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Autoantibodies to CD59, CD55, CD46 or CD35 are not associated with atypical haemolytic uraemic syndrome (aHUS)

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Abstract

Autoantibody formation against Factor H (FH) is found in 7–10% of patients who are diagnosed with atypical haemolytic uraemic syndrome (aHUS). These autoantibodies predominately target the C-terminal cell binding recognition domain of FH and are associated with absence of FHR1. Additional autoantibodies have also been identified in association with aHUS, for example autoantibodies to Factor I. Based on this, and that there are genetic mutations in other complement regulators and activators associated with aHUS, we hypothesised that other complement regulator proteins, particularly surface bound regulators in the kidney, might be the target for autoantibody formation in aHUS. Therefore, we assayed serum derived from 89 patients in the Newcastle aHUS cohort for the presence of autoantibodies to CD46 (membrane cofactor protein, MCP), CD55 (decay accelerating factor, DAF), CD35 (complement receptor type 1, CR1; TP10) and CD59. We also assayed 100 healthy blood donors to establish the normal levels of reactivity towards these proteins in the general population. Recombinant proteins CD46 and CD55 (purified from *Escherichia coli*) as well as soluble CR1 (CD35) and oligomeric C4BP-CD59 (purified from eukaryotic cell media) were used in ELISA to detect high responders. False positive results were established though Western blot and flow cytometric analysis. After excluding false positive responders to bacterial proteins in the CD46 and CD55 preparations, and responses to blood group antigens in CD35, we found no significant level of patient serum IgG reactivity with CD46, CD55, CD35 or CD59 above that detected in the normal population. These results suggest that membrane anchored complement regulators are not a target for autoantibody generation in aHUS.

Keywords

Autoantibodies; Complement; Surface regulators; Ahus

1. Introduction

Atypical haemolytic uraemic syndrome (aHUS) is an ultra-rare disorder characterised by the clinical triad of thrombocytopenia, haemolytic anaemia and acute renal failure (Scully and Goodship, 2014). Atypical HUS is the term given to define HUS not caused by Shiga toxin-producing bacteria, in particular *Escherichia coli* (*E. coli*) O157:H7 (Mele et al., 2014). The disease can be triggered through viral infection, pregnancy, transplantation and drugs (Kavanagh et al., 2013) and frequently arises in association with mutations in genes encoding complement proteins associated with the alternative pathway – such as factor H (FH), factor I (FI), CD46 (or membrane cofactor protein), factor B and C3 (Kavanagh and Goodship, 2010, 2011). Although mutations in complement genes play a major role in susceptibility to aHUS, incomplete penetrance of around 50% for many of these mutations suggests other factors are also necessary for the disease to arise (Bresin et al., 2013).

Acquired defects in the form of autoantibodies against FH have become recognised as another key mechanism whereby the complement system can become deregulated (Kavanagh and Goodship, 2010). FH autoantibodies (FH aAb) are found in approximately 10% of aHUS patients (Dragon-Durey et al., 2005; Jozsi et al., 2007; Moore et al., 2010); they predominately target the C-terminal domain of FH, an area that is critical for FH binding to host cells during complement attack and unsurprisingly, a common site for mutations in FH that associate with aHUS (Dragon-Durey et al., 2005; Saunders et al., 2006). The importance of the C-terminal binding region of FH in its overall function has been confirmed using an animal model, where mice express FH devoid of the C-terminal region and develop a renal thrombotic microangiopathy (Pickering et al., 2007). In man, complete absence of factor H related proteins (FHR), in particular FHR-1, is also associated with the formation of FH aAb in aHUS (Jozsi et al., 2008; Moore et al., 2010). However, the mechanism that links these two is not yet clear. Thus, human mutations, antibody binding and animal models all show that appropriate and robust cell surface control of complement activation (particularly of the alternative pathway) in the kidney is critical to prevent the development of aHUS.

Autoantibodies to other complement proteins have been reported in association with inflammatory conditions, including FH aAb in rheumatoid arthritis (RA), C1q aAb in systemic lupus erythematosus (SLE), and, most notably, autoantibodies to the alternative pathway C3 convertase (C3Nef) in association with C3 glomerulopathies (Dragon-Durey et al., 2013). Therefore, it seems plausible that autoantibodies to other complement regulatory proteins could exist in association with aHUS.

