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Title:

Rapid isolation of pure Complement Factor H from serum for functional studies by the use of a monoclonal antibody that discriminates FH from all the other isoforms

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ABSTRACT

Several mutations have been identified in the gene coding for Complement Factor H (FH) from patients with atypical Hemolytic Uraemic Syndrome (aHUS), Age-related Macular Degeneration (AMD) and Membranoproliferative Glomerulonephritis (MPGN). These data allow for a precise description of the structural changes affecting FH, but a simple test for specifically assessing FH function routinely is not yet of common use. We have produced and characterised a monoclonal antibody (5H5) which discriminates between FH and the smaller FH-like 1 and FH-related proteins and show here that it specifically binds to FH without detecting the smaller isoforms. We therefore used this mAb for a quick, one-step micro-purification of FH directly from control sera and showed that this affinity chromatography procedure is not disruptive of its cofactor function. We also developed a modified sheep erythrocytes haemolysis test using our antibody and affinity-purified FH. These tests can be used in conjunction for assessing the function of FH purified from patients affected by FH-related diseases. Moreover we used this mAb to develop a FH-specific ELISA test.

Keywords

Complement Factor H, functional assays, purification, affinity chromatography, immunoassays.

Abbreviations

AP, alternative pathway

aHUS, atypical haemolytic uraemic syndrome

AMD, age-related macular degeneration

BCIP, 5-bromo-4-chloro-3-indolyl-phosphate

CCP, complement control protein

CFH, complement factor H gene

DAA, decay acceleration activity

FB, complement factor B

FH, complement factor H

FHL-1, factor H–like protein 1

FHR, factor H–related

FI, complement factor I

GAGs, glycosaminoglycans

IP, immunoprecipitation

NBT, nitro-blue tetrazolium

MPGN, Membranoproliferative Glomerulonephritis

RCA, regulators of complement activation

SCR, short consensus repeat

1. INTRODUCTION

Factor H (FH) is the main regulator of the alternative pathway (AP) of complement activation and was first identified in 1965 by Nilsson and Mueller-Eberhard as β1H globulin (Nilsson and Mueller-Eberhard, 1965).

FH acts in down-regulating AP in three different ways: it competes with Factor B (FB) for binding to C3b, thus preventing formation of C3 and C5 convertases of the AP (Conrad et al., 1978); it possesses decay acceleration activity (DAA) and promotes the dissociation of these convertases once they have formed (Weiler et al., 1976); and finally it acts as an essential cofactor for Factor I (FI) in the proteolytic inactivation of C3b to iC3b (Pangburn et al., 1977). In the absence of FH, spontaneous activation of AP occurs in plasma and leads to consumption of complement components C3 and FB (Pangburn and Müller-Eberhard, 1983).

The regulatory function of FH, which is mainly due to its interaction with C3b, is performed both in the fluid phase and on self-surfaces where C3b is bound. However, while FH binds and inactivates C3b promptly in the fluid phase, inactivation of surface-bound C3b by FH is dependent on the chemical composition of the surface. FH is able to distinguish between self and foreign surfaces thanks to its capacity to bind polyanions such as sialic acid and glycosaminoglycans (GAGs) such as heparin. The presence of these markers on self surfaces increases the affinity of FH for surface-bound C3b as a consequence of the simultaneous recognition of both types of molecules by the same FH molecule (Fearon, 1978; Kazatchkine et al., 1979; Pangburn et al., 1983). Thus, FH acts by selectively limiting the progression of the complement cascade on the surfaces of self-tissues and allowing amplification of the AP only on foreign surfaces.

Qualitative and quantitative deficiencies of FH are frequently associated with a number of human diseases, such as membranoproliferative glomerulonephritis (MPGN) type I, II and III, atypical haemolytic uraemic syndrome (aHUS) and age-related macular degeneration (AMD) (Caprioli et al., 2003; Dragon-Durey et al., 2004; Edwards et al., 2005; Pérez-Caballero et al., 2001).

FH is a plasma glycoprotein of about 150 kDa, it is a single polypeptide chain with a beads-in-a-string like structure composed of 20 homologous domains named short consensus repeat (SCR) or complement control protein (CCP) domains (Ripoche et al., 1988). SCRs are small domains, of approximately 60 amino acids each, which are characterized by a high degree of conservation. They all contain four invariant cysteines that form two disulphide bridges essential to maintain a globular bead-like structure of the domain (Barlow et al., 1991; Norman et al., 1991).

Different parts of the protein contain binding sites for different ligands. Three binding sites for C3b have been identified; two main binding sites located at each end of the molecule and a third one in the central part of FH (Jokiranta et al., 2000). In addition, two binding sites for heparin in SCR 7 and SCRs 19-20 have been (Blackmore et al., 1998, 1996).

FH is encoded by a single gene (*CFH*), located on chromosome 1q32 in the "regulators of complement activation" (RCA) gene cluster, where genes coding for other important regulators of the complement system are also located. Two alternative transcripts are produced by the *CFH* gene, FH and a 43-kDa protein named FH-like 1 (FHL-1) which shares with FH the first 7 SCRs but has a short C-terminal tail of four amino acids (Estaller et al., 1991).

Both proteins are mainly expressed by the liver (Schwaeble et al., 1987), but extra-hepatic synthesis has been demonstrated in a variety of tissues including endothelial and epithelial membranes, glomerular mesangial cells, platelets, mesenchymal stem cells and retinal pigment epithelium (Brooimans et al., 1990; Chen et al., 2007; Licht et al., 2009; Tu et al., 2010; van den Dobbelsteen et al., 1994).

FH plasma levels in humans are very variable (116-562 μg/ml) and are influenced by environmental factors such as age and smoke, although the genetic component accounts for the major part (~62%) (Esparza-Gordillo et al., 2004). FHL-1 is less abundant in plasma with a concentration of 10-50 μg/ml (Friese et al., 1999).

FHL-1 has complement regulatory functions, such as cofactor activity for FI and convertase C3bBb dissociation activity. FHL-1 is also capable of binding to heparin, various microbial proteins, and

cell surfaces, being involved in cell motility processes (Kühn and Zipfel, 1996; Pangburn et al., 1991).

