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Research paper

Functional assessment of mouse complement pathway activities and quantification of C3b/C3c/iC3b in an experimental model of mouse renal ischaemia/reperfusion injury ☆

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ABSTRACT

The complement system is an essential component of our innate immunity, both for the protection against infections and for proper handling of dying cells. However, the complement system can also contribute to tissue injury and inflammatory responses. In view of novel therapeutic possibilities, there is an increasing interest in measurement of the complement system activation in the systemic compartment, both in the clinical setting as well as in experimental models. Here we describe in parallel a sensitive and specific sandwich ELISA detecting mouse C3 activation fragments C3b/C3c/iC3b, as well as functional complement ELISAs detecting specific activities of the three complement pathways at the level of C3 and at the level of C9 activation. In a murine model of renal ischaemia/reperfusion injury (IRI) we found transient complement activation as shown by generation of C3b/C3c/iC3b fragments at 24 h following reperfusion, which returned to base-line at 3 and 7 days post reperfusion. When the pathway specific complement activities were measured at the level of C3 activation, we found no significant reduction in any of the pathways. However, the functional complement activity of all three pathways was significantly reduced when measured at the level of C9, with the strongest reduction being observed in the alternative pathway. For all three pathways there was a strong correlation between the amount of C3 fragments and the reduction in functional complement activity. Moreover, at 24 h both C3 fragments and the functional complement activities showed a correlation with the rise in serum creatinine. Together our results show that determination of the systemic pathway specific complement activity is feasible in experimental mouse models and that they are useful in understanding complement activation and inhibition *in vivo*.

1. Introduction

The complement system is a robust and tightly regulated first line of defence against invading pathogens, essential in proper clearance of injured host cells (Walport, 2001). However, loss of complement regulation, due to extensive damage or inadvertent activation, is central in several systemic and organ-specific diseases (Lesher and Song, 2010; McCullough et al., 2013). Activation of the complement system is initiated by specific

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pattern recognition molecules; the classical pathway (CP) is activated for instance *via* C1q binding to surface deposited immunoglobulins (Kishore and Reid, 2000), and the lectin pathway (LP) is activated *via* mannan binding lectin (MBL) or Ficolins that recognise specific carbohydrate moieties on pathogens (Petersen et al., 2001) and altered self-structures (Collard et al., 2000). The alternative pathway (AP) can be initiated directly through C3 deposition on damaged or unprotected surfaces, or through properdin acting as a specific pattern recognition molecule (Spitzer et al., 2007). Activation for terminal pathway produces C5a, which promotes local inflammation and recruits inflammatory cells, and mC5b-9 (terminal complement complex), which can lyse unprotected cells and promote apoptosis of damaged host cells (Nauta et al., 2002; Walport, 2001).

Renal ischaemia/reperfusion injury (IRI) is a multifactorial condition, where local and systemic factors contribute to the development of acute kidney injury and tubular necrosis. Complement activation has been observed following renal IRI in human renal biopsies, and evidence from experimental animal models suggests prominent contribution to the overall injury (Arumugam et al., 2004; Danobeitia et al., 2014; McCullough et al., 2013). Studies with knockout mice and selective blocking of complement factors such as fB, have shown that AP is the major activation pathway in experimental renal IRI (Miwa et al., 2013; Thurman et al., 2006; Zhou et al., 2000). Furthermore, inhibition of C5a and C5b-9 formation reduces the renal injury, confirming the central role for complement mediated damage in renal IRI (De Vries et al., 2003a, 2003b).

