then added to each well of a filtration plate (MultiScreen$_{HTS}$; Millipore, Saint-Quentin-en-Yvelines, France). The plate was washed five times with washing buffer (50 mM Tris-HCl, 50 mM NaCl, 1% Tween 20 [pH 7.2]) using a vacuum manifold (Millipore). Liquid scintillation fluid was added, and the quantity of precipitated labeled protein was counted in MicroBeta TriLux (PerkinElmer, Courtaboeuf, France). All samples were tested in duplicate. Each experiment was performed three times. The samples were considered to be positive when their value was greater than the mean + 2 SDs obtained by analyzing 20 healthy donor plasma samples. Average linkage hierarchical clustering was applied, and the results were displayed using Genesis software version 1.7.5 to perform Pearson uncentered hierarchical clustering (Genesis; University of Graz, Graz, Austria).

Immunoprecipitation of plasma CFHR proteins by anti-FH IgG

Patient IgG samples were purified using protein G (GE Healthcare). After a control of IgG purification (data not shown), 100 μg purified IgG was immobilized on protein G-agarose beads overnight at 4°C. After washing, the IgG-coupled beads were incubated with a pool of plasma from 100 healthy donors or with CFHR1/3-deficient plasma. Immunoprecipitated proteins were then eluted, and Western blot analysis using a polyclonal anti-FH Ab (Quidel, Chilly-Mazarin, France) was performed.

Determination of the circulating FH–anti-FH IgG complexes

Purified anti-FH polyclonal Ab (1:400; The Binding Site, Grenoble, France) was incubated overnight at 4°C to coat a microtiter plate (Nunc microtiter microplates). After blocking with 1% BSA in PBS, patient plasma samples diluted 1:100 in PBS/1% BSA were added to the plate and incubated for 1 h at room temperature. The binding of the FH-containing immune complexes was revealed using an anti–IgG-HRP (Sigma-Aldrich). Titers of positive samples were expressed as arbitrary units (AU) per milliliter and calculated using a calibration curve obtained with serial dilutions of a circulating immune complex (CIC)-positive plasma taken as reference (1:100 to 1:3200), giving an arbitrary titer from 62.5 to 2000 AU/ml. The positive threshold was calculated by the mean + 2 SDs of those obtained with plasmas from 20 individual healthy donors. This titer was determined to be 110 AU/ml, and titers above this value were considered to be positive. Noncoated wells and wells incubated without a plasma sample (blank) were used to measure the background reactions. Each sample was tested in duplicate.

Study of the binding avidity of anti-FH for FH

Purified FH (Calbiochem) was coated overnight at 4°C on a plate at 1 μg/ml (Nunc microtiter microplates). After washing with PBS containing 0.5% Tween 20, serial dilutions of plasma samples from 20 patients corresponding to anti-FH IgG titers from 250 to 1000 AU/ml were added to the plate and incubated at room temperature for 5, 10, 15, 30, and 60 min for a time-dependent kinetic assay. Binding of anti-FH IgG to FH was visualized with an anti–IgG-HRP Ab (Sigma-Aldrich). Avidity for FH was determined by the slope of the dose–response curve (absorbance versus time).

Alternatively, the resistance of the FH–anti-FH IgG complex to increased ionic strength was assessed by ELISA. Microtiter plates were coated overnight at 4°C with FH (CompTech) at 0.01 mg/ml in PBS. After blocking with 1% BSA in PBS for 1 h at 37°C, the plates were washed four times with 0.1% Tween 20-PBS. Increasing concentrations of NaCl (150, 250, and 500 mM) were diluted in 0.1% Tween 20-PBS containing samples from patients diluted 1:50 in PBS-Tween 20. After a 30-min incubation at room temperature, the plates were washed four times with 0.1% Tween 20-PBS, and human IgG labeled with HRP (SouthernBiotech) diluted 1:2000 was used to measured the IgG binding.

Statistical analysis

All experiments were repeated in triplicate. Statistical analysis was performed using GraphPad software (version 5.0). The Mann–Whitney *U* test was applied to compare two unpaired groups. In all cases, statistical significance was accepted at $p < 0.05$ between groups.

Average linkage clustering was also applied using Genesis software (version 1.7.5).

Results

Complement measurements

Nineteen patients (17 children and 2 adults) presenting with clinical and biological criteria of aHUS were studied. Blood samples were collected at the acute phase, before any treatment. The median age at disease onset was 8 y. All patients were positive for anti-FH IgG. The anti-FH titers were established between 664 and 35,000 AU/ml (positive threshold, 110 AU/ml) (6). All 19 patients had anti-FH Abs of IgG3 isotype, and 25% (5 of 19) also had IgG1 anti-FH Abs.

Low C3 plasma levels were observed in 48% (9 of 19) of patient samples, suggesting complement activation (range of patient values, 376–1310 mg/ml; normal values, 660–1290 mg/ml). Genetic analysis revealed the presence of a homozygous deletion of *CFHR1* and *CFHR3* in 90% (17 of 19) of patients. These data are summarized in Tables I and II.

Nonsensitized sheep erythrocyte lysis by anti-FH IgG

We tested plasma samples collected at the acute phase and during remission from six patients (Fig. 1). We observed a high level of lysis in the samples from the acute phase, which was significantly reduced in samples from remission (Fig. 1A). The amount of lysis observed in samples collected at remission remained significantly higher than that in the control group (Fig. 1A). The addition of purified FH in anti-FH IgG-containing samples prevented lysis in a dose-dependent manner (Fig. 1B).

To directly address the role of IgG, we compared the lysis induced by plasma containing anti-FH IgG and the same samples after IgG depletion by preincubation with protein G-coupled Sepharose beads. IgG-depleted samples lost their lytic activity (Fig. 1C). IgG-depleted plasma from one patient with a mutated FH without anti-FH IgG was used as a control to test that the IgG depletion procedure did not impair complement-mediated hemolysis (Fig. 1D). Furthermore, no lysis was detected in CFHR1/3-deficient plasma lacking anti-FH autoantibodies (Fig. 1A).

