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ISOLATION OF MYELIN BASIC PROTEIN AND DETECTION OF ITS IMMUNOLOGICAL PROPERTIES

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system of presumed autoimmune etiology. One of the best animal models of demyelinating diseases is experimental autoimmune encephalomyelitis (EAE), which can be induced in a variety of animals by injection of a target antigen such as myelin basic protein (MBP). The immune responses against the target amino acids cause tissue damages such as demyelination in the CNS.

In this study, after isolation of myelin basic protein from bovine cord, we examined its purity and molecular weight by SDS-PAGE and in order to investigate its immunological properties, two varieties of guinea pigs were injected with different amounts of the isolated protein.

Clinical signs of EAE and also histological changes were detected. Delayed type hypersensitivity, and anti-MBP antibodies to the isolated MBP were also investigated. Another form of EAE which has been called chronic-relapsing EAE (CR-EAE), was induced by injection of cord homogenate. EAE and CR-EAE are the best models for MS investigations.
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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS). It is the most common crippling neurological disorder of young adults.[1,2] It is thought to be an autoimmune disease mediated by T-lymphocytes that recognize myelin components of the CNS.[1-3]

Clinical onset of MS is frequently associated with weakness or visual disturbances, and in advanced disease, the clinical picture may be dominated by spastic paralysis,or by wild, uncontrollable intention tremor, urinary and/or bowel incontinence, and varying degrees of dementia. Most cases of MS start between 10 and 60 years of age. Women outnumber men by a factor of which varies from 5 to 7 in different series.[4,5]

Because of its inflammatory nature and evidence for immune activation, clinical trials have attempted to ameliorate the disease by immunosuppression.[5]

Therapies in current use or under investigation which are attempting to suppress immunoresponses, prevent the presumed viral triggering,or modify immune regulation, and reduce inflammation.[6,7]

Because the majority of immunosuppressive agents used are non-specific and have toxic side effects, the goal of immunotherapy is to develop an antigen-specific, non-toxic method to suppress the immune response that

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is postulated to be central to these diseases.[8,9]

In MS, myelin basic protein (MBP) and proteolipid protein (PLP) are probably the major targets of cell-mediated autoimmune responses.[10-12] When animals are injected with MBP or PLP with complete Freund's adjuvant (CFA), experimental autoimmune encephalomyelitis (EAE) is induced, which is a T-cell mediated autoimmune disease of the central nervous system, characterized clinically and pathologically to MS. EAE is the best model to examine the demyelinating diseases especially MS and also to assess the new drugs or the modern ways to suppress it.[7]

In this study, we attempted to induce EAE in guinea pigs to find the optional encephalitogen amount. Our goal was to observe the clinical signs of the disease and the pathological disorders, especially infiltration of inflammatory cells to CNS, which is the best sign of encephalomyelitis, and also detect the immunological changes in affected and unaffected animals.[1] firmed that the isolated protein was MBP, or if it was not the complete protein, could be the encephalitogenic peptide of it. This isolated protein can be used in the related investigations or immunosuppressive therapies.

MATERIAL AND METHODS

Myelin basic protein was purified from bovine spinal cord, which had been collected at the slaughterhouse and transferred to the laboratory under refrigeration. After cleaning and stripping of meninges and rinsing with saline, the cord was cut into small pieces.

Purification was done in the following manner according to Eylar et al:[13,15,16]

Approximately 100 g of bovine cord was used for each preparation. All steps were done at 0-4°C.

Defatting with chloroform-methanol (2:1)

Pieces of cord were mixed in 500 mL of cold chloroform and 250 mL of cold methanol. The suspension was poured into a decanter and remained at 4°C to separate lipids. The lower layer was discarded and the residue was filtered through Watman No. 4 paper and then was washed with 100 mL of cold acetone. After drying, it was suspended in 500 mL of cold distilled water.

Acid extraction

Adding 6N HCl, the suspension was adjusted to pH 2.1, and stirred at 15000g for 15 minutes. The precipitate was discarded and the supernatant was collected.

Neutralization

The supernatant fluid was adjusted to pH 5.5 with 15N NH_4OH and stirred for 1 hour, during which a small precipitate was formed. It was removed by centrifugation at 15000 g for 15 minutes.