Moreover, in the RCA cluster just downstream of the *CFH* gene, five other genes (*CFHR1, CFHR2, CFHR3, CFHR4, CFHR5*) coding for the FH-related proteins (FHRs) are present. These proteins share a high degree of sequence identity with FH and, together with FH and FHL-1, form a family of glycoproteins ("Factor H protein family") which are structurally and functionally related (Zipfel et al., 2002).

The high amino acid identity among the family members is also reflected by the fact that antibodies raised against FH also detect different FHRs in plasma and that the antibodies generated against specific FHRs cross-react with other FHRs. Moreover, autoantibodies against FH in aHUS recognize not only FH but also certain FHRs (Kopp et al., 2012). This cross-reactivity is a challenge for FH and FHRs purification from plasma, determination of their concentration and in depth analysis.

Quantification of FH serum levels and assessment of its functionality are crucial for patients' characterisation in all FH-associated diseases but, due to the high degree of sequence similarity between FHR proteins and FH, tests specific only for FH are difficult to develop.

Quantification methods of serum FH are often not specific as antibodies can recognise both FH and FHR proteins, thus leading to an overestimation of real FH levels. Methods based on the use of mAb are generally more reliable, but specificity is not always fully guaranteed.

We previously generated a mouse mAb (5H5) against full length FH (Pintér et al., 1995b). We have here further characterized this antibody to verify its specifity to FH alone and used it to rapidly purify FH from small amount of human serum and to develop an erythrocyte haemolysis assay and a FH-specific ELISA.

2. MATERIALS AND METHODS

2.1. Antibodies

In the present work we used two antibodies against full length FH generated in our lab, mAb 5H5 (Pintér et al., 1995a) and a chicken polyclonal antibody. Some commercial antibodies were also used for FH, a sheep polyclonal antibody (The Binding Site, San Diego, CA) and a mouse monoclonal antibody, OX-24 (Life Technologies, Carlsbad, CA, USA) which is directed against the SCR-5 of FH and detects also FHL-1 (Sim et al., 1983). Commercially available antibodies against FHRs were used: rabbit anti-FHR1 and rabbit anti-FHR4 were from Abcam (Cambridge, UK), rabbit anti-FHR2 and rabbit anti-FHR5 from Abnova (Taipei City, Taiwan). For FHR3 a rabbit polyclonal antibody recognising also FHR4 and FH (described in Skerka et al., 2013 and kindly provided by Prof. P. Zipfel) was used. Finally secondary alkaline phosphatase-conjugated antibodies, anti-mouse IgG (A3688) and anti-rabbit IgG (A3812 – Sigma-Aldrich, Milan, Italy), pre-adsorbed with human serum proteins were used to avoid unspecific signal due to cross-reactivity with human immunoglobulins.

2.2. Polyclonal antibody production

A young hen at the start of oviposition was immunised with decreasing amounts of human plasma FH, purified by affinity chromatography on Sepharose-bound monoclonal antibody 5H5). Briefly, a first dose of 100 μg FH dissolved in PBS and emulsified in Incomplete Freund Adjuvant (IFA) (Sigma-Aldrich) was injected subcutaneously in the neck. Subsequently, every two weeks and until the fourth injection, the dose of FH was reduced to 50, 25 and 12 μg, this last dose being maintained as far as the antibody titre in the egg yolk reached 1:5,000. The protein fraction of the egg yolk was extracted by diluting each yolk 1:3 with PBS and adding 1 volume of chloroform, with a 5-min vigorous shaking to generate an emulsion. The emulsion was centrifuged at 2,500 x g for 30 min. The upper fraction, containing the yolk proteins, was then recovered and analysed in SDS-PAGE before and after separation on a Bio-Rad anion exchange HPLC chromatography preparative column (MA7P, Bio-Rad, Hercules, CA). The single IgY peak was identified by ELISA and the

fractions were recovered and concentrated by microdialysis. A small amount of this antibody preparation was affinity-purified on FH-Sepharose to check whether the purified pAb and the unpurified IgY fraction had similar or different background reaction when used in western blots on whole serum or plasma samples for the detection of FH. Since no significant differences were found, we decided to use the IgY fraction as our final reagent.

2.3. mAb 5H5 production

The murine hybridoma producing the mAb 5H5 against human FH was cultivated at high density using the CELLine Bioreactor (CL1000) system (INTEGRA Bioscience, Zizers, Switzerland). The medium compartment was filled with 1 L of RPMI1640 medium containing 1x PenStrep and 4 mM L-glutamine (Euroclone, Milan, Italy) which was completely changed every 14 days. $2.5x10^7$ hybridoma cells were resuspended in 15 ml of RPMI1640 medium containing 10% FBS, 1x PenStrep and 4 mM L-glutamine (Euroclone) and seeded in the cell compartment. Every 7 days almost all liquid (12 ml) was collected from the cell compartment, the remaining cells were splitted 1:5 into fresh medium and reseeded into the same chamber. The supernatant recovered from the 12-ml culture was checked for the presence of antibody by an ELISA test before storing it for subsequent antibody purification, and the cells were discarded.

2.4. mAb 5H5 purification and preparation of 5H5-affinity column

5H5 mAb was purified from culture supernatant on Protein G. Briefly, Protein G-Agarose (Sigma-Aldrich) was prepared as indicated by the manufacturer and packed into a column, the column was attached to the Econo System (Bio-Rad, Hercules, CA). Supernatant was loaded onto the column and antibody was eluted in 0.1 M Glycine pH 2.7, monitoring OD. Eluted fractions were analysed by SDS-PAGE, pooled together and concentrated using Vivaspin-20 (30kDa) concentrators (Sartorius, Goettingen, Germany).

To prepare the affinity column, concentrated antibody preparations were desalted using EconoPac DG10 columns (Bio-Rad), subsequently coupled with Cyanogen Bromide-Activated Sepharose 4B resin (Sigma-Aldrich) and put into a column.