FH cofactor activity for the cleavage of C3b by FI

To test whether anti-FH IgG affects the FI cofactor activity of FH, we performed an in vitro cofactor test with purified IgG from 10 patients. C3 fragments were detected by Western blot (Fig. 2,

Table I. Summary of the results for each patient: functional consequences

	Lysis	FI Cofactor	C3(H_2O) Binding	C3c Binding	C3d Binding	CIC-FH Acute Phase	CIC-FH Remission
P1	+	Normal	Normal	Normal	Normal	+	−
P2	+	Normal	Normal	Normal	Normal	+	+
P3	+	Normal	Decreased	Decreased	Decreased	+	−
P4	+	Normal	ND	ND	ND	ND	ND
P5	+	Delayed	Decreased	Normal	Decreased	+	−
P6	+	Normal	Decreased	Normal	Normal	+	−
P7	+	Normal	ND	ND	ND	ND	ND
P8	+	Normal	Decreased	Decreased	Normal	+	+
P9	+	Normal	ND	ND	ND		ND
P10	+	Normal	Normal	Normal	Decreased	ND	ND
P11	+	Normal	Normal	Normal	Decreased	+	−
P12	+	Normal	ND	ND	ND	+	+
P13	+	Normal	ND	ND	ND	+	−
P14	+	Normal	Normal	Normal	Normal	+	−
P15	+	Normal	ND	ND	ND	ND	ND
P16	+	Normal	Decreased	Decreased	Decreased	+	+
P17	+	Normal	Decreased	Normal	Decreased	+	−
P18	+	Normal	Normal	Normal	Decreased	+	−
P19	+	Inhibited	Normal	Normal	Decreased	+	+

+, Positive; −, negative.

Table I). Purified IgG from healthy donors and from 8 of 10 aHUS patients did not affect the generation of the C3b cleavage fragment (43-kDa α-chain), indicating normal cofactor activity under our experimental conditions (Fig. 2A, *lanes 4* and *5*). For P19, no C3 43-kDa α-chain band could be generated (Fig. 2B, *lane 3*), indicating a perturbation of the FI cofactor activity induced by anti-FH IgG. For P5, a time-dependent delay was observed in the generation of the C3 43-kDa α-chain band (Fig. 2C, 2D) in comparison with IgG from healthy donors (Fig. 2B, *lanes 1* and *2*, Fig. 2C).

Study of FH binding to C3(H_2O), C3c, and C3d

The binding of FH to C3(H_2O), C3c, and C3d was tested in presence of purified IgG from 13 patients, each of them in at least in three independent assays (Fig. 3). Significantly reduced binding of FH to C3(H_2O) was observed in six patients (Fig. 3A) in an IgG dose-dependent manner (Fig. 3D). Purified IgG from 3 of 13 patients was able to induce a decrease of FH binding to the C3c part of C3 (Fig. 3B). In 8 of 13 patients IgG induced a significant reduction in FH binding to C3d, reaching up to 50% (Fig. 3C). In two patients (P3 and P17), IgG induced a reduction in FH binding to all C3 fragments (Fig. 3, Table I).

Study of CFHR1 binding to C5

The effect of anti-FH IgG on the binding of CFHR1 to C5 was studied by ELISA (Supplemental Fig. 1). We observed that CFHR1 binds to C5 in a dose-dependent manner (Supplemental Fig. 1A). Purified IgG from the two CFHR1-sufficient patients was not able to disturb this binding. The L20/3 Ab, interacting with the CFHR1 (and FH) C-terminal domains, was also not able to disrupt this binding (Supplemental Fig. 1B).

Epitope mapping and determination of antigenic sites

ELISA using FH constructs. An ELISA assay was performed using FH and FH constructs (Supplemental Table I) containing the N-terminal (SCR 1–4) or the C-terminal domains (SCR 19–20) (Fig. 4, Table II). Binding to the entire protein was observed in plasma from all 14 patients (Fig. 4A), to the N-terminal domains

Table II. Summary of the results for each patient: characterization of anti-FH autoantibodies

	Level of C3	Level of FH	Titer Ab Anti-FH (Onset)	Isotypes	CFHR1 Deletion	Binding to FH				Binding to CFHR2		
						N-terminal ELISA (1–4)	N-terminal RLA (1–7)	C-terminal RLA (19–20)	C-terminal ELISA (19–20)	IP 29 kDa	IP 24 kDa	RLA
P1	N	N	750	IgG3	+	ND	Pos	Pos	ND	ND	ND	Pos
P2	L	L	1,000	IgG3	+	ND	Pos	Pos	ND	ND	ND	Pos
P3	D	N	20,000	IgG3	+	Pos	Pos	Neg	Pos	ND	ND	Pos
P4	D	D	>32,000	IgG3	+	Pos	Pos	ND	Pos	Pos	Pos	Pos
P5	D	N	1,000	IgG3	+	Pos	Pos	Neg	Pos	ND	ND	Pos
P6	D	D	2,000	IgG1 + IgG3	+	ND	Pos	Neg	ND	Pos	Pos	Pos
P7	N	N	3,000	IgG3	+	Pos	Pos	Pos	Neg	ND	ND	Pos
P8	N	D	1,130	IgG1 + IgG3	+	Pos	Pos	Pos	Neg	Pos	Neg	Pos
P9	D	D	664	IgG1 + IgG3	+	ND	Pos	Neg	ND	Pos	Pos	Pos
P10	D	N	4,480	IgG3	+	Pos	Pos	Pos	Neg	Pos	Pos	Pos
P11	N	N	3,000	IgG3	+	Pos	Pos	Pos	Neg	Pos	Neg	Pos
P12	N	N	7,700	IgG1 + IgG3	+	Pos	Pos	ND	Pos	Pos	Pos	Pos
P13	N	N	1,770	IgG1 + IgG3	+	Pos	Pos	Pos	Pos	Pos	Pos	Pos
P14	N	D	5,800	IgG3	−	Pos	Pos	Pos	Neg	Pos	Neg	Pos
P15	D	N	12,240	IgG3	+	ND	Pos	Neg	ND	Pos	Neg	Pos
P16	D	N	13,300	IgG1 + IgG3	+	Pos	Pos	Neg	Pos	Pos	Neg	Pos
P17	N	N	1,130	IgG3	+	Neg	ND	Neg	Pos	Pos	Neg	Pos
P18	D	D	976	IgG3	+	Pos	Neg	Neg	Pos	Pos	Pos	Pos
P19	D	L	5,176	IgG3	−	Pos	Pos	Neg	Neg	Pos	Neg	Pos

