Original Paper

Clinical and Histopathological Assessment on an Animal Model with Experimental Autoimmune Encephalomyelitis

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ABSTRACT: Multiple sclerosis (MS) is a disease of the Central Nervous System (CNS) which alters over 2 million people, and involves an abnormal autoimmune response directed against the brain, nerves and spinal cord. The antigen or the autoimmune target still remains unknown, a fact for which MS is considered to be an immune mediated disease. The pathology involves mainly the white matter, but the gray matter demyelination plays an important role in its pathogenesis. In 80% of the cases with MS, the disease develops relapses. Experimental autoimmune encephalomyelitis (EAE) is the most used model to study MS and for assessing potential treatments. In the present study we report on the histopathological characterization of an EAE model in C57BL/6 mice immunized by injection with myelin oligodendrocyte glycoprotein, MOG35-55 in complete Freud's adjuvant supplemented with pertussis toxin. On a group of 10 immunized animals and on 5 control animals, we followed the development and grading signs of motor deficiency, and after a survival of 34 days, the study aimed to evaluate the histopathological changes in the telencephalon, brainstem, cervical spinal cord, the optic nerve and retina. We utilized histochemistry, immunohistochemistry, and densitometric image analysis methods to assess myelin loss [Luxol fast blue, immunohistochemistry for the presence of microglia (Iba1) and reactive astrocytes (GFAP)]. Moreover, the study includes a first analysis of the detailed histopathological changes of the optic nerve and retina on an EAE model, all of these as the background for testing drugs with potential therapeutic role in MS.

KEYWORDS: *Multiple sclerosis, autoimmune, myelin, astrocytes, animal model*

Introduction

EAE is the most widely used model for reproduction of MS in humans and is an elaborated form in which the interplay of neuropathological and immunopathological mechanisms reproduces as close as possible the pathological features of MS: inflammation, demyelination, neuronal loss and gliosis.

Multiple sclerosis (MS) is the central nervous system's (CNS) inflammatory demyelinating disease. It is believed to alter up to two million people around the world [1].

Clinically, the manifestations usually begin around 30 years old and particularly affects women with a female: male ratio of 3: 1.

Thus, MS is a primary cause of neurological disability for young adults.

From the clinical point of view, MS frequently presents a relapsing-remitting form that can be completely solved or can let the patients with residual deficits. Deficiency may interest any part of the CNS such as somatosensory manifestations, pyramidal and visual pathways, ultimately due either to inflammation-mediated demyelination of the visual pathways (optic neuritis) or to the visual pathways (ocular motility disturbance such as inter-nuclear ophthalmoplegia).

Unfortunately, many patients with relapses develop over time a gradual neurological progression.

There are four forms of MS, namely: Relapsing-Remitting MS, Secondary Progressive form, Primary Progressive type and Progressive-Relapsing MS. Persons who presented one typical inflammatory demyelinating episode that suggests an MS attack but who did not have another episode present a clinically isolated syndrome (CIS).

Experimental autoimmune encephalomyelitis (EAE) is the most comprehensive used

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experimental animal model for the reproduction of multiple sclerosis (MS) in humans.

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease mediated by T-helper (Th) cells characterized by infiltration of monocytes and T cells of the CNS and associated with local inflammation. The target and identified target cells are proteins expressed by CNS oligodendrocytes that produce myelin. The result is primary demyelination of axons with damage to CNS axonal conduction [2].

The mechanisms of inflammation reduction and remyelination occur in the EAE, so this model may serve as a model for these activities. Furthermore, EAE is frequently used as a pattern for cell mediated cellular autoimmune diseases. EAE presents an elaborated neuropharmacology so a lot of drugs used in MS have been tested or validated by studies on this animal disease model.

Due to its heterogeneity in the process of induction of the disease and its reaction to various neuropharmacological or immunological operations, it makes the EAE a versatile method but which still needs to be adapted to the needs of the projects. While producing difficulties and underlining the immanent deficiency of this MS model, this variability is also a chance to explore various sides of the immune-mediated immune processes and demyelinating immune mechanisms, as well as intrinsic protective processes.