2.5. Purification of factor H

FH was purified from human serum by a one-step affinity chromatography on 5H5-Sepharose column attached to the Econo System (Bio-Rad). Briefly, total human serum was brought to a final concentration of 0.5 M NaCl, filtered through a 0.45 µm filter and loaded onto the column. The flow-through fraction (FH-depleted serum) was collected. After washing with PBS/0.5 M NaCl, FH was eluted from the column with 0.1 M Glycine pH 3.0 and immediately neutralised with Tris-HCl 1.5 M pH 8.8. Eluted fractions were analysed by SDS-PAGE under reducing and non-reducing conditions. Positive fractions were pooled together and concentrated using Vivaspin-20 (30kDa) concentrators (Sartorius).

2.6. Factor H Immunoprecipitation (IP)

To isolate FH from a small amount of sample an IP micro-method was developed using 5H5-Sepharose resin. The final standard IP protocol consisted in incubation of 50 µl of serum with 100 µl of 5H5-sepharose resin (1:2 slurry in PBS) in a total volume of 500 µl of PBS, followed by washes in PBS/TW and elution in 0.1 M Glycine pH 3.0. The tubes were centrifuged at each step at 10,000 x g for 30 sec. to precipitate the resin and aspirate the supernatant. The method was further improved by the use of Micro Bio-Spin™ Columns (Bio-Rad) which allow for a faster procedure. The protocol was essentially the same except that the samples were collected from the bottom of the micro-columns. Samples were analysed by SDS-PAGE on 4–12% NuPAGE® Novex® Bis-Tris Protein Gels (Life Technologies) in MOPS 1X buffer at 200 V for 60 min, followed by Coomassie staining with BioSafe Coomassie (Bio-Rad).

2.7. Albumin and IgG depletion

In order to better visualize bands of specific proteins on Western Blot, Albumin and IgG were depleted from some samples of normal human serum. Depletion was performed using Hi-Trap™ Albumin & IgG depletion columns (GE Healthcare, Buckinghamshire, UK) according to the manufacturer protocol with minimal modifications.

2.8. Heparin chromatography

Heparin purification was performed by the use of a HiTrap Heparin HP Column (GE Healthcare, Buckinghamshire, UK) connected to a peristaltic pump. Briefly, 300 µl of human serum were diluted 1:10 in binding buffer (10 mM sodium phosphate, pH 7 with 50 mM NaCl) and filtered through a 0.45 µm filter immediately before to be applied to the column. Sample was loaded at a flow rate of 0.5 ml/min, the column was then washed with binding buffer at 1 ml/min and FH or FHRs proteins were eluted using 10 mM sodium phosphate, pH 7 with increasing NaCl concentrations (100-500 mM).

2.9. Western blotting (WB)

Samples were run on 4–12% NuPAGE® Novex® Bis-Tris Protein Gels (Life Technologies) in MOPS 1X buffer at 200 V for 60 min.

Gels were blotted on a PVDF membrane using iBLOT (Life Technologies) with protocol P3 for 7-10 min. Membranes were blocked overnight in TBST with 3% BSA at 4°C. Primary antibodies were typically diluted in TBST with 1% BSA and applied to the membranes 1h RT in constant agitation. Membranes were then washed 3 times with TBST and incubated 1h RT in constant agitation with the corresponding alkaline phosphatase conjugated secondary antibody diluted in TBST with 1% BSA. After 3 washes in TBST, specific signal was revealed with SigmaFast™ BCIP/NBT (Sigma-Aldrich).

2.10. Trypsin digestion

5H5-affinity purified FH was incubated at 37°C with TPCK-Trypsin (Sigma-Aldrich) at a substrate/enzyme ratio of 100:1 (w/w) in TBS pH 7.5. Digestion was carried on for 5 min to achieve about 50% of cleavage and stopped by adding a two-fold weight excess of soybean trypsin inhibitor (Sigma-Aldrich).

2.11. Cofactor Assay

In the test we used purified C3b and FI (Merck Millipore, Billerica, MA, USA) and 5H5-affinity purified FH. All the reagents were previously diluted into PBS buffer. A 20-µl reaction containing 500 ng of C3b and 250 ng of FI in PBS was incubated for 30 min at 37°C alone or in the presence

of different amounts of FH (0.1 – 50 ng). After incubation, samples were put on ice and SDS sample buffer was immediately added to stop the reaction. Samples were analysed by SDS-PAGE under reducing condition followed by Coomassie staining. The cofactor activity was calculated as a decrease of C3b-α' chain compared to C3b-β chain that remains constant.

2.12. Haemolytic assay

Due to difficulties in obtaining sodium barbital (veronal) in our country, we used an HEPES-based buffer (H-AP) adapted from Moreno-Indias et al (Moreno-Indias et al., 2012).

Sheep erythrocytes were stored in Alsever's solution and prepared in H-AP buffer (4.2 mM HEPES, 145 mM NaCl, 6 mM $MgCl_2$ and 10 mM EGTA, gelatine 0.1%, pH 7.4) just prior to use.

A total of 45 µl of normal serum containing approximately 20 µg of FH were used in each sample, mAb 5H5 was added at 1:1, 1:2, 1:4 molar ratio and 20 µg of 5H5- affinity purified FH were added in some samples, volume was brought to 100 µl with H-AP buffer.

100 µl of sheep erythrocytes ($5x10^7$ E/ml) were added to each sample and the mixtures were incubated 1h at 37°C. After centrifugation, the absorbance of supernatant was measured at 415 nm. Duplicates of each sample prepared with heat-inactivated serum (1h at 56°C) were used as a blank whereas erythrocytes diluted in distilled water were taken as 100% lysis.