+, Positive; −, negative; D, decreased; IP, immunoprecipitation; L, low; N, normal; Neg, negative; Pos, positive; RLA, radioligand assay.

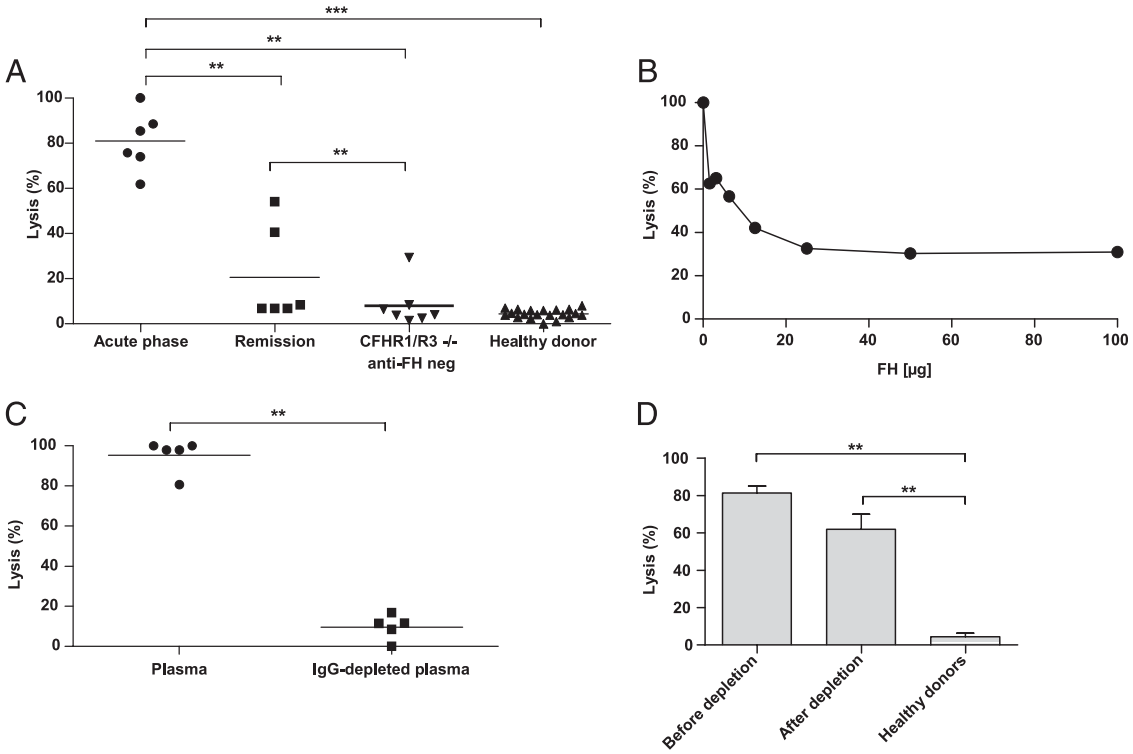

FIGURE 1. Sheep erythrocyte assay. (**A**) Sheep erythrocytes were incubated with diluted plasma (25%) from AI-aHUS patients collected during the acute phase and in remission and from patients with complete CFHR1/3 deficiency but without anti-FH Abs and from healthy donors. (**B**) The lysis of the sheep erythrocytes induced by patient (P3) plasma diluted 25% was corrected by the addition of increasing doses of purified FH. (**C**) Lysis caused by patient plasma was prevented by protein G-IgG depletion. (**D**) IgG-depleted plasma from one patient with a mutated FH was used as a positive control. All experiments were performed three times independently, and representative data are shown. The results are compared using a Mann–Whitney *U* test. $^{**}p < 0.001$, $^{***}p < 0.0001$.

for 13 of 14 patients (93%) (Fig. 4B), and to the C-terminal for 8 of 14 patients (57%) (Fig. 4C). Plasma from 7 of 14 patients (50%) was able to recognize both constructs (Fig. 4B, 4C).

Immunoprecipitation of plasma CFHR proteins using IgG from anti–FH-positive patients. We studied the recognition of FH and CFHR proteins by the purified IgG from 10 patients by an im-

