ELECTROLUMINESCENT TCC, C3dg AND Fb ASSAYS FOR PROFILING COMPLEMENT CASCADE ACTIVATION IN VITRO USING ACTIVATED COMPLEMENT SERUM CALIBRATION STANDARD

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**Abstract**

Electroluminescent assays for the complement components C3dg, terminal complement complex (TCC) and factor B (fB) have been developed with capture and detection antibodies to produce detection limits C3dg = 91 ± 9 ng/mL, TCC=3 ± 0.1 ng/mL and fB= 55.7 ± 0.1 ng/mL. The assay performance was assessed against a series of zymosan and heat aggregated IgG (HAlgG) *in vitro* activations of complement using a calibrated activated complement serum (ACS) as calibration standard. The ACS standard was stable within 20% accuracy over a 6-month period with freeze-thaw cycles as required. Differential activation of the complement cascade was observed for TCC showing a pseudo-first order formation half-life of 3.5 hrs after activation with zymosan. The C3dg activation fragment indicates a 10% total activation for both activation agents. The kinetic-epitope analysis for fB indicates the capture epitope is on the fB/Bb protein fragment and since it becomes covered during the time course.

Keywords: Complement, cascade, activation, electroluminescent, assays, epitope
Introduction

Complement (C) activation through all four pathways, classical, lectin, alternative and terminal can be monitored by measuring both consumption of constitutive proteins such as C3 and C4 as well as concentrations of activation products. A full profile of activation and consumption would provide differential flux markers in each of the pathways. Ideally, a multiplexed, sensitive assay platform would measure a number of analytes simultaneously from the same serum sample to derive the profile at a single point in time. The electroluminescent platforms offer such an assay performance although the assay design and optimisation, especially the choice of capture and detection epitopes, is critical, as is the need for a reliable calibration standard.

Many C activation studies have been performed, where combinations of C components and fragments were used to establish C activation both in vivo and in vitro. A multi-analyte study was conducted which monitored levels and ratios of CRP, C3, C4, C9, the terminal complement complex (TCC), C3a and C4d in pregnant women with preeclampsia (Derzsy et al., 2010). A larger set of markers, C3, C3a, C5a, C4d and Factor B (fB) was investigated for the diagnosis of patients with severe sepsis (Wolbink et al., 1998). A detailed analysis of C activation following coronary artery grafting (Hoedemaekers et al., 2010) considered the components C3, C4, C3bc, C4bc, MBL and C1inh-C1rs as well as the C3 convertase C3bBbP and observed a biphasic response of the cascade. Many studies have looked at smaller sets of makers, some preferring TCC and comparisons with CRP and interleukins for specific diseases states such as rheumatoid arthritis (Mollnes et al., 1986; Gu et al., 1999; Molenaar et al., 2001) and acute ischaemic stroke (Pedersen et al., 2004), as well as cardiopulmonary bypass extra-corporal surface activation (Gu et al., 1999; Hoedemaekers et al., 2010). A disease state firmly linked with C deficiencies is systemic lupus erythematosus (SLE) (Mollnes et al., 2007; Truedsson et al., 2007) and C activation has been observed following the anaphylatoxins C3a and C5a (Belmont et al., 1986) although the low-mass products are quickly cleared by the kidneys, first-pass, owing to the filtration cut-off of ~43 kDa (Anderson and Anderson, 2002). Consequently, urine analysis of fragments, specifically C3a in SLE (Manzi et al., 1996), is obvious diagnostic with functioning kidneys whereas larger fragment concentrations in the urine, such as TCC, may point to disease states such as proteinurea (Morita et al., 2000).

The problems of clearance and regulation of C activation in vivo present additional challenges to profiling C activation to the design of efficient immune assays that reflect faithfully the change in the fragment concentrations. Formally, detection and capture antibodies monitor the change in the concentrations of the epitope in the serum requiring
precise knowledge of the epitope locations to correlate with target fragments. Ideally, neo epitopes, specific to the fragment and not the parent proteins are required to monitor fragment concentrations (Mollnes et al., 1985a; Mollnes et al., 1993; Harboe et al., 2004). Further, epitopes that are hidden on protein surfaces following complex formation (Mollnes and Tschopp, 1987) would show a decrease in the concentration of a component. C activation in vivo provides an ideal assay optimisation protocol for epitope validation and fragment identification using activation agents such as zymosan and HA IgG. The activation may be frozen at each time point with effective sample handling procedures (Stöve et al., 1995; Pfeifer et al., 1999) for effective time course mechanistic kinetic analysis. Some studies have focused on single pathway activation by removing key proteins in other pathways (Stephens et al., 1977), therefore halting C activation via that route, and another constructed the alternative pathway using six isolated proteins, activating the pathway with erythrocytes (Schreiber et al., 1978).