2.13. ELISA

An ELISA test for the specific quantification of FH from biological fluids was set up using the reagents here produced. The mAb 5H5 was used as the catcher in 96-well Microtiter plates; after applying the samples the presence of FH was revealed with the chicken anti-FH antibody followed by a rabbit, HRP-conjugated anti-chicken IgY (Promega, Fitchburg, WI, USA). The reaction was developed by exposure to TMB (3,3′,5,5′-Tetramethylbenzidine) peroxidase substrate (KPL, Gaithersburg, MD, USA), stopped with 2 M sulphuric acid and OD_{450} was monitored by the use of a Microplate Reader Model 680 (Bio-Rad). For the quantification 5H5-affinity purified FH was used as a standard at six serial dilutions (250 – 7.8 ng/ml). The software Microplate Manager 5.2.1 was used to generate the standard curve and quantify the samples.

3. RESULTS

3.1. 5H5 is specific for FH

Human serum samples, total as well as IgG and albumin-depleted, were run on a SDS-PAGE gel both under reducing and non-reducing conditions and analysed by Western Blot using different antibodies against FH: 5H5, a chicken polyclonal, a sheep polyclonal (The Binding Site) and mAb OX-24.

5H5 under non-reducing condition detects a single band around 150 kDa corresponding to FH, no other bands are visible on the membrane. The antibody does not recognise FH after disulphide bridges reduction. (Fig. 1A)

The chicken pAb recognises both non-reduced (150 kDa) and reduced (180 kDa) FH, unspecific bands are also present in the total serum but not in the IgG and albumin-depleted serum (Fig. 1A). These unspecific bands are due to cross-reactivity of anti-chicken IgY secondary Ab with human immunoglobulins as demonstrated by secondary Ab-only control (data not shown).

The sheep pAb recognises FH both under non-reducing (150 kDa) and reducing (180 kDa) conditions, two additional bands around 75 kDa are present in the non-reduced samples (Fig. 1A).

The mAb OX-24 is known to recognise also FHL-1: under non-reducing conditions both FH (150 kDa) and FHL-1 (37 kDa) are visible on the membrane, whereas upon reduction the signal is fainter and only FH can be seen (Fig. 1A).

A 5H5-Sepharose resin was used to purify FH from human serum, samples were run on a gel and immunoblotted with different antibodies to FH, FHL-1 (OX-24) and FHRs. To exclude the presence of FHL-1 and FHRs in 5H5-affinity purified FH, the samples derived from the affinity column were compared with eluates obtained by a heparin-affinity chromatography where also FHRs co-elute with FH. In the lane corresponding to 5H5-affinity purified FH only the band corresponding to FH is present, whereas FHL-1 (Fig. 1B) and FHRs (Fig. 1C) are present in the total serum, in the FH-depleted serum and in the eluates from heparin column, or in the flow-through from heparin column in the case of FHR-4.

3.2. 5H5 is very efficient in binding FH

The mAb 5H5 was shown to be very efficient in binding FH. A single step of affinity chromatography is sufficient to almost totally deplete the sample of FH, less than 1 µg/ml is still present in depleted serum as demonstrated by ELISA assay (data not shown).

For the purification of FH from small samples, a micro-method was developed in which efficient purification was possible starting from 50 µl of serum. Samples before and after purification were analysed by SDS-PAGE on a gradient gel followed by Coomassie staining. The band corresponding to FH is visible in total serum but it is no longer detectable after purification in the FH-depleted serum. In the eluates a clear band corresponding to FH is present (Fig. 2).

3.3. 5H5 recognises both cleaved and uncleaved FH

FH can be cleaved by plasma proteinases to a form composed of two chains (38 and 142 kDa) held together by a disulphide bridge. These two additional bands are visible on a reducing gel after some purification procedures of FH (Pintér et al., 1995a). This cleavage can be mimicked *in vitro* by trypsin. To check the ability of our antibody to recognise the cleaved form of FH, affinity purified FH was cleaved *in vitro* with trypsin in order to obtain about 50% of cleavage and subsequently immunoprecipitated with 5H5-sepharose resin. Analysis of immunoprecipitated samples on reducing SDS-PAGE followed by Coomassie staining revealed that the antibody is able to bind and immunoprecipitate both forms (Fig. 3A).

Moreover, to assess if the binding capacity of 5H5 was the same for the two forms, gels were analysed by densitometry with TotalLab Quant software (TotalLab Ltd, Newcastle upon Tyne, UK) and intensity of the bands prior and after IP was compared. We quantified the ratio between the intensity of the uncleaved FH and the major band of the cleaved form (120 kDa) before and after IP. These two ratios were similar (ratio[before]/ratio[after] = 1.12 ± 0.06), confirming that the mAb is able to bind both forms with the same efficiency (Fig. 3B).

3.4. 5H5-affinity purified FH is fully functional

To test the functionality of the 5H5-affinity purified FH, a cofactor assay was performed as

described in Material and Methods. In the absence of FH, FI cannot cleave C3b and only C3b-α' and C3b-β chains are present on a SDS-PAGE gel upon reduction. In the presence of FH, α'-chain can be cleaved and the cleavage products α68 and α43 chains appear. The purified FH resulted to be fully functional, a minimum of 0.25 ng of FH was sufficient to induce the FI-mediated cleavage of C3b, as shown by the presence of α68 and α43 bands. Increasing the amount of FH the cleavage of C3b-α' chain is also enhanced (Fig. 4).

3.5. Haemolytic assay using 5H5

In order to test the functionality of the C-terminal of FH, where the majority of mutations causing aHUS are located, we developed a sheep erythrocytes haemolytic assay using mAb 5H5.
Endogenous FH of a control serum was blocked using 5H5 and 5H5-affinity purified FH was added to restore the protective function.
A dilution of a normal serum containing approximately 20 µg of FH was used in the assay, and lysis of sheep erythrocytes without or with different amounts of 5H5 was compared. 5H5 induces erythrocytes' lysis in a dose dependent manner; 20 µg of 5H5, corresponding to an equal amount of endogenous FH, induce a 45% lysis. 20 µg of FH purified by affinity chromatography on 5H5 were added to the same samples. This amount of FH was able to restore the protective function of normal serum and no lysis was observed (Fig. 5).