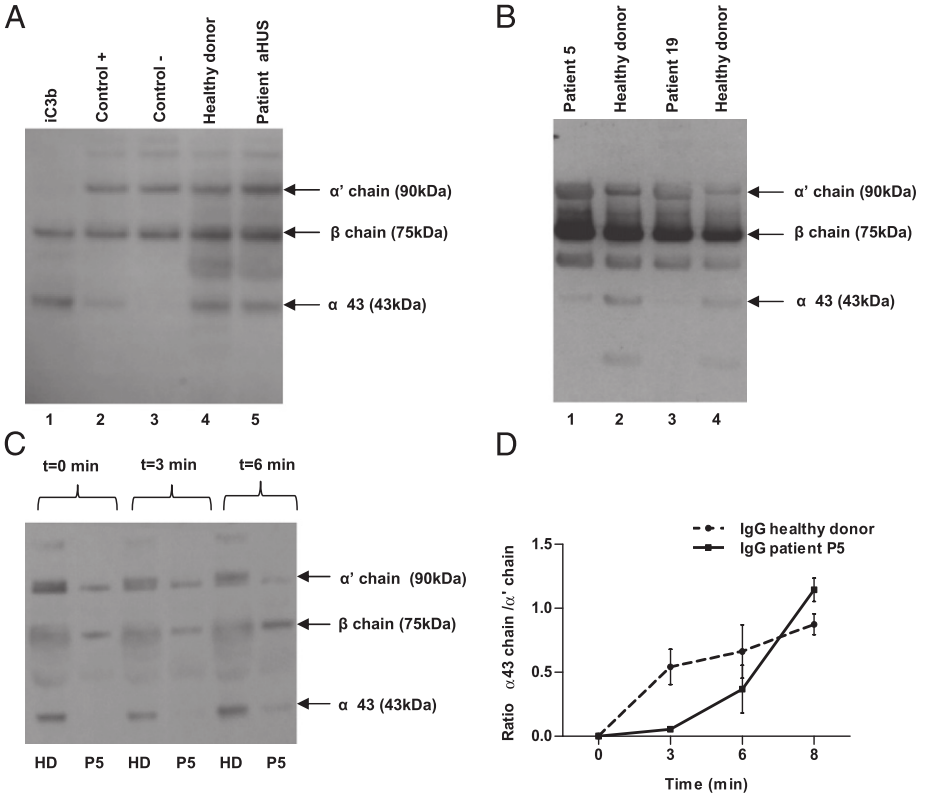

FIGURE 2. FI cofactor activity. Purified C3, FH, and FI were incubated at 37˚C with IgG from AI-aHUS patients or normal donors. The enzymatic reaction was stopped at different time points by addition of reducing sample buffer. The C3 cleavage was revealed by Western blot using anti-C3 specific antiserum. (**A**) No inhibition of C3 cleavage by IgG from a healthy donor (*lane 4*) and for eight patients with Abs against SCR 1–7 (the result of one representative patient among eight tested is shown in *lane 5*) compared with a positive control (no IgG, *lane 2*) and negative control (no FI, *lane 3*). (**B**) Inhibition of C3 cleavage by IgG from patients P5 and P19. (**C**) A time-dependent perturbation of C3 cleavage is observed in presence of patient P5 IgG compared with control IgG from a healthy donor (HD). (**D**) Quantification of the C3 α43-chain generation over time by densitometry quantification of scanned bands (ImageJ software version 1.44 developed at the National Institutes of Health) and calculation of the α43/α′-chain ratio. All experiments were performed three times independently, and representative data are shown.

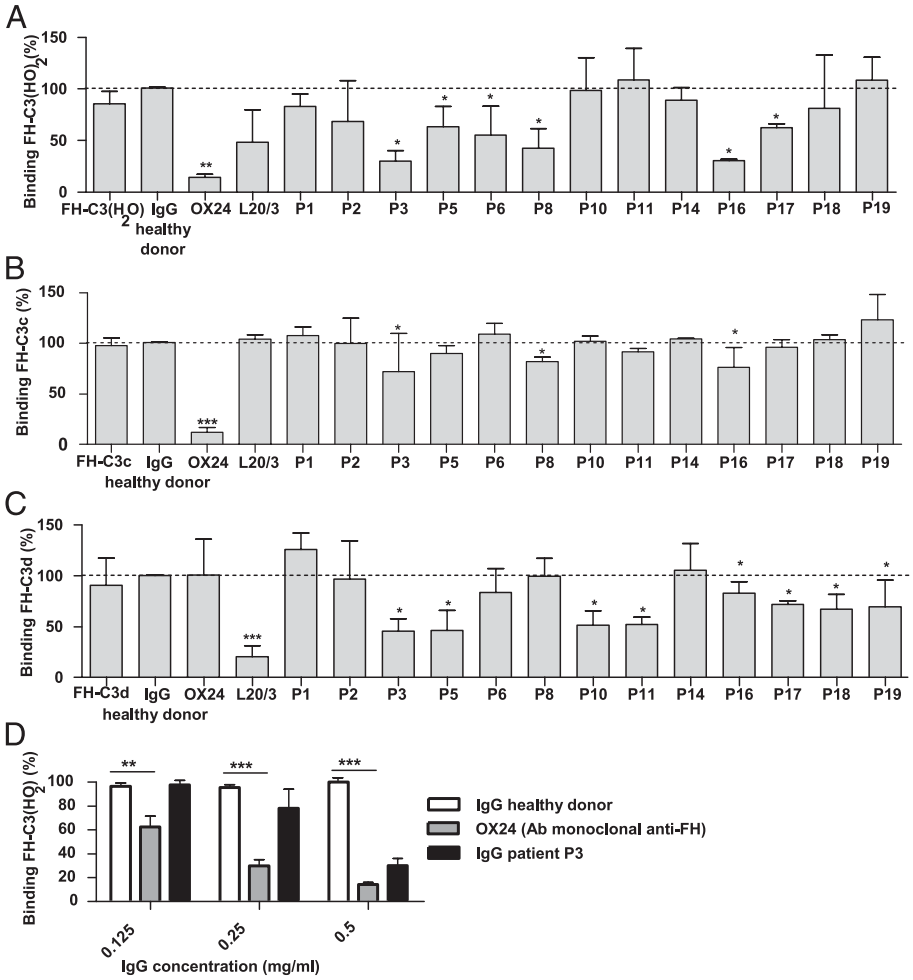

FIGURE 3. Inhibition of the interaction between FH and C3(H_2O) or the C3 degradation fragments C3c and C3d, as measured by ELISA. (**A**) C3(H_2O), (**B**) C3c, or (**C**) C3d was coated on a microtiter plate and left to interact with FH in presence of 0.5 mg/ml IgG purified from patient plasma. Bound FH was detected, and the percentages of inhibition were calculated by defining the OD obtained in presence of the normal donor IgG to be 100%. (**D**) A dose-dependent (0–0.5 mg/ml) inhibition of the interaction between C3(H_2O) and FH by P3 IgG is represented. Monoclonal anti-FH Abs Ox24 (against FH N terminus) and L20 (against FH C terminus), known to inhibit FH function, were used as positive controls. All experiments were performed three times independently, and representative data are shown. The results are compared using a Mann–Whitney *U* test. $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