Following the concept that complement proteins with mutations known to associate with aHUS, e.g. FI and CD46, would be realistic targets for functionally neutralising autoantibodies, we have previously identified autoantibodies to FI (a serine protease involved in regulating the complement system) in approximately 2% of aHUS patients in the Newcastle aHUS cohort (Kavanagh et al., 2012). However, in these three cases, we did not find compelling evidence to suggest that the presence of aAb to FI was critical to the development or the severity of aHUS. Nonetheless, these data supported our initial hypothesis and led us to assess other targets, the first being CD46, because mutations in the

gene encoding this protein are found in ~15% of aHUS patients (Kavanagh and Goodship, 2011; Kavanagh et al., 2008). Following this, we extended our analysis to other surface bound regulators which play an important role in protecting the kidney from excessive or unwanted complement activation. These are CD35 (or complement receptor 1), CD55 (or decay accelerating factor) and CD59. Both CD35 and CD55 are potent regulators of the C3 and C5 convertases in both the alter-native and classical pathways (Fearon, 1979, 1980; Medof et al., 1984; Nicholson-Weller et al., 1982). Expression of CD35 is more limited in the kidney than CD55, with CD35 only being found on glomerular podocytes, although expression on erythrocytes could contribute to complement regulation within the kidney (Kinoshita et al., 1986; Medof and Nussenzweig, 1984; Wilson et al., 1987). CD55, on the other hand, is widely expressed on endothelial and epithelial surfaces and likely plays a key role in the control of complement activation at these surfaces (Kinoshita et al., 1985; Medof et al., 1987; Nicholson-Weller et al., 1985). Unlike CD46, CD55 and CD35, CD59 is not a member of the regulator of complement activation (RCA) gene family but is ubiquitously expressed including on the endothelial, epithelial and mesangial cells of the glomerulus (Nangaku, 1998). CD59 is a key regulator of the membrane attack complex (MAC) by preventing binding of C9 in the nascent C5b-9 complex (Morgan and Harris, 1999). The success of Eculizumab, a recombinant monoclonal antibody that blocks C5 function, MAC formation and C5a generation, in the treatment of aHUS (Wong et al., 2013) might infer levels of MAC are potentially important in progression or outcome in aHUS. Any neutralisation of CD59 function could potentially exacerbate aHUS and potentially autoantibodies (aAbs) to CD59 may associate with the disease.

Herein, we describe the screening of 89 patients from our New-castle aHUS cohort for the presence of aAbs to CD46, CD55, CD35 or CD59. These patients were selected based on the strength of the clinical certainty that these patients had aHUS. Patient samples were screened against highly purified proteins by ELISA and Western blotting. Our data strongly suggest that aAbs to these surface-bound regulators of complement are not associated with aHUS.

2. Materials and methods

2.1. Subjects

Stored serum/plasma samples from a cohort of 89 aHUS patients (median age = 31 years, normally distributed, ranging from 4 to 90 years of age; 64% female; 12% have FH autoantibodies, 61% possess a known mutation in a complement gene associated with disease; 88% White-Caucasian; samples are labelled P1–P89 chronologically with respect to entry to the cohort) and 100 healthy blood donors (blood donor controls, BDC; normally distributed, ranging from 17 to 72 year of age, median age 46, 54% female, 98% White-Caucasian). Serum samples were stored at –70°C. All individuals gave their informed consent in accordance with the Declaration of Helsinki.

2.2. Antibodies

Purified GB24 (mouse monoclonal raised against human CD46) was provided by Prof John Atkinson/Paula Bertram (Washington University School of Medicine, St. Louis, MO, USA).

Purified MBC-1 (mouse monoclonal raised against CD55) was a gift from Prof Claire Harris (Cardiff University School of Medicine, Cardiff, UK). MEM-258 (mouse anti-Human CD46) was obtained from Abcam (Cambridge, UK). Hybridoma producing the mouse monoclonal antibody 543 was obtained from EACAC and purified from cell supernatant in house using protein G affinity column and standard protocols. Culture supernatant containing BRIC229 (mouse monoclonal raised against CD59) and anti-serum with reactivity to the Knops blood group antigen (Kn^a) was obtained from the International blood group reference laboratories (NHS BTS, Bristol, UK). Goat or donkey anti-Human IgG specific – [horse radish peroxidase] HRPO; (#109-035-008), phycoerythrin conjugated (#705-116-147) and sheep anti-mouse-IgG-HRPO (#515-035-071) were obtained from Stratech Scientific, Ltd. (Newmarket, UK).

2.3. Recombinant MCP

Two purified, *E. coli* generated, recombinant human CD46(rhCD46) protein samples, herein listed as ‘St. Louis’ and ‘London’ preparations, were provided by Prof John Atkinson/Paula Bertram and Dr Claudia Kemper (MRC Centre for Transplantation, King’s College London, England), respectively.