3.6. Specific FH-ELISA test

An ELISA assay for the specific quantification of serum FH was developed making use of mAb 5H5 as the catcher and our FH-specific chicken antibody as the tracer. Serial dilutions of 5H5-affinity purified FH are used to generate a standard curve.
The specificity of the test is ensured by the use of mAb 5H5 in the solid phase, whose ability to bind only FH was demonstrated by IP.
The dynamic range of the assay was 250 ng/ml – 7.8 ng/ml, linear regression of a six points standard curve with two-fold serial dilutions in this range gives a correlation coefficient around 0.997. Intra-assay precision was assessed by testing two samples with different FH concentrations

in three different dilutions (1:2,000, 1:4,000, 1:8,000) in twelve replicates in one assay. CV was <5% in the first two dilutions, greater variations were present in the 1:8,000 dilution but CV was less than 5.5% (Table I).

Table I

Intra-assay precision	***CV***
Sample 1	
Dil. 1:2000	2.79%
Dil. 1:4000	3.73%
Dil. 1:8000	5.26%
Sample 2	
Dil. 1:2000	4.01%
Dil. 1:4000	4.55%
Dil. 1:8000	5.36%

Inter-assay precision was assessed by testing different dilutions of two samples with different FH concentrations in three independent assays; CV was ≤5.2% (Table II).

Table II

Inter-assay precison	***Sample 1***	***Sample 2***
Mean Concentration (µg/ml)	235.90	167.10
Standard Deviation	11.04	8.59
CV	4.68%	5.14%

This test was used to screen a small group aHUS patients (n=20) selected by the Center for HUS Control, Prevention and Management, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico of Milan on the basis of aHUS symptoms, low alternative pathway haemolytic activity, low levels of C3 and normal levels of C4 (Supplementary Table 1) and a group of sex- and age-matched normal controls (n=20). Range values of normal controls were 212-441 µg/ml whereas for aHUS patients the range was significantly ($p<0.0001$) lower (48-279 µg/ml) (Fig. 6)

4. DISCUSSION

Complement Factor H (FH) is a crucial regulator of complement activation and mutations in the *CFH* gene or presence of autoantibodies are frequently associated with a number of human diseases.

However, the study of this protein is complicated by the presence of a shorter isoform (FHL-1) and FHR proteins belonging to the "FH-protein family". The high amino acid identity among the family members is a cause of antibody cross-reactivity. Antibodies raised against FH often detect also different FHRs and antibodies generated against FHRs can cross-react with FH or other FHRs. This cross-reactivity is thus a challenge for correct quantification and/or purification of FH from plasma. Some methods of purification of FH by affinity chromatography based on the use of mAbs are available, but they are often challenging and time-consuming, involving different steps prior to the affinity passage to achieve a pure fraction (Alsenz et al., 1984; Yu et al., 2014). However, a perfect separation of FH from the other family members is not always accomplished and further purification steps are thus necessary.

Determination of FH levels in serum or plasma is also complicated by the presence of related proteins, thus quantifications of FH by ELISA are often not specific and can overestimate real quantities. A really FH-specific ELISA test would therefore be useful to exclude any unwanted cross-reaction in the measurements (Fülöp et al., 2015).

We here characterised a mouse mAb (5H5), previously produced in our laboratory, and demonstrate its specificity to FH alone. This antibody was shown to specifically recognise FH but not the shorter isoform FHL-1 neither any of the FHR proteins. No cross-reaction with FHL-1 or FHRs was detected in WB using this antibody. Moreover, FH purified by affinity chromatography on 5H5-sepharose was shown to be highly pure and no contaminating FHL-1 or FHRs were present.

This antibody can therefore be used to isolate FH without any contaminating proteins for use in functional assays. The purification method based on mAb 5H5 consists in a single step chromatography starting from total plasma without any need for other manipulations neither before

nor after this single chromatographic step. This purification method is thus very fast, convenient and efficient.

In order to purify FH from patients from whom availability of sample is sometimes a limiting factor, especially when dealing with children, we adapted our purification method for small sample volumes. This micro-method makes use of the same resin used for the affinity-chromatography and small spin-column supports. It was possible to achieve purification and obtain sufficient amounts of FH for functional studies, starting from just 50 µl of serum sample. Thus this antibody can be used as a potent tool to isolate and study FH directly from patients.

In order to check if the function of FH was retained after the purification method, the purified protein was tested in a cofactor assay and shown to be fully active.

Moreover, the mAb 5H5 was able to recognise and bind with the same efficiency both the intact and the cleaved form of FH. The cleaved form of FH, as mimicked by tryptic digestion, appears to be generated upon multiple chromatographic steps, most probably as a consequence of separation of alpha-1 antitrypsin and FH, thus rendering FH sensitive to the serum protease(s) responsible for this cleavage, which might co-purify with FH in the classical chromatographic procedures. In fact we never observed the cleaved form after long-term incubation at 4°C, RT or 37°C incubation of whole plasma or serum (data not shown). The biological function of cleaved FH is not yet fully understood. The cleaved form retains the cofactor activity for fluid phase C3b, as this activity is located within the N-terminal 38-kDa tryptic fragment which was also shown to work alone (Alsenz et al., 1984), and some authors report even an enhanced cofactor activity of the cleaved form (Hong et al., 1982). On the contrary, the cofactor activity for the membrane-bound C3b is diminished (Hong et al., 1982; Koistinen, 1992), indicating the importance of the integrity of FH structure for the binding to cell surfaces. So, this test might be conveniently applied as a quality control check in case of classic FH purification procedures to assess the integrity of FH and its degree of cleavage, and might then be used to address the problem of measuring the biologic activity of the cleaved form.

We developed a modified sheep erythrocytes haemolytic assay making use of mAb 5H5 to block endogenous FH of a control serum and adding 5H5-affinity purified FH to restore the protective function. This assay could be used in conjunction with the micro-purification method to test the functionality of patients' FH in an independent manner. Haemolysis tests for aHUS patients are normally performed with patient's sera and are dependent on the complement components present in the serum. Storage and manipulation of serum are critical when performing functional complement assays and the same serum treated in different ways can give different results. Moreover aHUS patients may have a major consumption of complement caused by their defect; therefore their sera would have a lower amount of "active" complement thus leading to false-negative or less positive results. Using the same system for all the patients, based on the use of a normal serum, will enable to overcome these limits and to test the "real" contribution of FH for protective function.

