Molecular Immunology 89 (2017) 111–114

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Eculizumab-C5 complexes express a C5a neoepitope *in vivo*: Consequences for interpretation of patient complement analyses

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ARTICLE INFO

Keywords:
Complement
Eculizumab
C5
C5a
Neoepitope

ABSTRACT

The complement system has obtained renewed clinical focus due to increasing number of patients treated with eculizumab, a monoclonal antibody inhibiting cleavage of C5 into C5a and C5b. The FDA approved indications are paroxysmal nocturnal haemoglobinuria and atypical haemolytic uremic syndrome, but many other diseases are candidates for complement inhibition. It has been postulated that eculizumab does not inhibit C5a formation *in vivo,* in contrast to what would be expected since it blocks C5 cleavage. We recently revealed that this finding was due to a false positive reaction in a C5a assay. In the present study, we identified expression of a neoepitope which was exposed on C5 after binding to eculizumab *in vivo*. By size exclusion chromatography of patient serum obtained before and after infusion of eculizumab, we document that the neoepitope was exposed in the fractions containing the eculizumab-C5 complexes, being positive in this actual C5a assay and negative in others. Furthermore, we confirmed that it was the eculizumab-C5 complexes that were detected in the C5a assay by adding an anti-IgG4 antibody as detection antibody. Competitive inhibition by anti-C5 antibodies localized the epitope to the C5a moiety of C5. Finally, acidification of C5, known to alter C5 conformation, induced a neoepitope reacting identical to the one we explored, in the C5a assays. These data are important for interpretation of complement analyses in patients treated with eculizumab.

1. Introduction

Complement inhibitory therapy has reached the clinic as an increasing number of patients are treated with the humanized monoclonal IgG2/4 chimeric anti-complement C5 inhibitor eculizumab, particularly those with the FDA approved diagnoses of paroxysmal nocturnal haemoglobinuria and atypical haemolytic uremic syndrome (Hillmen et al., 2006; Legendre et al., 2013). A number of assays have already been developed by our and others' groups, to detect complement activation *in vivo* by antibodies to neoepitopes (Harboe et al., 2011)' (Mollnes et al., 2007). In patients, these techniques are important for evaluating ongoing complement activation, and thus whether complement inhibitory treatment is indicated and if treatment is effective. Previously, we documented that a C5a neoepitope appeared in plasma from patients treated with eculizumab, but without cleavage of C5 into C5a and C5b (Volokhina et al., 2015). Remodelling of C5, independent on cleavage, with exposure of neoepitopes, appears in C5 upon e.g. serum acidification or treatment with reactive oxygen metabolites (Hammer et al., 1983; von Zabern et al., 1987). Here, we document that C5a detected in plasma from eculizumab treated patents was due to false positive reaction in the BD C5a assay, not recognizing C5a, but a neoepitope exposed after a conformational change of native C5 upon binding to eculizumab.

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http://dx.doi.org/10.1016/j.molimm.2017.05.021
Received 5 May 2017; Received in revised form 24 May 2017; Accepted 25 May 2017
Available online 10 June 2017

2. Methods

2.1. Serum preparation

Whole blood was collected, with informed consent according to the Declaration of Helsinki, from twenty healthy individuals, from a genetic C5-deficient individual (Lappegard et al., 2009), and from a patient with antiphospholipid syndrome prior to and after administration of eculizumab. Serum was prepared by clotting for 60 min and immediately stored at −80 °C.

2.2. Size-exclusion chromatography and detection of C5, eculizumab and complexes thereof

Patient serum (PS) pre- and post-eculizumab administration were fractionated on an ENrich™ size-exclusion column 650 (Bio-Rad Laboratories Inc., Hercules, CA) using a NGC Quest™ 10 (Bio-Rad) chromatography system. Dulbecco's PBS (Sigma-Aldrich, St. Louis, MO) with 10 mM EDTA was used as running buffer at 1 mL/minute; 0.5 mL fractions were collected. Elution volume was related to a molecular weight standard (#1511901, Bio-Rad). Serum fractions were incubated overnight on microtiter plates (Nunc MaxiSorp, Nunc, Roskilde, Denmark) and C5 and IgG4 were detected in coated fractions by specific antibodies (rabbit anti-human C5, Complement Technologies, Tyler, TX and mouse anti-human IgG4, clone HP6025, Southern Biotech, Birmingham, AL). C5a was detected in fractionated serum by C5a ELISA kits (BD OptEIA™ from BD Bioscience, San Jose, CA; and Hycult Biotech, Uden the Netherlands). For reference, complement activated NHS (Bergseth et al., 2013) was fractionated as for PS described above. To prove that it was eculizumb-C5 complexes that were detected in the BD C5a assay, we replaced the anti-C5a detection antibody with the anti-IgG4 antibody.

