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Rotarod motor performance and advanced spinal cord lesion image analysis refine assessment of neurodegeneration in experimental autoimmune encephalomyelitis

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HIGHLIGHTS

- The rotarod provides a quantitative and reproducible way of measuring motor performance in EAE.
- Clinical score and rotarod performance are highly correlated.
- Rotarod performance is correlated with demyelination in the motor systems of the spinal cord.

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ABSTRACT

Background: Experimental autoimmune encephalomyelitis (EAE) is a commonly used experimental model for multiple sclerosis (MS). Experience with this model mainly comes from the field of immunology, while data on its use in studying the neurodegenerative aspects of MS is scarce.
New method: The aim of this study is to improve and refine methods to assess neurodegeneration and function in EAE. Using the rotarod, a tool used in neuroscience to monitor motor performance, we evaluated the correlation between motor performance, disease severity as measured using a clinical scale and area covered by inflammatory lesions.
Results: The included parameters are highly correlated in a non-linear manner, with motor performance rapidly decreasing in the intermediate values of the clinical scale. The relation between motor performance and histopathological damage is exclusively determined by lesions in the ventral and lateral columns, based on a new method of analysis of the entire spinal cord. Using a set of definitions for distinct disease milestones, we quantified disease duration as well as severity.
Comparison with existing methods: The rotarod measures motor performance in a more objective and quantitative manner compared to using a clinical score. The outcome showes a strong correlation to the surface area of inflammatory lesions in the motor systems of the spinal cord.
Conclusions: These results provide an improved workflow for interpreting the outcome of EAE from a neurological point of view, with the eventual goal of dissecting neurodegeneration and evaluating neuroprotective drugs in EAE for application in MS.

1. Introduction

Multiple sclerosis (MS) is the most common cause of neurological disability in young people. It affects 0.1–0.2% of the population with onset of disease at a mean age of 28 years (Goodin, 2014). As of yet, no curative treatment has been developed, despite very good progress in reduction of the inflammatory component, mostly by biological anti-inflammatory drugs (Ransohoff et al., 2015). MS is a complex disease entity, with characteristics of both an immunological and a neurodegenerative disorder (Stys et al., 2012). Research into the causes of MS is difficult, partially because a large discrepancy exists between biological and clinical onset with patients

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developing symptoms after years of subclinical disease. As more time passes, epidemiological or histological traces of any causative factor will be harder to detect. Also, the processes leading to disability in MS occur in the brain and spinal cord, areas of the central nervous system (CNS) that are hard to probe or study at the microscopic level from the outside. The majority of MS patients are initially diagnosed with relapsing-remitting MS, a form of the disease in which short periods of attacks are followed by timeframes in which disease activity is nearly absent. Although almost all individuals eventually show progression to a continuously active form of the disease as damage accumulates (Ebers, 2001; Tremlett et al., 2008). The variation seen in MS patients supports the growing conviction that MS should be considered a collection of several disorders with different causes and pathways involved, but with a relatively similar set of symptoms as result (Lassmann, 2005).

All these challenges stress the importance of an accurate experimental model for MS. Ever since it was first described in the 1920s, as a chance finding during vaccination trials (Koritschoner and Schweinburg, 1925), Experimental Autoimmune Encephalomyelitis (EAE) has been the most widely used model system for MS (Mix et al., 2010; Baker and Amor, 2015). In EAE animals are inoculated with myelin components, triggering an immune response against the injected compound which also targets the native myelin sheath. The resulting disease depends on the species and strain of animals selected, as well as the molecule or cell used to evoke an immune response and any adjuvants used to stimulate cell-mediated immunity (Gold et al., 2006). Although EAE has greatly contributed to our understanding of MS (Baxter, 2007; Steinman and Zamvil, 2006), several disadvantages limit its use (Steinman and Zamvil, 2005; Ransohoff, 2012). The first and foremost of these is that in many protocols, EAE is an inflammatory disease of the CNS, only partially mimicking the neurodegenerative process seen in MS (Sriram and Steiner, 2005). Another concern involves the type of immune response, which in rodent EAE is mainly $CD4^+$ T-cell mediated, while active MS lesions in human patients tend to show a higher involvement of $CD8^+$ cells (Friese and Fugger, 2009; 't Hart et al., 2011).

