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Antibodies reactive to cleaved sites in complement proteins enable highly specific measurement of soluble markers of complement activation

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ABSTRACT

An emerging number of diseases and therapeutic approaches with defined involvement of the complement system justify a need for specific markers reflecting activation of particular effector arms of the complement cascade. Measurement of such soluble markers in circulation is a challenge since the specificity of antibodies must be limited to activated complement fragments but not predominant and ubiquitous parental molecules. Existing assays for the measurement of soluble, activated complement proteins are based on the detection of conformational neoepitopes. We tested an alternative approach based on detection of short linear neoepitopes exposed at the cleavage sites after activation of the actual complement component. Obtained antibodies reactive to C4d and C5b fragments enabled us to set up highly specific sandwich ELISAs, which ensured trustful measurements without false positive readouts characteristic for some of the widely used assays.

1. Introduction

The complement system consists of more than 30 proteins organized in a cascade of proteolytic events and conformational changes, which support sequential interactions of activated complement components eventually leading to lysis of target cells (Walport, 2001). Proteolysis takes place during the initiation of the alternative complement pathway (cleavage of C3 and factor B) and classical/lectin pathway (C2 and C4), during generation of C3a and C3b by C3 convertases, during inactivation of C3b and C4b opsonins by factor I (FI) (Nilsson et al., 2011), and finally at the transition from opsonic to lytic stage, *i.e.* splitting of C5 molecule to C5a and C5b (Walport, 2001). Proteolysis triggers conformational changes resulting in exposure of binding sites for complement components placed downstream in the cascade, which in turn also adopt new conformations allowing interaction with the next component. The best-known soluble complement activation products are anaphylatoxins C3a and C5a (and their desArg forms lacking the C-terminal arginine). However, free anaphylatoxins are rapidly cleared from circulation (Webster et al., 1982) thus making the interpretation of their systemic measurement problematic. Notably, not all of the cell-bondable, activated complement components bind target cells but a certain amount remains in their soluble, stable forms, *e.g.* Bb, C4d, sC5b-9 (Bergseth et al., 2013). Although this gives an opportunity to monitor complement activation in clinical material like plasma and body fluids, measurement of such soluble markers is challenging, as it demands highly specific antibodies not reactive to abundant, non-activated complement components.

Most of the existing assays make use of antibodies against conformational neoepitopes but these sites may be mimicked or

Abbreviations: aa, amino acid; CAU, complement activation unit; FI, complement factor I; ICS#2, International Complement Standard #2; HRP, horseradish peroxidase; NHS, normal human serum; HI-NHS, heat inactivated NHS; OPD, *o*-phenylenediamine dihydrochloride.

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Table 1
Sequences of peptides used for immunization and purification of antibodies.

Antigen	Immunization	Purification step 1	Purification step 2
C-terminal C4d fragment	Cys-GGSSTGR	Cys-NVTLSSTGR	Cys-NVTLSSTGRNGFK
N-terminal C4d fragment	TLEIPGGG-Cys	Not performed	Not performed
C5b	LHMKTGGG-Cys	LHMKTLLPV-Cys	QLGRLHMKTLLPV-Cys

decayed in the course of sample handling. Manufacturers seem to be aware of the problem as they recommend very strict sampling guidelines and discourage prolonged storage or heat-inactivation of the sample before measurement as well as repetitive freezing and thawing of the samples to be measured; a precaution not always easy to follow, especially with archival clinical material. Therefore, we tested a novel strategy of raising antibodies specific to very short linear neoepitopes exposed in activated complement components.

2. Methods

2.1. Reagents, sera, antibodies and purified complement proteins

Normal human serum (NHS) was prepared as described (Blom et al., 2014) and heat inactivation was performed by heating at 56 °C for 30 min. C6 and C9-depleted sera were purchased from Complement Technology (Tyler, TX). Function-blocking antibody against FI was bought from Quidel (San Diego, CA). The C5-blocking antibody eculizumab was purchased from Alexion Pharmaceuticals (Lausanne, Switzerland). Zymosan was purchased from Sigma (St Louis, MO). Mouse monoclonal antibody mk54 against protein S was prepared as described (Dahlback et al., 1990).