The potential for an activation profile captured in time as a marker for a disease state or following in vitro activation is clear and requires a multi-analyte detection platform that is highly sensitive and, critically, and activation calibration standard. Electroluminescent assay (ELA) platforms such as the Meso Scale Discovery platform have been compared directly with conventional enzyme-linked immunosorbent assays (ELISA) using the same capture and detection antibodies for each assay. Thullier et al have performed the ELA and ELISA comparison studies for ricin B chain and Clostridium botulinum type B neurotoxin (Guglielmo-Viret et al., 2005; Guglielmo-Viret and Thullier, 2007) reporting an 8-fold increase in sensitivity (Guglielmo-Viret and Thullier, 2007) in the ELA format over the ELISA. In addition, the electroluminescent platform has the potential for in-well multiplexing with up to 20 assays printed on the surface to allow for massively parallel assay profile C activation. The location of the assay on the well surface allows the camera to collect and process the luminescence from each activation assay.

In this paper, we report the preparation of three C activation marker assays for TCC, Bb and C3dg using an electroluminescent detection platform, calibrating the assays against a totally activated complement serum (ACS). The location of the epitopes on each of the capture and detection antibodies is inferred from the time course mechanistic kinetic analysis. The assays are optimised for serial dilution, matrix effects, intra-assay and inter-assay stability variations. The assays are used to monitor the in vitro activation of a standard serum using two activation agents zymosan and heat aggregated IgG. The concentrations of C3, C4, TCC, Bb and C3dg are observed over a time course of 24 hours.
Materials and Methods

In vitro serum complement activation protocol

The protocol has been adapted from the protocols of Bergseth et al. (Bergseth et al., 2013) and Harboe et al. (Harboe et al., 2004), tailored to the 24 hr time-course study using EDTA stabilisation at each of the target time points. In addition, lower concentrations of the activation agents zymosan and HAIgG were used to allow detection of intermediate activation kinetics.

A stock of pooled human serum (Biochemed) was divided in two and temperature stabilised at 37°C: to one half zymosan A (Sigma-Aldrich) was added to give a final concentration of 0.1 mg/ml; and to the second half HAIgG was added to make a final concentration of 0.1 mg/ml and a total volume of 250 mL. Both activators were added to their respective stocks of serum simultaneously at \( t = 0 \). A 1 mL aliquot was removed from each sample 5 minutes prior to the addition of zymosan and HAIgG as an initial value and then subsequently over the time course \( t = 0, 0.5, 1, 2, 4, 6, 10, 16 \) and 24 hours. Each sample was added to a concentrated solution of EDTA (Fluka) in PBS (Sigma-Aldrich) to make a final concentration of 10mM. The samples were centrifuged for 30 minutes in a Microfuge (Thermo Espresso Centrifuge) at 14400 RCF, after which two 300µL aliquots were taken from the top of the vial, leaving a zymosan pellet. These aliquots were frozen immediately using liquid nitrogen and kept at -80°C until the assays were performed.

Activated complement serum calibration standard

All assays were calibrated using a stock solution of fully activated C serum (ACS) (prepared in Cardiff) following the protocol described by Mollnes et al. (Bergseth et al., 2013). In addition to the known concentrations of fragments within the ACS, ELISA calibrations were performed for TCC (ELISA, TCC mAb, clone aE11, Hycult Biotech) 1 µg/ml and Bb (Quidel reference protein concentration) from which the following concentrations were determined: Bb concentration in ACS 55 ± 6 µg/mL, TCC concentration in ACS of 2.1 ± 0.1mg/mL Concentrations of other components are reported in Table 1.

Capture and detection antibody preparation protocol

A neoepitope antibody raised against the assay fragment was immobilised overnight at 4°C onto the MSD high-bind plate (L11XB-6) surface using a protein concentration of 30 µL, 1µg/ml in PBS. The surface was then blocked at room temperature for 1hr using MSD Blocker A in PBS-T (PBS with 0.05% Tween-20), after which 25 µL of the sample or assay standard, ACS (prepared in 10mM EDTA, 1% Blocker A PBS-T solution) was added to the surface for 2hr at room temperature to bind any fragment present in the sample to the
immobilised neoepitope antibody. The plate is then washed 3 times with 150 µL PBS-T, after which 25 µL of the SULFO-Tag labelled (following the standard MSD protocol, see below) detection antibody (1/500 dilution) is added to the wells for 1hr at room temperature. The different assays are performed in different wells. The plate is then washed a final time before 150 µL of 1× MSD Read Buffer is added to the wells for reading in the instrument. The capture and detection antibodies for each of the assays are summarised in Table 1 together with the assay performance parameters and the ACS calibration standard concentrations.

