



Eculizumab treatment during pregnancy does not affect the complement system activity of the newborn



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ARTICLE INFO

Article history:

Received 1 November 2014

Received in revised form 4 November 2014

Accepted 4 November 2014

Available online 13 November 2014

Keywords:

Complement

Therapy

Placenta

Eculizumab

Paroxysmal nocturnal hemoglobinuria

ABSTRACT

Eculizumab is a humanized IgG2/4 chimeric anti-complement C5 antibody used to treat patients with paroxysmal nocturnal hemoglobinuria (PNH) or atypical hemolytic uremic syndrome. The aim of this study was to evaluate whether or not the complement activity in newborns from pregnant women who receive eculizumab is impaired. A novel eculizumab-C5 complex (E-C5) specific assay was developed and revealed that two newborns carried only 6–7% of the E-C5 detected in their eculizumab-treated PNH mothers. Serum from the pregnant women completely lacked terminal complement pathway activity, whereas the complement activity in the serum of the newborns was completely normal. Data from the pregnant women and their newborns were compared with that of healthy age-matched female controls and healthy newborns, as well as a non-treated pregnant woman with PNH and her newborn. These all showed normal complement activity without detectable E-C5 complexes. Furthermore, absence of eculizumab or E-C5 in the newborn could not be explained by lack of eculizumab binding to the neonatal Fc receptor (FcRn), as eculizumab bound strongly to the receptor *in vitro*. In conclusion, despite binding to FcRn neither eculizumab nor E-C5 accumulates in fetal plasma, and eculizumab treatment during pregnancy does not impair the complement function in the newborn.

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Introduction

Complement is part of the innate immune system, recognizing microbes and thus protecting the host against invasion of pathogens. Although discovered more than hundred years ago, as a factor in serum that induced lysis and killing of bacteria in

the presence of antibodies, it is only during the last couple of decades that the functional role of complement in human diseases has been explored. Previously regarded as a pure defense system, complement is now appreciated as crucially important for tissue homeostasis by clearance mechanisms, tissue regeneration and repair (Ricklin et al., 2010). The main effectors of complement activation to serve these functions are induction of a number of downstream inflammatory networks as well as phagocytosis and cell lysis (Walport, 2001). Complement activation products like the anaphylatoxins C3a and C5a are potent inflammatory inducers (Klos et al., 2009). Assembly of the terminal C5b-9 complement complex (TCC) on a lipid membrane leads to insertion of the

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membrane attack complex (MAC), which in sublytic doses induces inflammation (Morgan, 1989; Tegla et al., 2011), or in the case of red blood cells and neisserial bacteria leads to lysis (Bhakdi, 1991). When the TCC is formed in the fluid phase, the soluble sC5b-9 complex is generated, which is a useful indicator of complement activation.

Complement activation is a double-edged sword. By inducing inflammation and cell lysis as response to both microbes and damaged host structures, there is a potential for complement to damage self-tissue (Mollnes et al., 2002). In order to avoid self-damage, there is an absolute need for strict control by a number of fluid-phase and membrane-bound regulatory proteins (Meri, 2007; Sjöberg et al., 2009; Zipfel and Skerka, 2009). Many of the complement components are spontaneously activated in the absence of activating stimuli. Thus, the lack of such a regulatory protein *per se* is sufficient for a pathologic complement activation leading to tissue damage in various disease conditions.

Genetic deficiencies or mutations of regulatory proteins like factor H, factor I and membrane cofactor protein (CD46) are associated with diseases like atypical hemolytic uremic syndrome (aHUS) (Le et al., 2010), membranoproliferative glomerulonephritis (MPGN) type II (dense deposits disease) (Servais et al., 2012) and age-related macular degeneration (Shaw et al., 2012). Paroxysmal nocturnal hemoglobinuria (PNH) is a disease characterized by spontaneous complement-mediated red blood cell lysis due to a somatic mutation in the *PIG-A* gene coding for the glycosylphosphatidylinositol anchor, which is required for the attachment of complement regulators decay accelerating factor (DAF; CD55) and CD59 (Takeda et al., 1993). The phenotype of PNH is similar to genetic deficiency of CD59 (Yamashina et al., 1990), suggesting that this protein, which protects against insertion of C8 and C9 into the TCC and, thus, against cell lysis, is crucial for the pathogenesis of PNH.

Efficient therapeutic alternatives for these conditions are limited. Eculizumab (Soliris®), a humanized IgG2/4 kappa anti-C5 antibody is the only specific complement inhibitor approved by the Food and Drug Administration (FDA) (Dmytrijuk et al., 2008). It blocks the enzymatic cleavage of C5 to C5a and C5b, leaving the initial activation of the three complement pathways up to and including C3 intact. Eculizumab is in routine clinical use on the indication of PNH and aHUS. Many years of experience have shown that eculizumab reduces the need for blood transfusions and improves the prognosis in patients with PNH (Parker, 2009; Brodsky, 2009; Kelly et al., 2011; Luzzatto et al., 2011; Roth and Duhrsen, 2011; Palmeira et al., 2012; Hillmen et al., 2013; Varela, 2013; Heitlinger, 2013). Recently, eculizumab was also documented to be effective and without adverse effects in children (Reiss et al., 2014). Thus, treatment with eculizumab is generally safe, the main concerns being high costs and a small risk of neisserial infection, requiring vaccination prior to treatment. Recently, it was suggested that inhibition of C3 would reduce lysis more than C5 inhibition, since some PNH cells are opsonized with C3 fragments and undergo extravascular hemolysis (Risitano et al., 2014). Whether C3 or C5 should be the target for systemic complement inhibition over a long period of time in PNH patients will depend on relative the risk of infections due to lack of C3 opsonisation of pathogens as compared to the benefit on the hemolysis.