Protein precipitation

The remaining proteins were precipitated with ammonium sulfate 50% (356 g/lit).The suspension was stirred for 4 hours at 4°C. After centrifugation at 15000 g for 20 minutes, the supernatant was discarded and the precipitate was washed with saturated ammonium sulfate and centrifuged at 15000 g for 15 minutes. The collected precipitate was dissolved in 30 mL cold water. Acid acetone was added to the solution (12N HCl in 1 liter acetone), and remained for 1 hour to form a white folliculated precipitate, which was collected by centrifugation at 15000 g for 20 minutes. It was dissolved in 10 mL cold water.

Dialysis

The solution was dialysed overnight against water which was changed after 6 hours. Generally a small precipitate was formed during the dialysis . It was removed by centrifugation at 15000g for 15 minutes.The dialysed solution was collected in 1-1.5 mL aliquots and stored at -20°C.

The amount of protein was determined by the Lowry method.[17] amounts (serial dilutions) of bovine serum albumin. To obtain different dosages of protein, it was diluted in distilled water.

Purity and molecular weight of the isolated protein was determined by SDS-PAGE.[18] The calibration curve was drawn using three standard proteins: albumin from bovine serum, carbonic anhydrase from bovine RBC, and cytochrome C from horse heart.

The best way to confirm a protein as MBP is to assess its encephalitogenic properties. So the immunological activities of the isolated protein was tested in guinea pig which is the most sensitive animal to the encephalitogenic activity of myelin basic protein.[19] varieties of guinea pigs were taken from Razi Institute and Pasteur Institute, strains of guinea pigs were not known, so we called them Red-eye and Black-eye, based on their phenotypes which can result from their genotypes. Animals from each variety were divided into control and test groups, each group consisting of 6 animals. Different dosages of isolated protein (25,50,75,100 and 150 µg) were used to achieve the best encephalitogenic amount. Each dilution was emulsified with an equal volume of complete Freund's adjuvant (CFA) in double syringes. Test animals were injected intradermally with 0.2mL mixture of protein and CFA. Control animals received the same volume of emulsion without the protein.

Animals so treated were weighted and observed daily

for development of clinical signs of experimental autoimmune encephalomyelitis (EAE), which generally appeared between days 13-18. The clinical signs were scored by the following manner according to Swanborg:[19]

1- Loss of hind leg tonicity
2- Hind leg weakness
3- Hind leg paralysis
4- Definite hindquarter paralysis
5- Hindquarter paralysis and death sometimes without clinical signs at the time of onset of disease.

The relation of antigen dosage to the disease severity, the day of onset of the disease, and the day of death were compared between the two varieties of animals using chi-square test.[21,22]

The brain and spinal cord of dead animals and animals not showing clinical signs were taken for histological examinations. After fixation in 10% formalin, slices from different areas of brain, as well as cervical and thoracic regions of the spinal cord were embedded in paraffin, and sections were stained with hematoxylin and eosin. Cellular infiltration, edema and perivascular cuffing in the brain and spinal cord were observed. Histological lesions were scored according to Lewine - Wenk in the following manner[19]:

0- No inflammatory lesion
1- About 5 lesions in all fields
2- Lesions in some fields
3- Many lesions in many fields

To examine the immunological properties of the isolated protein, delayed type hypersensitivity to the isolated protein was determined in the animals before and after challenge by protein. The animals were injected subcutaneously with 20 μg of protein.[15,16]

To assay anti-MBP antibodies, double diffusion precipitation in gel by serial dilutions of serum against 100 μg of isolated protein were done. The serum of challenged and non-challenged animals and those with clinical signs and those not showing clinical signs, all were tested.[23]

The encephalitogenic activity of nervous system tissues, were examined by injection of homogenate of cord. A group of 6 black-eye guinea pigs were injected with 200 μg of bovine cord homogenate, emulsified in CFA.

RESULTS AND DISCUSSION

The yield of isolated protein from each 100 g of bovine cord in each preparation was 10-15 mg/mL.

Gel electrophoresis of the proteins showed one distinct protein band with a molecular weight of 17.8 KD which is very close to the molecular weight of myelin basic protein (18.3 KD).[15,16]

Two faint bands above the major protein band were also detectable which probably represented aggregated protein (Fig.1).

