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Probenecid-treatment reduces demyelination induced by cuprizone feeding

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ABSTRACT

Recent experiments showed that a pannexin-1 inhibitor, probenecid, reduced clinical symptoms in the murine experimental autoimmune encephalomyelitis when applied during the initial phase of neuronal inflammation. An inflammatory component is also present in a toxically induced inflammation and demyelination using cuprizone diet. Probenecid is a pannexin-1 antagonist and a probenecid therapy was investigated. Mice were fed for 10 days with a cuprizone diet. In the following, the diet was continued but combined with a daily injection of a low dose of probenecid or solvent for 10 days. Electron microscopy revealed demyelination in the optic nerve. The demyelination as measured by the axonal diameter was significantly reduced in the animals treated with 100 mg per kg body weight probenecid. In comparison to controls, the number of leukocytes and lymphocytes in the peripheral blood was reduced in all cuprizone groups including the treatment group. In conclusion, early demyelination in the optic nerve was moderately reduced by 10 days treatment with a low dose probenecid. This is a hint for the involvement of pannexin-1 modulated inflammation in cuprizone feeding induced toxic demyelination. Thus, probenecid is a candidate for the treatment of neuro-inflammation and multiple sclerosis.

1. Introduction

In experimental research on inflammation and demyelination in multiple sclerosis, two murine models are commonly used: the experimental autoimmune encephalomyelitis and the cuprizone diet (Gudi et al., 2014; Praet et al., 2014). In experimental autoimmune encephalomyelitis, mice develop a specific immunity against molecules of the myelin sheaths in the central nervous system. This results in neuronal inflammation, which leads to the destruction of myelin sheaths and axons. Many approaches of treatment have been applied and tested, both clinically and experimentally. As recently reported, a daily application of low dose probenecid with 100 mg per kg body weight prevented the onset and reduced the symptoms of experimental autoimmune encephalomyelitis (Hainz et al., 2016). Therefore, it was a challenging approach to investigate a potential effect of the same treatment in an alternative disease model of multiple sclerosis. Anti-inflammatory effects and the prevention of tissue damage were also reported for probenecid in murine pneumonia caused by *Pseudomonas aeruginosa* (Wonnenberg et al., 2014). The reduced activity of the NLRC4-inflammasome was assumedly responsible for the effect (Wonnenberg et al., 2016). A diet containing cuprizone leads to loss of oligodendrocytes, glial activation and inflammation and to a demyelination of axons in the central nervous system (Gudi et al., 2014). Cuprizone is a chelator of copper but the pathogenesis is not fully understood. The deprivation of copper presumably has somehow a toxic effect. Surprisingly, it was shown recently that the Nlrp3 inflammasome is also involved in the cuprizone-induced inflammation (Jha et al., 2010). The aim of this study was now to evaluate whether probenecid can prevent early demyelination in the cuprizone model of multiple sclerosis.

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2. Material and methods

2.1. The cuprizone model and treatment

C57BL/6J mice (male, 8 weeks old) were kept under standard conditions (12 h day/night, 20 °C, humidity 50%). The chow was mixed with 0.2% cuprizone (ssniff special diets, Soest, Germany). It was stored at 4 °C and replaced daily. Chow and water were ad libitum. The experiments were approved by the Saarländische Landesregierung (Saarland State Government). The whole experiment lasted 21 days from the beginning to the analysis (Fig. 1). The cuprizone/cuprizone (CPZ/CPZ) group (n = 11) was fed for 21 days with the cuprizone containing chow. The CPZ/CPZ + solvent group (n = 10) received the same diet but was also injected i.p. daily with the treatment solvent (800 μl 1N NaOH, 2320 μl Tris buffer, 12 ml NaCl, pH 7.38) from day 11 to 20. The CPZ/CPZ + probenecid (n = 12) group was additionally injected i.p. daily with probenecid (100 mg per kg body weight) from day 11–20 (150 mg probenecid, 800 μl 1 N NaOH, 2320 μl Tris buffer, 12 ml NaCl, ph 7.34). The control group (n = 6) was fed with normal chow for 21 days (for overview, please, see the schematic drawing of the experimental set ups and groups in Fig. 1). The animals were under constant observation.

2.2. Electron microscopy and analysis of axonal morphology in the optical nerve

The optic nerve of three animals of each experimental group was carefully excised and immersed in fixation solution (1% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer) at 4 °C before for 24 h further processing. After repeated rinsing in 0.1 M cacodylate buffer, samples were osmicated for 2 h in 2% osmium tetroxide (OsO4) in 0.1 M cacodylate buffer. After three rinses in distilled water, the specimens were dehydrated in an ascending series of ethanol (70%, 80%, 90%, 96%, and 100%) and acetone (100%, water-free). Embedding in Epon resin followed. Semi-thin (300 nm) and ultra-thin sections (65 nm) were cut using an ultra-microtome (Leica EM UC7, Leica Microsystems, Wetzlar, Germany). Semi-thin sections were stained with Richardson blue staining solution, while ultra-thin sections were stained with lead citrate for 5 min. Ultra-thin sections were investigated using a transmission electron microscope (Tecnai G2, FEI, Hillsboro, OR, USA) and areas were chosen at random and documented.