A third rhCD46, termed ‘Newcastle’ was prepared from *E. coli* (provided by Prof John Atkinson/Paula Bertram) as described previously in Frémeaux-Bacchi et al. (Frémeaux-Bacchi et al., 2008) with modifications. Briefly, 500 ml of recombinant *E. coli* broth (optical density 0.6–0.8) was induced with 1 mM Isopropyl β -D-1-thiogalactopyranoside. Following centrifugation, the pellet was resuspended in 50 ml of lysis buffer with 0.8 ml lysozyme and 1250 units of benzonase nuclease, sonicated briefly and EDTA was added prior to centrifugation. The resulting inclusion body pellet was washed in lysis buffer with and then without Triton X-100. The inclusion body slurry was then split into 1 ml aliquots and centrifuged. The supernatant was removed and replaced with 1 ml of guanidine solubilisation buffer. The solubilised pellets were spun to remove any residual solids. The supernatant was removed and added drop-wise into 1 l of refolding buffer in three equal volumes, every 12 h for 36 h. The protein in the refolding buffer was then dialysed against 5 l of PBS containing 0.05% sodium azide overnight. Dialysed samples were then applied to a GB24 affinity column (generated in house using 2.5 mg of GB24 applied to a N-hydroxysuccinimide ester activated HF HiTrap column according to manufacturer’s instructions (GE Healthcare, UK)) using an AKTA purifier (GE Healthcare, UK). After extensive washing with PBS, bound rhCD46 was eluted using 0.1 M glycine pH 3.0 into 1 M Tris pH 8.0. Protein containing fractions were analysed by SDS-PAGE and rhCD46 containing fractions pooled, dialysed against PBS-0.05% NaN₃ and concentrated using 5000 MW cut off spin columns (Sartorius Sedum, UK).

2.4. Elisa

2.4.1. MCP autoantibody ELISA—Following a template based on the ‘Paris assay’ in Watson et al. (2014); 96-well plates were coated with 2 μ g/ml (50 μ l per well) of rhCD46 in phosphate buffer saline (PBS) and incubated overnight at 4°C. The rhCD46 plate was washed once with PBS. The plate was then blocked with 200 μ l PBS-0.1% Tween 20 (PBST) and left for 1 h at room temperature (RT). A ‘blocked only’ plate was also setup at

this time. After blocking, the plate was then washed three times with PBST. A dilution of 1/50 sera in PBST was loaded in triplicate onto both plates and incubated for 1 h at RT. The plates were then washed three times with PBST and the goat anti-human IgG-HRPO (1/20,000) was added to each well and incubated for 1 h at RT. The plates were washed three times with PBST. Tetramethylbenzidine (TMB; AbD serotec) was added to each well for 7 min, before 10% sulphuric acid was added to stop the reaction. The plates were read at an absorbance of 450 nm (SpectraMax 190; MDS Analytical Technologies, Ltd., Coventry, UK). Readings from the blocked only plate were subtracted from those generated on the rhCD46 plate and triplicate data were averaged. Monoclonal antibody GB24 was used as a positive control in a titration curve starting at 1/5000 (0.5 µg/ml followed by sheep anti-mouse-HRPO at 1/5000). Negative controls (pooled normal healthy donor serum) and secondary only (goat anti-mouse and goat anti-human IgG HRPO only) were run alongside the patient sera. All data was standardised through calibration to the average value for positive and negative control values across a given assay. Relative Units (RUs) were established using four parameter non-linear regression curve on a titrated positive control sample in triplicate. This analysis was performed using Graphpad Prism3 software.

2.4.2. Modifications for the anti-CD55 and -CD35 autoantibody assay—This assay was performed as above except flexible ELISA plates (Thermo, UK) were coated with 2 µg/ml of recombinant CD55 (CCP1–4, gift from Prof Susan Lea, Oxford, UK) or recombinant soluble CD35 (TP10, gift from Prof B. Paul Morgan, Cardiff, UK) in the coating step (essentially as previously described (Dhillon et al., 2010)). Proteins were coated in a pH 7.6 coating buffer (AbDserotec) (overnight, 4°C) and washes were with PBS-0.01% Tween. Blocking buffer was 1/5 ultrablock solution (AbDserotec). The positive control for CD55 was the mouse monoclonal MBC-1 at 1 µg/ml and the positive control for CD35 was either mouse monoclonal antibody 543 (produced in house) or antisera for the knops antigen blood group (titrated from 1/25).

2.4.3. Modifications for the anti-CD59 ELISA assay—Nunc Maxisorp plates were coated with 2 µg/ml of an oligomeric CD59-C4bp construct (gift of Dr Timothy Hughes, Cardiff, UK; who also provided C4bp backbone as control and a rabbit anti-C4bp to allow its detection) in a carbonate coat buffer pH 9.6 overnight at 4°C. PBS with 0.05% Tween 20 was used for all wash steps (and sample dilution) and a 5% solution of non-fat milk powder in PBS-0.05% Tween 20 was used for the blocking steps in this assay.