EAE is not a trivial experimental procedure, and the consistency of measurements can vary depending on the experience of the person who implements the complex protocol (Stromnes and Goverman, 2006). Even seemingly minor decisions -such as the adjuvant used for immunization (Smith et al., 2011) and the dose and timing of pertussis toxin- will influence the disease course. Similar attention should be paid to the acquisition and analysis of results (Baker et al., 2011). It is common for EAE experiments to measure disease severity using a clinical score running from 0 to 5, with 0 indicating no symptoms and 5 death (e.g. Kassiotis et al., 1999). This scale is observer-dependent, non-linear and produces a categorical variable, implying that any statistical analysis involving a mean score has to be interpreted with caution (Baker et al., 2014). Two animals with a mean score of 2.5 can both be moderately sick or one animal is dead while the other shows no symptoms. Representing the results of an EAE experiment accurately requires a thorough statistical analysis (Fleming et al., 2005). Failure to take the proper precautions may contribute to publication bias (Tsilidis et al., 2013).

In the field of neuroscience, it has been common for several decades to measure motor performance of rodents using a rotarod, a bar rotating at a slowly increasing speed. The animal is forced to increase its walking speed to keep up with the bar, until it can no longer maintain its balance and drops on a switch, connected to a clock. The latency time to fall can then be used as an objective and non-invasive measurement of motor performance (Jones and Roberts, 1968). As common as this method is in neuroscience, relatively few studies have explored its application in EAE (Jones et al., 2008; Al-Izki et al., 2012; Moore et al., 2014). In this paper, we demonstrate the use of a rotarod to evaluate the results of EAE induction in mice. We compare the rotarod measurements to a clinical score as well as to the number of inflammatory lesions found in the spinal cord of these animals. Using a set of definitions for crucial disease events based on clinical score and rotarod, we also provide a quantifications of EAE disease course over time. The clinical impact of any CNS lesion is determined by its location as well as its size, where a minor lesion in a critical system can lead to severe clinical disability. Furthermore, we develop a novel image analysis approach in which for each spinal cord lesion the information on anatomical context is preserved, allowing for a better understanding of the three-dimensional distribution of inflammatory lesions and their relation to clinically observed disease severity.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Government of The Netherlands. All animal experiments were approved by the Animal Ethical Review Committee (DEC) of the Erasmus Medical Centre under protocol number 128-12-01. All experiments were performed in the animal facilities of the Erasmus Medical Centre.

2.2. EAE induction and assessment

C57BL/6Tac animals were originally obtained from Xenogen Biosciences (currently: Taconic Farms, Inc. NJ, USA). At the start of experiments, the strain had been in our facility for four generations. Animals were housed in specified pathogen-free conditions. Both male and female animals were used for the experiments. The mice were 12 weeks old at the start of the experiment, all animals were born within 3 days of eachother. EAE was induced by subcutaneous immunisation at 4 sites in the flanks with 200 μg MOG_{35-55} peptide in total, emulsified in CFA (Difco/Voigt, Lawrence, KS, USA) on day 0 as described previously (Stromnes and Goverman, 2006). 500 μg heat-killed *Mycobacterium tuberculosis* H37RA (Difco/Voigt, Lawrence, KS, USA) was added per 100 μl CFA to evoke a better immune response and more extensive neuropathology. At day 0 and 2 mice were injected i.p. with 150 ng pertussis toxin (PTX, Sigma-Aldrich, MO, USA). After EAE induction, mice were monitored daily, and weight and clinical status were recorded. A clinical score was used as defined in Table 1. If an animal was scored higher than 2, gel packs and soft food pellets were provided. In addition,

Table 1
Scale for Clinical Score. During this experiment, all animals with a score higher than 2.0 were provided with gel packs and soft food pellets. If an animal reached a score of 4.5, it was euthanized. Scores of 4 and above are rare, only animal reached 4.5 and was euthanized.

Score	Symptoms
0.0	None
0.5	Weakness of the tail.
1.0	Paralysis of the tail without weakness of the limbs.
1.5	Weakness of the limbs without paralysis of the tail.
2.0	Weakness of the limbs with weakness or paralysis of the tail.
2.5	Complete paralysis of one of the hind limbs, or weakness of three or four limbs while retaining the ability to walk.
3.0	Complete paralysis of front or hind limbs, or weakness of three or four limbs with the loss of walking ability.
3.5	Complete paralysis of the hind body, animal cannot turn its body.
4.0	Complete paralysis of front and hind body.
4.5	Complete paralysis with inability to eat and drink.
5.0	Death due to experiment.