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Prevention of experimental autoimmune myasthenia gravis by rat Crry-Ig: A model agent for long-term complement inhibition in vivo

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Abstract

Despite its vital role in innate immunity, complement is involved in a number of inflammatory pathologies and has therefore become a therapeutic target. Most agents generated for anti-complement therapy have short half-lives in plasma, or have been of mouse or human origin, thereby limiting their use either to murine models of disease or to short-term therapy. Here we describe the generation of a long-acting rat therapeutic agent based on the rat complement inhibitor, Crry. Characterisation of various soluble forms of Crry demonstrated that the amino-terminal four short-consensus repeat domains were required for full regulatory and C3b-binding activities. Fusion of these domains to rat IgG2a Fc generated an effective complement inhibitor (rCrry-Ig) with a circulating half-life prolonged from 7 min for Crry alone to 53 h for rCrry-Ig. Systemic administration of rCrry-Ig over 5 weeks generated a weak immune response to the recombinant agent, however this was predominantly IgM in nature and did not neutralise Crry function or cause clearance of the agent from plasma. Administration of rCrry-Ig completely abrogated clinical disease in a rat model of myasthenia gravis whereas soluble Crry lacking the immunoglobulin Fc domain caused a partial response. rCrry-Ig not only ablated clinical disease, but also prevented C3 and C9 deposition at the neuromuscular junction and inhibited cellular infiltration at this site. The long half-life and low immunogenicity of this agent will be useful for therapy in chronic models of inflammatory disease in the rat.

Keywords: Complement; Crry; EAMG; Myasthenia gravis; Therapy

1. Introduction

The complement (C) system forms part of the innate immune system; activation brings about target cell death or damage, opsonisation of pathogens, induction of inflammatory responses and clearance of immune complexes. The critical activation step is enzymatic cleavage of C3 or C4 by multi-molecular enzymes termed convertases, and deposition of the active components (C3b or C4b, respectively) on the target membrane. Nascent C3b and C4b bind indiscriminately to pathogens and adjacent host cells. To prevent inappropriate damage, self-cells express on their membranes complement regulatory proteins (CReg). These function by inactivating the convertases formed during C activation or by preventing formation of the membrane attack complex (MAC). In humans, membrane cofactor protein (MCP; CD46), decay accelerating factor (DAF; CD55) and complement receptor 1 (CR1; CD35) inactivate the convertases by accelerating their natural decay or by acting as a cofactor for the factor I (fI) mediated cleavage of C3b and C4b (Kim and Song, 2006). Rodents have a unique regulator of the convertases called Crry which both increases decay of the convertases as well as acting as a cofactor for fI (Wong and Fearon, 1985; Quigg et al., 1993; Takizawa et al., 1994; Li et al., 1993; Kim et al., 1995). All

Abbreviations: AChR, acetylcholine receptor; ADEAE, antibody-augmented demyelinating EAE; AP, alternative pathway; BuTx, Bungarotoxin; C, complement; CHO, Chinese hamster ovary; CP, classical pathway; CR1, complement receptor 1; CRegs, complement regulators; DAF, decay accelerating factor; EAE, experimental autoimmune encephalitis; EAMG, experimental autoimmune myasthenia gravis; FACS, fluorescent activated cell sorting; HRPO, horseradish peroxidase; MAC, membrane attack complex; NMJ, neuromuscular junction; OPD, 1,2-phenylenediamine dihydrochloride; PBS, phosphate-buffered saline; sCR1, soluble recombinant CR1; SCR, short-consensus repeat; sCrry, soluble recombinant form of rat Crry containing the four N-terminal SCRs; SPR, surface plasmon resonance

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these CReg are comprised from short-consensus repeats (SCR), domains of approximately 60 amino acids in which the functional activity of the protein resides. CR1 consists of 30 SCR domains, DAF and MCP have four whilst rat Crry is comprised from either 6 or 7 SCRs due to alternative splicing of the gene.

In health, CReg are sufficient to protect the host from complement damage. However, in disease, dysregulated C activation can damage self-cells and exacerbate pathology. Complement is implicated in ischaemia/reperfusion injury, transplant rejection and a number of inflammatory diseases, such as systemic lupus erythematosus, autoimmune arthritis, multiple sclerosis and myasthenia gravis where activation triggers and sustains a 'vicious cycle' of inflammation and tissue damage (Morgan and Harris, 2003). In myasthenia gravis, an antibody-mediated disease of the neuromuscular junction (NMJ), C activation products have been found at the motor end plate (Lennon et al., 1978; Engel and Arahata, 1987; Sahashi et al., 1980). Involvement of C in pathology is demonstrated in rodent models by protective effects of C5 or C6 deficiency (Christadoss, 1988; Chamberlain-Banoub et al., 2006), amelioration of disease by anti-C therapeutics targeted either to activation pathways or to C5 (Piddlesden et al., 1996; Hepburn et al., 2007), and enhancement of disease in mice deficient in CReg (Morgan et al., 2006).

