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MAKING AN EXPERIMENTAL ANIMAL MODEL FOR MULTIPLE SCLEROSIS

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ABSTRACT

To understand the mechanism of Multiple Sclerosis (MS), an autoimmune demyelinating disease, the researchers developed an experimental animal model for MS, which is called EAE (Experimental Allergic Encephalomyelitis). There are several methods for inducing this animal model. In this research the active EAE, which is developed by injecting bovine myelin antigens into genetically susceptible animals, was used. Proteolipid protein (PLP), which is a prominent neuroantigen, was extracted from fresh bovine brain, and used for inducing EAE in female Balb/C and Guinea pig. Animals were weighed and examined daily for clinical symptoms. Also histological sections from EAE brains were prepared. These sections showed infiltration, congestion and demyelination.

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Keywords: Multiple Sclerosis disease, EAE animal model, PLP.

INTRODUCTION

Myelin is a lipid - protein membrane construct within the central and peripheral nervous system (CNS and PNS) of vertebrates. Dehydrated myelin is made up of approximately 70-75% lipid and 25-30% protein.[1] PLP (Proteolipid protein) and DM-20 are the major myelin proteolipids accounting for almost 50% of the total myelin proteins. PLP is composed of 276 amino acids and has an apparent molecular weight of around 26kd by gel electrophoresis.[2]

EAE (Experimental Allergic Encephalomyelitis) is an inflammatory disease of the central nervous system (CNS), induced in genetically susceptible rodents and other species by injecting CNS myelin antigens hemogenized in an adequate adjuvant.[3,4,5,6,7] Two major myelin proteins, MBP (Myelin Basic Protein) and PLP, have been known for many years to induce EAE and are considered to be potential autoantigens in MS.[8] The CNS inflammation induced in EAE results in paralysis and other neurological abnormalities, resembling the human disease Multiple Sclerosis (MS) in some aspects. EAE is characterized by perivascular infiltrates of mononuclear cells accompanied by varying degrees of demyelination.[3,4]

EAE has been shown to be mediated by autoreactive T lymphocytes specific for myelin proteins but B cells or antibody-mediated mechanisms are probably also implicated in the disease process. Suppression of the EAE episodes has been one of the major goals of research in this area. Therapeutic strategies used to suppress this disease include treatments with immunosuppressive drugs, the use of monoclonal antibodies directed against the CD4 molecule or MHC class II glycoproteins. The main aspect of this area is indicating whether methods of tolerance for EAE therapy with oral administration of antigen, the administration of soluble neuroantigens either intravenously or intraperitoneal administration of antigen emulsified in adjuvant are effective in PLP-induced disease or not and screening the cDNA library prepared from PLP-induced EAE animal brains by CSF (Cerebrospinal Fluid) and serum of MS patients.[9,10]

In this study, we describe the induction of EAE with cow brain PLP in Balb/C and guinea pig. Of course, we also checked the potential of serum and CSF of MS patients in developing EAE.

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MATERIAL AND METHODS

PLP Extraction

Bovine brain was obtained from freshly sacrified animals at the Ziaran animal house and transported to the laboratory on ice (the cows of the Ziaran animal house have a mixed breed of Holstein Friesian and domestic). The white matter was dissected, washed with ice - cold normal saline and either stored at -70°C or used immediately. Bovine white matter was hemogenized in 10 volumes of Chloroform: Methanol: 0.1N HCl (2:1:0.1 v/v/v) and stirred at 4°C for 30 min. The insoluble material was removed by gravity infiltration using Whatman filters. The filtrate was extracted with 0.2 volume of distilled water. Phase separation was carried out by centrifuging the mixture in 8000 rpm for 30 min at 4°C. The organic phase was re-extracted with Chloroform: Methanol: water (3:47:48, v/v/v) and the phases were separated as before. The proteolipid proteins were precipitated by adding 4 volumes of diethyl ether to the organic phase and placing the solution at -20°C for overnight. The protein was recovered by centrifugation at 8000 rpm for 30 min and methanol washing was carried out for partial delipidation. The precipitated brain proteolipid proteins were solubilized in Chloroform: Methanol: 0.1N HCl (9.5:9.5:1, v/v/v). Any insoluble material was removed by centrifuging at 5000 rpm for 10 min at room temperature and then was loaded directly onto a 2×80 cm size exclusion LH-20 resin column. This chromatography was performed for complete delipidation of PLP. After determining the concentration of the final PLP, it was stored at -70°C. In this way it has immunogenic potential for at least 2 years.[6,7,11]