The clinical signs of EAE were observed in some of the animals in each test group, which generally appeared between days 13-18 after injection. The severity of clinical signs (shown by the mean score of each group), were different related to the amount of the protein injected. The animals were scored as mentioned above (Fig. 2). Death occurred at days 12-14 after injection in some of the animals. Similar findings have been reported by Eylar[16] et al. Statistical evaluation by calculating of Chi square(γ^2 =8.7), showed that with more than 95 percent of confidence, black-eye guinea pigs were more sensitive than red-eye guinea pigs to even low dosage of protein (Table I). Previously, it has been reported by Swanborg et al. that there are variations of EAE among different species and strains of an animal.[24]

The relative resistance of red-eye guinea pigs can be due to not-having or having low numbers of encephalitogen-reacted T-cells. Because in autoimmune encephalomyelitis the autoreactive T-cells are the effector cells which cause tissue damages. As it has been reported, the MBP-specific T-reactive cells have regulatory or inhibitory effects on the other cells involved in EAE.[25,26]

Relation of severity (shown by the mean score of disease), the mean day of onset of the disease and the mean day of death to the antigen dosage in different groups of Black-eye guinea pigs showed that with more than 95 percent of confidence, increasing the amount of injected protein resulted in an increase of severity of the disease and death but it had no significant effect on the day of onset of disease (Tables II-IV), however, the results revealed that the disease initiated sooner by higher doses of protein. In some animals unexpected results were observed which had effects on the calculated mean amounts.

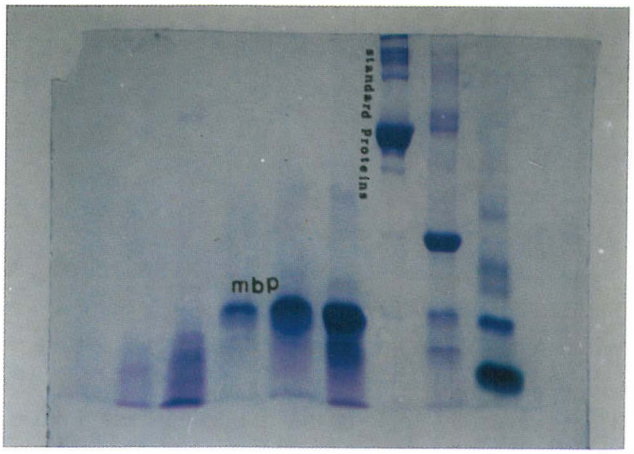
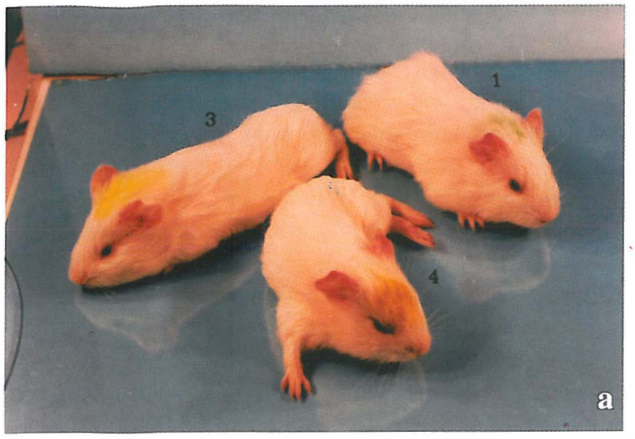
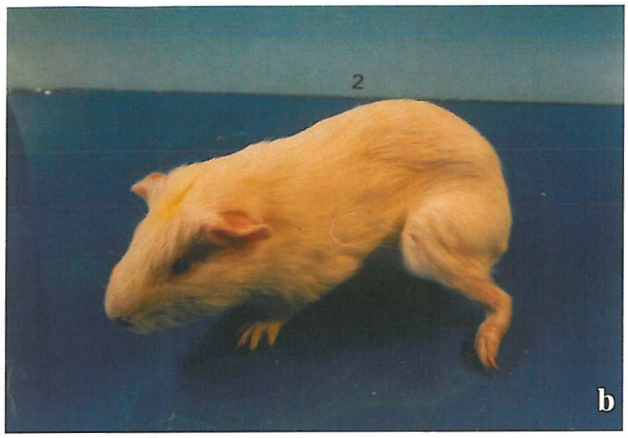


Fig. 1. The protein bands in SDS-PAGE from isolated and standard proteins.