2.5. SDS–PAGE and Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed based on the method described by Laemmli (Laemmli, 1970). Gels of the appropriate percentage were freshly prepared for each experiment. The samples analysed were prepared by mixing with non-reducing or reducing sample buffer as indicated, and boiling for 5 min at 95°C. As required, gels were submerged in coomassie blue staining solution for 10 min and then destained as required. For strip blots, purified proteins were diluted in solubilizing buffer (20 µg/ml), and 200 µl was loaded onto a 10% SDS–PAGE preparative gel and transferred to nitrocellulose, which was then cut into 1–2 cm-wide strips. After blocking in 5% nonfat milk/PBS, strips were then incubated with individual sera samples (1/100)

overnight at 4°C. After extensive washing in PBS-0.01% Tween, bound autoantibody was detected using goat anti-human IgG-HRPO. Appropriate positive controls were used to identify the presence of recombinant proteins in *E. coli* and cell culture preparations. Blots were developed using an enhanced chemiluminescence substrate according to the manufacturer's specifications (Pierce; Thermo Scientific) and developed using an Xograph compact 4(Xograph, Stonehouse, UK).

2.6. Statistics

Normal distribution of the data was established by skew, kurtosis and the Shapiro–Wilks tests. Mann–Whitney U test was used to determine the significance of population data, (p 0.05 was considered significant). These were calculated using the Graphpad Prism version 3 software.

3. Results

3.1. Testing and purification of *E. coli* produced recombinantCD46 (membrane cofactor protein, MCP)

We originally received a batch of recombinant human CD46 (rhCD46) protein, generated in *E. coli* from Dr Claudia Kemper's group (London, UK) and used this to screen our BDC and aHUS cohorts for the presence of autoantibodies to CD46. From this analysis we identified one patient (P20) with significant reactivity with this sample of rhCD46, as shown in Fig. 1 (Fig. 1c). During the study, we required additional protein. This was provided by Prof John Atkinson (St. Louis, USA) in the form of *E. coli* expressing rhCD46 as well as additional purified rhCD46. We produced rhCD46 as previously described (Fremaux-Bacchi et al., 2008). rhCD46 was dialysed from refolding buffer into PBS and analysed by Westernblotting under non-reducing conditions. A band at approximately 23 kDa was visualized in the dialysed sample, similar to the positive control, confirming the presence of rhCD46 in the Newcastle preparation (Fig. 1a). The signal strength was comparable to the positive control, suggesting the concentration was approximately 20 µg/ml. As our rhCD46 had a marginally lower molecular weight (23 kDa) than the rhCD46 from London (25 kDa), we carried out an additional Western blot (Fig. 1b), with rhCD46 from St. Louis included. A clear difference in molecular weight between the three rhCD46 samples was noted with GB24 staining under non-reducing conditions. London derived rhCD46 is the largest (~25 kDa), followed by St. Louis rhCD46 (~24 kDa) and then Newcastle rhCD46 (~22 kDa). The difference in relative molecular weight between the Newcastle preparation and the St. Louis preparation was expected as a longer rhCD46 protein was given as positive control compared to the minimal construct gifted in the *E. coli* for expression at New-castle. The London rhCD46 had been modified to contain a BirA sequence to enable tetramer formation of this protein for a previous study and therefore all proteins were of the predicted size and all rhCD46 'isotypes' were judged to have folded as expected according to binding by mouse monoclonal antibodies GB24 and MEM218 (both antibodies do not bind reduced CD46, data not shown).

We next assessed the binding of our putative CD46 autoantibody positive patient sera to all three rhCD46 preparations on Western strip blots. Using this analysis, only rhCD46 derived

from London showed reactivity with P20 sera, even on a long exposure film (Fig. 1c). Any reactivity with the rhCD46 preparations from St. Louis and Newcastle were not at the correct molecular weight. These data suggested the putative anti-CD46 'positive' patient (P20) was not interacting with CD46 and was likely reacting with an epitope related to the addition of BirA component in the London preparation (Fig. 1c). The analysis of this data also suggested that contaminating bands in the Newcastle rhCD46 preparation would be an issue for screening of patient populations. Therefore, we generated a GB24 affinity column and applied our rhCD46 preparation to the column, eluted and produced a pure minimal domain New-castle rhCD46 preparation (Fig. 1d).