munoprecipitation assay using normal plasma (Fig. 5A). The immunoprecipitated proteins were examined by Western blot using a polyclonal anti-FH Ab able to recognize FH and CFHR1 and CFHR2. For all IgGs, one band detected at 150 kDa was identified as FH (Fig. 5A, *lanes 1–4*). Two bands of 43 and 39 kDa were detected from the normal plasma (Fig. 5A, *lanes 1* and *3*). The immunoprecipitation assay performed with plasma from a CFHR1/3-deficient subject showed that the 43- and 39-kDa bands were absent (Fig. 5A, *lanes 2* and *4*), indicating that these bands were the two CFHR1 isoforms, that is, CFHR1β and CFHR1α, respectively. Another band of 29 kDa was detected in all cases (Fig. 5A, *lanes 1–4*), and an additional band of 24 kDa was also observed in 44% of patients (Fig. 5A, *lanes 3* and *4*, Table II). No protein was immunoprecipitated normal subject IgG (data not shown).

Radioligand assay. To delineate the recognition sites of the Abs by a fluid phase approach, radiolabeled FH, CFHR1, CFHR2, and CFHR3, or fragments of these, were tested by a radioligand assay (RLA) in the 19 patients. Fig. 5B illustrates the binding of each patient's IgG to FH and the CFHRs (Pearson uncentered algorithm and Genesis software). In all 19 patients, multiple binding sites were identified along the FH protein. IgG from 100% of patients bound to the C-terminal domains of FH (SCR 15–20) and to the SCR 19–20 in 47% (8 of 17). IgG from 88% (17 of 18) and 78% (14 of 18) of patients bound to FH SCR 1–7 and SCR 6–8, respectively (Fig. 5B, Supplemental Fig. 2). Binding to the central part of FH SCR 8–11 was also observed in 28% (5 of 18) of patients. Finally, binding of SCR 4–5 to CFHR1 was observed in 80% (12 of 15) of patients (Fig. 5B), to CFHR2 in 100% (19 of 19), and of SCR 1–3 to CFHR3 in 0%.

Fig. 6 and Table II summarize the results of the epitope mapping performed by ELISA and by RLA.

FH–anti-FH complexes and IgG anti-FH avidity assessment

The detection and quantification of the CIC FH–anti-FH IgG (CIC-FH) were performed in plasma from 14 patients, and the avidity to anti-FH IgG was studied by two ELISAs using a kinetic assay and increasing concentrations of NaCl (Fig. 7, Supplemental Fig. 3, and Table II) for 6 patients. We found that all patients had CIC-FH at the acute phase of the disease and that their titers decreased significantly in remission (mean at acute phase 36,400 AU/ml versus 1500 AU/ml at remission; $p = 0.01$, Student *t* test) (Fig. 7A). Among the samples collected during disease remission, 8 of 14 (57%) were negative for CIC-FH, and all were persistently positive for free anti-FH IgG (Fig. 7A). Moreover, the presence of CIC-FH correlated better with the spontaneous lysis of sheep erythrocytes than the Ab titers (Spearman test: $\rho = 0.615$, $p = 0.033$ versus $\rho = 0.491$, $p = 0.120$, respectively). We then tested the anti-FH IgG avidity. Because the presence of CIC-FH impaired the determination of the avidity using surface plasmon resonance (data not shown), we applied a kinetic ELISA and correlated the slope of the time–response curve with the presence of CIC-FH (Fig. 7B, Supplemental Fig. 3A). Patients with these immune complexes had anti-FH autoantibodies of higher avidity for free FH compared with those from patients without CIC-FH

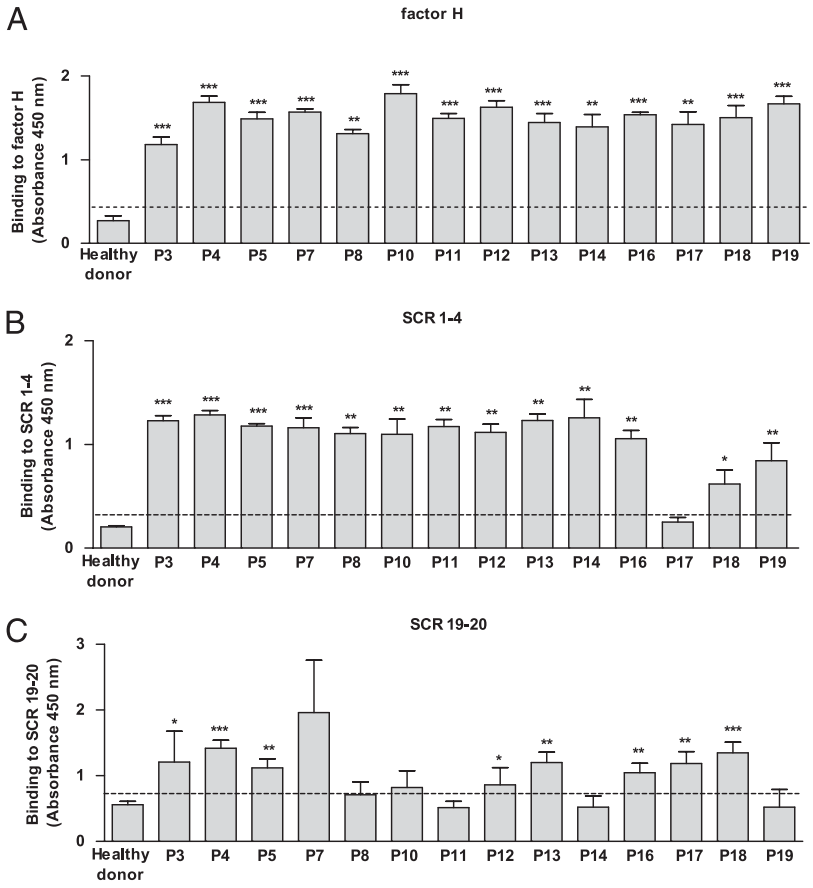

FIGURE 4. Binding of patient IgG to FH as measured by ELISA. Binding to (**A**) FH, (**B**) SCR 1–4, or (**C**) SCR 19–20 by purified IgG from AI-aHUS patients. FH or the recombinant FH fragments were coated on the ELISA plate at equal molarity and incubated with plasma from the aHUS patients or normal donors. The IgG bound was detected by anti-human IgG-HPR. The results are presented as the means and SDs of three different experiments. All experiments were performed three times independently, and representative data are shown. The results are compared using a Mann–Whitney *U* test. $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