Little is known about possible passage of eculizumab or E-C5 over the human placenta and, consequently, how such passage could affect the complement system of the newborn. This is of particular importance in PNH since without treatment, the risk of complications is increased for both the pregnant woman and her child during pregnancy and delivery (de Guibert et al., 2011). Only a few cases have been examined with respect to the amount of free eculizumab in pregnant women and their newborns, reviewed by

Kelly et al. (Kelly et al., 2010). Of four newborns from three mothers with therapeutic levels, eculizumab was undetected in two and detected in low amounts in two (twins).

There is a need for sensitive and specific methods to detect E-C5, combined with novel functional assays for evaluation of the total complement activity, in order to study the balance between free eculizumab and free C5. Thus, the aims of the present study were to establish such assays, and to investigate whether or not eculizumab given to pregnant women affects the complement system of the newborn.

Methods

Patients

Three pregnant women with PNH and their newborns were included. Two of the patients were treated with eculizumab. **Patient 1 (P1)**, born 1981, was diagnosed with PNH in 2007 during the sixth week of her first pregnancy. Fluorescein-labeled proaerolysin (FLAER) negative monocytes and granulocytes were 80%, lactate dehydrogenase (LD) 840 Units/L (U/L) and hemoglobin (Hb) 11.2 g/dL. Low molecular weight heparin (LMWH) was started as thrombosis prophylaxis. Emergency cesarean section was performed at 35th gestational week due to fetal distress. The Apgar score was 5 and placenta showed immaturity without thrombi. During her next pregnancy in 2010, she was treated with LMWH from week nine. FLEAR negative monocytes and granulocytes were 91% and 94%, respectively. Hb fell gradually to 7 g/dL and LD increased to 3300 U/L. Fatigue gradually increased. The patient received meningococcal vaccine and was started with eculizumab during week 34 (600 mg/week for four weeks, thereafter 900 mg every second week). The last infusion in the pregnancy was given six days before delivery. She improved clinically, Hb increased to 9 g/dL and LD decreased to 300 U/L. At 41 gestational week she delivered a healthy child. Vaginal birth was complicated by postpartum hemorrhage (the lowest postpartum Hb level was 5.8 g/dL) and she received four red blood cell units.

Patient 2 (P2), born 1986, was diagnosed with classical PNH in 2009. Initially flow cytometry of red cells showed 30% CD59 negative cells (type III) and 47% type II cells. In 2012, FLAER showed 92% clone of GPI negative granulocytes. Despite chronic hemolysis and LD ranging 400–600 U/L, Hb levels were close to normal (11–12 g/dL) and she did not need any transfusions. Aspirin was used for primary thromboprophylaxis. Her first pregnancy resulted in early spontaneous miscarriage at week 10 in February 2013. Aspirin had been switched to LMWH prophylaxis since the detection of the pregnancy. LD and Hb remained stable. She conceived again six months later. LMWH was used for prophylaxis and she started with eculizumab during her second trimester in November 2013, initially dosed 600 mg once weekly for four doses followed by 900 mg every two weeks. In addition to receiving meningococcal vaccine prior starting eculizumab, continuous oral penicillin prophylaxis was also used. There were no complications during pregnancy, Hb was 10–12 g/dL and LD decreased to normal levels. Delivery was electively induced due to hyperglycemia in pregnancy week 39, April 2014. A healthy baby was delivered by emergency cesarean section due to obstructed labor. Blood loss due to uterine atony was 2500 mL and the patient received two units of PRBCs, eight units of platelets and three units of plasma on the day of delivery. She had received eculizumab on schedule 9 days prior to delivery and received the next dose the day before discharge. Her Hb was 9.6 g/dL and LD was 387 U/L at discharge. Twelve days after discharge the patient developed uterine infection and a mesenteric vein thrombosis was diagnosed. Prophylactic LMWH was increased

to therapeutic dose with plans of long-term anticoagulation with warfarin. Eculizumab was planned to continue for at least 3 months post-partum.

Patient 3 (P3) was not treated with eculizumab. She was born 1981 and diagnosed with classical PNH in 1998. Clone size from granulocytes has been 50–60%. She had a history of pulmonary embolism two years after the PNH diagnosis and warfarin anticoagulation was then started. She has not required transfusions, but has been iron deficient. Her obstetric history includes a spontaneous early miscarriage in 2008 and two prior successful pregnancies and deliveries in 2009 and 2011, for which she required multiple red cell transfusions. Her most recent pregnancy was detected in July 2013. Eculizumab treatment was offered, but she declined as her Hb levels stayed at 10–12 g/dL and there were no signs of increased hemolysis (LD 350–450 U/L). She received LMWH at treatment doses. She had an uncomplicated vaginal term delivery of a healthy baby. There were no signs of increased hemolysis and the puerperium was uneventful.

Blood sampling and preparation

Serum samples were obtained from P1 and P2 before starting eculizumab treatment and at delivery. Serum from P3 was obtained during pregnancy and at delivery. Blood samples from the umbilical cords were carefully obtained by needle puncture of a placental vein to avoid contamination from mothers' blood and Wharton's jelly. Serum was prepared by drawing whole blood into empty tubes, clotting was allowed for 60 min, followed by 15 min centrifugation at $3220 \times g$. The samples were immediately stored in multiple aliquots at -80°C and repeated thawing and freezing was avoided.