3.2. Screening of serum samples from aHUS patients using column purified CD46 provides no evidence of autoantibodies to CD46

Using the consensus ELISA protocol established in our recent FH autoantibody standardisation paper (Watson et al., 2014) as a template, we analysed 100 BDC samples and 89 aHUS patient samples for reactivity with the GB24 column purified Newcastle generated rhCD46. We found relatively low reactivity of patient or control sera with coated plates ELISA (Fig. 2b). Transformation of background subtracted OD450 value to RU using a titrated GB24 antibody as a standard curve (similar to (Watson et al., 2014)) confirmed no samples reached the nominal 100 RU cut off for positivity used for determining positive titre in the FH autoantibody ELISA. However, this cut off may be artificially high as comparing a mouse monoclonal to human serum has many technical pitfalls. Using the 0.975 fractile of the BDC group to determine autoantibody positivity as recommended by the International Federation of Clinical Chemistry for data with non-normal distribution (39), i.e. 40 RU, only one patient is above this threshold, P19 (Fig. 2b), which remains below the reactivity of the highest responder in the BDC population (B91). Notably, P20 only generated a signal of 10 RU in this ELISA assay. Our standard practice on samples giving high responses, on or around this cut off, is to attempt to gain additional evidence of binding to the target antigen and therefore, Western blot analysis of the highest samples was carried out and showed no CD46 specific reactivity (Fig. 2c). These data suggest that the patient samples with higher responses are still within the normal variance for background. Indeed, the mean population response was essentially the same with the BDC group having a mean of 15 RU and the aHUS a having a mean of 10 RU.

3.3. No evidence of significant autoantibodies to rhCD55 (SCR1–4) found in aHUS patients

At least one mutation in CD55 is known to associate with aHUS in a cohort of 46 patients, but functional analysis of the mutant protein failed to demonstrate any significant effect on CD55 function (Kavanagh et al., 2007). The authors concluded that the mutation found was unlikely to be a causal factor in disease, especially as the individual also possessed a factor H mutation. Furthermore, no CD55 mutations were established in an additional cohort of 41 aHUS patients (Esparza-Gordillo et al., 2005). Despite this, a functional deficit in CD55 could conceivably result in aHUS and, therefore, and in parallel with our initial studies on CD46, we began to investigate whether aAbs to CD55 might associate with aHUS. To facilitate this, we obtained purified recombinant human CD55 (CCP1–4, Fig. 3a; a gift from Prof Susan Lea, Oxford, UK) and carried out analysis of serum using a modification of our now routine ELISA technique for measuring FH aAbs (Dhillon et al., 2010). The rhCD55

based assay was highly reproducible and generated low serum reactivity from both the patient and control cohorts, with average OD 450 values after non-specific binding was subtracted was 0.065 and 0.072, respectively. A standard curve generated by the mouse monoclonal antibody MBC-1 was used to assign RU and this provided a 97.5 percentile cut off of 100 RU (Fig. 3b). Average RU between the patient and control samples was 30 RU and 34RU, respectively and the highest responder in the patient cohort, P40, was only marginally above this cut off (Fig. 3c). Nevertheless, we decided to confirm the reactivity of the sample with rhCD55 in a strip blot (Fig. 3d). In order to detect any definitive reactivity with the sample, we used 5 µg/ml of the rhCD55 preparation per well of the gel (with a matched BSA sample as negative control for non-specific binding). A faint signal is noted in the BDC sample at the correct molecular weight for rhCD55 but this is not present in the patient sample. Indeed, this data suggests that reactivity of the highest BDC and aHUS patient responders in the ELISA is with proteins of a higher molecular weight than the rhCD55. This protein is likely a trace contaminant in the rhCD55 preparation. Furthermore, exposure of patient and control serum to rhCD55 expressing CHO cells did not indicate any specific reactivity beyond background binding of human Ig in flow cytometric analysis (data not shown). Therefore, no evidence of specific rhCD55 aAbs was identified in either the aHUS cohort or BDC samples.