(Fig. 7B). No significant difference was observed between the bindings of patient IgG to the N-terminal or C-terminal domains of FH in presence of increasing concentrations of NaCl (Supplemental Fig. 3B).

Discussion

The main protein implicated in the pathogenesis of aHUS is the plasma regulator FH. The present work presents an extensive characterization of the functional consequences of AI-aHUS anti-FH autoantibodies by several functional and binding assays performed on samples collected at disease onset from 19 patients, 17 of whom exhibited the homozygous *CFHR1/R3* deletion. The functional consequences were correlated to the anti-FH autoantibody binding sites on FH and related proteins, determined by fluid and solid phase approaches, using plasma proteins and recombinant constructs. The autoantibodies bound to several epitopes localized in different domains of FH. Our work revealed that during the acute phase of the disease, anti-FH autoantibodies neutralized multiple functions of the protein, extending their role beyond the impairment of cell-surface protection.

aHUS is considered to be a paradigm for diseases related to impaired cell-surface protection against complement activation. Abs from all of the tested patients perturbed cell-surface protection as measured in vitro by the hemolysis of nonsensitized sheep erythrocytes (21), in accordance with previous data in the literature (19, 20). Lysis was not observed in plasma from patients with a complete CFHR1/3 deficiency but negative for anti-FH Abs. Furthermore, lysis was rescued by addition of purified FH or by IgG depletion. Therefore, lysis was indeed due to the presence of anti-FH IgG in the plasma. In 46% of cases, anti-FH IgG inhibited the binding of FH to C3(H_2O) in a dose-dependent manner, as reported previously for three patients (18). Furthermore, IgG from 60% of the patients caused a diminution of FH binding to the cleavage fragment C3d, whose binding site is located in SCR 19–20 of FH (9). Recent studies demonstrated an important role of C3d in enhancing the binding of FH to cell surfaces, allowing better host cell protection by a feedback control activity (10, 11). Our results suggest that this activity could be impaired by the anti-FH autoantibodies.

In aHUS, a "hot spot" for FH mutations is mapped to the C-terminal domains of FH (SCR 19–20) (24, 25); these mutations are associated with spontaneous lysis of nonsensitized sheep erythrocytes (21), similar to the action of anti-FH Abs. Epitope mapping using a solid phase approach revealed that IgGs from 57% of patients were able to recognize the FH SCR 19–20 in agreement with previously published data (13, 18, 19). We confirmed our results with another assay of fluid phase epitope mapping consisting of immunoprecipitating radiolabeled recombinant constructs derived from FH. Using this approach, we observed IgG binding to FH SCR 15–20 in all tested patients but recognition for the SCR 19–20 in only 47%. The fluid-phase (RLA) and solid-phase (ELISA) assays gave complementary information, as some patients were positive for one and negative for the other. This result suggests that epitopes are more conformational than linear in this C-terminal region.

Previous studies suggested that AI-aHUS anti-FH Abs are directed primarily against SCR 19–20 with very little (13) or no recognition (19) of the N-terminal domains. Our results demonstrated the perturbation not only of the C terminus but also of the FH N-terminal domain-related functions. We previously reported that the plasma FH decay activity, a function also driven by the N-terminal domains of FH, was diminished in three anti-FH IgG-