2.2. Raising of neoepitope antibodies

The whole process of custom antibody production was performed by Agrisera AB (Vännäs, Sweden). Briefly, synthetic peptides embracing five terminal amino acids (aa) residues adjacent to the cleavage site of a given complement component followed by two glycine and one cysteine residue spacer were linked to keyhole limpet hemocyanin and used for rabbit immunization (two rabbits per peptide construct) according to the following schedule: 200 μg of peptide with complete Freund's adjuvant followed by another 200 μg of peptide with incomplete Freund's adjuvant four weeks later and 100 μg of peptide every four weeks. Bleeding was performed two weeks after booster injections starting from round two. Sequences of the peptides used for immunization are given in Table 1.

2.3. Purification of antibodies by affinity chromatography

Synthetic peptides (Genscript, Piscataway, NJ) corresponding to nine aa residues before the cleavage site were immobilized with added Cys residue to Sulpholink beads (Thermo Scientific, Waltham, MA) according to manufacturer's instruction and loaded onto a 10 ml column, further equilibrated with PBS buffer. Antiserum was loaded and bound antibodies were eluted with linear gradient of 0.1 M glycine pH 2.5. After neutralization with 1 M Tris–HCl pH 8.0, the eluate was directly applied onto another column containing Sulpholink beads conjugated to the similar peptide but extended 4 aa beyond the cleavage site. At this step we collected flow through and all antibodies with affinity to extended peptide motif, and therefore cross-reactive to the parental complement molecule, were absorbed. Pooled flow through was dialysed against PBS and concentrated. Sequences of the peptides used for antibody purification are given in Table 1.

2.4. Complement activation in vitro

Serum at concentration of 2% was diluted in $DGVB^{++}$ buffer (2.5 mM veronal buffer pH 7.3, 72 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$) and 0.1 mg/ml zymosan was added. After 45 min incubation at 37 °C mixture was diluted five times (up to 0.4% final concentration) with AG buffer (0.2% Tween 20, 20 mM EDTA, 0.02% NaN_3 in PBS). Zymosan particles were discarded by centrifugation for 3 min at 5000 × *g* prior to analysis by ELISA. Alternatively, 8% of serum diluted in $DGVB^{++}$ was activated with 0.8 mg/ml human IgG (Immuno, Vienna, Austria) aggregated at 63 °C for 20 min. After 45 min incubation the supernatant was diluted twelve times (up to 0.66% final serum concentration) with AG buffer and prepared for ELISA.

2.5. ELISA detecting soluble C4d

Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at room temperature with 3.5 μg/ml of affinity-purified C4d neoepitope antibody diluted in PBS + 0.02% NaN_3. Wells were blocked with 3% fish gelatine (Norland Products, Cranbury, NJ) in washing buffer (50 mM Tris–HCl, 150 mM NaCl, 0.2% Tween 20). Blocking step and subsequent incubation with samples and standards followed by detection with antibodies were performed for 1 h at 37 °C. Serial dilutions of ICS#2 (International Complement Standard #2, a pool of sera from healthy volunteers activated with aggregated IgG and zymosan (Bergseth et al., 2013)) were used as a standard. Since ICS#2 is a source of every possible complement activation product, their content was defined as 1000 complement activation units (CAU) per one milliliter of ICS#2. Detection was performed by anti C4d #253 Ab (Quidel) diluted 1:1500 in PBS + 0.2% Tween 20 followed by goat anti-mouse, HRP-conjugated Ab (Dako, Glostrup, Denmark) diluted 1:1000. Assay was developed with OPD tablets (Dako) according to the manufacturer's instruction and absorbance at 490 nm was measured using Cary50 MPR microplate reader (Varian, Palo Alto, CA).

2.6. ELISA detecting sC5-9 and its soluble intermediates

Quantification of sC5b-9 and its intermediates was performed similarly to C4d ELISA but affinity-purified C5b neoepitope antibody was used for coating and samples/standards were incubated at 4 °C. For detection, goat polyclonal antibodies against C5 or C6 or C8 or C9 (Complement Technology) or C7 (Quidel) were used at dilution 1:2000 with exception of anti C8, which was diluted 1:500. Secondary antibody was rabbit anti-goat, HRP-conjugated (Dako).

2.7. Hemolytic assay

Hemolytic assay was performed as described previously (Blom et al., 2014). Briefly, sheep erythrocytes were sensitized with anti-sheep IgM (amboceptor), washed in veronal buffer ($DGVB^{++}$) and incubated with 5% NHS in the same buffer in the presence or absence of C5-blocking antibody. Complement-mediated lysis was assessed by measurement of hemoglobin released to supernatant at 405 nm. Readout of the sample of erythrocytes lysed with distilled water was considered as maximal attainable (full) lysis.