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Membrane attack complex of complement is not essential for immune mediated demyelination in experimental autoimmune neuritis

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ABSTRACT

Antibody deposition and complement activation, especially membrane attack complex (MAC) formation are considered central for immune mediated demyelination. To examine the role of MAC in immune mediated demyelination, we studied experimental allergic neuritis (EAN) in Lewis rats deficient in complement component 6 (C6) that cannot form MAC.

A C6 deficient Lewis (Lewis/C6−) strain of rats was bred by backcrossing the defective C6 gene, from PVG/C6− rats, onto the Lewis background. Lewis/C6− rats had the same C6 gene deletion as PVG/C6− rats and their sera did not support immune mediated haemolysis unless C6 was added. Active EAN was induced in Lewis and Lewis/C6− rats by immunization with bovine peripheral nerve myelin in complete Freund's adjuvant (CFA), and Lewis/C6− rats had delayed clinical EAN compared to the Lewis rats. Peripheral nerve demyelination in Lewis/C6− was also delayed but was similar in extent at the peak of disease. Compared to Lewis, Lewis/C6− nerves had no MAC deposition, reduced macrophage infiltrate and IL-17A, but similar T cell infiltrate and Th1 cytokine mRNA expression. ICAM-1 and P-selectin mRNA expression and immunostaining on vascular endothelium were delayed in Lewis C6− compared to Lewis rats' nerves.

This study found that MAC was not required for immune mediated demyelination; but that MAC enhanced early symptoms and early demyelination in EAN, either by direct lysis or by sub-lytic induction of vascular endothelial expression of ICAM-1 and P-selectin.

1. Introduction

Immune mediated demyelination is a major pathological feature of a number of diseases including multiple sclerosis (MS) (Raine, 1994), Guillain–Barre Syndrome (GBS) (Hartung et al., 2002) and chronic inflammatory demyelinating polyneuropathy (CIDP) (Hahn, 1996). Antibody activation of MAC is widely considered to mediate demyelination in these diseases and deposits of antibody and complement components, including MAC, are present in areas of demyelination in multiple sclerosis (Compston et al., 1989; Lucchinetti et al., 2000; Prineas and Graham, 1981; Storch et al., 1998), GBS (Hafer-Macko et al., 1996; Koski, 1987; Lu et al., 2000; Putzu et al., 2000; Wanschitz et al., 2003) and CIDP (Daeron, 1997; Dalakas and Engel, 1980). MAC has been found in CSF (Mollnes et al., 1987; Yam et al., 1980), at sites of active demyelination, on oligodendrocytes as well as degraded myelin (Lucchinetti et al., 2000; Storch et al., 1998). A role for antibody-mediated injury is supported by the ability of antibodies from patients with GBS and CIDP to induce peripheral nerve injury in animals either by systemic (Yan et al., 2000) or local injection (Pollard et al., 1995). Antibody activation of MAC is considered a major mediator of demyelination in animal models such as experimental autoimmune encephalomyelitis (EAE) (Pender, 1988) and experimental autoimmune neuritis (EAN) (Lampert, 1969; Wisniewski et al., 1969). Deposition of MAC preceded demyelination in EAN (Stoll et al., 1991). While EAE and EAN are studied as models of multiple sclerosis and GBS respectively, the active forms of these models are principally $CD4^+$T cell and macrophage mediated, and may not replicate the antibody component of demyelination seen in human diseases. Even so, there is debate about whether MAC is essential for induction of demyelination in active EAE and EAN.

The complement system is activated via the lectin, classical and alternate pathways (Barnum, 2002; Morgan, 1999), that converge to activate C5 to C5a and C5b (Guo and Ward, 2005). C5b sequentially binds C6, C7, C8 and multiple C9 molecules to form the C5b-9 complex, known as MAC (Barnum, 2002; Morgan, 1999; Turner, 1996). C8 and C9 insertion into lipid bi-layers causes cell lysis (Hadders et al., 2007). Absence of any components of MAC prevents its assembly and function.

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Host cells, including oligodendrocytes (Zajicek et al., 1995), are protected from MAC mediated injury by CD59, which binds to C8 and C9 and prevents pore formation (Hadders et al., 2007), so that EAE is more severe with CD59 deficiency (Mead et al., 2004). MAC also has sub-lytic actions including pro-inflammatory activation of vascular endothelium that promotes T cell and macrophage infiltrate (Kilgore et al., 1995; Tedesco et al., 1997; Tran et al., 2002). In immune mediated models of neurological disease MAC also has neuro-protective actions, including induction of cell cycle (Rus et al., 1996) and prevention of oligodendrocyte apoptosis (Soane et al., 1999).

Animals deficient in C6, such as the PVG/C6− (Leenaerts et al., 1995) and Lewis/C6− strains (Chamberlain-Banoub et al., 2006) do not form MAC, and allow direct examination of the role of MAC without the confounding effects of loss of earlier components of complement that occurs in C3 (Calida et al., 2001; Nataf et al., 2000) and C5 (Weerth et al., 2003) deficient animals. Severity of active EAE in PVG/C6− rats is reduced (Tran et al., 2002), as sub-lytic MAC does not activate endothelial cells to express P-selectin and ICAM-1, which are required to promote T cell and macrophage migration (Tran et al., 2002). Demyelination is not a prominent feature of active EAE unless anti-MOG antibodies are present, and anti-MOG antibodies do not induce demyelination in PVG/C6− rats (Mead et al., 2002; Tran et al. unpublished data).