SDS-PAGE

PLP was dissolved in sample buffer containing 2% SDS and 2ME (2-Mercaptoethanol) and placed at 4°C overnight to solubilize and boiled for 10 min. Slab gels (12×15 cm with 0.7mm thickness) were run, according to the procedure of Laemmli, using a 13% polyacrylamide gel. The running buffer was composed of 0.05M Tris base, 0.35M Glycine and 0.1% SDS (PH 8.4). Samples were loaded onto a 6% polyacrylamide stacking gel. Current was set at 25mA. Gels were stained with Coomasie briliant blue R-250.

Induction of EAE and tissue sectioning

Female Balb/C mice and guinea pigs (purchased from the Razi institute, Tehran, Iran), and Ratus ratus µgrown at NRCGEB animal house, Tehran, Iran), aged 6-8 weeks, were immunized 2 or 3 times with 100-300 µg PLP or brain white matter emulsified in the same volume of complete and incomplete Freund's adjuvant per animal. Injection was carried out subcutaneously (SC) in the abdominal part of the animal body, intramuscularly (IM) on hind limb muscle or intraperitoneally (IP) in a total volume of 0.1-0.3 mL. There were some negative control animals by injecting PBS buffer, adjuvant and none of them. Some of the animals were immunized by injecting serum or cerebrospinal fluid (CSF) obtained from MS patients. For preparing the patients' serum, 5mL peripheral blood was held at 37°C water bath for 2 hours and kept at 4°C for overnight, then centrifuged at 10000 rpm at 4°C for 10 min. The serum, which is in the supernatant, was stored at -20°C. The CSF of MS patients was prepared by the physicians of Shariati hospital with lumbar puncture and stored at -20°C. Animals were weighed and examined daily for clinical symptoms. The clinical symptoms such as tail atony, hind limb then forelimb paralysis, ataxia, sensory loss, blindness, dysarthria and finally death were seen.[10,12,13] The dissected brain tissues were fixed with 10% formalin in PBS for one week. Histological sections from EAE brains were prepared by microtome from paraffin embedded tissues and stained with hematoxylin-eosin. A pathologist confirmed the infiltration and demyelination in the sections.

RESULTS

Isolation of cow brain proteolipid proteins

The Chloroform: Methanol soluble fraction consisted of a mixture of proteolipid proteins and lipids. The proteins should be cleaned from the lipids by methanol washing and size exclusion chromatography using LH-20 resin. SDS-PAGE analysis of extracted protein showed one major band corresponding to a protein migrating at 26 kd molecular weight and in some preparations there was a second faint band at around 20 kd, which presumably is DM - 20.[5,7] Fig. 1 shows this electrophoresis.

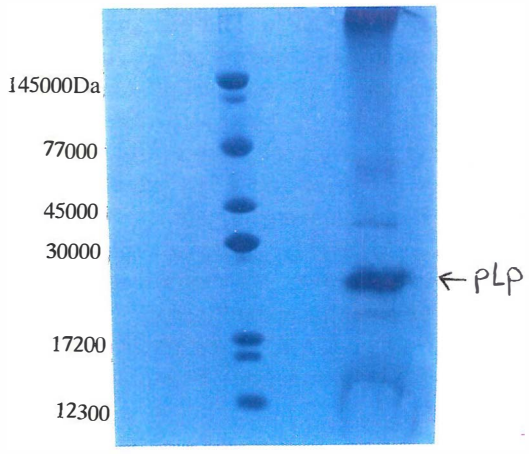

Fig. 1. Analysis of purified PLP by SDS - PAGE on 13% polyacrylamide gel stained with Coomasie blue. Molecular weight markers are indicated on the first lane.

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Table I. Summary of EAE results.