There are currently many pathophysiological forms of EAE with different clinical models depending on animal species, proteins/peptides used for disease induction and immunization pathway used.

EAE in mice was initially induced 60 years ago by active immunization with homogenates from the spinal cord [3].

Subsequent research led to the discovery of numerous peptides that could induce encephalitis, and the mice remained the most abundant animal used, both because of the use of transgenic mice and the high availability of knockout mice.

In the present study we report on the histopathological characterization of EAE model in C57BL/6 mice immunized by injection with myelin oligodendrocyte glycoprotein, MOG35-55 in complete Freud`s adjuvant supplemented with pertussis toxin.

Material and Methods

Animal model

The study was performed on 15 C57BL/6 female mice of 10-11 weeks of age (18.22±1.98g) (The National Institute of Research and Development for Microbiology and Immunology "Cantacuzino", Bucharest, Romania). The animals were housed in groups of three in a controlled 12h light/12h dark schedule, and free access to water and food until the beginning of the experiments. Animals were randomly assigned to one of the two groups: EAE induction group (n=10), and control group (n=5).

This project was approved by the ethics committee of the University of Medicine and Pharmacy of Craiova, Romania (203/24.10.2017).

Immunization kits based on MOG_{35-55}/CFA emulsion and Pertussis Toxin (PTX) (code EK-2110) were purchased from Hooke Laboratories, Inc, Lawrence, MA, USA, with the protocol being developed as indicated by the producer [4-6].

In the beginning of the experiments, in the first day, the animals received initially the MOG_{35-55} antigen emulsion in two subcutaneous injections (0.1 mL/site) on the upper and lower back areas.

At 2 hours after the last antigen injection, the first PTX dose was injected intraperitoneally (150ng diluted in 0.1ml glycerol/PBS buffer).

In the next day, the second PTX dose was injected intraperitoneally in the same concentration. Control animals received saline of the same volume.

The animals were then was followed for 34 days, with their motor dysfunction being scored depending of the following EAE scoring guide [7]: 0 (no disease), 0.5 (distal limp tail, tip of the tail drops), 1 (normal gait, tail drops), 1.5 (hind limb paresis, uncoordinated gait), 2 (uncoordinated gait with one hind paw dragging, tail limps), 2.5 (uncoordinated gait with both hind paws dragging, tail limps), 3 (hind paws paralyzed, weakness in forepaws), 3.5 (hind paws paralyzed, one forepaw paralyzed), 4 (hind paws paralyzed, both forepaws paralyzed), 4.5 (moribund), 5 (the mouse is only rolling or is found dead).

After the beginning of the induction, all animals were fed with freshly prepared 2% agarose-glucose pellets.

Histopathology and Immunohistochemistry

After 34 days of survival, the animals were deeply anesthesiated, perfused with saline and 10% neutral buffered formalin (NBF), and then the brain, cervical spinal cord, and eyes were dissected and fixed in NBF for 2 days at room temperature.

All tissues were next processed for paraffin embedding and cutting using rotary microtome HM355S equipped with a section-transfer system (Thermo Scientific Inc., Walldorf, Germany), in the department of histology of U.M.F. of Craiova.

Paraffin blocks were next cut as 4µm-thick sections.

Sections were processed for Luxol fast blue staining for myelin visualization.

For this protocol, the sections were deparaffinized, re-hydrated to 95% ethanol, and then incubated in a 0.1% Luxol fast blue solution, overnight at 60˚C.

Next day, the sections were differentiated in 90% ethanol, re-hydrated to distillate water, counterstained for 3 minutes with a 0.2% nuclear red solution, then cleared, and mounted utilizing a xylene-based mounting medium.

For immunohistochemistry, seriate blank sections were rehydrated, processed for antigen retrieval by microwaving in citrate buffer pH6 for 20 minutes, incubated in 1% hydrogen peroxide for 30 minutes, and incubated for another 30 minutes in 3% skimmed milk.