Due to the implication of C in inflammatory diseases a number of different anti-C therapeutics has been developed. Some of these reagents have reached clinical trials. A soluble, recombinant form of CR1, sCR1, has been tested in numerous animal models and reached clinical trials for acute conditions such as adult respiratory distress syndrome and cardiopulmonary bypass (Zimmerman et al., 2000; Rioux, 2001). Pexelizumab, a single chain Fv (scFv) which binds human C5 and prevents its cleavage, is in advanced stages of development for use in myocardial infarction (Whiss, 2002; Fleisig and Verrier, 2005). These reagents have been limited to acute therapies as they have short circulating half-lives necessitating constant administration in vivo. Eculizumab, an intact monoclonal antibody that binds C5, has a longer half-life in vivo and has been particularly successful in treatment of paroxysmal nocturnal haemoglobinuria and is being trialled in other chronic autoimmune indications (Hill et al., 2005; Kaplan, 2002).

A number of different approaches have been used to increase the circulating half-lives of therapeutic reagents. One approach has been generation of antibody-like molecules where the therapeutic moiety replaced the Fab arms of antibody. This approach was used to increase the circulating half-life of the C inhibitor, DAF, from 20 min to 33 h and to create a murine Crry-Ig with a half-life of 40 h (Harris et al., 2002; Quigg et al., 1998). Such reagents have proved therapeutically effective in antigen-induced arthritis, glomerulonephritis and intestinal ischaemia–reperfusion injury (Harris et al., 2002; Quigg et al., 1998; Rehrig et al., 2001). Reagents generated to date have been based upon human or mouse proteins, limiting their use in rat models due to their immunogenicity. In order to enable the testing of such reagents in rats we generated rCrry-Ig, a rat CReg-rat Fc protein consisting of rat Crry and the Fc of rat IgG2a. We compared rCrry-Ig to soluble Crry lacking an Fc domain both in vitro and in vivo. Tethering Crry to the Fc created a reagent with a greatly increased in vivo half-life that was capable of protecting against disease in a model of myasthenia gravis. This result paves the way for testing such reagents in chronic disease models in the rat.

2. Materials and methods

2.1. Materials

Chemicals and reagents were from Fisher Scientific (Loughborough, UK) or Sigma (Poole, UK) unless otherwise stated. All tissue culture reagents and plastics were from Invitrogen Life Technologies (Paisley, UK). pDR2ΔEF1α was a gift from Dr. I. Anegon (INSERM U437, Nantes, France; Charreau et al., 1994). The cell line expressing the monoclonal antibody, TLD1C11, was from Prof. W. Hickie (Dartmouth, USA). Chinese hamster ovary (CHO) cells expressing surface-bound rat Crry were from Dr. O.B. Spiller (Cardiff, UK). Sheep erythrocytes in Alsever's solution were from TCS microbiology (Claydon, UK), guinea pig erythrocytes and rat serum were from the local animal facility. Rabbit anti-rat Crry polyclonal antiserum and polyclonal sheep anti-human C9 were prepared in-house using standard immunisation procedures. The hybridoma expressing mouse anti-human C3b (C3-30) was a gift from Novartis (Horsham, UK; Kemp et al., 1992). Polyclonal goat anti-rat C3c was from Nordic Laboratories (Copenhagen, Denmark) and polyclonal sheep anti-human C3c antiserum was obtained from The Binding Site (Birmingham, UK). The rat hybridoma TIB-175, secreting the anti-AChR mAB35 was obtained from the American Tissue Culture Collection; antibody was purified as described previously (Tzartos et al., 1987; Chamberlain-Banoub et al., 2006). Mouse anti-rat CD68 was obtained from Serotech (Oxford, UK).

PBS is 8.1 mM Na_2PO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4. CFD (Complement fixation diluent; Oxoid) is 2.8 mM barbituric acid, 145.5 mM NaCl, 0.8 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.9 mM sodium barbital, pH 7.4. Alternative pathway (AP) buffer is 5 mM sodium barbitone, 150 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA, pH 7.4. FACS buffer is PBS containing 0.1% sodium azide, 1% BSA and 10 mM EDTA. Borate-buffered saline is 100 mM boric acid, 25 mM sodium borate, 75 mM NaCl, pH 8.3.

2.2. Generation of cell lines expressing recombinant proteins

DNA encoding the signal peptide and the three (C-terminal residue Leu193) or four (C-terminal residue Lys256) N-terminal SCR of rat Crry was amplified by PCR from rat oligodendroglioma cell cDNA (33B; obtained from ECACC), the antisense primer encoded a stop codon. DNA was ligated into the pDR2ΔEF1α expression vector. To generate the rCrry-Ig protein, DNA encoding the Fc (hinge, CH2 and CH3 domain) of rat IgG2a was ligated into pDR2ΔEF1α to generate the pDR2-Fc vector and DNA encoding the signal peptide and four SCR of rat