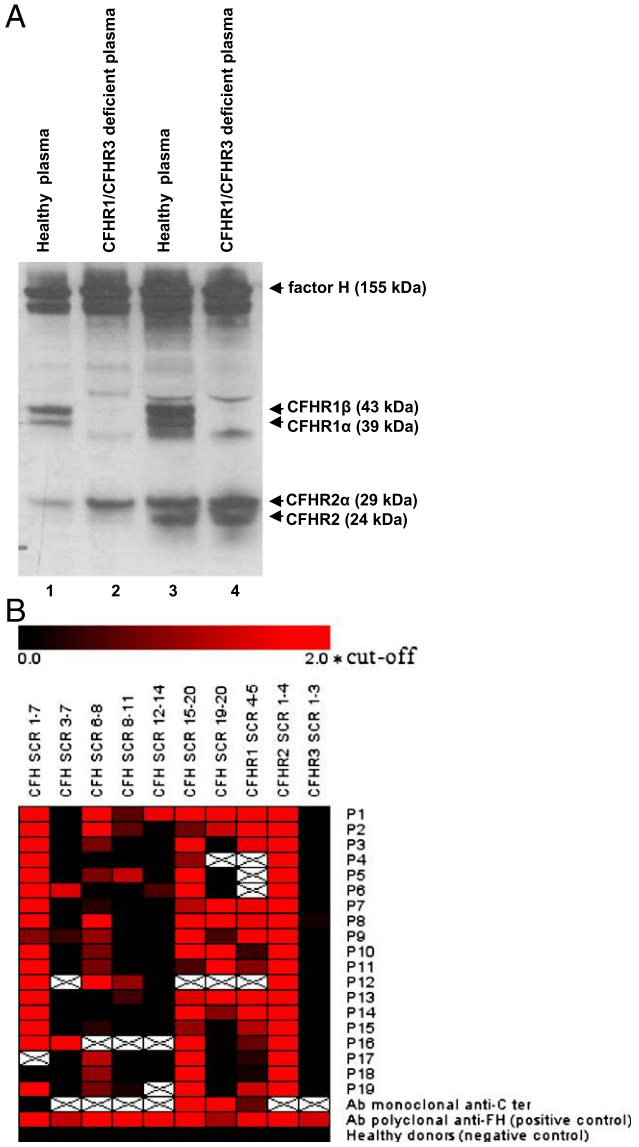

FIGURE 5. Immunoprecipitation of plasma proteins and recombinant fragments of FH, CFHR1, CFHR3, and CFHR2. (**A**) IgG isolated from 16 aHUS patients were used and incubated with plasma from a healthy donor (*lanes 1* and *3*) and plasma without CFHR1/3 (*lanes 2–4*). (**B**) RLA. The radioactivity count above the mean + 2 SD of those obtained from plasma of 20 individual healthy donors was considered to be positive. Values obtained from the 19 patients are displayed using the Genesis program to perform Pearson uncentered hierarchical clustering. The intensity of the binding is represented by red shading. White squares with an X correspond to no available data. A mAb against the C-terminal domain of FH was used as a positive control for SCR 19–20 and as a negative control for SCR 1–7. A polyclonal anti-FH Ab was used as a positive control for all recombinant proteins. All experiments were performed three times independently, and clustering data are shown.

positive patients (5). In this study, we observed that the FI cofactor activity of FH was disturbed in vitro by IgG from two patients. In accordance with these results, IgG from both patients had reactivity against SCR 1–4 and SCR 1–7, and we found a high frequency of FH N-terminal recognition by anti-FH Abs. Indeed, FH SCR 1–4 was recognized by 93% of patient IgGs by ELISA, and the FH SCR 1–7 by 94% by RLA. Our approach using samples collected at the acute phase of the disease could explain this difference, although Moore et al. (13) reported N terminus recognition only for 1 of 12 patients tested shortly after presentation, and the time of the sample collection was not precise in the other studies (18, 19). Collectively, our results demonstrate that the impairment of FH functions by anti-FH IgG was not restricted to self surface protection and revealed a more extended role related to inhibition of the N-terminal domain functions. These findings may explain the low C3 plasma levels observed in 48% of our cohort. We previously reported that such low C3 levels were associated with a pejorative prognostic factor for relapse and renal survival in AI-aHUS patients (6).

A C3c binding site has been mapped to the central portion of FH (26), and IgG from three patients induced a significant decrease in the binding of FH to C3c. We observed reactivity against the central part of the protein in 28% of the patients in accordance with previously published studies revealing such binding in 20 and 25% of patients (12, 13).

In summary, for all tested patients, multiple binding sites were identified along the FH protein. This polyclonal reactivity to several parts of the FH could explain the high levels of plasma FH–anti-FH IgG immune complexes found at disease onset for all patients. The presence of these complexes could explain the quantitative FH deficiency (low FH plasma levels) observed in 28% of our cohort. Indeed, the mechanisms of immune complex removal could induce an excessive elimination of FH and thereby low FH plasma levels. Normally, immune complex elimination is mediated notably by their opsonization by C3b followed by their interaction with the complement receptors complement receptor 1 and complement receptor of the Ig on erythrocytes, monocytes, and macrophages (27–29). Nevertheless, it remains unclear why some patients are able to eliminate immune complexes and others are not.

Other related autoimmune diseases are also linked to the generation of polyclonal autoantibodies leading to the neutralization of the target protein by immune complex formation, such as thrombotic thrombocytopenic purpura with the anti-ADAMTS 13 autoantibodies (30), or acquired hemophilia A with the anti-factor VIII autoantibodies (31). As in these diseases, plasma exchange is efficient in AI-aHUS (6), probably because this treatment allows a quick and efficient removal of immune complexes.

The anti-FH Abs from patients in the acute phase of the disease form stable immune complexes. We show in this study that the presence of these immune complexes correlates with high avidity binding of IgG to FH. We observed that CIC-FH decreased between the acute phase and the remission and were no longer detectable in 50% of the samples collected at remission, suggesting that they could be related to the disease activity. Moreover, we showed that the hemolytic test was better correlated with CIC-FH titers than with free anti-FH IgG titers, as previously suggested by Strobel et al. (19). Therefore, the presence of FH immune complexes could be a better marker of the AI-aHUS activity than the free Ab titers.

Ninety percent of the tested patients had a homozygous deletion of two genes structurally related to *CFH*: *CFHR1* and *CFHR3*. This genetic condition is found in 2–8% of the normal population and has already been reported to be associated with the generation of anti-FH Abs (12, 14, 17). The role of this particular genetic background for the generation of anti-FH Abs remains unclear. The deletion appeared to have no role by itself in the cell protection function because we observed no lysis induced by CFHR1/3-deficient plasma samples. CFHR1 is composed of five SCRs, the last three of which are highly homologous to the FH C-terminal region (100, 100, and 98%, respectively). This homology explains why some anti-FH Abs are also able to recognize CFHR1 (20). We confirmed this binding by plasma protein immunoprecipitation and by RLA in which reactivity with the SCR 4–5 of CFHR1 was observed for 80% of the patient samples. Strobel et al. (20) showed