Animal strain	No. of animals	Mean Weight (on day 0) (gr)	The injected material	Type of injections	Number of injections	Duration between injections	Total dose in 3 injections	Total dose per weight	Affected percentage
Balb/C	2	20	PBS	SC	3	4 days	600 µl	30 µl/gr	0
Balb/C	1	20	PBS + adj	SC	3	4 days	600 µl	30 µl/gr	0
Balb/C	2	20	MS Serum	IM	3	4 days	600 µl	30 µl/gr	0
Balb/C	1	20	MS Serum	SC	3	4 days	600 µl	30 µl/gr	0
Balb/C	7	20	PLP + adj	3IM, 4SC	3	4 days	450 µg	22 µg/gr	57% (4/7)
Balb/C	2	20	PLP	SC	3	4 days	450 µg	22 µg/gr	50% (1/2)
Balb/C	4	20	CSF	SC	3	4 days	600 µl	30 µl/gr	75% (3/4)
Balb/C	7	20	W.M. hemog+ adj	3IM, 4SC	3	4 days	450 µg	22 µl/gr	0
Balb/C	4	20	W.M. hemog+ adj	2IM, 2SC	3	4 days	450 µg	22 µl/gr	43% (3/7)
Ratus ratus	2	250	W. M. hemog+adj	IP, SC	3	4 days	750 µg	3 µg/gr	0
Ratus ratus	2	250	CSF + adj	IP, SC	3	4 days	750 µl	3 µl/gr	0
Ratus ratus	2	250	PLP + adj	IP, SC	3	4 days	750 µg	3 µg/gr	0
G. Pig	2	380	PLP + adj	SC, IP	3	4 days	900 µg	2.3 µg/gr	100% (2/2)
G. Pig	1	400	PBS	IP	3	4 days	1200 µl	3 µl/gr	0
G. Pig	2	400	W.M. hemog + adj	IP, SC	3	4 days	900 µl	2.2 µg/gr	50% (1/2)

Abbreviations: adj / adjuvant, SC / subcutaneous, IM / intramuscular, CSF / cerebrospinal fluid, hemog. / hemogenate, W.M. hemog. / white matter hemogenate, G. Pig / guinea pig.

Animal experiment results

The Ratus ratus didn't show any sign of disease with different amounts of neuroantigens. So it was excluded from our experiment. Balb/C mice and guinea pigs developed moderate to severe EAE following immunization with brain white matter homogenate, PLP and CSF of the patients but the result of injection of MS patient serum was negative. Some of the weight progression graphs of EAE animals are shown in Fig. 2. The pictures in Fig. 3 show some of the EAE animals and the results of the experiments are summarized in Table I.

None of the control mice inoculated with Freund's adjuvant and PBS buffer developed any clinical sign of EAE. While control animals showed a gradual increase

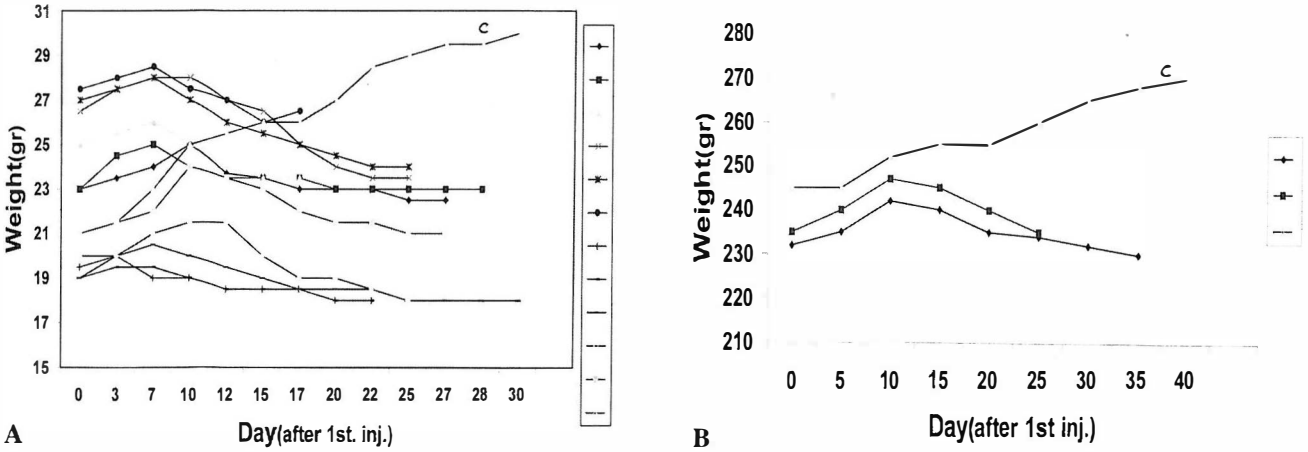

Fig. 2. Weight progression of some EAE and control animals (→ shows the negative control)

A) Weight progression of 9 affected and one control Balb/C

B) Weight progression of 2 EAE and one control guinea pig.