2.3. Induction of C5a neoepitope

NHS and genetic C5 deficient serum were acidified to pH 6.8 and 6.4 by addition of 5% (v/v) 0.33 M and 0.50 M sterile HCl respectively. After 15 min incubation in room temperature, C5a was quantified in acidified and unmodified serum (pH 7.4) by the C5a ELISAs from BD and Hycult. In separate experiments different well characterized monoclonal antibodies (20 µg/mL) against C5 were preincubated with NHS (pH 6.4–7.4): clones 137-30 and 137-26 (Fung et al., 2003) (a kind gift from Michael Fung), clone C17/5 (Oppermann et al., 1991) (a kind gift from Martin Oppermann) and mAb anti-C5 #A217 (Quidel, San Diego, CA), in order to localize the site of the neoepitope by competitive inhibition.

3. Results and discussion

Eculizumab binds C5 and prevents cleavage into C5b and C5a, possibly by sterically hindering the association of C5 to the C5-convertase (Jore et al., 2016; Schatz-Jakobsen et al., 2016). We recently showed that administration of eculizumab to three patients induced a false-positive C5a signal in plasma detected with BD C5a ELISA (Volokhina et al., 2015). No C5a was detected in corresponding C5a ELISAs from Hycult or RnD. Also sC5b-9 showed baseline values, clearly supporting fully blocked C5 cleavage. Here, we characterize the actual epitope reacting in the BD C5a assay and show that eculizumab binding to C5 *in vivo* induces a conformational change with exposure of a C5a neoepitope without C5 cleavage, but detected in the actual assay.

Patient serum (PS) before (PS-PRE) and after (PS-POST) first time infusion of eculizumab were fractionated by size-exclusion chromatography. IgG4 and C5 eluted as single peaks in both samples corresponding to their molecular weight (IgG 150 and C5 190 kDa) (Fig. 1A–B). In PS-POST, eculizumab-C5 complexes, detected by both anti-IgG4 and anti-C5 antibodies, eluted as a high-molecular weight peak (rage 340–530 kDa), consistent with both monovalent and bivalent C5 binding (Fig. 1A–B).