Reagents

Chemicals and buffers

2,2'-Azino-di(3-ethylbenzthiosoline sulphonate) (ABTS), ethylene diamine tetra-acetic acid (EDTA) and polyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich (St. Louis, MO). $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$, NaHCO_3 , KH_2PO_4 , Na_2HPO_4 , NaCl and $\text{NaC}_2\text{H}_3\text{O}_2$ were obtained from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) consisted of 0.068 M phosphate buffer, pH 7.2, containing 0.077 M NaCl. Coating buffer consisted of 0.05 M carbonate buffer, pH 9.6. Antigens and antibodies for ELISA analyses were diluted in PBS containing 0.2% Tween 20. Washing buffer consisted of PBS containing 0.1% Tween 20. Substrate buffer consisted of 0.15 M sodium-acetate buffer, pH 4.0.

Antibodies and proteins

Monoclonal antibody against human C5 was obtained from Quidel (San Diego, CA). Horseradish peroxidase (HRP)-conjugated mouse anti-human IgG4 antibody (clone HP 6025) was obtained from Southern Biotech (Birmingham, AL). Eculizumab (Soliris®), a humanized monoclonal antibody (IgG2/4) inhibiting cleavage of C5, was obtained from Alexion Pharmaceuticals (Cheshire, CT). Purified human complement protein C5 was obtained from Quidel.

Functional complement activity assay

Functional complement activity was measured using the Complement system Screen Wieslab® (Euro Diagnostica AB, Malmö, Sweden) detecting complement activation through the classical and alternative pathways, with the complete terminal pathway activation as readout. Thus, samples without C5 activity will give zero % activity in this assay, due to blocking of the terminal pathway, although the activity of the initial pathways up to and including C3 is intact.

Enzyme-immunoassay for the detection of eculizumab-C5 complexes

A new sandwich ELISA to measure eculizumab-C5 (E-C5) complexes was designed by using anti-C5 and anti-IgG4 as capture and detection antibodies, respectively, and using *in vitro*-generated E-C5 complexes as standard. The standard of E-C5 complexes was made *in vitro* by adding eculizumab to a normal serum pool from 20 healthy blood donors. Eculizumab was diluted in PBS twofold in 10 steps starting at 1 mg/mL. Five μL from each dilution were added to new tubes, each containing 95 μL of the standard serum pool. Optimal dilutions of the reagents in the different steps were found after careful titration. Nunc Maxisorp® 96-wells Immunoplates (Thermo Scientific Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with a monoclonal antibody against human C5 (1.1 $\mu\text{g}/\text{mL}$ in 0.05 M carbonate buffer). Standards and serum samples were added in duplicates, diluted 1:100 in PBS containing 0.2% Tween 20 and incubated at room temperature for 60 min. Samples from the patients receiving eculizumab needed further dilution, up to 10 times, in order to obtain absorbance values at the optimum of the standard curve. HRP-conjugated mouse anti-human IgG4 antibody was diluted 1:2500 in PBS containing 0.2% Tween 20, added to the plate and incubated at room temperature for 60 min. The plates were automatically washed three times after each incubation (Hydrospeed, Tecan Trading AG, Männedorf, Switzerland) using PBS containing 0.1% Tween 20. As substrate, H_2O_2 was used at a final concentration of $2.4 \times 10^{-3}\%$, in 0.15 M sodium acetate buffer, pH 4.0, containing 180 mg/L ABTS. The wells were filled with 100 μL in all steps, except for the washings steps (250 μL). Optical density (OD) was determined at 405 nm (Dy nex MRX^c reader, DYNEX Technologies, Inc, Chantilly, VA, USA) when the highest standard had reached an OD of approximately 1.0 (10–15 min).

Validity and specificity of the assay was verified using C5 deficient serum reconstituted with purified C5. The reliability was investigated by calculating inter- and intra assay variation. The sensitivity and accuracy was examined by spiking normal human serum as well as the patient serum samples were eculizumab (1, 10, 30 and 100 $\mu\text{g}/\text{mL}$) and/or C5 protein (100 $\mu\text{g}/\text{mL}$). The concentration of the E-C5 complexes is given as eculizumab-equivalent amount in $\mu\text{g}/\text{mL}$.

Immunofixation

The anti-kappa antibody for immunofixation electrophoresis was obtained from Sebia (Evry Cedex, France). Immunofixation electrophoresis of serum samples, eculizumab, free C5 and E-C5 complexes in PBS was performed using a HYDRASYS Focusing electrophoresis and immunofixation system from Sebia (Evry Cedex, France). Eculizumab-C5 complexes were generated *in vitro* by mixing eculizumab and purified C5 at different molar ratios. Immunofixation was performed using 12 μL anti-kappa antibody and Hydragel 4 IF kits (Sebia). After electrophoresis, the gels were stained with Coomassie brilliant blue and air-dried. The gels were photographed using a ChemiDoc XRS+ gel imaging system with Image Lab software from BioRad (Hercules, CA).