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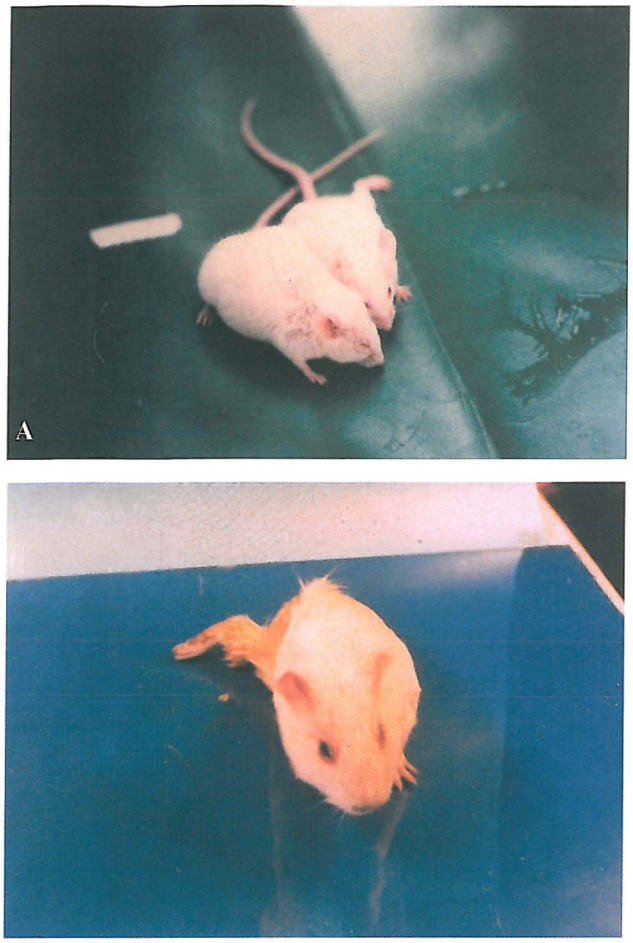

Fig. 3. Pictures of some of the EAE animals.
A) The left mouse has EAE and the right one is normal.
B) EAE guinea pig.

in their body weight, PLP treated mice and guinea pigs showed a similar slower behavior just before the onset of the neurological signs and weight loss when the clinical signs were apparent.

Histological findings

CNS tissue sections from EAE mice revealed widespread infiltration and congestion throughout the CNS, which were absent in control animals. Infiltration was accompanied by mild to moderate demyelination. By the conventional hematoxylin - eosin staining, the treated animals showed the marked histological changes. The images in Fig. 4 are some of the histopathological sections from EAE brains.