The sections were next incubated at 4°C for 18h with the primary antibody (rabbit anti-GFAP, Dako, diluted as 1: 30.000), and the next day the signal was visualized with an anti-rabbit peroxidase polymer detection system adsorbed for mouse immunoglobulins (Nikirei-Bioscience, Tokyo, Japan) and 3,3'-diaminobenzidine (DAB) (Dako, Glostrup, Denmark).

Finally, the slides were coverslipped with DPX medium (Sigma-Aldrich, St. Louis, MO, USA).

Image analysis

All the slides were imaged at 10× and 20× objectives on a Nikon 90i microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands) equipped with a Nikon DS-Ri2 CMOS 16Mp color camera and the Nikon NIS-Elements Advanced Research imaging software.

For Luxol fast blue assessment, images were captured utilizing the same exposure and illumination settings of the microscope and software.

For Luxol fast blue stained sections, the area of the blue signal and its intensity were assessed using the integrated optical density measuring tool in the Image ProPlus AMS software (Media Cybernetics, Bethesda, MD, United States).

For this purpose, the regions of interest of the blue color dye were automatically selected in each image utilizing a single predefined RGB profile of the color.

Finally, all resulting behavioral and imaging data were plotted in Microsoft Excel as average ±standard error of the means (SEM), and statistical differences were sought utilizing a student's t test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Clinical evolution confirmed the occurrence of EAE clinical changes starting with the 10th day of evolution following the immunization (day 0) (Fig.1A, B).

As expected, the deficit occurred first time at the level of the tail then extended to one hind limb, then to both, and in our setup, it extended up to moderate deficits in the forelimbs.

The pathology developed in 7 out of 10 animals injected, and only these animals were considered for this analysis.

The scores followed an upward slope until the day 20, (2.41±0.91), and then begun to gradually drop till the day 27th (1±0.93), after which the values did not change until the end of the experiments (Fig.1C).

Body mass showed a constant evolution during the experiments, and somehow this concurred with the relative mild pathology obtained with this PTX dosage.

In all the 3 animals that did not develop EAE signs, and in 1 further EAE animal, we noticed the development of small necrotic-granulomatous lesions at the level of the subcutaneous injection sites, a fact that has been previously reported for this model.

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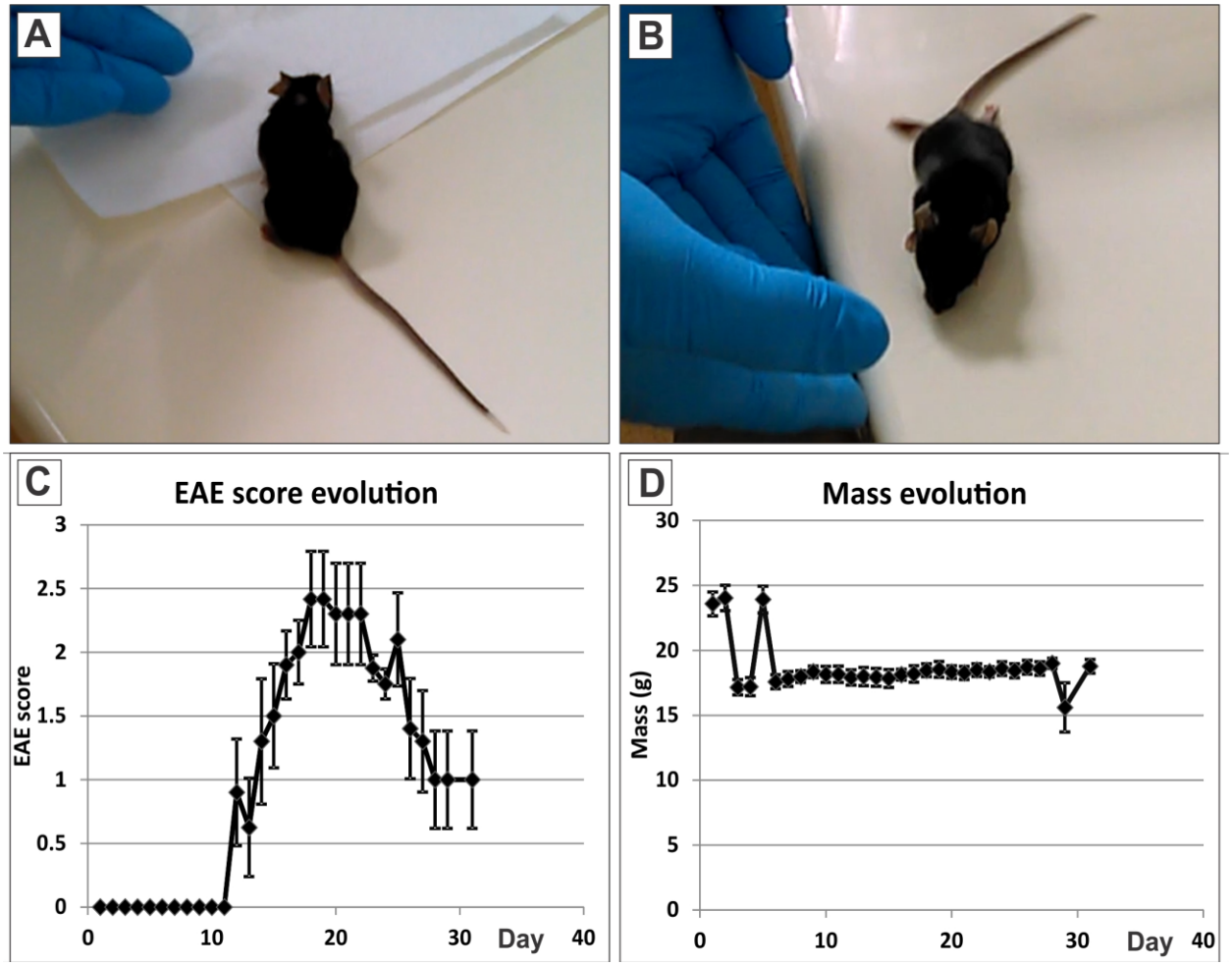