Enzyme-immunoassay for FcRn binding

Nunc MaxiSorp® microtiter wells were coated with titrated amounts of eculizumab, rituximab (anti-CD20, Roche, Basel, Switzerland) or infliximab (anti-tumor necrosis factor α , Janssen Biologics, Leiden, The Netherlands) diluted to 4 $\mu\text{g}/\text{mL}$ in PBS. Following incubation overnight at 4°C , the plates were blocked with 4% skimmed milk/PBS/Tween20 (S/PBS/T) for 2 h at room temperature and washed four times in PBS/T. Recombinant glutathione-S-transferase (GST)-tagged soluble human FcRn

(shFcRn-GST) (Andersen et al., 2008) was diluted to 2 $\mu\text{g}/\text{mL}$ in S/PBS/T and incubated for 1 h prior to washing as above. Binding was detected by a HRP-conjugated goat anti-GST antibody (GE Healthcare, Oslo, Norway). Plates were developed by adding 3,3',5,5'-Tetramethylbenzidine substrate (Calbiochem, San Diego, CA) and the reaction was terminated by addition of 100 μL 1 M HCl. Absorbance was measured at 450 nm using a Sunrise TECAN spectrophotometer (Tecan Trading AG, Männedorf, Switzerland).

Statistics

Student's *t*-test was used to compare the complement activity in eculizumab-treated patients and their newborns with the respective controls.

Ethics

The study was approved by the regional ethical committees. Written informed consent was obtained from the patients and controls.

Results

A novel assay for quantification of eculizumab-C5 (E-C5) complexes in human serum

Assay design, reliability and validity

The E-C5 assay was designed as a double-antibody enzyme-immunoassay using an anti-C5 antibody for capture and an anti-IgG4 antibody for detection. An E-C5 standard was produced by adding eculizumab to a normal human serum pool, and the standard curve was designed by two-fold dilution starting at 100 $\mu\text{g}/\text{mL}$ (Fig. 1A). The amounts of E-C5 are given in eculizumab-equivalents, i.e. 1 $\mu\text{g}/\text{mL}$ E-C5 corresponds to 1 μg eculizumab added to 1 mL of serum. The lower detection limit (defined as background + 2 SD) was approximately 1 $\mu\text{g}/\text{mL}$ (Fig. 1A). The intra- and inter-assay coefficients of variation were 8.9% and 4.9%, respectively.

The assay detected E-C5 in human serum with high specificity as demonstrated using serum from a genetic C5-deficient individual (Lappegard et al., 2009) with and without addition of purified C5 (Fig. 1B). No signal was detected upon addition of up to 100 μg eculizumab/mL to C5-deficient serum, whereas E-C5 was detected in a dose-dependent manner after reconstitution of the deficient serum with C5. During spiking experiments using normal human sera, the amounts of E-C5 detected corresponded exactly to the exogenously added 1, 10 and 30 μg eculizumab per mL serum (Fig. 1C). This indicates that there was no interference in the assay.

E-C5 complexes in human serum samples

Serum samples were added in optimal dilutions (see Section "Methods") and the results were corrected for the dilution to give the E-C5 concentration in undiluted serum (Fig. 1D). Using this assay, 133 $\mu\text{g}/\text{mL}$ and 115 $\mu\text{g}/\text{mL}$ of E-C5 were detected in serum from two eculizumab treated mothers (P1 and P2). In contrast, only 8.1 $\mu\text{g}/\text{mL}$ and 7.8 $\mu\text{g}/\text{mL}$ were detected in their newborns, corresponding to 6–7% of that in the mothers' sera. In control sera ($n=6$ for adults and newborns) and in the serum of a non-eculizumab-treated PNH patient (P3) and her newborn, E-C5 was not detected (Fig. 1D).

Effect of eculizumab on functional complement activity

Assay for functional complement activity

Serum samples from the eculizumab-treated PNH mothers (P1 and P2) and their newborns, as well as from the controls,