Finally, the antibody was used to develop an ELISA assay for the specific quantification of FH from human samples. The specificity of the test is ensured by the use of mAb 5H5 in the solid phase. Several ELISA assays for the quantification of FH are available, but they are often not fully specific for FH and may give inconsistent results when FH concentrations are measured in different labs. Most of them rely on the use of mAbs for specificity. However, due to the high degree of similarity among FH family members, ensuring that FH is the only species to be quantified is a hard task. Some ELISA assays for the specific quantification of FH have been described, mainly using antibodies directed against the central part of FH (Bernabéu-Herrero et al., 2015). It has been shown that different results in the quantification of FH can be obtained using two monoclonal antibodies (OX-24 and A229) recognising different part of the molecule in patients with anti-FH autoantibodies or with homozygous FI deficiency, due to formation of complexes between FH and autoantibodies or C3b (Nozal et al., 2014). A specific ELISA test able to discriminate between the two variants of the Y402H polymorphism is also available, thanks to the use of two specific mAbs for the Y402 or the H402 variant (Hakobyan et al., 2008). These antibodies cannot discriminate

between FH and FHL-1 and thus these assays measure the combined levels of these two proteins. The epitope recognised by mAb 5H5 is still unknown; our data indicate that the antibody is able to recognise FH only in the unreduced form: in fact, the epitope is lost upon reduction of disulphide bridges, thus indicating that it is a conformation-dependent epitope. This is probably the reason why 5H5 cannot discriminate between full-size and cleaved FH, enabling the purification of both forms with the same efficiency.

Studies to map the epitope by competition from a collection of other available mAbs or correctly conformed recombinant fragments of FH are currently going on.

Furthermore, mAb 5H5 is potentially able to recognize all FH variants, since it was shown to react with all the sources of FH tested so far: FH from healthy controls as well as from a collection of sera from subjects with mutations at different positions are recognised with the same efficiency (data not shown).

In summary, we have fully characterised the FH specificity of our mAb 5H5 and used it to purify FH from human serum and to develop a quantitative and a functional assay for FH. This method can be used to assess FH cofactor and protective function directly from small amounts of patients' serum samples, independently from the presence of FHL-1 and FHRs. This test is here proposed as a quick and reliable tool to complete the genetic data with a specific functional description of FH activity in patients with aHUS, AMD and MPGN.

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FOOTNOTES

1. S.B. designed the study, performed experiments, and wrote the manuscript; A.C. advised on experimental design and writing the manuscript
2. The nomenclature of complement proteins is according to Kemper et al. (Kemper et al., 2014)

FIGURES

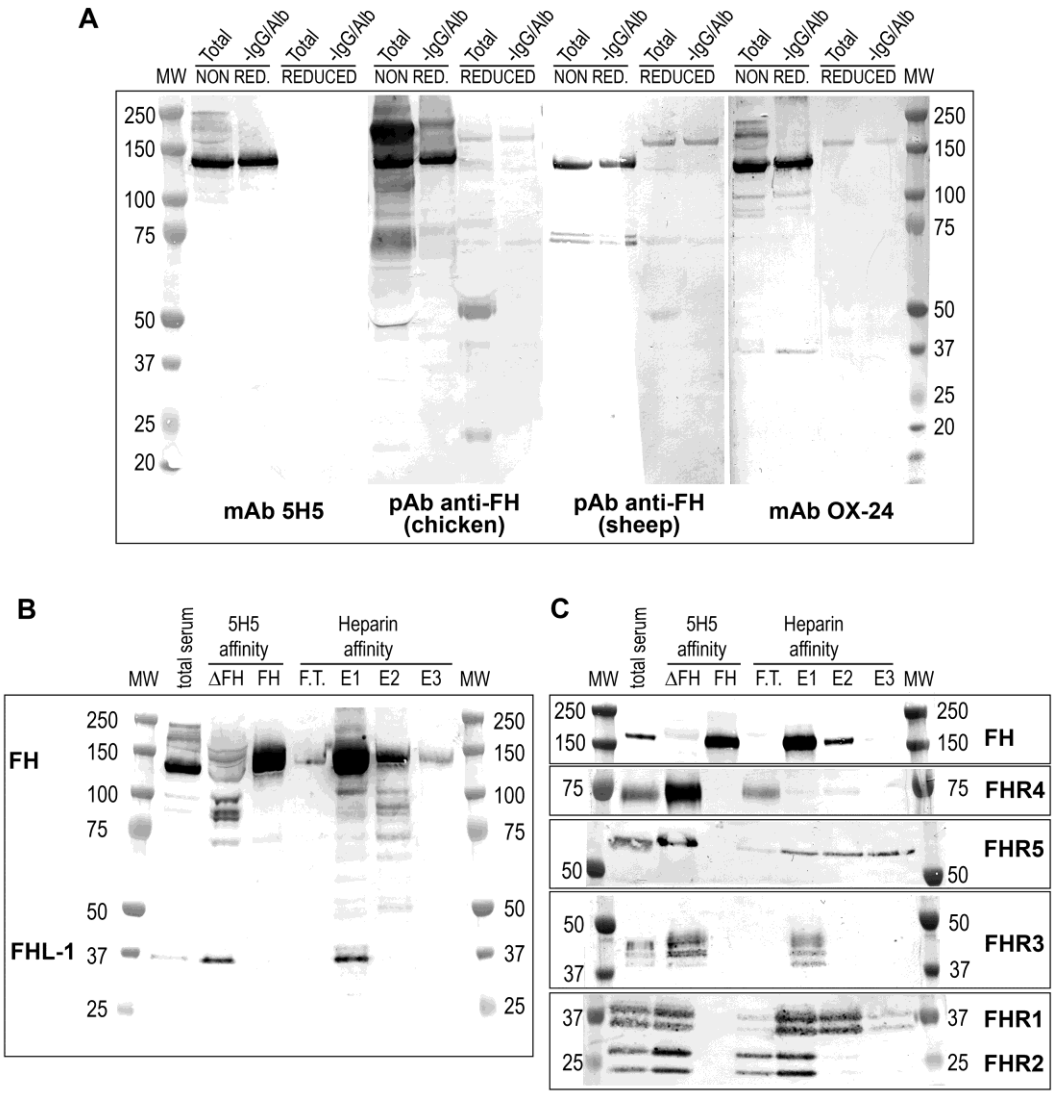

Figure 1. 5H5 is specific for FH

A. Western Blot for FH from total and IgG/Albumin depleted serum both under non-reducing and reducing conditions developed with different antibodies: mAb 5H5, chicken pAb to FH, sheep pAb to FH and mAb OX-24. MW: Molecular weight marker.