**Detection antibody SULFO-TAG NHS ester labelling protocol**

The antibody labelling protocol was used as supplied by MSD. A 1-2 mg/ml solution of the antibody to be labelled is prepared in preservative-free PBS, pH 7.9. After equilibrating the antibody solution to room temperature, the MSD SULFO-TAG NHS ester is reconstituted in water and immediately added to the antibody. The reaction vessel is incubated in the dark for 2 hours at room temperature, after which the free, unbound tag is removed from the reaction mixture by centrifuging three times at 1000 g for three minutes using a Zeba Spin Desalting Column with a 40 kDa MW cut off at 4°C. Once the unbound ester is removed, an optimal dilution of the antibody may be found for the respective assay.

Concentrations of capture and detection antibodies were optimised by testing a variety of capture antibody and detection antibody concentrations, then selecting the combination that yielded the greatest dynamic range. Consideration was also given to different dilution buffers at this stage.
Results

The assay performance over the period of the investigation was calibrated against the ACS standard producing calibration curves shown in Figure 1 for all three components, TCC, C3dg and Bb. The error bars on the calibration curves are derived from 10 separate experiments performed over a 6-month period with storage of samples between analysis points. The assays show good dynamic range over typically three orders of magnitude and detection limits of order ng/mL, Table 1. Dilution invariance was achieved with in a 18% error over the concentration range of the calibration curves using serum and PBS producing inter-assays errors typically of less than 5 % with intra-assay errors typically 15%, Table 1.

The ELAs together with the two turbidimetric assays from the clinical chemistry laboratory (at the Royal Devon and Exeter Hospital) were performed on the C activation time course over a 24hr period. The time course activations for zymosan and HAIgG were performed separately. The C3 and C4 concentrations for either activation agent show no significant
deviation within the accuracy and sensitivity of the assay,

Figure 2. By contrast, the TCC concentration profile shows a pseudo-first order rise in the concentration following activation with a half-life of $t_{1/2} = 3.5$ hrs following zymosan activation but no production was observed during the HAlgG activation. The TCC reached 80% of the ACS concentration within the 24 hr activation time course.

The kinetic traces following the zymosan or HAlgG activations profiles for both Bb and C3dg are the same within the accuracy of the assays. Bb has an initial concentration of 275 µg/mL which is comparable to the concentration of fB in serum (typically, 180 µg/mL (Morley and Walport, 1999)). The Bb concentration falls to two-thirds of its initial value in an essentially monotonic decrease although shows some complex kinetic behaviour in the early part of the time course close to the assay accuracy. C3dg rises during the 24hr time course to 60% of its value in ACS $114 \pm 22$ µg/mL, showing a similar monotonic increase and potentially some more detailed kinetic information regarding the activation process.

**Discussion**

Activation assays implemented on an electroluminescent platform have been used to observe the *in vitro* activation of serum using both zymosan and HAlgG. Assays for Bb, C3dg and TCC were performed with defined capture and detection antibodies to assess their detection limits and kinetic behaviour. The assays were calibrated against an activated complement serum standard that has stable concentrations over a period of six months.
when stored appropriately. TCC shows a first-order activation profile with time with a half-life of 3.5 hours, C3dg shows a monotonic increase whereas Bb shows a decreasing trend. Formally, each is the concentration kinetic profiles is a measure of the concentration of the epitope whether becoming available as a results of the location of some is well known becoming available for binding following protein complex formation or covered. The ACS calibrations standard and a set of parallelised assays has potential to screen epitope efficacy and C activation flux.

Electroluminescent assay platforms have the potential for extremely low detection limits (Leng et al., 2008) and multiplexed assays up to 20, within a single well of a 96-well plate. The technology will allow the 20 assays to be printed at the base of each well and read imaging the fluorescence from each assay with the CCD camera to produce pM sensitivity. A single serum sample can then be used to determine the concentration of a number of C fragment species simultaneously allowing an activation flux profile of the C cascade to be determined. However, the integrity of the activation profile and the activation pathway flux determinations depends critically on the successful choice of capture and labelled detection antibodies as well as a multi-component calibration standard. The current study consider the development of assays for three components of the C cascade and assed the use of ACS as a multi-component calibration standard.