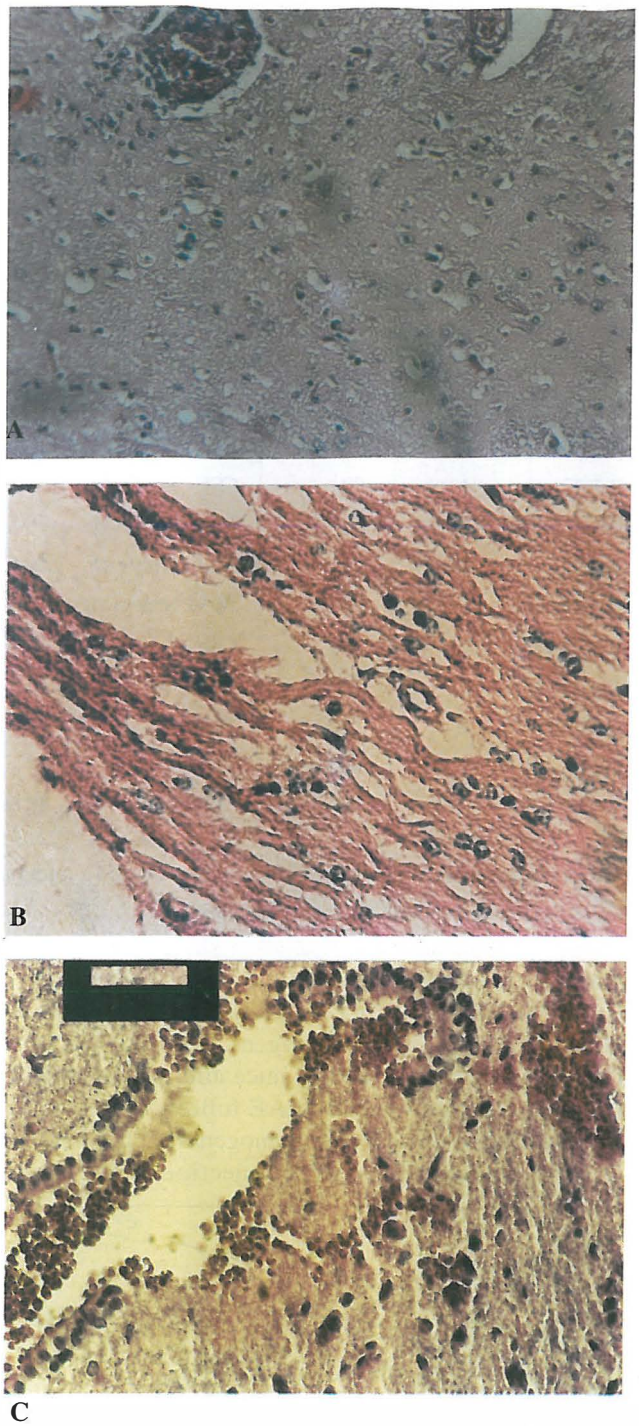

Fig. 4. Some histopathological data of EAE animals.
A. Infiltration in guinea pig
B. Demyelination in guinea pig
C. Congestion in Balb/C.

DISCUSSION

Animal models have had a critical role in solving problems in different fields of biology and medicine. For example, transgenic mice, containing one oncogene, have an important role to find the mechanism of tumorigen-

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esis. To understand the initiation and progression of MS disease and investigation of the cellular and molecular basis of MS, the researchers developed an EAE model. There are several methods for developing this animal model. We used active EAE, which is made by injecting autoantigens into susceptible animals.[3,4-9]

When a foreign antigen confronts the immune system, two processes can take place: tolerance or immunity. Whether tolerance or immunity develops depends on many factors including the route of administration, whether adjuvants are used, and the concentration and physical state of the antigen.[10]

There are many reports on the encephalitogenic potential of PLP.[3,4-12] So we extracted this protein for inducing EAE. The typical PLP recovery from 50 g of bovine brain ranged from 40-80 mg depending upon the amount of myelin in the portion of brain utilized. PLP prepared in this study is 20 mg from 50 gr bovine brain. A minor contaminant having slightly higher electrophoretic mobility (around 20 kd) than PLP, suggested previously to be DM-20, has been detected in some of our preparations.[1]

Preferentially, the researchers use SJL/J, PL/J mice and Lewis rats for developing the EAE model but since these species were not accessible in Iran, Balb/C, guinea pigs and Ratus ratus were used in this research. Of course, Ratus ratus did not show any sign of disease.

We established murine and Guinea pig models of EAE in which the disease was moderate to severe. Balb/C mice are less susceptible to the induction of EAE than SJL/J and PL/J, which is observed in the incidence of the disease. The efficiency of inducing EAE in different reports is very versatile.[10,12] In our experiments 40% of the immunized animals had clinical symptoms. However, the rest of the animals manifested a subclinical state of the disease, and presented some degree of histological alterations.

Cow PLP results corresponds to the cow MBP that previously has been reported to be encephalitogenic in Balb/C. Our research showed that introducing the antigen subcuteously or intraperitoneally is the most effective method of inducing EAE. The result of intramuscular administration of antigen was negative. So the animal strain, the encephalitogenic potential of introduced antigen and the method of immunization are important parameters for determining the efficiency of experiment.

In summary, we have described a method which allows the isolation of PLP from bovine brain and then the development of an experimental animal model for MS disease by introducing bovine brain PLP onto some rodent strains.

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