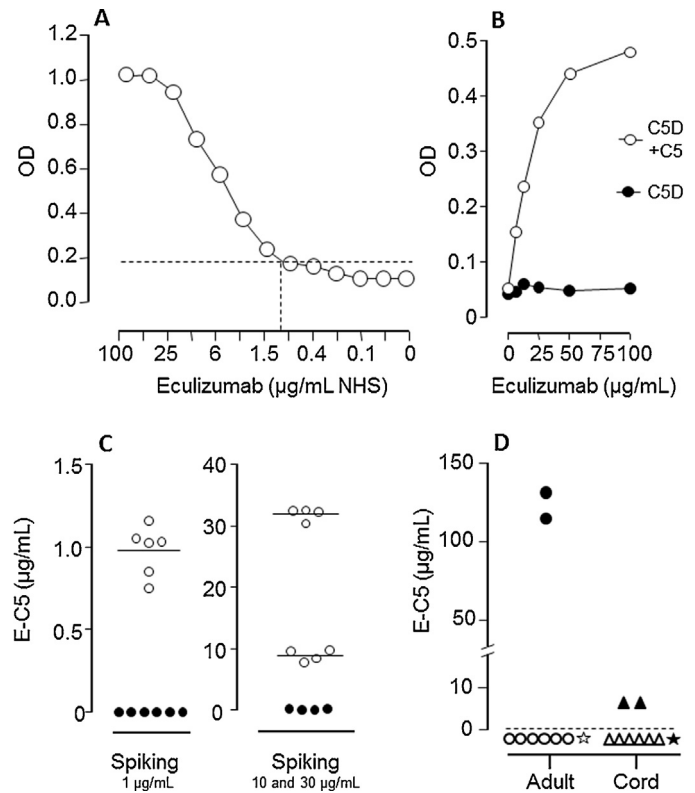


Fig. 1. Assay for quantification of eculizumab-C5 (E-C5) complexes. (A) The standard was made by adding eculizumab (0–100 $\mu\text{g}/\text{mL}$) to a normal human serum pool. This standard showed a dose-dependent curve read as optical density (OD) and was used as standard curve for the assay. The amount of E-C5 complexes is indicated in eculizumab equivalents, i.e. corresponding to the amount in $\mu\text{g}/\text{mL}$ of eculizumab added per mL of serum. Broken lines indicate lower detection limit. (B) Specificity of the assay was examined using C5-deficient serum (C5D, closed circles). Eculizumab was added to C5D without any increase in OD. In contrast, a dose response curve was obtained with formation of E-C5 after reconstitution of the deficient serum with purified C5 (C5D+C5, open circle). (C) Normal human serum was spiked with 1, 20 and 30 μg eculizumab per mL and measured in the E-C5 assay. The obtained values corresponded closely to those expected. (D) The concentration of E-C5-complexes was 133 and 115 $\mu\text{g}/\text{mL}$ in P1 and P2 serum (closed circles), whereas the newborns' corresponding serum contained 8.1 and 7.8 $\mu\text{g}/\text{mL}$, respectively (closed triangles). Adult (open circles) and newborn (open triangles) control sera ($n=6$ of each) did not contain detectable E-C5 complexes, as was the case for P3 (PNH without eculizumab, open asterisk) and her newborn (closed asterisk). Broken line indicate lower detection limit.

were examined in the Total Complement Functional Screen ELISA (Wieslab®), which is superior to the traditional CH50 based on red cell lysis with respect to sensitivity and reproducibility (Seelen et al., 2005). The assay specifically detects complement activity of the three initial pathways using terminal assembly of C5b-9 as readout. Thus, complete inhibition of C5 would abolish activity of the terminal pathway irrespective of which pathway that is initially activated, whereas the initial pathway activation up to and including C3 is intact. We here used the classical pathway (CP) and alternative pathway (AP) assays for evaluation of their suitability for evaluation of eculizumab. Serum from P1 and P2 had no detectable activity in CP and AP (Fig. 2A), consistent with complete inhibition of C5. The six healthy controls and P3 showed normal complement activity (significant difference between P1 and P2 versus controls: $p < 0.0001$ for CP and $p = 0.018$ for AP). Serum from the newborns of P1 and P2 showed completely normal complement activity (Fig. 2B), with no significant difference from the controls ($p = 0.54$ for CP and $p = 0.30$ for AP).

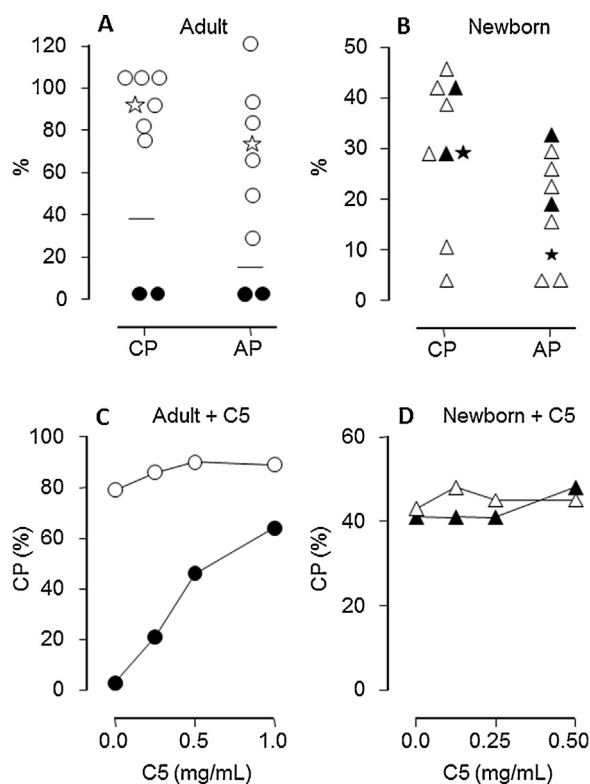


Fig. 2. Complement functional activity. (A) Serum complement activity was measured in P1 and P2 at delivery (closed circles), in six healthy controls (open circles) as well as in P3 at delivery (PNH without eculizumab, open asterisk) in the classical (CP) and alternative (AP) pathways. Consistent with full inhibitory effect on the terminal pathway by eculizumab, there was no activity in the P1 and P2 sera. Horizontal lines indicate lower reference values. (B) Newborns have lower complement activity and greater range than adults (cfr. scale on y-axis) and reference ranges for the functional assay is not established. The newborns of P1 and P2 showed high activity in both pathways (closed triangles), consistent with a fully active complement system. Controls include healthy normal newborns (open triangles) and newborn from P3 (PNH without eculizumab, closed asterisk). (C) Effect of adding C5 in increasing concentrations on serum functional activity of the terminal pathway measured by the classical pathway assay from a normal adult serum (open circles) and serum from P1. (D) Same experiment as described in panel C, using serum from a normal healthy newborn (open triangles) and serum from the newborn of P1 (closed triangles).

Effect of addition of C5 to the serum on functional complement activity

The effect of adding increasing amounts of purified C5 to the serum was studied using the CP assay. The average normal serum concentration in adults is 75 $\mu\text{g}/\text{mL}$. Addition of 1 mg/mL of C5 to serum from P1 restored functional complement activity (Fig. 2C), whereas no additional complement activity was observed in serum from her newborn (Fig. 2D), suggesting efficient suppression of C5 and the presence of free eculizumab in the mother, but no free eculizumab available for C5 suppression in her newborn.