**ACS as a large-scale complement research standard**

The *in vitro* activation of a mixed serum sample of C by the known activation agents zymosan and HAIgG in excess leads to composition-stable totally activated serum that has been demonstrated to be stable over extended freeze-thaw cycles. The fully activated C serum has been proposed as a C calibration standard by Bergseth *et al.* (Bergseth *et al.*, 2013), ideal for the multi-component activation profile analysis. Calibration of ACS for each of the required components during ten separate experiments (duplicate measurements in each) performed over a period of six months produced a stable calibration standard for TCC, C3dg and Bb. The long-term stability of the calibration may be assessed from the 95% confidence intervals derived from the mean of all of the individual calibration curves over the 6-month period. The variations in the measurements for TCC, C3dg and Bb over a period of six months were: TCC = 22.9%, C3dg = 20.2% and Bb = 17.9%. The average value for the overall reproducibility of the ACS calibration was then determined to be 20.3% using data from all three assays. These activation products appear to be stable in the ACS throughout suggesting a single ACS sample could be prepared for a study for distribution to multi-centres.
The ACS standard may be used in two ways for the calibration of the C cascade activation in vitro: either calibrated for each of the individual components, requiring primary calibration against pure protein sources or expression the activation as a percentage of the total activation. A simple epitope integrity comparison can be made for possible capture and detection antibodies where concentrations larger than 100% of the activation concentrations would indicate epitope specificity concerns. With the current epitopes in this study TCC was always found to be less than the ACS value throughout all activation experiments reaching typically <90% ACS concentration. This indicates epitope homology between the current antibodies and the calibration standards. Similar observations and therefore epitope specificity may be attributed to the C3dg assay < 60% of the ACS value. By contrast, the Bb concentration was measured to be > 550% of the ACS Bb calibration value indicating the capture antibody epitope does not discriminate between fB and Bb and, further, the epitope becomes hidden in the protein complexes containing Bb component resulting in a decrease in the epitope concentration during the in vitro activation.

The kinetic response of an epitope can be determined by measuring the change during an in vitro activation. Measurements were performed on the activation of C in a pooled serum sample for zymosan and HA IgG separately at concentration of 0.1 mg mL⁻¹, ten-fold lower than the ACS preparation protocol, changing both the concentration and surface area of the activating species. The alternative pathway is triggered by (nearly) all foreign surfaces resulting in the consumption of C3 and fB through the amplification loop. The C3dg fragment and others C3 would rise in concentration from both zymosan and HA IgG activation agents. HA IgG is expected to activate the classical pathway from the specific antibody complex interactions and the alternative pathway through the foreign surfaces. Zymosan will activate both the lectin and alternative pathways. Both activations will form the C3 convertases in the cascade leading to lower pathway activation and the formation of TCC. The activation steps provide a well understood kinetic mechanism against which to test epitope location and efficiency.

The C3 and C4 with concentrations were measured in using the turbidimetric assay in the clinical chemistry laboratory with a quality-controlled accuracy of 3% and showed no
Figure 1. The C3 assay is formally detecting an epitope on the C3c fragment. Two conclusions may be drawn from this: the change in concentration of the C3 and C4 is rather small given the concentration of the activation species both zymosan and HALgG or the detection epitopes are present on C3c and its fragments and C4 and its fragments, the total epitope concentration is not changing during the activation. The rising concentration of C3dg neo-epitope, however, indicates production of the fragment at a rate of 2.6 µg hr$^{-1}$ or 72 pmol hr$^{-1}$ which must correlate stocimetrically with consumption of C3: the measured change in the number of moles of the C3dg neo-epitope 1.3 ± 0.2 nmoles which mechanistically equivalent to the number of C3 molecules consumed. From the initial concentration of C3 in the plasma sample, 12.6 nmoles, ACS has 10% of the available C3 activated. The change is significantly different with respect to the assay accuracy indicating the epitope is located on the C3c-C3 fragment and the epitope concentration does not change during the activation.

The epitope concentration for Bb falls during the activation indicating the consumption of 0.9 nmoles in the production of 1.3 nmoles of the C3dg neo-epitope. However, the initial epitope concentration of Bb indicates the epitope is not a neo-epitope for Bb but is detecting both fB and Bb the activation. The Bb epitope concentration falls over the time course indicating a
an epitope that becomes hidden during the formation of the C3bBb and stabilised C3bBbP complexes. The early time kinetic variations may be indicative of the complex kinetic mechanisms associated with the concentration-limited kinetics of fD which is required to catalyse the conversion of C3bfB to C3Bb. Rising concentrations of C3bfB will reduce the fD concentration slowing the production of C3bBb and C3bBbP indicating the role of fD as a flux control molecule in the cascade.

