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The murine gammaherpesvirus-68 chemokine-binding protein M3 inhibits experimental autoimmune encephalomyelitis

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ABSTRACT

Chemokines are critical mediators of immune cell entry into the central nervous system (CNS), as occurs in neuroinflammatory disease such as multiple sclerosis. Chemokines are also implicated in the immune response to viral infections. Many viruses encode proteins that mimic or block chemokine actions, in order to evade host immune responses. The murine gammaherpesvirus-68 encodes a chemokine-binding protein called M3, which has unique biochemical features that enable it to bind to and inhibit an unusually broad range of chemokines. We applied a replication-defective adenoviral vector encoding M3 (AdM3) directly to the CNS to evaluate the capacity of this protein to inhibit neuroinflammation using the experimental autoimmune encephalomyelitis (EAE) model. Treatment with the AdM3 vector significantly reduced the clinical severity of EAE, attenuated CNS histopathology, and reduced numbers of immune cells infiltrating the CNS. These results suggest that M3 may represent a novel therapeutic approach to neuroinflammatory disease.

1. Introduction

The migration of immune cells from the periphery into the central nervous system (CNS) is a key feature of neuroinflammation, as seen in the disease multiple sclerosis (MS) and the animal model experimental autoimmune encephalomyelitis (EAE). Chemokines are abundantly expressed during both MS and EAE, and they play an essential role in disease pathogenesis (Ransohoff, 2002; Sospedra and Martin, 2005).

Experimental interventions using chemokine-blocking antibodies and chemokine or chemokine receptor knockouts have contributed much to our understanding of the biology of chemokines in CNS inflammation (Glabinski et al., 1995, 1997, 1998, 2003; Godiska et al., 1995; Hulkower et al., 1993; Ransohoff et al., 1993; Tran et al., 2000a). Nevertheless, the literature on chemokines and EAE is fraught with complexities and can often be difficult to interpret, due in part to the variety of EAE models used, the constraints of conventional knockout approaches to readily address timing, and the limitations of peripheral treatments to penetrate the CNS. As well, these studies typically examine the role of single chemokines or receptors, and while this is to some extent a limitation of the tools available, such an approach does not address the high degree of redundancy which is a hallmark of the chemokine system. There is therefore a need to employ an experimental strategy that can target multiple chemokines simultaneously. Viral chemokine-binding proteins offer one possibility for such a strategy.

The chemokine system fulfils a crucial role in coordinating host immune responses to viral infection by directing the composition and distribution of responding immune cells. Consequently, several viruses have evolved sophisticated strategies to evade the host immune system and facilitate their propagation by circumventing or co-opting chemokine responses (Seet and McFadden, 2002). These strategies generally take three forms: the production of proteins which mimic chemokines themselves; proteins which have structural similarity to chemokine receptors; and proteins which bind chemokines and inhibit their interactions with endogenous receptors and glycosaminoglycans (GAGs) (Alcami and Koszinowski, 2000; Holst and Rosenkilde, 2003).

One example of this is the murine gammaherpes virus-68 (MHV-68), a natural pathogen of wild rodents, which has homology with other members of the gammaherpesvirus family, including Epstein–Barr virus, herpesvirus saimiri, and Kaposi's sarcoma-associated herpesvirus (HHV-8) (Simas and Efstathiou, 1998; Virgin et al., 1997). MHV-68 encodes a secreted 44 kDa protein called M3, translated from a 1.4 kb early–late lytic transcript (van Berkel et al., 1999). Although a number of

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herpesviruses encode homologues of chemokines and cellular chemokine receptors, this is the first protein identified in the herpesvirus family that has chemokine-binding activity (Parry et al., 2000; Seet et al., 2003). In contrast to most chemokine receptors and other viral chemokine-binding proteins, M3 can bind chemokines of all four classes: CC, CXC, C and CX3C (Parry et al., 2000; van Berkel et al., 2000). This is achieved by interactions with specific amino acid residues that are required for chemokine homodimerization and binding to endogenous receptors (Alexander et al., 2002). Conformational flexibility of M3 as well as electrostatic interactions facilitates binding to a wide range of chemokine targets (Alexander et al., 2002). Furthermore, M3 has been shown to prevent chemokine binding to GAGs, and to disrupt established chemokine gradients (Webb et al., 2004).

To evaluate the effects of broad spectrum chemokine blocking in EAE, we used a replication-defective adenoviral vector encoding the *M3* gene to administer this chemokine-binding protein directly to the CNS via intrathecal injection. Treatment with the AdM3 vector led to a significant reduction in the clinical severity of EAE. This was accompanied by a significant reduction in spinal cord inflammation, and reduced entry of immune cells to the CNS. This represents the first demonstration of clinical efficacy of MHV-68 M3 in the EAE model.