For each animal, nine photographs were recorded. The g-ratio and the axonal diameter were measured at 15 locations and summarized using medians for each photograph. The g-ratio was determined as the quotient of the axon diameter without the myelin sheath, divided by the whole diameter. A low g-ratio represents normal myelination whereas a g-ratio close to 1.0 represents a high degree of demyelination. Per animal at least 100 single g-ratios of single axons were determined on nine original electron microscopic photographs. Per photograph, a median g-ratio was calculated. The approach of generalized estimating equations (GEE) were used to model g-ratio and axonal diameters statistically as continuous dependent variables (for details see 2.5).

2.3. Flow cytometry

Samples of blood and spleen of at least 6 animals per group were analysed. Blood was taken by heart puncture and 100 μl was analysed per animal. Cells of whole spleens were obtained through rinsing in 70 μm cell sieves. Cell suspensions were incubated with a lysis buffer to remove erythrocytes. Nucleated cells were counted using a Luna-II automated counter (Biozym Scientific, Hessisch Oldendorf, Germany). All antibodies (anti-CD3/FITC 1:500, anti-CD4/APC 1:800 and anti-CD25/PE 1:200) were purchased from BioLegend (Fell, Germany) and used according to the manual instructions. Briefly, 10^6 cells were incubated at room temperature in the dark for 45 min and washed twice using phosphate buffer solution (centrifugation with 400*g*). Cells were analysed using a FACS Calibur (BD Biosciences, Heidelberg, Germany). The lymphocyte fraction was determined and all subsets were calculated.

2.4. Determination of cytokine concentrations

The concentrations of the inflammatory cytokines IL-1β and TNFα were measured from brain homogenate using an enzyme-

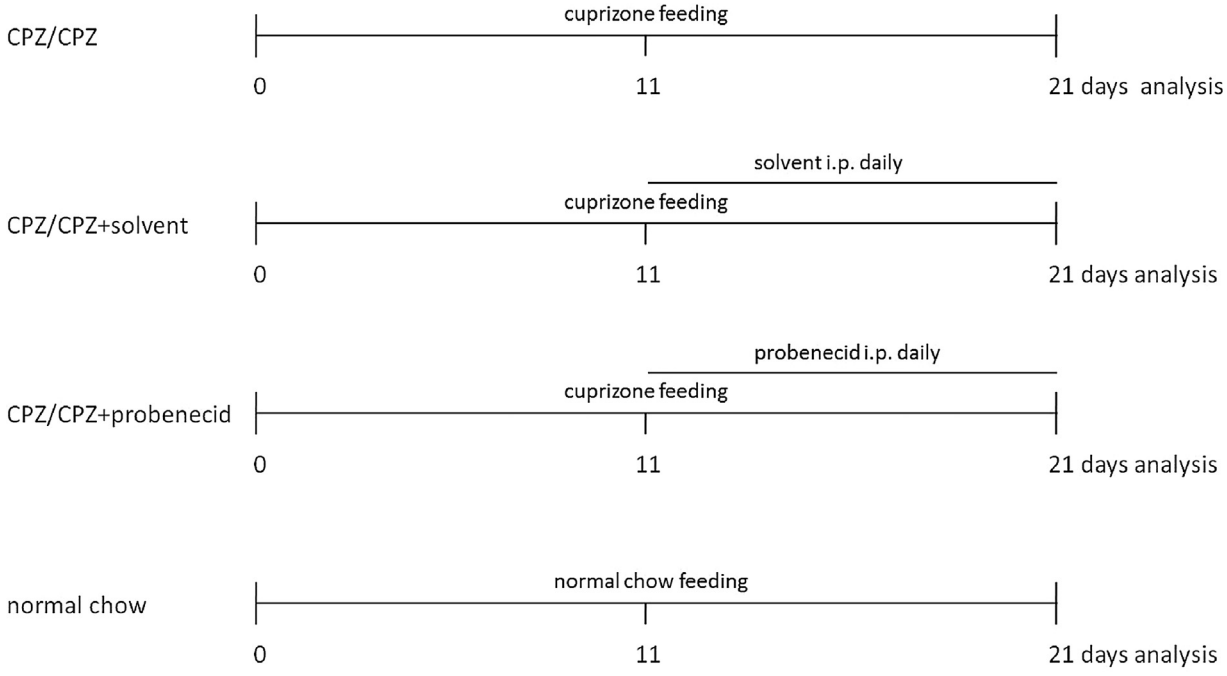

Fig. 1. Schematic drawing of the experimental set up and the investigated groups.