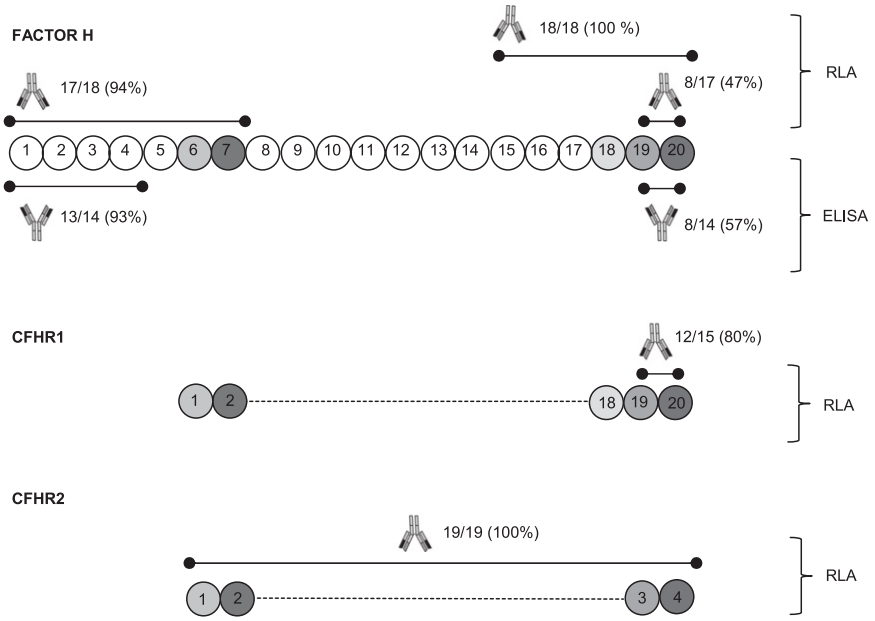

FIGURE 6. Synthesis of the recognition sites of the anti-FH autoantibodies to FH, CFHR1, and CFHR2 by ELISA and RLA. The percentage of binding and the number of patients are shown.

that the lysis induced by plasma samples containing anti-FH IgG may be prevented by addition of purified CFHR1. This is probably due to the competition between FH and CFHR1 for the anti-FH IgG binding, resulting in the neutralization of the autoantibodies and the release of free FH. Heinen et al. (15) showed that CFHR1 is a regulator of the alternative pathway C5-convertase. The anti-FH IgG could affect this regulation as another functional consequence in the CFHR1-sufficient patients; however, we failed to detect by ELISA any perturbation of the binding of CFHR1 to C5 by IgG purified from two CFHR1-sufficient patients.

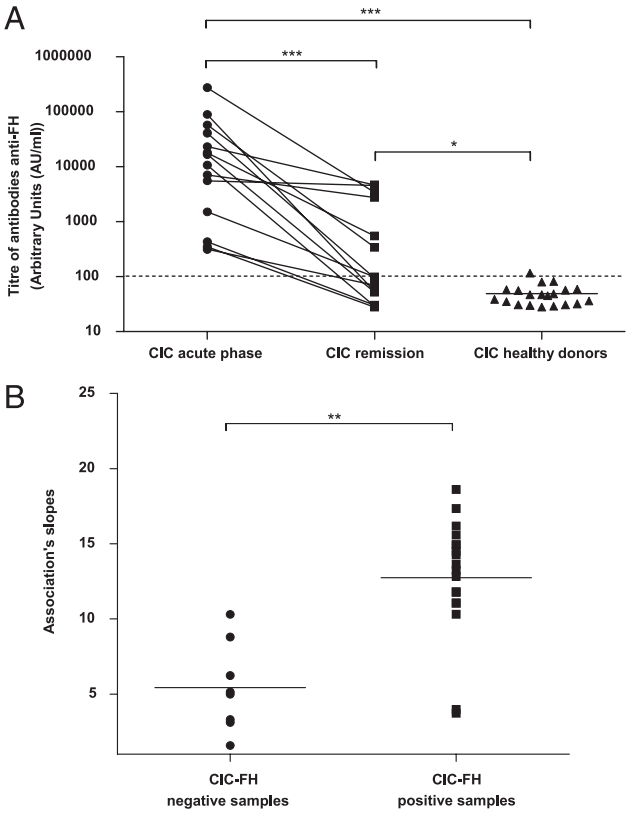

FIGURE 7. Levels of CIC containing FH–anti-FH IgG in patients during the acute phase and in remission. (**A**) Samples collected during the acute phase exhibited higher levels of FH immune complexes than samples collected during remission. (**B**) The presence of FH immune complexes in samples was correlated with anti-FH IgG of higher avidity for FH. $*p < 0.01$, $**p < 0.001$, $***p < 0.0001$.

We also confirmed that purified IgGs from all patients were able to immunoprecipitate a plasma protein of 29 kDa, and 44% a 24-kDa protein. These last two proteins could be the glycosylated and unglycosylated forms of CFHR2, respectively (CFHR2α and CFHR2). This result was confirmed by RLA with the recombinant CFHR2 protein. This protein shares homologous sequence with CFHR1 in a region showing 89 and 61% homology with the SCR 3 and SCR 4 of FH, respectively, and was recently identified as a regulator of the alternative C3-convertase pathway (16). This function could also be affected by the anti-FH IgG. We did not find any reactivity with CFHR3 SCR 1–3, confirming the recent results of Strobel et al. (20).

In conclusion, our results improve the knowledge of the physiopathological mechanisms of AI-HUS and reveal that anti-FH autoantibodies are responsible for FH neutralization, causing a perturbation of both cell-surface and fluid-phase complement control. However, questions remain unanswered concerning the mechanisms of immunization against FH that lead to the production of these autoantibodies. Interestingly, the homozygous *CFHR1* deletion is only associated with AI-aHUS and not with the other forms of aHUS (14). This genetic characteristic is a strong predisposing factor for the generation of anti-FH autoantibodies regardless of ethnic origins (32). However, it is not sufficient because ~2–8% of the normal population exhibit this genetic condition (14, 17). Therefore, other genetic or environmental factors are likely to play a role in anti-FH autoantibody production. A specific HLA haplotype may be an additional factor influencing the risk of developing anti-FH IgG in predisposed (i.e., CFHR1-deficient) subjects. A trigger event, such as an environmental factor, is likely to occur, leading to a lower tolerance against FH. Considering that most patients are children, microbiological agents may be suspected. Additional studies are now necessary to identify these factors, which could help to better understand the mechanisms of autoantibodies generation but also perhaps to prevent this autoimmune disease.

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Disclosures

The authors have no financial conflicts of interest.

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