mAb 5H5 is specific for FH and recognises only the unreduced form whereas with the other antibodies other bands are present.

B-C. Western Blot from different fractions of 5H5-affinity and heparin-affinity purifications developed with mAb OX-24 (B) and different antibodies for FHRs (C). Bands corresponding to FHR proteins from different blot have been cropped and put together in the same picture for clarity.

FHL-1 and FHRs are present in total serum, FH-depleted serum (ΔFH) and elution fractions (E1-E3) or flow-through (F.T.) in the case of FHR4 from heparin column, but are completely absent in the 5H5-affinity purified FH (FH).

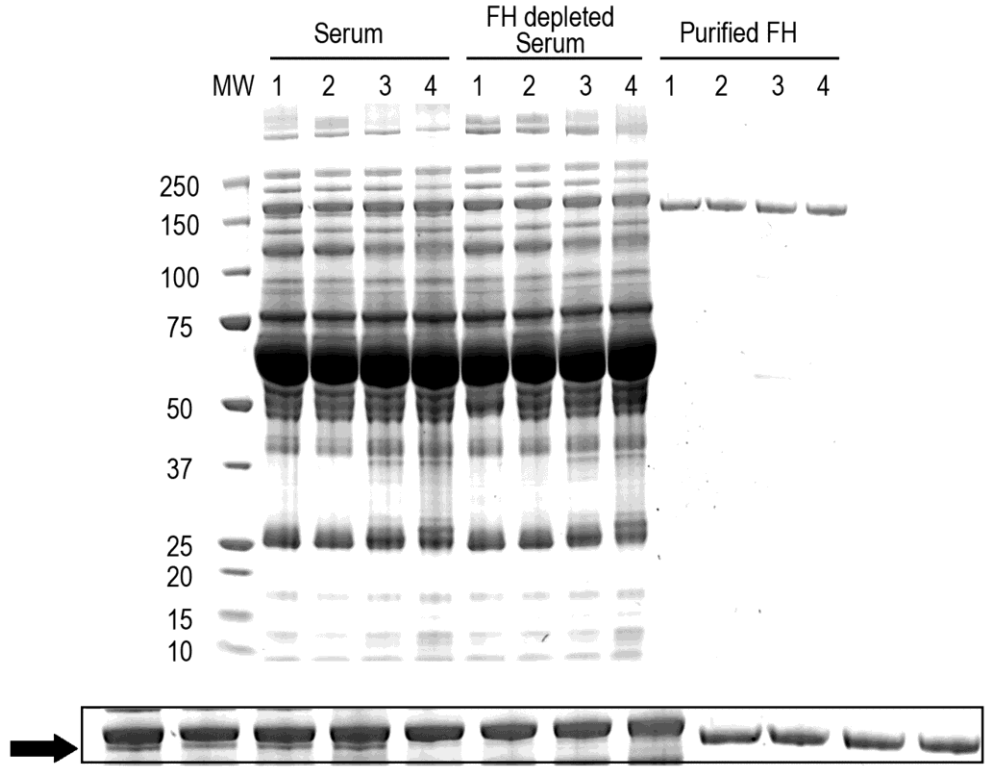

Figure 2.

IP from 4 serum samples (1-4); total serum, FH-depleted serum and immuoprecipitated FH are shown. For total and depleted serum a quantity corresponding to 0.3 µl of serum were loaded, whereas for immuoprecipitated FH 15 µl of eluates, corresponding to 1/13 of total volume, were used.

In the lower panel a magnification of the bands corresponding to FH, depletion of FH from plasma is complete as indicated by the arrow.

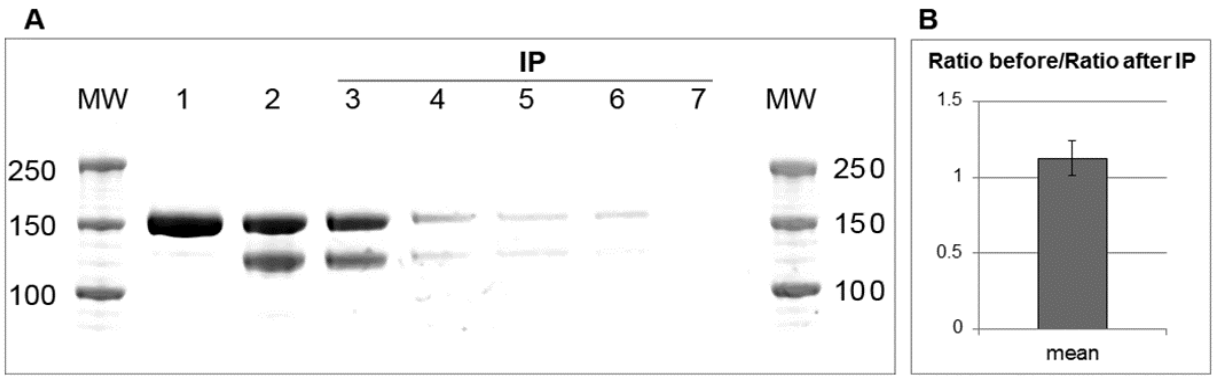

Figure 3. 5H5 recognises both cleaved and uncleaved FH

A. A typical gel in which purified uncleaved (1) and trypsin-cleaved (2) FH were run together with fractions obtained from IP with mAb 5H5 is shown. Two elution fractions (3,4), unbound fraction (5) and two washes (6,7) are shown. A band around 150 kDa corresponding to intact FH and a band around 120 kDa corresponding to the major band of the trypsin-cleaved FH are present, the smaller fragment of the trypsin-cleaved form is not visible in these conditions.

