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Cerebrospinal fluid levels of neurofilament light in chronic experimental autoimmune encephalomyelitis

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Abstract

Experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendrocyte glycoprotein (MOG) is a chronic relapsing–remitting animal model of multiple sclerosis (MS). Neurofilament light (NF-L), a structural protein expressed in neuronal cells can be used to quantify the amount of neuronal damage in MS patients. An immunoassay was used to measure levels of neurofilament light in cerebrospinal fluid (CSF) in rats with myelin oligodendrocyte glycoprotein-induced EAE. Significantly increased levels of neurofilament were found in the immunized animals compared to the controls, strengthening the similarities in the diseases and the progression pattern between the animal model and MS. The turnover of NF-L during this disease is increased since significantly elevated levels also were identified in the spinal cord of the diseased animals and immunohistochemistry gave support for this observation. Monitoring neurofilament levels in EAE can be used to follow disease progression and effects of therapy.

Keywords: Encephalomyelitis; Cerebrospinal fluid; Autoimmune

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease affecting the central nervous system. The disease typically initiates as a relapsing–remitting phase followed by a transition to a secondary progressive disease type with more severe functional deficit [20]. Animal models of this disease have been developed to study MS at controlled conditions. Experimental autoimmune encephalomyelitis (EAE) was established in the 1930s by intramuscular injections of rabbit brain emulsions [18] and has thereafter been adapted for several rodent species. The most commonly studied form of EAE is an acute T-cell-mediated disease, which displays little or no demyelination. A chronic relapsing–remitting subtype of the disease with subsequent demyelination can, however, be induced [9,21,23]. The myelin oligodendrocyte glycoprotein (MOG) is located in the myelin sheaths encapsulating the neuron and is a strong encephalitogen. By immunizing the animals with purified MOG or recombinant peptides derived from the protein, a chronic relapsing–remitting disease type can be induced [1,3,5].

The neurofilaments constitute an important structural group of neuronal cytoskeleton proteins [8] and have been shown to be reliable markers of neuronal degeneration and disease [13,15,16,19]. Patients with MS display significantly elevated levels of neurofilament light (NF-L) early in the disease progression [11,14]. We have recently developed a sensitive ELISA assay for measurement of NF-L levels in cerebrospinal fluid (CSF) [12,13] and our aim was to investigate if MOG-induced EAE causes neuronal breakdown and release of neurofilaments into the CSF. Dark Agouti rats were immunized by recombinant rat MOG (aa 1–125) and the disease development was monitored and scored. After approximately 1 month the animals were sacrificed and the spinal cords and CSF, obtained by autopsies and cisterna magna puncture were analyzed for NF-L levels.

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2. Material and methods

2.1. Animals

Female dark Agouti rats were purchased from Scanbur (Uppsala, Sweden) and were maintained in the animal house in a controlled light environment with water and food ad libitum.

2.2. Induction and clinical evaluation of EAE

The rats, aged 12–13 weeks, were immunized with MOG intradermally in the tail tip under halothane anesthesia according to Dahlman et al. [3]. Each rat was given a 200 μl injection containing 20 μg of recombinant MOG (aa 1–125) kindly provided by Prof. T. Olsson (Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden), mixed with 100 μl IFA (Difco, USA) and 100 μl of phosphate-buffered saline (PBS). The rats were weighed and clinically scored daily from day 1 post-immunization (p.i.) to day 27 p.i. when the animals were sacrificed using excess anesthesia with Hypnorm–Dormicum (Hypnorm–Janssen Pharmaceuticals, Beerse, Belgium, Dormicum—Roche, Stockholm, Sweden). The clinical signs of disease were scored as follows: (1) tail paralysis or tail weakness; (2) hindlimb gait disturbances; (3) hindlimb hemiplegia; (4) tetraplegy, urinary and/or fecal incontinence. The investigation was approved by the institutional animal ethical committee following the European Community Council directives.