Although detection of complement deposition in mouse tissues is well established (Mastellos et al., 2004; Trouw et al., 2005), determination of the systemic complement activity in mouse is still challenging. Haemolytic assays for mouse are impaired by their sensitivity (De Vries et al., 2009; Klerx et al., 1983), whereas C3a and C5a are extremely labile *ex vivo* and are rapidly cleared from circulation by cellular receptors *in vivo* (Kirschfink and Mollnes, 2003; Oppermann and Götze, 1994). Several groups have recently described the development and use of antibodies specific for neo-epitopes on C3 activation fragments to quantify C3 activation in mouse (Lesher et al., 2013; Møller-Kristensen et al., 2005; Thurman et al., 2013). However, assay sensitivity, analysis of sample type preference and the stability of mouse C3 activation fragments have not been extensively described. We have recently described methodology for specific ELISAs measuring functional complement pathways in rats, in analogy to described human assays (Seelen et al., 2005). The functional complement ELISAs are analogous to the haemolytic complement assays in that each pathway may be activated independently with a specific ligand. In functional complement ELISAs the resulting formation of intermediate or terminal complement activation products is quantified using specific antibodies, and the degree of product deposition on ELISA plate reflects the activity in biological sample. Together with sensitive measurement of soluble C5b-9 (sC5b-9) we showed that functional complement ELISAs are ideal in validating *in vivo* inhibition, but that experimental rat renal IRI alone did not lead to significant systemic consumption of complement components, and that generation of sC5b-9 was a relatively late event after IRI (Kotimaa et al., 2014; van der Pol et al., 2012). Until now ELISAs to measure specific pathways of complement activity in mice have been limited to the detection of C3 deposition (Trouw et al., 2005), whereas human rat assays measure the pathway activities to the level of C5b-9 deposition.

To better understand changes in systemic complement following experimental renal IRI in mouse, we developed in parallel an ELISA for C3 activation fragment (C3b/C3c/iC3b) using the neoepitope-specific monoclonal antibody (mAb) clone 2/11 (Mastellos et al., 2004), and six functional complement ELISAs for pathway specific activity measurement at the level of C3 and C9. We provide information on the specificity of these assays and the requirements for sample handling. Together these assays allowed us to profile systemic complement changes after experimental renal IRI in mouse, showing major complement consumption in line with previously published results.

2. Methods

2.1. Animal materials

The Animal Care and Use Committee of the Leiden University Medical Center (LUMC) approved all animal experiments. The C57bl/6 mice were purchased from Charles River, the $C3^{-/-}$ and $C4^{-/-}$ mice (both on C57bl/6 background) were provided by Marina Botto (Imperial College, London, U.K.) and Mike Carroll (Harvard Medical School, Boston, MA) and bred as described previously (Otten et al., 2009). A/J mice with natural C5-deficiency (Wetsel et al., 1990) were purchased from Jackson laboratories (Bar Harbour, ME). The CD1 serum (NMS) was purchased from Innovative Research (Novi, MI). NMS and plasmas were prepared from CO_2 euthanized mice *via* heart puncture, stored on ice and prepared as described previously (Kotimaa et al., 2014). Briefly; serum (NMS) was let to clot 1 h at 4 °C and supernatant was collected. EDTA and Lepirudin plasmas were prepared by adding 10 mM EDTA or 50 μg/ml Lepirudin (r-hirudin; Pharmion, Germany) to the collected blood. To remove clot and cells, the samples were centrifuged twice at 3000–5000 *g* for 10 min at 4 °C, and the supernatant was pooled, aliquoted and stored at −80 °C.

2.2. Anti-mouse C9 polyclonal antibody

Polyclonal antiserum (pAb) against recombinant mouse C9 (rC9) was obtained by immunization of male New Zealand White rabbits (Harlan) with rC9 (kind gift of Prof. Piet Gross, Utrecht, The Netherlands). Injection of 30 μg rC9 in 100 μl complete Freund's adjuvant (Difco, Detroit, MI) subcutaneously was followed by three boosts with 30 μg mouse rC9 in 100 μl incomplete Freund's adjuvant (Difco) at 2-week intervals. Rabbit pAb anti-mouse C9 was prepared as described previously (Trouw et al., 2004), with minor modifications: fractions were tested for the presence of anti-mouse rC9 reactivity with a direct ELISA. ELISA plate was coated with purified mouse rC9 at 2.5 μg/ml, serial dilutions of the fractions in PBS/0.05% Tween/1% BSA were tested and binding of rabbit IgG was demonstrated using goat anti-rabbit IgG conjugated to HRP (Jackson ImmunoResearch Laboratory Inc., PA). Mouse rC9 reactive fractions were pooled, concentrated and dialysed against PBS.