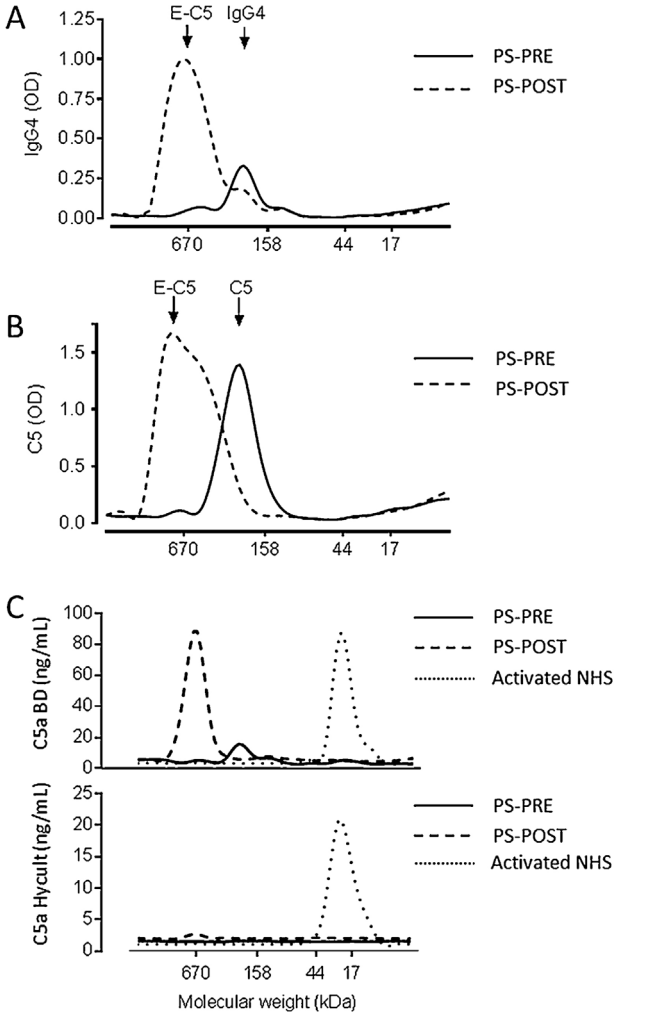

Fig. 1. Size-exclusion chromatography for the identification of C5a in patient treated with eculizumab. (A–C). A patient serum (PS) samples pre- and post- (PS-PRE and PS-POST, respectively) first time infusion of eculizumab were subjected to fractionation by size-exclusion chromatography. Fractions (0.5 mL) were collected at a flow rate of 1 mL/minute and coated on microtiter plates. Polyclonal antibodies against IgG4 (A) and C5 (B) were employed for detection of eculizumab (IgG2/4 chimeric antibody), C5 and eculizumab-C5 complexes (E-C5) in the coated fractions. (C) The fractions were tested in the BD and Hycult C5a-ELISAs, showing reactivity of the E-C5 complexes in the PS-POST sample only in the BD assay (upper panel). A positive control to show true C5a reactivity in human serum was included by fractionating normal human serum (NHS) activated with zymosan and heat aggregated IgG (activated NHS), being positive in both assays (these values were diluted 10 times compared to the factions of patient sera in order to get comparable peaks).

All fractions were analysed for C5a by using the BD and Hycult C5a ELISAs (Fig. 1C). C5a (10 kDa) was not detected in any of the low molecular weight fractions (< 150 kDa) but the eculizumab-C5 complex rendered a distinct signal in the BD C5a-ELISA, which was not detected in the Hycult C5a-ELISA, documenting that the BD C5a-ELISA reacts to a C5a neoepitope exposed in the eculizumab-C5 complex. Replacing the anti-C5a detection antibody in the BD assay with an anti-IgG4 antibody confirmed that it was the eculizumab-C5 complex that was detected, since the optical density was 2–3 logs higher in the sample from the eculizumab treated patient than the values observed in normal serum. Conformational remodelling in proteins upon ligand interaction is not unexpected. Jore et al. recently showed that two small protein-based C5-inhibitors, both binding distal to the C5a domain, induced remodelling of the C5a domain (Jore et al., 2016). Our data indicates that eculizumab does the same, and assays used for