Quantification was done comparing intensity of the two bands (150 kDa and 120 kDa) in lane 2 and 3 corresponding to the sample before and after IP.

B. Quantification of the ratio before and after IP from three independent experiments.

The ratio between uncleaved (150 kDa band) and cleaved (120 kDa band) form was calculated in both lanes corresponding to the sample before and after IP and the two ratios were compared. The ratio before IP/ ratio after IP was nearly 1 (1.12 ± 0.06), confirming that 5H5 binds to the cleaved and uncleaved forms of FH with approximately the same efficiency.

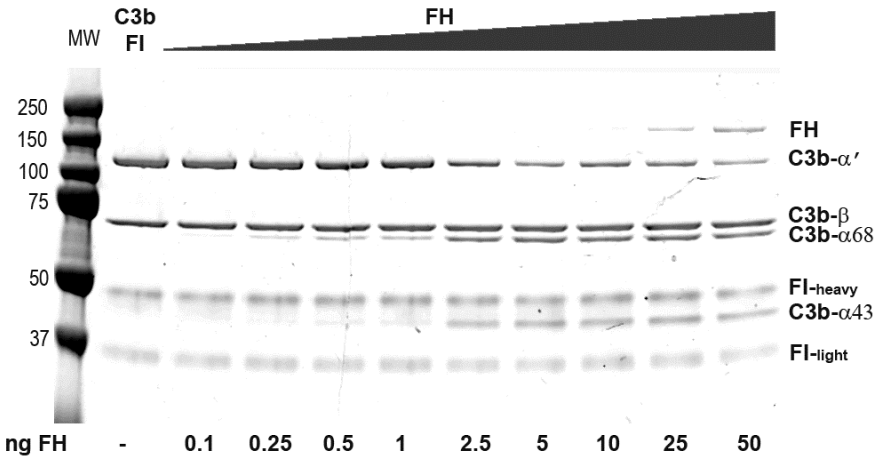

Figure 4. 5H5-affinity purified FH is fully functional

Cofactor assay with 5H5-affinity purified FH. C3b and FI are incubated with different amounts of FH and subsequently run on a reducing SDS-PAGE. FI alone is not able to cut C3b and only C3b-α' and C3b-β chains are visible, whereas in the presence of FH also C3b-α68 and C3b-α43 bands are present. Increasing the amount of FH the cleavage increases and the lower cleaved fragments become more visible. Bands corresponding to FI and FH are also present and are indicated.

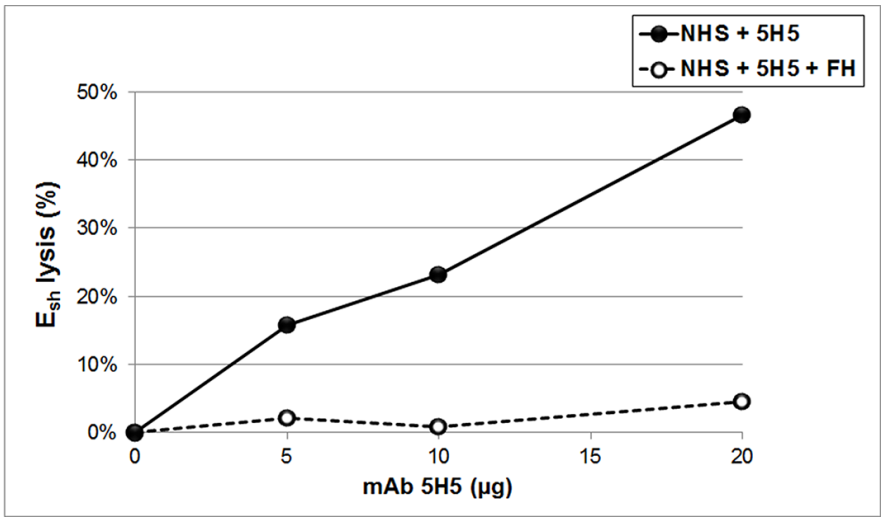

Figure 5. Haemolytic assay using 5H5

Sheep erythrocytes' lysis is induced in normal serum (NHS) by the addition of increasing amounts of mAb 5H5 (continuous line) that blocks endogenous FH. Protective function is restored by the addition of 20 µg of 5H5-affinity purified FH (dotted line).

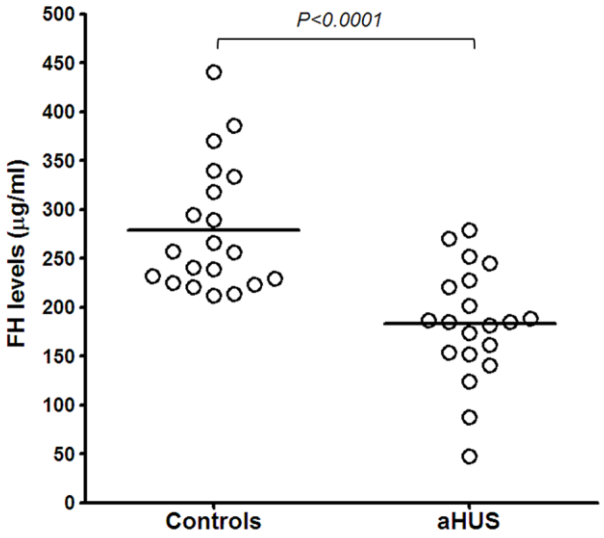

Figure 6.

FH serum levels of normal controls (n=20) and aHUS patients (n=20) detected by home-made ELISA based on 5H5 antibody. The mean value within each group are illustrated with horizontal bars. FH levels in aHUS patients were significantly lower (p<0.0001) than in controls.