3.4. Antibody responses to rhCD35 (TP10) are equivalent in BDC and aHUS patients

CD35 is another potent cell membrane bound C regulator of the RCA gene family and after the finding that mutations in FH and CD46 associated with aHUS, it became a candidate in patients with no identified mutations. However, no mutations in CD35 have been reported in association with aHUS. Analysis has been carried out on a small cohort of 18 aHUS patients (Noris et al., 2003), with no abnormalities being identified. We had historical preliminary results in our cohort suggesting that aAbs to CD35 had been identified in some of the original patients entered into the Newcastle aHUS cohort. Therefore, we obtained a batch of TP10 (soluble complement receptor 1, sCR1, recently renamed CDX-1135; (Weisman et al., 1990)) as a gift from Prof B. Paul Morgan (Cardiff, UK) (Fig. 4a) and carried out analysis of serum again using a modification of our standard aAb ELISA screen (Dhillon et al., 2010). Here, we also had the advantage of being able to use anti-serum from an individual with reactivity to the Knops blood group antigen found on CD35, which gave consistently high responses when exposed to TP10 coated on the ELISA plates. This allowed a representative RU standard curve to be generated based on human IgG, alloanti-body reactivity with TP10 (Fig. 4b). On this ELISA, a broad range of serum reactivity, likely reflecting some alloreactivity, to TP10 was detected in both BDC and aHUS patients. Average RU responses were 76 and 62 RU respectively. The same serum dilution of Knops antigen positive sera gave an RU value of 1000 (Fig. 4c). As positives in the normal population are expected, we set a mean plus 2S.D. cut off based on the BDC controls (276 RU). Six BDC samples were deemed positive and only two aHUS patients. Overall, we conclude from these data that the presence of alloantibody responses to CD35 is as expected in the healthy blood donors and that reactivity measured in aHUS patients does not correlate with aHUS.

3.5. No evidence of significant autoantibodies against rhCD59 were found in aHUS patients

It is now well established that the terminal complement path-way plays a pivotal role in the pathogenesis of aHUS and therefore autoantibodies against CD59 could play a role in the development of the disease. For this analysis, we took advantage of an existing recombinant protein, an oligomeric CD59 (CD59 fused to C4bp-CCPs 1–4) provided as a gift from Dr Tim Hughes (Cardiff, UK, purity is shown in Fig. 5a). In this assay, our standard approach did not give reproducible results and we therefore used an assay based on the 'London' FH aAb method described in our FH autoantibody standardisation paper (Watson et al., 2014). There was consistent low level serum reactivity from both the patient and control cohorts in this assay, with average subtracted OD 450 values being 0.151 and 0.110, respectively. A standard curve was generated to allow assignment of RU using mouse monoclonal antibody Bric229 (Fig. 5b). The highest responder to the rhCD59 was in the BDC cohort (Fig. 5c) but repeated Western blot analysis did not show evidence of reactivity with rhCD59 in this format (data not shown). Analysis of reactivity of both high responding aHUS and BTS samples with U937 cells treated with or without Phosphatidylinositol-specific Phospholipase C or Bric229 prior to exposure to patient serum showed no evidence of specific binding to CD59. Indeed, in this assay the BTS gave higher background binding (data not shown). We conclude that these samples have high reactivity to the trace levels of non-specific proteins in the oligomeric rhCD59 preparation.

4. Discussion

In this study we have screened 89 aHUS patients for the presence of aAb to membrane bound complement regulators under the premise that if autoantibodies to FH and FI contribute/associate with this disease, then potentially aAbs to other complement regulators may exist. However, our data clearly do not support this hypothesis. 89 aHUS patients, including 11 already known to possess autoantibodies to FH and 2 with low level FI aAbs as well as 35 patients with unknown cause of disease did not show evidence of a significant titre of aAb to recombinant human CD35, CD55, CD46 or CD59 proteins. We therefore opine that it is unlikely that aAb to surface regulators play a major role in either the development or progression of aHUS.

There has been a concerted effort to understand the role that aAb to FH play in aHUS since their discovery (Dragon-Durey et al., 2005). FH aAbs are generally regarded as pathogenic. *In vitro* functional analysis confirms they have the ability to block FH binding to cells and thus reduce its effectiveness to regulate complement activation on cell surfaces (Jozsi et al., 2008, 2007; Skerka et al., 2009). B cell depleting therapies such as Rituximab which reduce FH aAb titres have been reported to be beneficial (Kwon et al., 2008; Le Quintrec et al., 2009; Sana et al., 2014). The presence of aAb in association with aHUS offers an acquired mechanism by which the function of the alternative pathway of complement can be impaired. Approximately 40% of aHUS patient do not have either an acquired or inherited complement abnormality. It is possible that as of yet unrecognised autoantibodies to complement regulators may play a role in the pathogenesis of the disease in these patients. Our identification of autoantibodies to FI, albeit in only three cases (Kavanagh et al., 2012), supports this hypothesis.