The zymosan activation of the alternative and lectin pathways produced a rapid rise on in the concentration of the TCC epitope, locating it firmly as a neo-epitope on SC5-9 complex. The epitope is well characterised (Mollnes et al., 1985b; Tschopp and Mollnes, 1986) and is exposed on C9 when in the poly-C9 complex. Some epitope may be slightly exposed in activated C8α given the sequence homology. It is kinetically well defined as a neo-epitope of TCC and all epitopes to this complex must behave in a similarly quantitative way. The pseudo-first order rise indicates the production of 1.78 nmoles of TCC indicating 1 molecule of C3 produces one molecule of TCC. The half-life, \( t_{\frac{1}{2}} = 3.5 \) hrs is a measure of the response time of the C cascade, top-to-bottom, for the size of the stimulation. Zymosan contains ‘ghost’ cells of 3µm in diameter which at a mass of 0.1mg mL\(^{-1}\) corresponds to a cell number of approximately \( 10^{12} \) cells.

The in vitro activation analysis is an excellent, quantitative method to test the location of an epitope on a capture antibody pair, requiring very similar kinetic parameters as well-known epitopes. The HAIG activation however, reveals similar upper-cascade level activity for C3, C4, fB and C3dg but not lower cascade activation. Propagation of classical pathway activation through the cascade to TCC formation requires the stability of the C4b2a C3-convertase to be comparable with the C3bBbP convertase in the serum. The concentration of the C4b2a convertase depends on its rate of production and rate of decay to trigger TCC formation, indicating its concentration is approximately 100-fold less in the classical pathway and alternative pathway activation by HAIG.

**Conclusion**

The electroluminescent platform produced assay with detection limits not significantly better than conventional ELISA assays with the choice of capture and detection antibodies although preparation of the assays was relatively straight forward. The potential however to locate up to 10 assays in a single well would allow for a parallel multi-analyte profiling of C components. The ACS calibration standard performed well over a 6-month period providing a mult-analyte calibration standard for a systematic analysis of the mechanism and kinetics of the C in vitro activation pathways. Differential activation of the pathways may with zymosan and HAIG may prove mechanistically useful. The mechanistic concentration
behaviour of the epitope provides addition information on the location of epitopes on particular protein fragments. A careful choice of epitope would provide detailed mass-balance and flux analyses of the C cascade.
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Figure Captions

Figure 1 Calibration Curves for the analytes: (a) C3d, (b) TCC and (c) Bb against the ACS standard. The error bars refer to ten identical calibration experiments performed over a 6 month period. ACS Units – undiluted ACS = $10^6$
Figure 2 C3 (■) and C4(●) ACS concentrations determined by the immunoturbidometric assay. Error bars are 3% determined in the routine quality control for the assay in the clinical laboratory application.
Figure 3 TCC time course following (a) zymosan and (b) activation by HAlgG activations is not observed at this 0.1 mg/mL concentration.
Figure 4 Bb activation time course following both zymosan and HAlgG activation. The kinetic response of this epitope is the same for both activations, within error of the assay.
Figure 5 C3d concentration kinetic response following both zymosan and HAIgG activations
Table 1. Electroluminescent and turbidimetric assay properties

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Capture Antibody</th>
<th>Detection Antibody (Labelled)</th>
<th>Detection Limit</th>
<th>Dynamic Range (orders of magnitude)</th>
<th>Intra-assay, Inter-assay Error</th>
<th>ACS Calibration Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3dg</td>
<td>Anti C3dg neo (Quidel), 1 µg/ml</td>
<td>Anti-C3b clone C3/30 (Cardiff)</td>
<td>91 ± 9 ng/mL</td>
<td>&gt;3</td>
<td>8.8% 3.6%</td>
<td>190 ± 10 µg/mL (Bergseth et al., 2013)</td>
</tr>
<tr>
<td>fB/Bb</td>
<td>Anti Bb neo (Quidel), 2 µg/ml</td>
<td>Anti-fB clone JC1 (Cardiff)</td>
<td>55.7 ± 0.1 ng/mL</td>
<td>~1½</td>
<td>13.7% 3.9%</td>
<td>55 ± 6 µg/mL</td>
</tr>
<tr>
<td>TCC</td>
<td>TCC mAb, clone aE11 (Hycult Biotech), 1 µg/ml</td>
<td>aC8 (Cardiff)</td>
<td>2.3 ± 0.1 ng/mL</td>
<td>~3</td>
<td>18.2% 5.7%</td>
<td>2.10 ± 0.04 mg/mL</td>
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<tr>
<td>C3</td>
<td>Cobas Tina quant® Complement C3c ver.2</td>
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<td>40 ± 1 mg/L</td>
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<td>C4</td>
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