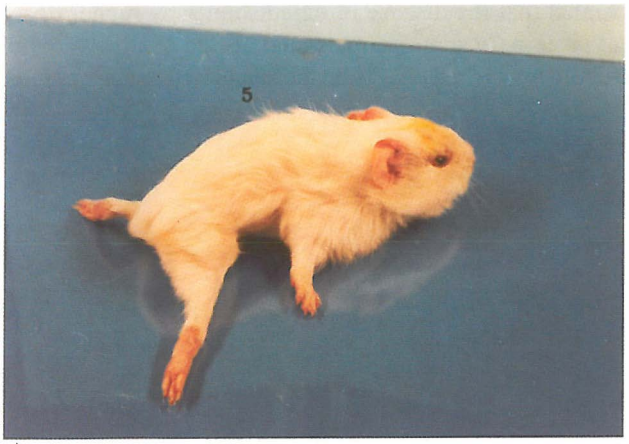


Fig. 2. EAE induced by injection of different amounts of isolated protein with CFA. Scores 1,3,4(A), score 2 (B) and score 5(C).

These variations could be due to the genetic and physiologic differences of individual animals or caused by the conditions of experiments.[24]

According to these results, the best amount of protein for induction of EAE which did not cause death at the early days after injection was obtained at the level of 50 μg.

Table I. Relationship between the severity of the disease and the amount of injected protein in two groups of guinea pigs.

Severity of disease	Amount of protein 25μg	50μg	100μg	150μg
Red-eye guinea pigs	0	3	0	3.5
Black-eye guinea pigs	3	4	5	5

Table II. Relationship between the severity of the disease and the amount of injected protein in black-eye guinea pigs.

Amount of protein (μg)	25	50	75	100	150
Severity of disease	3	4	4.5	5	5

Table III. Relationship between the day of onset of the disease and the amount of injected protein in black-eye guinea pigs.

Amount of protein (μg)	25	50	75	75	100	150
Day of onset of disease	17	13.5	14	16	15.5	12

Table IV. Relationship between the day of death and the amount of injected protein in black-eye guinea pigs.

Amount of protein (μg)	25	50	75	100	150
Day of death	19	17	18	18	13.5

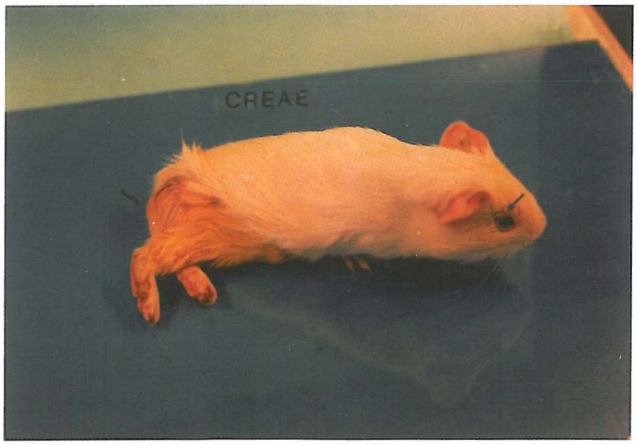


Fig. 3. CREAE induced by injection of cord homogenate. Note the infection of eyes and paralysis of the lateral parts.