2.3. CSF collection from the cisterna magna

The CSF collection was made by a modification of the method described by Consiglio et al. [2]. In brief, 28 animals were deeply anaesthetized with an intraperitonal injection of 1–1.5 ml Hypnorm–Dormicum (equal amounts of Hypnorm and Dormicum prediluted 1:1 with PBS, pH 7.4) and an incision were made from the occipital bone and downward the neck separating the musculature. The head of the animal was tilted downwards at a 90° angle. A small syringe U-100 insulin (BD, Stockholm, Sweden) was inserted between the occipital protuberance and the spine of the atlas gently penetrating the atlanto–occipital membrane. CSF was slowly aspirated yielding between 50 and 100 μl of clear liquid with no visual signs of blood contamination. The cervical part of spinal cords together with the whole brain including the medulla oblongata was carefully dissected out from four EAE rats and four controls and immediately put on ice. Tissue samples were removed both for immunohistochemistry and quantitative determination of tissue levels.

2.4. CSF assay

NF-L was analyzed in rat CSF using a previously described in-house ELISA assay [13]. In brief, a microtiter plate was coated with 3 μg/ml monoclonal antibody (mAb) 47:3 overnight at 4 °C. The samples were added to a volume of 25 μl and diluted to a total volume of 50 μl with phosphate-buffered saline supplemented with Tween-20 (PBS-T). Internal control and standard samples were added in duplicates. The biotinylated detection antibody mAb 2:1 was added followed by the subsequent addition of streptavidin horseradish peroxidase (SA-HRP) (Pharmacia, Uppsala, Sweden). Color substrate OPD (Dako, Älvsjö, Sweden) was added and the plates were read at $\lambda = 450$ nm.

2.5. Protein analysis by SDS-PAGE and Western blot

Spinal cords from controls and rats with chronic EAE were homogenized and purified using a minor modification of the method described by Funchal et al. [4]. In brief, 100–200 μg of tissue was homogenized in a glass tissue homogenizer in a high salt buffer at a ratio of 1 ml buffer to 15 mg tissue. The high salt buffer used contained 5 mM KH_2PO_4, 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA and 1% Triton X-100, pH 7.4. After centrifugation at 13,200 × *g* for 10 min at 4 °C the pellet obtained was resuspended in 400 μl high salt buffer and the centrifugation was repeated once. The intermediate filament-enriched fraction was finally diluted in a 20 mM Tris buffer containing 4 mM urea and 0.4 M NaCl, pH 7.4. A standard BCA protein assay (Pierce, Stockholm, Sweden) was performed and a total protein content of 40 μg was loaded onto a 7.5% SDS-PAGE gel according to Laemmli et al. [7]. The Western blot was performed according to Towbin et al. [22]. Nonspecific binding sites were blocked by overnight incubation with 3% (w/v) non-fat milk powder. Blots were washed in PBS containing 0.05% Tween-20 and the primary antibody mAb 2:1 was added at a concentration of 1 μg/ml diluted in wash buffer. The antibody was allowed to interact with the blot for 1 h at room temperature (RT) and after washing HRP-conjugated rabbit anti-mouse IgG diluted 1:2000 was added. The membrane was developed using HRP color development system (Bio-rad, Sundbyberg, Sweden) according to the manufacturer.

2.6. Tissue assay

NF-L levels were analyzed in homogenized rat brain tissue using the above-described ELISA. The same intermediate filament-enriched fraction used in the Western blot was used to quantify the neurofilament amount. A protein amount of 50 μg (total) was diluted to a total volume of 100 μl with PBS-T and added in duplicates to the plate. The absorbance was measured at $\lambda = 450$ nm using OPD substrate.

2.7. Tissue fixation and immunohistochemistry

The animals were deeply anaesthetized with a Hypnorm–Dormicum solution as indicated above, and perfused through the left ventricle, initially with a 0.1 M phosphate buffer, pH 7.4, followed by fixative solution