Fig.1. Motor deficits in this EAE model ranged from a limp tail (A) to a limp tail with hind limb paralysis (B). EAE score evolution registered a peak around the 20-th day after immunization, while the scores dropped around the day 30 (C). Body mass did not record significant changes during these clinical changes (D). (Error bars represent standard error of the means)

At the level of the tissue there were no gross morphological changes that could be observed after EAE induction. Microscopically, we first evaluated the density of the myelin in the spinal cord, optic nerves, and brainstem based on the Luxol fast blue integrated optical density measurements (Fig.2A).

We did not find any differences regarding the myelin staining in the layers of the retina (Fig.2B, C).

In all the other parts of the CNS that we analyzed, there was a clear-cut tendency for lower myelin densities in the immunized animals, but these differences attained statistical significance only for the brain stem and the optic nerves (Fig.2 A, D-I).

There did not seem to be a difference between the peripheral region of the optic nerves and their proximal segments.

In any of the cases, we could not find classical demyelinating plaques with adjacent inflammatory infiltrate, but only a lower density of an apparent unchanged myelin fibers arrangement.

In any of the measurements, we did not consider the immediately sub-meningeal denser myelin arrangements, as their felt work was too dense to be quantified (Fig.2H, I).

Also at the level of the brain stem, the most affected fibers seem to be the diffuse connections and not the thicker pathways composed of larger bundles of tracts.