Effect of eculizumab on functional complement activity in titrated sera

Titration of sera in the functional assay

Since the functional complement activity examined in the experiments presented in Fig. 2 was measured as end-point at a fixed dilution of the serum, we investigated the effect of eculizumab on the complement activity over a long dilution range (Fig. 3). By using both CP (Fig. 3A) and AP (Fig. 3B) the terminal pathway activation was completely abolished in P1 and P2 regardless of the dilution, whereas complement activity in the healthy controls decreased in accordance with titration. Notably, the newborns from P1 and P2 showed a similar decrease in terminal pathway

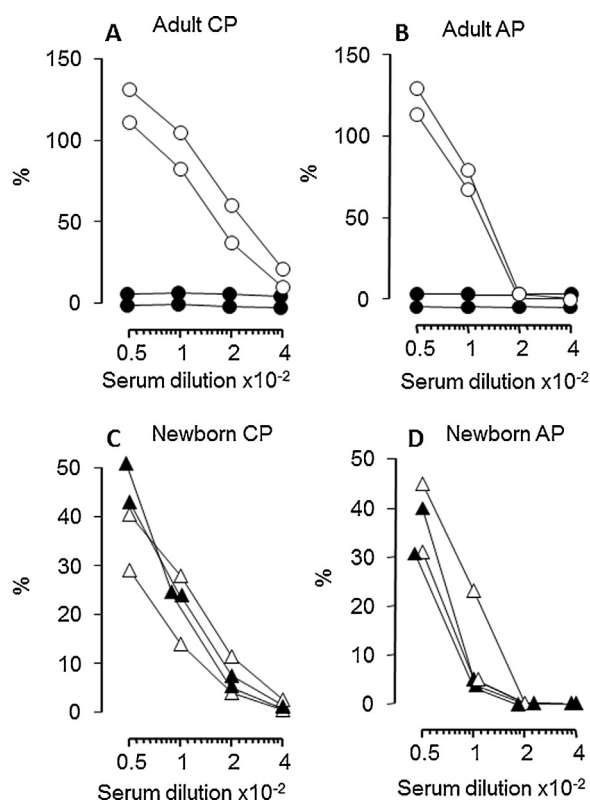


Fig. 3. Complement activity in titrated sera. (A and B) Serum from P1 and P2 at delivery (pregnant women with eculizumab, closed circles) and serum from two healthy controls (open circles) were titrated in the functional complement assay. (C and D) Serum from the newborns of P1 and P2 (closed triangles) and sera from two healthy newborns were titrated in the functional complement assay. CP, classical pathway; AP, alternative pathway.

activity as their controls as measured by the CP (Fig. 3C) and AP (Fig. 3D). Collectively, these data demonstrate that the newborns from eculizumab treated mothers had completely normal complement activity independent of serum dilution.

Detection of E-C5 in serum from P1 and her newborn with and without substitution with eculizumab or C5

In order to control for sufficient therapeutic C5 inhibition in the eculizumab-treated mother and free C5 in her newborn, the presence of E-C5 was measured in serum from P1 and her newborn before and after addition of eculizumab or C5 (Fig. 4). Consistently, the E-C5 concentration in P1 was unaffected by the addition of eculizumab or C5 (Fig. 4A). Addition of eculizumab to the P1 newborn serum substantially increased the E-C5 levels, consistent with binding of eculizumab to free C5 (Fig. 4B).

Visualization of eculizumab, C5 and E-C5 in serum samples from P1 and her newborn by immunofixation

Detection of purified C5, eculizumab and E-C5 by agarose gel electrophoresis and immunofixation

Purified E-C5 was identified by protein staining (Fig. 4D, lane 1) and compared to IgG κ -specific immunofixation using different molar ratios between C5 and eculizumab (Fig. 4C, lanes 2–6). The anti-kappa antibody specifically detects free eculizumab (IgG2/4 κ) and E-C5. When E-C5 was formed in the presence of an excess of free eculizumab, the latter appears in a lower molecular weight band (Fig. 4C, lanes 2–4), which corresponds to the band observed