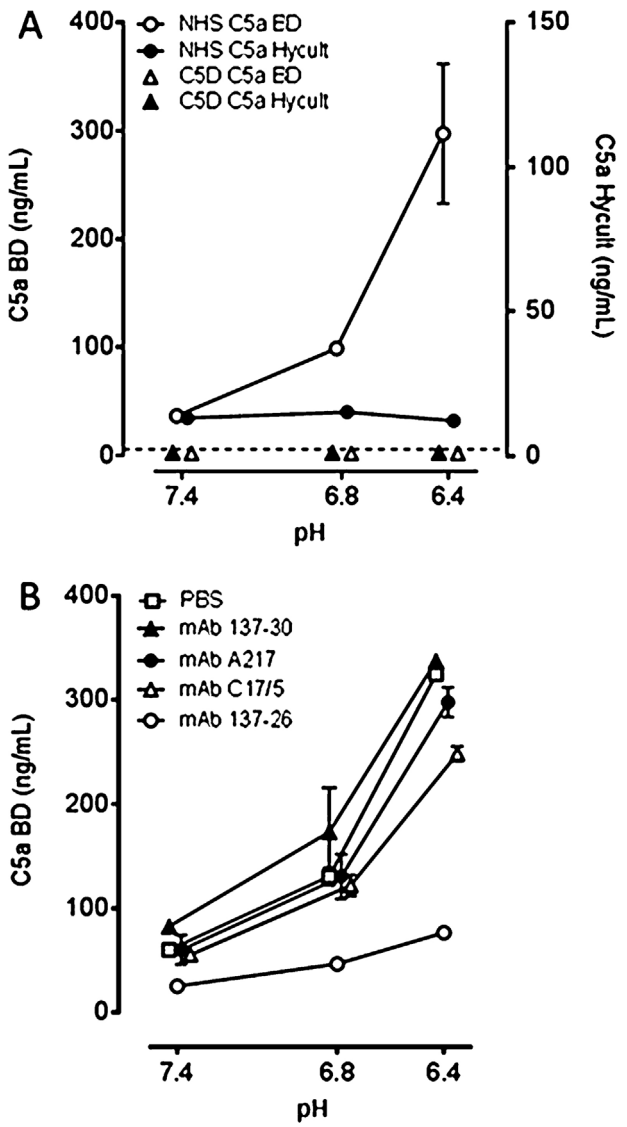

Fig. 2. Detection of C5a neoepitope in acidified serum. (A) Normal human serum (NHS) (circles) and C5-depleted serum (C5D) (triangles) were acidified to pH 6.8 and 6.4 by addition of HCl. C5a was quantified by using the C5a ELISAs from BD (left y-axis) and Hycult (right y-axis). Dashed line represents the lower detection limit of the assays. (B) Neutral and acidified sera were preincubated with mABs binding C5b (filled symbols) or C5a (open symbols) before quantification of C5a in the BD C5a ELISA. The PBS control is indicated by grey square symbols. Data are presented as mean of 3 experiments +/− SD.

quantification of C5a should be carefully checked for reactivity against C5 bound to eculizumab and be avoided when analysing samples from patients treated with C5 inhibitors.

C5 is prone to conformational change during stress, induced *e.g.* by acidification (Hammer et al., 1983), freeze-thawing (Dessauer et al., 1984), or by oxidative reagents (von Zabern et al., 1987) thus adopting a "C5b-like" conformation which is able to bind C6, however without liberation of C5a (Vogt et al., 1989). Here we quantified C5a in NHS and in C5 deficient serum at pH 7.4 and in pH-modified serum (pH 6.8 and 6.4). Acidification of C5 caused a pH-dependent exposure of a C5a neoepitope in C5, which was detected as C5a in the BD C5a-ELISA (Fig. 2A). This was dependent on C5 since no detection occurred in C5-deficient serum at any pH. Detection was independent of liberation of C5a since there was no signal in the Hycult C5a-ELISA. Four mAbs, binding to the C5b- or C5a-part of C5, were preincubated with normal and acidified sera before analysis in the BD C5a ELISA. One of four antibodies, clone 137-26, competitively inhibited detection (Fig. 2B). The 137-26 clone binds to a C5a epitope exposed in the native C5 molecule, and neutralizes C5a before C5a is released (Fung et al., 2003). These data indicate that the epitope detected by the BD C5a ELISA is in close proximity to the 137-26 C5a epitope and explains the false positive C5a results, by a conformational change in the C5a moiety of C5 when binding eculizumab.

Treatment of patients with eculizumab is approved for the indications of PNH and aHUS, but eculizumab is also regularly administered to patients in various off-label use (Castaneda-Sanabria et al., 2016). There are solid data on efficient blockade of C5-cleavage by eculizumab in patients with common polymorphisms in C5, however, patients carrying any of the C5 Arg885His/Cys mutations are non-responders (Nishimura et al., 2014) and recently, residual C5-cleavage was observed *ex vivo* upon forceful complement activation with high surface densities of C3b (Harder et al., 2017). Therefore, an accurate monitoring of complement inhibition upon treatment is critical, which in the case of eculizumab is further more complicated by individual variations in clearance rate (Jodele et al., 2016; Wehling et al., 2016). Here, we show that one of the most commonly used assays for C5a-quantification, the BD OptEIA™ Human C5a ELISA Kit II, is not applicable for monitoring of eculizumab C5 inhibition since the eculizumab-C5 complex induces a C5a neoepitope formation in the C5a-domain of uncleaved C5, falsely detected as C5a in the assay.

Authorship

PHN, GB, EV, LPH and TEM designed the research and considerably contributed to the scientific concept; PHN, AMT and GB performed the experiments; PHN, GB, AG, ABD and TEM analysed data; PHN and TEM wrote the paper. All authors critically revised the paper, and approved the final manuscript.

Conflict-of-interest statements

All authors declare no competing financial interests.

Acknowledgements

This study was financially supported by The Odd Fellow Foundation and the European Community's Seventh Framework Programme under grant agreement n° 602699 (DIREKT).

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