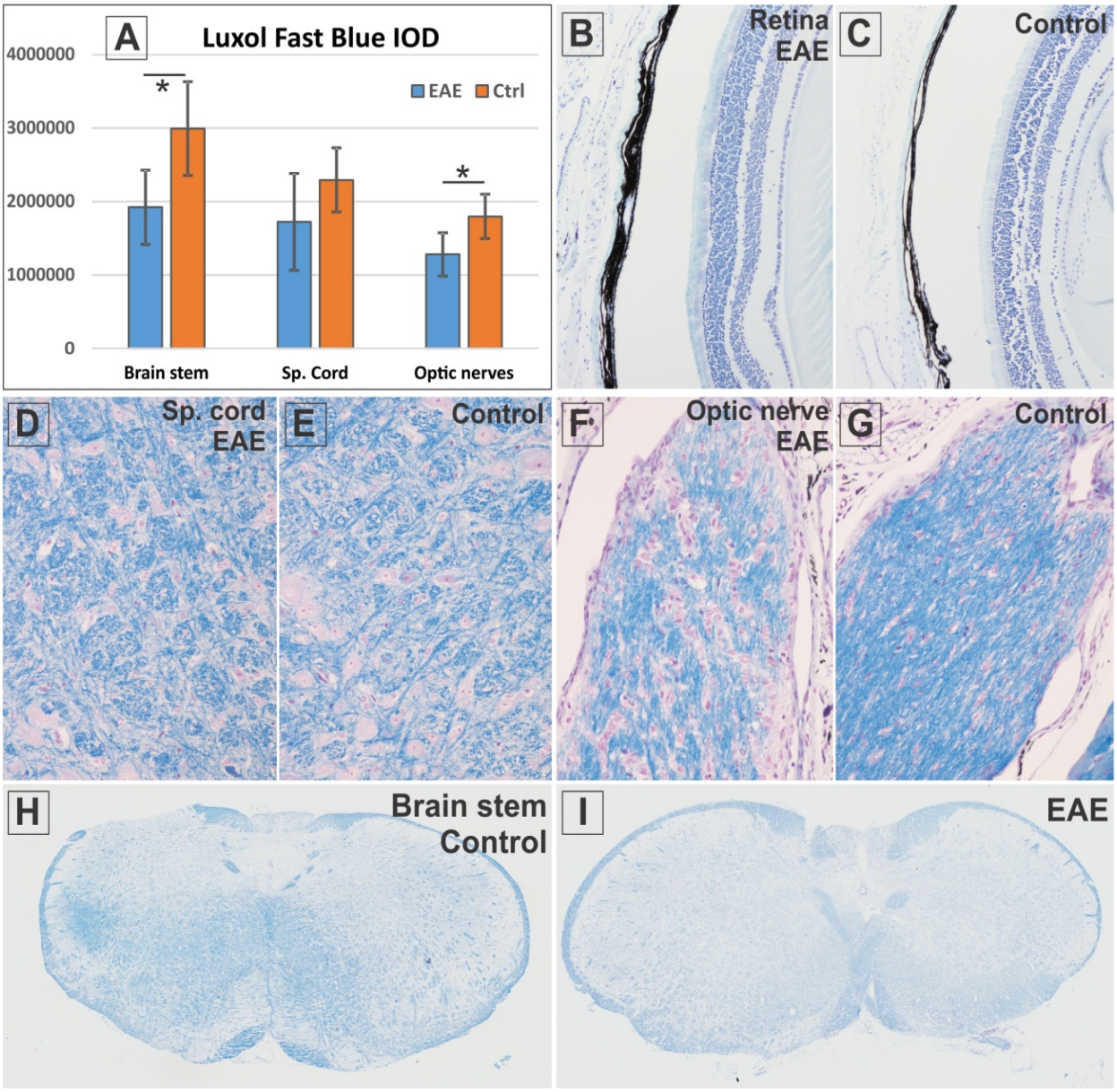

Fig.2. Myelin density staining, assessed as the integrated optical density (IOD) of the Luxol fast blue staining (A-I). There were apparently no changes in the retina, based on Luxol fast blue staining (B, C). Except the spinal cord region (D,E), there were significant drops in myelin density for the optic nerves (F,G) and medulla (H, I). Error bars represent standard error of the means, * p<0.05. B- I, Luxol fast blue staining, B,C-10×, D-G-20×, H, I 10× scan

Lastly, we were interested to see if there was an increased glial reaction after the experiment (Fig.3).

We could not find any change at the level of the retina, with minimal and invariable GFAP reactivity in what most probably were retina Müller cells (data not showed).

There was a constant sub-meningeal increased GFAP expression in the external glia limitans for both treated and control animals (Fig.3A, B).

However, at the level of the internal glia limitans (around the vessels and the ventricular system), there was a reduced reactivity in control animals compared to immunized ones.

