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Cuprizone inhibits demyelinating leukomyelitis by reducing immune responses without virus exacerbation in an infectious model of multiple sclerosis

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ABSTRACT

Multiple sclerosis is one of the most common demyelinating central nervous system diseases in young adults. Theiler's murine encephalomyelitis (TME) is a widely used virus-induced murine model for human myelin disorders. Immunosuppressive approaches generally reduce antiviral immunity and therefore increase virus dissemination with clinical worsening. In the present study, the progressive course of TME was significantly delayed due to a five-week cuprizone feeding period. Cuprizone was able to minimize demyelinating leukomyelitis without virus exacerbation. This phenomenon is supposed to be a consequence of selective inhibition of detrimental inflammatory responses with maintained protective immunity against the virus.

1. Introduction

Multiple sclerosis (MS) is one of the most common neurological disorders in young adults characterized by immune mediated demyelination in the central nervous system (CNS). Although the primary cause is unknown, several environmental factors, including virus infection are currently discussed as initiating events (Kurtzke, 1993). Major aims in the field of neuroscience are the prevention and attenuation of clinical signs and inflammation (Compston and Coles, 2008; Thompson et al., 2010). To achieve these goals, animal studies based on different etiologies are used and serve as translational models for human myelin disorders. Myelin-specific auto-aggressive lymphocytes can be found in experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis (TME), which contribute to spinal cord demyelination (Brown et al., 1982; Oleszak et al., 2004; Tsunoda and Fujinami, 2010). However, while EAE is a primary autoimmune disease (Baxter, 2007), in TME autoimmunity develops secondary to viral persistence and epitope spreading (Miller et al., 1997). In addition, toxic demyelination models, such as the cuprizone model gain increasing interest because of their predictable course of de- and remyelination in the murine CNS (Matsushima and Morell, 2001). Interestingly and somehow unexpectedly, in experimental studies combining cuprizone and EAE, a clinical improvement and reduced CNS damage can be found, despite the increased generation of myelin-specific leukocytes in diseased mice (Kimberlin and Millson, 1976; Emerson et al., 2001; Mana et al., 2009). Presumptive explanations for this phenomenon might be a cuprizone-induced T cell-suppression (Emerson et al., 2001) and/or an induction of immunological tolerance against myelin proteins, possibly attributed to the expansion of myelin-specific regulatory T cells (Mana et al., 2009). Cuprizone primarily affects the corpus callosum, but current studies have focused upon region specific susceptibilities of different CNS compartments (Komoly, 2005; Skripuletz et al., 2008, 2010; Herder et al., 2011), which showed, that, although attenuated, cuprizone also induces oligodendroglial alterations in the murine spinal cord. However, the potential ability of the toxicant to modulate immune responses within the spinal cord has not been investigated so far and the underlying mechanisms of a beneficial effect upon the clinical course and CNS preservation in immune mediated demyelination are poorly understood.

These experiments have raised the questions about the impact of cuprizone in a virus-induced MS model. It remains to be investigated whether cuprizone exhibits beneficial effects by inhibiting immune mediated tissue damage as observed in EAE or detrimental effects by reducing antiviral immunity, which may lead to disease exacerbation. This topic is of special relevance, since immunosuppressive therapies of infectious myelin disorders are able to reduce immune mediated tissue damage, and, simultaneously can increase the risk of reduced pathogen-specific immune responses (Young et al., 2008; Welsh et al., 2010). In the present study, for the first time,

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TME was combined with the cuprizone model. Based on the hypothesis that cuprizone is able to specifically reduce autoimmune leukomyelitis in mice the goal was to investigate the impact of cuprizone on virus-induced demyelination. Results of the present studies demonstrate a clinical improvement and reduced myelin loss in Theiler's murine encephalomyelitis virus (TMEV)-infected mice as a consequence of cuprizone induced diminished immune responses in the spinal cord without increasing virus replication.

2. Materials and methods

2.1. Animals

Five-week-old female SJL/JCrl mice (Charles River Laboratories, Sulzfeld, Germany) were group housed in IVC cages (Tecniplast GmbH Deutschland, Hohenpeißenberg, Germany) separated randomly into four treatment groups: group 1 (TMEV/CPZ) received TMEV-infection and cuprizone-feeding, group 2 (TMEV) virus only, animals of group 3 were mock-injected (mock) and group 4 animals gained cuprizone-feeding as well as mock-injection (CPZ/mock). Study design is illustrated in Fig. S1. At five weeks of age (0 days post infection [dpi]) TMEV- or mock-infection was performed intracerebrally into the right hemisphere as described (Ulrich et al., 2008). Animals were permitted free access to water and fed for five weeks (35 dpi until 70 dpi) *ad libitum* 0.2% (w/w) cuprizone (Bis(cyclohexanone) oxaldihydrazone, C9012, Sigma-Aldrich, Germany) mixed into a powdered standard rodent chow (ssniff® R/M-H, Soest, Germany) as described (Lindner et al., 2008). The food was freshly prepared daily. Animals without cuprizone-feeding received powdered standard chow from 35 to 70 dpi. Before and after cuprizone-treatment all animals were put on the same pellet chow *ad libitum*. Weekly clinical scoring (Ulrich et al., 2006) and RotaRod-measurements (TSE Systems GmbH, Bad Homburg v. d. Höhe, Germany) on an accelerating rod (30 mm diameter) from 5 rounds per minute (rpm) to 55 rpm in 5 min were performed. The rpm value when the animal fell off the rod was recorded automatically and three measurements per animal were performed and averaged. Groups of four to six animals were euthanized at 42, 49, 70, 98, 147 and 196 dpi and samples were taken for histology, immunohistochemistry and RT-qPCR. Brains were immediately removed and fixed in 10% formalin. After formalin-fixation cervical and thoracic spinal cord segments encased in the vertebral bodies were treated for 48 h in 10% disodium-ethylenediaminetetraacetate (EDTA). All tissues underwent a routine paraffin embedding. Additionally, snap frozen tissues from the thoracic spinal cord were taken. This study was conducted in accordance with German law for animal protection and with the European Communities Council Directive 86/609/EEC for the protection of animal used for experimental purposes. All animal experiments were approved and authorized by the Local Institutional Animal Care and Research Advisory committee and permitted by the local authorities (Regierungspräsidium, Hannover, Germany, permission number: 33.9-42502-04-07/1331).