In this study, we examined the role of MAC in the active EAN model, because in EAN marked demyelination of peripheral nerves occurs without transfer of anti-MOG antibodies or other antibodies (Wisniewski et al., 1969). Antibody and MAC deposits appear on myelin and Schwann cells before cellular infiltrate in EAN (Stoll et al., 1991). Peripheral nerve demyelination can be induced by intraperitoneal injection of EAN serum and complement (Harvey et al., 1995; Harvey and Pollard, 1992), or intraneural injection of EAN serum combined with activated T cells (Harvey and Pollard, 1992). These findings can be interpreted as auto-antibody activation of MAC playing a central role in the demyelination of nerves in EAN.

Not all strains of rat are susceptible to EAN (Steinman et al., 1981) and we found that PVG and PVG/C6− rats developed a milder form of EAN compared to Lewis rats. Thus, we backcrossed PVG/C6− to Lewis rats and bred a Lewis/C6− strain. We induced active EAN in Lewis/C6− and demonstrated marked demyelination in the peripheral nerves. These studies demonstrated that MAC is not the sole or essential mediator of demyelination in EAN and that there are other immune mediators of demyelination.

2. Materials and methods

2.1. Animals

PVG, PVG/C6− (Merten et al., 1998), Lewis, and Lewis/C6− were bred at the Animal House of Liverpool Hospital. All experiments were approved by the University of New South Wales Animal Ethics Committee.

Lewis rats deficient in C6 (Lewis/C6−) were bred by backcrossing PVG/C6− to Lewis rats once. The progeny were inbred and those homozygous for C6 deficiency were then cross-bred with normal Lewis. This cycle of inbreeding then crossing with Lewis was repeated 11 times. To establish their genetic identity skin grafts were exchanged between Lewis/C6− and Lewis rats, as described (Roser and Ford, 1972). Skin grafts were observed for over 100 days, and were healthy with normal hair growth.

2.2. Haemolytic assay

Haemolytic complement assay was as described (Tran et al., 2002; Spicer et al., 2007) and to confirm C6 deficiency, human C6 (Sigma) was added (Spicer et al., 2007). Briefly, human red blood cells were incubated with rat anti-human red blood cell antibody that had been produced by immunization of Sprague Dawley rats and had been inactivated of complement at 56° for 60 min, as described (Tran et al., 2002; Spicer et al., 2007). 50 µl sera from individual rats were plated in triplicate to the wells of 96 well round bottom microtiter plates (Bio-Rad) and 150 µl of the RBC anti-red blood cell sera preparations was added to each well, before incubation at 37 °C for 1 h. Controls included known normal rat sera and C6− sera, and lysis was mediated by addition of 5% Triton X-100, which lysed all cells. Lysis was detected by red coloration of the supernatant and lack of a red cell pellet at the bottom of the well. With C6− sera there was a red blood cell pellet at the bottom of the well and no discoloration of the supernatant, as described (Tran et al., 2002; Spicer et al., 2007).

2.3. Experimental design

Bovine peripheral nerve myelin (PNM) was prepared as described (Norton and Poduslo, 1973). Ten to twelve week old female Lewis rats and Lewis/C6− were immunized in the footpads with 200 µl of emulsion containing 4 mg PNM and 1.5 mg of complete Freund's adjuvant (CFA) prepared by emulsifying heat killed *Mycobacterium tuberculosis* (strain H37RA; Difco, Detroit, MI) in 100 µl saline and 100 µl incomplete Freund's adjuvant (Difco). Animals were monitored daily for weight and clinical disease activity scored as: 1+ limp tail, 2+ hind leg weakness, 3+ paraplegia, and 4+ quadriplegia.

In each experimental group, extra rats were immunized to be used at days 14 and 21 post-immunization to obtain cauda equina for histology, ultrastructure, immunopathology and mRNA extraction as described (Tran et al., 2002). The samples were taken at day 14 as this is shortly after onset of clinical disease and demyelination is first evident. Day 21 is post peak of clinical disease and when demyelination is maximal. Combining animals from four experiments there were 5–6 rats in each group for these analyses including immunostaining, ultrastructure and RT-PCR analysis.

2.4. Anti-PNM antibody assay

Serum collected at day 14 post-immunization was assayed in an ELISA essentially as described (Tran et al., 2001) except that PNM antigen was used at a concentration of 25 µg/ml in coating buffer. Control serum was from a hyper-immunized Lewis rat that had recovered from EAN and had been re-immunized with PNM in incomplete Freund's adjuvant at day 30 and had sera collected 14 days later. Results were expressed as a percentage of the control hyperimmune sera.

2.5. Preparation of nerve specimens for light and electron microscopy

Cauda equina segments 5 mm in length were fixed for 15 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4. They were then embedded in epoxy resin. Transverse semi-thin 0.5 micron sections were cut for light microscopy and corresponding ultra-thin sections (130 nm) were cut for transmission electron microscopy.

Sections for light microscopy were stained with 2% methylene blue in 1% borax and were examined with an Olympus BH-2 microscope (Olympus Corporation, Tokyo, Japan) fitted with a Spot RT Slider digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Digital images of nerve fibre bundles were taken at ×20 objective and areas of demyelination in nerves were quantified using computer-based image analysis (Image-Pro Plus, Media Cybernetics, Silver Spring, MD).

Processing for electron microscopy was as described (Spicer et al., 2007). Briefly, ultra-thin sections were stained with uranyl acetate 2% and Reynolds' lead citrate 2.6% and samples were viewed with an FEI Morgagni 268D transmission electron microscope (FEI, Eindhoven, The Netherlands) at 80 kV.