While there were readily identifiable astrocytes throughout the neuropil in control mice, there was not too much signal around the Sylvian aqueduct. In immunized mice, however, there was a clear-cut gliosis with dense, inseparable astrocyte branches, mostly connecting the immediate sub-epithelial region of the aqueduct with the sub-meningeal glia limitans (Fig.3C, D).

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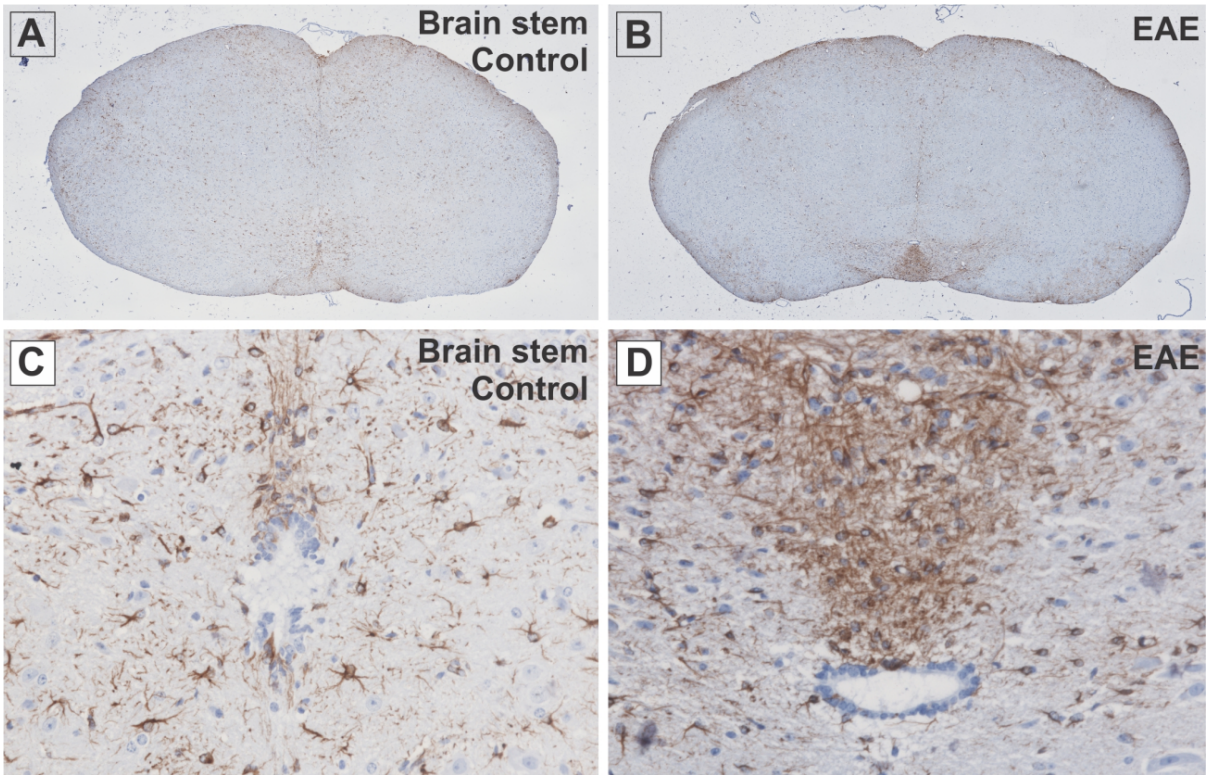

Fig.3. Glial reactivity after EAE induction. There was a constant increase of GFAP reactivity in all brain areas investigated, here illustrated in the brainstem (A,B), and on a closer view, around the cerebral aqueduct of Sylvius (C,D). Immunohistochemistry for GFAP, A,B-10×scan, C, D-20×

Discussion

For the study of EAE as a model for human demyelinating diseases, analogies/differences must be sought between the model and the actual disease cases.

There are two main ways to induce EAE in mice: (i) An animal model in which EAE can be initiated by active immunization with a myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and myelin proteolipid protein (PLP), and (ii) an animal model in which EAE can be initiated by passive immunization, by injecting myelin antigen-specific T cells. Notably, there is, also, a line of T cell receptor (TCR) transgenic mice directed against a myelin antigen that develops spontaneous EAE (sEAE) [8].