In determining which other complement regulators might be potential targets for aAbs associated with aHUS, CD46 was an obvious candidate. Mutations in *CD46* are found in up to 15% of aHUS patients (Kavanagh and Goodship, 2011) and therefore, aAbs might, in a similar fashion to FH aAbs mimic the functional effects of CD46 mutations. Early data in one patient supported this but it became apparent that the reactivity detected in this patient (Fig. 1) was to a bacterial protein and not CD46. This observation emphasised to us the need for a meticulous approach in determining the purity of the proteins used in the ELISA, particularly when using recombinant protein fragments. Confirmation by Westernblot analysis continued to provide evidence of specificity. The use of cells expressing CD46 in its native form can provide additional confirmation of specificity. Only through use of all these approaches can non-specific effects and off site binding in both Western and ELISA experiments be detected. Moreover, using GB24 purified rCD46 there were still traces of contaminating bacterial proteins present in the preparation, as can be seen with the reactivity of patient sera with high molecular weight bands in the long exposure film shown in Fig. 2c. This raises the possibility that bacterially produced proteins do not faithfully recapitulate the native protein and thus confound the analysis. Analysis of FH aAb binding specificity has been successfully analysed with both native and recombinant proteins. This suggests that recombinant proteins can be used in these assays but careful optimization is required. In this study, each ELISA protocol was modified to give the optimal signal to noise ratio and reduce non-specific signals. This approach may reduce sensitivity but maintains specificity which we believe to be paramount.

In contrast to CD46 mutations in the genes encoding CD35, CD55 and CD59 have not been reported in aHUS (Kavanagh et al., 2007; Noris et al., 2003). However, we did have preliminary data suggesting that antibodies to CD35 were present in some aHUS patients and aAb to CD35 had been reported in association with autoimmune and chronic inflammatory diseases including SLE (Sadallah et al., 2003). That study used the same rhCD35 as we have and showed that the Knops blood group Ab (an alloAb to a polymorphic variant of CD35) bound rhCD35, providing a positive control for our assays. We found that 6/100 of the serum samples from healthy blood donor controls showed strong reactivity with rhCD35, this was approximately double that found in the Swiss study (Sadallah et al., 2003), although titres were ~50% lower than an equivalent alloAb positive sample sourced in the UK. Reactivity in the aHUS patients was lower with 2/89 showing significant titres. Overall these data show nothing other than the expected alloAb response.

This study suggests that aAbs to the surface bound complement regulators are rare or absent. This is in contrast to readily identifiable aAbs to FH in both aHUS and C3 glomerulopathy cohorts (Dragon-Durey et al., 2005; Goodship et al., 2012; Jozsi et al., 2007; Moore et al., 2010; Zhang et al., 2012) or indeed the high prevalence of C3Nef in Dense Deposit Disease patients (Davis et al., 1977; Spitzer et al., 1969; Zhang et al., 2012). This data fits well with the accepted mechanisms surrounding clonal selection of mature tolerant B cells (Rajewsky, 1996) i.e. B cell interaction with membrane bound antigens during their development in the bone marrow environment should lead to their deletion whereas B cells with reactivity to soluble proteins can become anergic and reach the periphery (Goodnow et al., 1988). It is thought this mechanism exists to maintain diversity in the repertoire of mature B cells and

so, if the correct stimulus (Chackerian et al., 2008) or conditions are encountered (Gauld et al., 2005), anergic B cells can become re-activated/responsive to antigen. Therefore, in the highly inflammatory environment of the kidney during aHUS or C3 glomerulopathy there may be a heightened chance that existing autoreactive B cell clones receive signals or conditions that favour/result in reversal of their anergy (Fearon and Carter, 1995; Kemper et al., 2003; van der Touw et al., 2013). Any predisposition to generate autoreactive B cells, such as loss of appropriate BCR signalling during B cell development, could result in a heighten risk of developing autoantibodies to soluble proteins, such as FH and C3 (Prodeus et al., 1998). Thus, kidney diseases possibly provide the perfect storm of increased activation components, neo-epitopes of proteins, immune complexes and activated cells presenting a variety of antigens that can drive a break in peripheral tolerance to soluble complement proteins.

Our analysis of 89 well characterised aHUS patients for the presence of aAb to surface bound complement receptors and regulators suggest that aAb to FH are the only major aAb to associate with aHUS. It will be worthwhile confirming aAb to surface bound regulators is not common in other diseases such as C3 glomerulopathy to address the hypothesis that soluble complement factors are particularly vulnerable to aAb formation whilst membrane bound complement receptors and regulators are largely protected by mechanisms of central tolerance in these diseases.