2.2. Histology

Three micrometer thick transversal sections of the cervical and thoracic spinal cord as well as the corpus callosum (according to bregma: −0.94 mm) from each animal were stained with hematoxylin and eosin as well as Luxol-fast blue/cresyl violet staining (LFB). Leukomyelitis was evaluated using hematoxylin and eosin (HE)-stained transversal sections and a semiquantitative scoring system as follows: 0 = no changes; 1 = 1–25 inflammatory cells; 2 = 26–50 inflammatory cells; and 3 = >50 inflammatory cells in the entire white matter. Inflammatory cells include lymphocytes, plasma cells and macrophages. For evaluation of gitter cell-accumulation (presence of phagocytized myelin within microglia/macrophages) a semiquantitative scoring system was applied: 0 = no changes; 1 = gitter cells and loss of tissue structure up to 1/4 of the affected white matter; 2 = gitter cells and loss of tissue structure up to 1/2 of the white affected matter; and 3 = gitter cells and loss of tissue structure more than 1/2 of the affected white matter. Meningitis was evaluated as described (Ulrich et al., 2006). The scoring was performed blinded and separately on all 4 quarters of spinal cord transversal sections. For each animal the arithmetic average of leukomyelitis and gitter cell-accumulation was calculated and both parameters were investigated in cervical and thoracic spinal cord sections.

The scores of demyelination were assessed by the proportion of blue-stained (myelinated) *versus* pale (unmyelinated) fibers in the corpus callosum and spinal cord by LFB (Lindner et al., 2008). In addition, the medial and lateral regions (Taylor et al., 2009) of the corpus callosum were assessed. All slides were evaluated in a blinded manner.

2.3. Immunohistochemistry

The entire cross section of cervical and thoracic spinal cord segments was evaluated by immunohistochemistry detecting the following antigens: CD3, CD107b, CD45R, MBP, TMEV, non-phosphorylated neurofilament (npNF) as described (Gerhauser et al., 2007a, 2007b; Kummerfeld et al., 2009; Navarrete-Talloni et al., 2010; Seehusen and Baumgärtner, 2010). Foxp3 (regulatory T cells; eBioscience, San Diego, CA, USA) was used in a 1:20 dilution applying the ABC- (Vectastain Elite ABC Kit; PK-6100, Vector Laboratories Inc., Burlingame, USA) and DAB-method. For negative controls the primary antibodies were replaced by anti-isotype antibodies in the same concentration as the primary antibody. The absolute numbers of npNF-positive axons within the white matter of the thoracic spinal cord segment were counted in a blinded manner. For evaluation of CD3-, CD45R-, CD107b-, TMEV, and FoxP3-immunohistochemistry, the total number of immunoreactive cells was counted in cervical and thoracic spinal cord transversal sections in a blinded manner. Using the analySIS® 3.2 software (SOFT Imaging system, Münster, Germany) the MBP-positive white matter area was measured after manually outlining the total white matter area in case of spinal cord in a digitalized photomicrograph with 4-fold objective. For each antibody the same threshold settings of the software were used to determine the positive area. The MBP-positive white matter area of each animal was calculated as percentage of the outlined white matter area.

2.4. Quantitative polymerase chain reaction

For reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), RNA was isolated from the thoracic spinal cord segments at 42, 49, 70, 98, 147, and 196 dpi using the RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription was performed as described (Pringproa et al., 2008). Primer sequences for the detection of TMEV (Ulrich et al., 2006), Foxp3 (Hansenne et al., 2009), GAPDH, ß-actin and HPRT (Ulrich et al., 2005) mRNA were taken from the literature. Primers for the other genes were as follows: IL-1α sense: AAG CAA CGG GAA GAT TCT GA, IL-1α antisense: TGA CAA ACT TCT GCC TGA CG; IL-2 sense: GCA GGA TGG AGA ATT ACA GGA, IL-2 antisense: TGA AAT TCT CAG CAT CTT CCA A; IL-4 sense: CCT CAC AGC AAC GAA GAA CAC C, IL-4 antisense: CAT CGA AAA GCC CGA AAG AGT C; IL-10 sense: CCA AGC CTT ATC GGA AAT GA, IL-10 antisense: TTT TCA CAG GGG AGA AAT CG; IL-12 sense: AGG TGC GTT CCT CGT AGA GA, IL-12 antisense: AAA GCC AAC CAA GCA GAA GA; IFN-γ sense: CAC GGC ACA GTC ATT GAA AG, IFN-γ antisense: AAT CTG GCT CTG CAG GAT TT; TGFβ1 sense: TTG CTT CAG CTC CAC AGA GA, TGFβ1 antisense: TGG TTG TAG AGG GCA AGG AC; TNF-α sense: GCC TCT TCT CAT TCC TGC TT, TNF-α antisense: CAC TTG GTG GTT