Additionally, there are EAE models induced by toxins (cuprizone, lysophospholecithin) or by inoculation of an intracerebral virus (Theiler's murine encephalomyelitis virus) [9-12].

In what concerns sEAE, it enables a study of intrinsic signals and mechanisms involved in developing of MS without other interventions. Anyway, it is important to mark that spontaneous disease progression in this model of transgenic mice allows an accurate appreciation of the evolution and temporal development of EAE for each animal since it can occur at different times in different mice or in mice from the same line. In other words, onset may be controlled but the course of the disease is less predictable [8,13].

Ben-Nun's group showed that EAE can be caused in C57BL/6 mice using MOG35-55 [13], demonstrating that mice 129/Sv do not develop disease by immunization with MOG. Later, it was demonstrated that a pure line of this animal model can develop the disease by immunization with the MOG peptide [14]. Unlike the C57BL/6 model, are present obviously a lot of different 129/Sv sub-strains, and some of them distinguish from each other as C57BL/6 mice are different from the BALB /c model [15].

Although C57BL/6 mice remain the most used animal model for active induction of EAE, disease induction has a heterogeneous efficacy, the development of the disease being dependent on the used pertussis toxin but also on other less known factors. Thus, a high number of activated Th1-polarized MOG specific T cells are utilized. The most imported peptide derived from MOG is MOG p35-55. Another EAE active animal model in C57BL/6 mice is by injection with MOG 1-125 or MOG 79-96 without pertussis

toxin which determines an autoreactive T-cell response. From the histopathological point of view, the brain tissue of these mice exhibits infiltrative lesions with T cells, macrophages and associated demyelination. What makes it a useful model is its great susceptibility to MOG and dependency to an immunodominant peptide homologous areas as that involved in MS [16].

If the MOG35-55 is used for the study of the onset and developing of EAE, MOG1-25 is recommended for testing the therapeutics which specifically target B-cells [4,5].

EAE can also be determined by proteolipid protein (PLP) immunization on C57BL/6 mice or with myelin basic protein (MBP), the latter induction also requires administration of PTX.

In our present setup, with a 150ng PTX dose for EAE induction, we did not find a brisk inflammatory infiltrate, nor classical MS-type of demyelinating plaques. However, the fine meshwork of myelin fibers did change, with decreasing IOD values in these animals. Significant differences occurred for brain stem and optic nerves, confirming that optic nerves seem to be affected like in the corresponding human pathology. The most affected regions were the central parts of the brain stem, while no significant changes could be observed at the periphery, under the meninges. On the other side, gliosis could be identified mostly also in the inner areas, around the Sylvian aqueduct, paralleling somehow the myelin loss.

There is no apparent difference between induction of EAE development with MBP or MOG expect just the price. PLP is not commercial and therefore must be purified from bovine white matter which makes it hard to use experimentally [17].

Although the EAE study on rats is a popular method, this model has been gradually replaced with EAE on mice because they are smaller, and therefore lead to a lower cost, thus representing a more practical and productive model.

A parallel study of the EAE model in rodents (mice, rats) is significant for the pathophysiology of MS as well as for new MS therapies.

The animal model facilitates the study of the implication of environment factors in the initiation and progression of the disease. However, no animal model reproduces all the features of multiple sclerosis.

Choosing the right model depends on the study typology but also has an influence on the results.

However, there are important features of MS pathology and pathogenesis including the role of T and B cells and demyelination and tissue impaired in the progressive stage of the disease that are not currently reproduced by the animal model.

So there is a need for new animal models but also to better characterize the existent models in order to obtain more accurate results.

Conclusion

A better clinical and morphological characterization of the MOG_{35-55}/CFA emulsion and PTX EAE mouse models, both with the similitudes and differences to the human disease, will make them invaluable tools in the study of multiple sclerosis.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgment

Laura Emilia Toader, Bogdan Catalin and Oana Taisescu contributed equally to this manuscript.

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