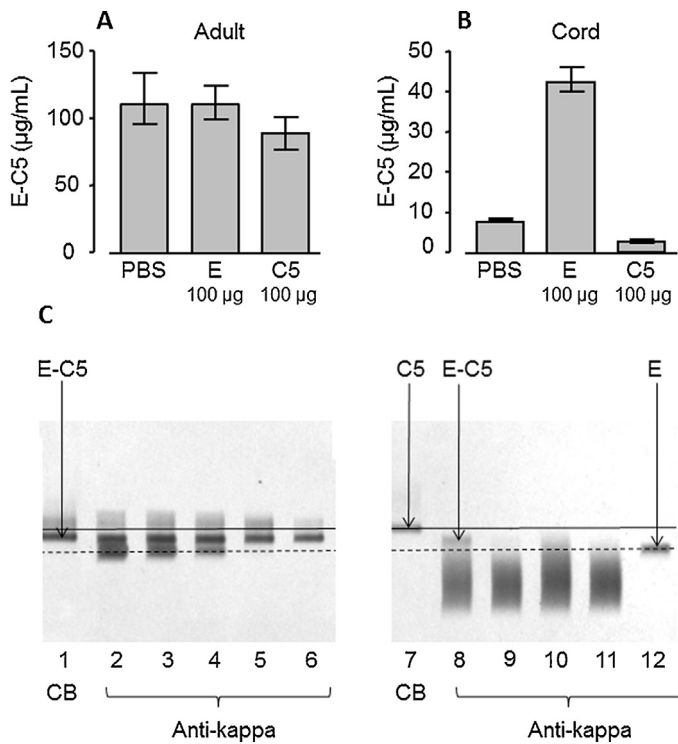


Fig. 4. Effect of adding eculizumab and C5 to the sera and detection of E-C5 in mother's serum using immunofixation electrophoresis. (A) E-C5 complexes were measured in P1 serum (PBS) and in P1 serum to which was added eculizumab (E) or purified C5 (C5) (mean with range of $n=3$). (B) Same experiment as described in panel A, but using serum from the newborn of P1 (mean with range of $n=3$). (C) Lanes 1 and 7 represent protein staining with Coomassie brilliant blue (CB) in electrophoresis, identifying the lines for purified E-C5 and purified C5, respectively. Lanes 2–6 and 8–12 represent immunofixation based on an antibody reacting with the kappa-chain (eculizumab has kappa light chains). Lane 12 identifies eculizumab alone. Lanes 2–6 shows EC-C5 complexes made *in vitro* and titrated from the left (eculizumab in excess) to the right (C5 in excess). Lane 8: Serum from P1. Lane 9: Serum from P1's newborn. Lane 10: normal adult serum. Lane 11: normal newborn serum. Solid horizontal line: indicating position of C5. Broken horizontal line: indicating position of eculizumab. E-C5 complexes appear between the lines.

after addition of eculizumab alone (lane 12). Purified free C5 was identified by protein staining (Fig. 4C, lane 7).

Detection of E-C5 complexes in serum samples from P1 and her newborn

The immunofixation revealed a distinct band in the serum from P1 (Fig. 4C, lane 8), corresponding to E-C5, while no such band was observed in serum from her newborn (lane 9). Also, serum samples from both a healthy adult control and a healthy newborn control did not contain detectable E-C5 (Fig. 4C, lanes 11 and 12).

Binding of FcRn to eculizumab

The therapeutic effect of IgG antibodies is dependent on their long serum half-life, which is regulated by FcRn-mediated recycling in endothelial and hematopoietic cells (Montoyo et al., 2009; Wani et al., 2006). Specifically, IgG binds FcRn within acidified endosomes that are subsequently recycled to the cell surface where exposure to the neutral pH of the blood triggers release of IgG (Ward, 2009; Roopenian and Akillesh, 2007). Thus, FcRn rescues IgG from intracellular degradation and extends its half-life. In addition, FcRn provides passive immunity to the fetus by transcytosing maternally-derived IgG across the placenta (Firan et al., 2001; Mathiesen et al., 2013). To address whether the humanized IgG2/4 κ eculizumab binds human FcRn with the same strength as human IgG1, we performed an ELISA where pH dependent binding to FcRn

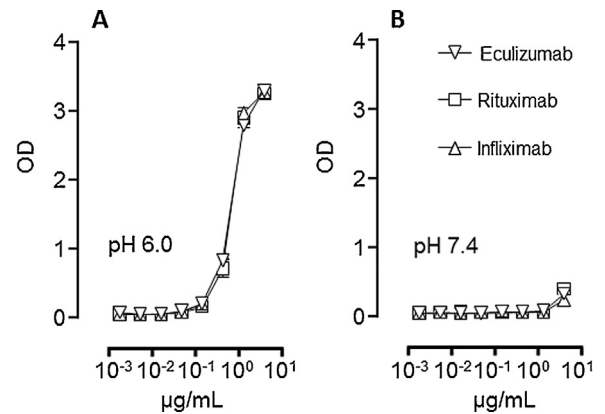


Fig. 5. Effect of pH on eculizumab binding to FcRn. ELISA binding responses of titrated amounts of eculizumab, infliximab and rituximab to GST-tagged hFcRn at pH 6.0 (A) and pH 7.4 (B). The data represents the mean of duplicates.

was compared with that of rituximab (chimeric IgG1 κ) and infliximab (chimeric IgG1 κ). Titrated amounts of the antibodies were coated in wells followed by addition of GST-tagged shFcRn at either pH 6.0 (Fig. 5A) or pH 7.4 (Fig. 5B). Eculizumab was shown to bind the receptor equally well as rituximab and infliximab, in a pH-dependent manner, with strong binding at pH 6.0 and no binding at neutral pH. Thus, the absence of eculizumab in fetal plasma cannot be explained by reduced binding to FcRn.

Discussion

We have here described a comprehensive set of experiments documenting the reliability and validity of a novel assay for detection of complexes between eculizumab and C5. Using this assay, as well as functional complement activity assays and immunofixation, we reveal that there is very little eculizumab as well as E-C5 in the serum of newborns of pregnant women treated with eculizumab. Consequently, the newborns have a fully functional complement system. The importance of the data is emphasized by the increasing number of pregnant women who are candidates for eculizumab treatment. PNH is the disease where most pregnant patients have been treated with eculizumab (de Guibert et al., 2011; Kelly et al., 2010; Danilov et al., 2010; Marasca et al., 2010), but there are also reports for aHUS and preeclampsia (Ardissino et al., 2013; Canigral et al., 2014; Burwick, 2012). Other conditions, in which pregnancy is an option, will likely come up in the future as candidates for complement inhibition, like the anti-phospholipid syndrome (Erkan et al., 2014). The safety of treatment and effect on the newborn will, however, be a main concern. Despite a limited number of patients available for the present study, robust methods were established and used to document that treatment with eculizumab during pregnancy would be of minor risk to the newborn. This could be individually evaluated in the future using the set of analyses presented here.