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Abbreviations

aHUS	Atypical haemolytic uraemic syndrome
BDC	Blood donor controls
CR1	complement receptor type 1
CHO	Chinese hamster ovary
DAF	decay accelerating factor
FH	Factor H
FHR	Factor H related
FH aAb	FH autoantibodies
Kn^a	Knops blood group antigen
MCP	membrane cofactor protein
TMB	Tetramethylbenzidine
SLE	systemic lupus erythematosus

OD	optical density
RU	Relative units
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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Fig. 1. rhCD46 preparations

(a) Newcastle preparation, after dialysis (Lane 1, 20 μ l and lane 2, 10 μ l), or St. Louis prep (lane 5), were loaded in non-reducing SDS-PAGE buffer (irrelevant lanes are not shown). (b) 400 ng London (1), St. Louis (2) and Newcastle (3) rhCD46 prep., or BSA (4) were run on 15% SDS-PAGE and blotted. rhCD46 was detected using GB24 and shee anti-mouse – HRP (both 1:1000) and a 1 min exposure is shown for each blot. (c) Again 400 ng of each preparation and BSA (1–4 as above) were loaded onto two identical 15% gels and blotted. The Nitrocellulose was divided in two and then probed with GB24 or P1 sera (1/300) or P1 IgG (1/50) or BDC sera (1/300) as indicated. The four ‘strips’ were aligned and exposed for 5 min. (d) Coomassie stained gel of GB24 purified Newcastle produced rhCD46. “Page ruler markers” are indicated throughout.

Fig. 2. Screening of aHUS patients for presence of autoantibodies to rhCD46

(a) A standard curve based on the mAb GB24 (1 $\mu\text{g}/\text{ml}$) binding to rhCD46 using ELISA is shown, allowing RU values to be assigned to test samples in the ELISA. (b) Indicates the relative unit (RU) values of 100 healthy blood donor controls (BDC) and 89 aHUS patient samples. Mean RU values are indicated by the solid line in each cohort and a dashed line representing the 97.5 percentile of the BDC group is also indicated. (c) The highest reacting BDC and aHUS samples, as indicated, were applied to nitrocellulose strips from a Western blotted rhCD46 prep gel. Each strip (delineated by vertical bars) was aligned prior to

exposure for 20 min. Irrelevant intervening strips have been removed in this picture. GB24 was used as a positive control for protein loading and position. Molecular weight markers are shown and results are representative of two experiments.

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Fig. 3. Screening of aHUS patients for presence of autoantibodies to rhCD55

(a) Coomassie stained gel of purified recombinant human CD55 (CCP1-4) with molecular weight markers. (b) Standard curve based on the monoclonal antibody MBC-1(1 µg/ml) binding to CD55 using ELISA is shown, allowing RU values to be assigned to test samples in the ELISA. (c) Indicates the relative unit (RU) values of 100 healthy blood donor controls (BDC) and 89 aHUS patient samples. Mean RU values are indicated by the solid line in each cohort and a dashed line representing the 97.5 percentile of the BDC group is also indicated. (d) A 15% SDS-PAGE gel was loaded with 5 µg/well of rhCD55 (lane 1, 4, 7, 10), BSA (lane 2, 5 and 8) and marker (lane 3, 6 and 9) under non-reducing conditions. After blotting, nitrocellulose was cut into four strips using the marker lanes as guide, as indicated by vertical lines. MBC-1 (positive control) or the highest reacting BDC (#38) or aHUS (#40) sample or secondary only, as indicated, was applied to the appropriate strip. Molecular weight markers are shown and results are consistent over several experiments.

Fig. 4. Screening of aHUS patients for presence of autoantibodies to CD35

(a) Western and Coomassie stained gel of 0.5 and 5 µg/ml of purified recombinant human CD35 (CCP1-30), respectively, with molecular weight markers. (b) Standard curve based on a Knops antigen positive antisera (starting from a 1/25 dilution) binding to rCD35 using ELISA is shown. (c) The relative unit (RU) values of 100 healthy blood donor controls (BDC) and 89 aHUS patient samples. Mean RU values are indicated by the solid line in each cohort and a dashed line representing the mean plus 2 times SD.

Fig. 5. Screening of aHUS patients for presence of autoantibodies to CD59

(a) A 7.5% SDS-PAGE was loaded with reduced (R) and non-reduced (NR) purified recombinant human CD59-C4BP (CCP1-4) at 5 µg/ml, a marker lane and lanes containing 0.5 µg/ml of NR and R sample as indicated. The gel was cut down the marker lane allowing Coomassie staining of the left hand side and Western analysis of the right handside using Bric229. (b) Standard curve based on the monoclonal antibody Bric229 (1/100) binding to rhCD59 using ELISA is shown. (c) Indicates the relative unit (RU) values of 100 healthy blood donor controls (BDC) and 89 aHUS patient samples calculated from

the standard curve shown. Mean RU values are indicated by the solid line in each cohort and a dashed line representing the 97.5 percentile of the BDC group is also indicated.

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