2. Materials and methods

2.1. Animals and EAE induction

Female wild-type C57BL6 mice were purchased from Taconic, Europe. EAE was induced by subcutaneous immunization with 100 µl of an emulsion containing 200 µg of $MOG_{35–55}$ (Dept. of Biochemistry and Molecular Biology, University of Southern Denmark) and complete Freund's adjuvant (Fisher Scientific, Slangerup, Denmark). Mice received 300 ng of Pertussis toxin (List Biochemicals) by intraperitoneal injection at the time of immunization and again 2 days later. Animals were monitored daily, and scored according to the following scheme: 0, asymptomatic; 1, loss of tail tonicity; 2, hind limb paresis (splayed hind limbs, disturbed righting reflex); 3, unilateral hind limb paralysis; 4, bilateral hind limb paralysis; and 5, moribund. All animal experiments were in accordance with the guidelines of the Danish Laboratory Animal Inspectorate (J. no. 2004/561-920).

2.2. Adenoviral vectors and intrathecal injections

The adenoviral vector encoding MHV-68 M3, AdM3, was generated as described (Hoegh-Petersen et al., 2009). Briefly, the *M3* gene was cloned from an M3 expressing plasmid into a pacCMV vector (Holst et al., 2008), and the adenovirus generated using standard methods. Infectious titre was measured using the Adeno-X Rapid Titer Kit (Clonetech) with HEK293 cells. Five days following immunization to induce EAE, mice were injected intrathecally with AdM3 or a control adenoviral vector encoding β-galactosidase (AdLacZ), as previously described (Millward et al., 2007). Under sterile conditions, a 30 gauge needle (bent at a 40° angle 3 mm from the tip) attached to a 50 µl Hamilton syringe was inserted into the intrathecal (subarachnoid) space of the cisterna magna (cerebellomedullary cistern). A 10 µl volume of sterile PBS containing the viral vector (10^7 infectious units) was slowly injected over approximately 30 s. Animals received post-operative analgesia.

2.3. Quantitative real-time PCR

RNA was extracted from brain and spinal cord using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was reverse-transcribed, and qPCR carried out as described (Wheeler et al., 2006). The following primer and probe sequences were used to detect MHV-68M3: forward primer, TGACCTAGCTGGCCTG-GATTCT; reverse primer, AAAGCTTGCTGGATGGTCTCA; MGB probe ACCTGTTTATGTGATGGCAGCA (Applied Biosystems). Data were acquired using an ABI Prism 7300 Sequence Detection System (Applied Biosystems), and are reported as the ratio of target gene expression over expression of 18S rRNA, which served as an endogenous reference.

2.4. Histology

Spinal cords were removed from PBS perfused mice and cut into 8 cross-sectional segments. The segments were embedded together in Cryo-Embed (Ax-lab), frozen in a methylbutane/dry ice mixture, then cut into 12 µm sections with a cryostat. Hematoxylin and eosin-stained sections were examined by light microscopy for the presence of inflammation. Semi-quantitative assessment was done by counting the number of quadrants which contained inflammatory infiltrates for each of the 8 segments. Quadruplicate sections for each mouse were assessed (blinded to treatment group) and data are presented as the percentage of total quadrants positive for inflammatory infiltrates.

2.5. Flow cytometry

Brain and spinal cord homogenates were prepared as described (Toft-Hansen et al., 2004). Cell suspensions were stained with rat anti-mouse CD45 (FITC), CD11b (PE), and Gr-1 (biotinylated followed by PerCP-conjugated streptavidin) for 20 min at room temperature, then washed. All antibodies were from BD Pharmingen (Brøndby, Denmark). Absolute cell numbers were determined with the use of BD TruCount beads added to each sample prior to data acquisition. Staining was detected with a Becton-Dickinson FACSCalibur flow cytometer, and analyzed with FlowJo version 7.1.3 (Tree Star Inc., Ashland, OR, USA).

2.6. Statistical analysis

Non-interval data (EAE clinical scores, histology scores) were analyzed with the non-parametric Mann–Whitney test. Flow cytometry data were analyzed using the Student's *t*-test. *p*-values less than 0.05 were considered significant. GraphPad Prism 4 (Graphpad Software) was used for the analysis.

3. Results

3.1. M3 expression detected after intrathecal administration of the AdM3 vector

In order to evaluate the potential for M3 to affect EAE, we administered a replication-defective adenoviral vector to the CNS via intrathecal injection into the cisterna magna. We and others have shown that this minimally-invasive approach can deliver a gene of interest directly to the CNS while avoiding tissue damage associated with an intraparenchymal injection into CNS tissue (Furlan et al., 2003; Millward et al., 2007). We verified expression of M3 mRNA in whole brain and whole spinal cord by quantitative real-time PCR, 25 days after administration of one dose of 10^7 ifu (Fig. 1). We have previously shown that message for genes transferred using adenoviral vectors and this approach can be detected as early as one day, and as late as six weeks after a single intrathecal administration. Message for M3 was not detected in brain or spinal cord of mice that received the control AdLacZ vector.

3.2. Administration of AdM3 attenuates the clinical severity of EAE

Mice that received one dose of 10^7 ifu of AdM3 5 days after immunization to induce EAE showed a significant reduction in disease severity as compared to mice that received the control AdLacZ vector (Fig. 2A). This effect was also reflected in a significant reduction in median cumulative disease activity in the AdM3 group, as determined by the area under the curve of clinical score plots for each individual