The amount of E-C5, complement blockage and complement activity in the newborn have to our knowledge not previously been described. In one review on seven pregnant patients receiving eculizumab during pregnancy, eculizumab was quantified in cord blood from four newborns (Kelly et al., 2010). In two, eculizumab was undetectable and in two (twins, cesarean section at week 35 of gestation) low levels were found, which according to the authors, "were within the background levels for the assay and insufficient to block complement". The pregnant women had therapeutic levels of eculizumab in their serum. Traditional hemolytic assays were used for evaluation of the complement activity. These assays do not have the same sensitivity and reliability as the novel assays recently used to evaluate aHUS patients treated with

eculizumab (Cugno et al., 2014; Noris et al., 2014). Using these robust ELISA-based assays for complement activity and the novel E-C5 assay developed in the present study, we consistently show that eculizumab in therapeutic doses in the pregnant women did not affect the complement system of their newborn.

The E-C5 assay we describe was documented to be highly specific and sensitive. Thus, the reactivity was nil in serum from a genetic C5-deficient individual, even when eculizumab was added to high concentrations. Addition of purified C5 to the C5-deficient serum restored a dose-dependent increase in E-C5. The sensitivity limit in serum was found to be below 1 µg/mL added eculizumab, which equals less than 1% of the amount detected in the patients treated with eculizumab. The E-C5 levels in the newborns, 7–8 µg/mL, were detected three titer steps above the lower detection limit, and were thus highly reliable.

Eculizumab is a humanized IgG2/4_K hybrid antibody aimed at minimizing the effector functions mediated by complement activation and Fcγ receptor engagement, as previously demonstrated (Lau et al., 2013). While the binding activity toward the classical Fcγ receptors are lacking or low, eculizumab binds the non-classical Fc receptor FcRn in a strictly pH dependent manner, similar to that of two commercially available human IgG1_K antibodies, infliximab and rituximab. This is as expected since binding to FcRn is dependent on two histidine residues (His310 and His435) at the C_H2–C_H3 elbow region, which are conserved in all subclasses except IgG3, which has Arg435 (Ward, 2009; Roopenian and Akilesh, 2007). FcRn is a homeostatic regulator of IgG, and hence responsible for the 3-week long half-life of endogenous IgG. However, the serum half-life of therapeutic IgG antibodies varies, and previous studies have shown the half-life of eculizumab, infliximab and rituximab to be 11.3, 9.5 and 22 days, respectively, the latter two being chimeric IgG1_K antibodies that cross the placenta and are found in the serum of the newborn (Malek et al., 1996; Kane, 2009; Regazzi et al., 2005; Klink et al., 2008; McKeage, 2011). As we here demonstrate that these three antibodies bind FcRn with equal binding strength, it explains their prolonged half-life, but not why they show different serum persistence in general and possibly different placental transfer and/or fetal serum persistence. The differences may be related to the amounts of therapeutic antibody given as well as the concentration, biophysical properties and biodistribution of their respective antigens. Furthermore, it could be due to differences in how the antibodies are transported and sorted by FcRn when bound to their cognate antigens. As the antigens for eculizumab and infliximab are both soluble with only one antibody binding site, these complexes should not cross-bind classical Fcγ receptors, which is a prerequisite for removal by phagocytosis.

The four human IgG subclasses are all actively transported across the placenta, and whereas IgG1, IgG3 and IgG4 are detected in the fetus in amounts similar to those found in the mother, IgG2 is detected in lower amounts (Palmeira et al., 2012; Hashira et al., 2000). It is generally accepted that FcRn plays a key role in shuttling of IgG to the fetus as substitution of the amino acids that are directly involved in FcRn, but not classical Fcγ receptor binding, abolishes transplacental transport in *ex vivo* perfusion model systems (Palmeira et al., 2012; Firan et al., 2001; Mathiesen et al., 2013). However, the reason why IgG2 is found in lower amounts is still not known. In this study, we demonstrate that very small amounts of the IgG2/4 chimeric eculizumab can be detected in the newborn. Still, eculizumab may well be transported across the placenta, and the observed low steady state level due to the relative rates of transport and clearance. This needs to be addressed in future studies.

Furthermore, the E-C5 detected in the serum of the newborn could be formed either after passage of eculizumab, or could be the result of E-C5 passage. We examined E-C5 concentration and complement activity in the serum from P1 in detail, also after addition

of purified C5. Normal physiological C5 concentration in adults is 75 µg/mL. Since full complement activity was obtained only after addition of at least 1 mg/mL C5, eculizumab was present in excess in the serum of the mother, consistent with the sample being obtained six days after an eculizumab infusion. Thus, our data show that both free eculizumab as well as E-C5 is present in mother, and available for transport to the newborn.

In conclusion, our data indicate no adverse effects of the eculizumab treatment of the pregnant women on the complement activity of her newborn. Thus, our study suggests that eculizumab should be regarded as safe with respect to the defense system of the newborn. We suggest that the combination of methods described in the present study is particularly suitable for further investigation of pregnant patients treated with eculizumab and their newborns.

Conflict of interest

None of the authors have any conflict of interest to declare.

Acknowledgements

Brith Sveia Kvarv is greatly acknowledged for excellent technical assistance.

Financial support: T.E.M. was supported by The Research Council of Norway (project no. 204874/F20), The Norwegian Council on Cardiovascular Disease, The Northern Norway Regional Health Authority (Project no. SFP926-10), The Southern and Eastern Norway Regional Health Authority (Project no. 2012060), The Odd Fellow Foundation, and the European Community's Seventh Framework Programme under grant agreement no. 602699 (DIREKT). I.S. and J.T.A. were supported in part by the Research Council of Norway through its Centers of Excellence funding scheme (project number 179573). J.T.A. was supported by the Research Council of Norway (Grant nos. 230526/F20 and 179573/V40) and the South-Eastern Norway Regional Health Authority (Grant no. 39375).

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