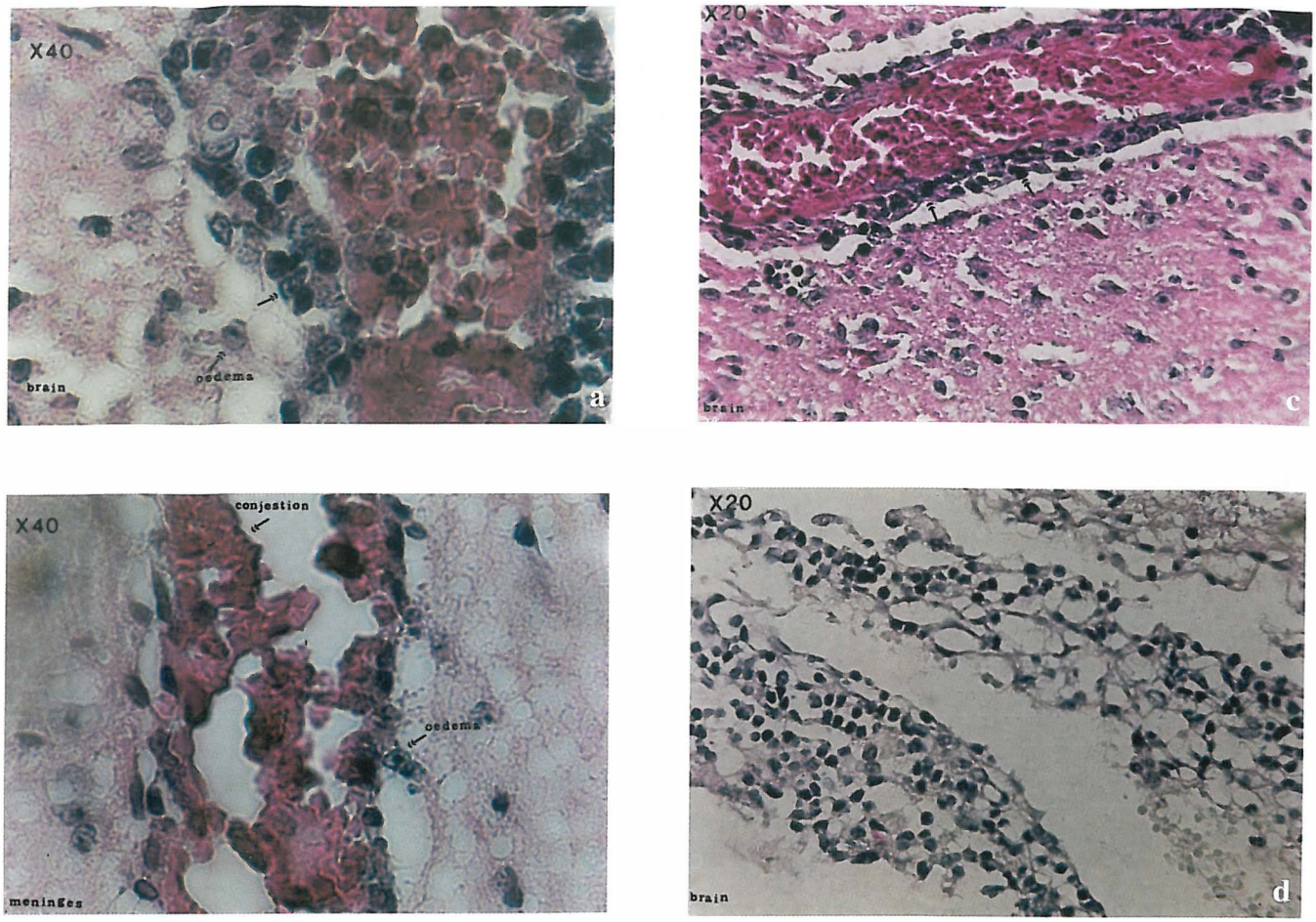


Fig. 4. The histological damages in the central nervous system, conjestion and edema in brain (a), meninges (b), (tissues stained with hemotoxylin & eosin, x40), infiltration of lymphocytes and monocytes in brain (c), spongiosis in brain (d). (tissues stained with hematoxylin & eosin, x 20).

The bovine cord as a whole has also been used to examine encephalitogenic activities of CNS tissues.

Two out of 6 animals which had been injected with 200 µg of cord homogenate, showed the clinical signs of encephalomyelitis after 40 days (Fig. 3).

The disease induced was different according to the day of onset of the disease and somehow in clinical signs, with the animals injected with purified protein. There was no evidence of death in this group and paralysis has been observed only in the lateral parts of the body such as hind legs. This type of EAE has been reported previously by Brod & Al Sabbagh as chronic relapsing-EAE (CREAE), which is similar to multiple sclerosis in man.[7]

Histological lesions included: lymphocyte meningitis, congestion, perivascular cuffing, intra- and extra-cellular infiltration, edema and status spongiosis, which is similar to demyelinated plaques in MS. These lesions were seen in all regions of the CNS in the animals which were injected with different amounts of protein (Fig. 4).

In the animals challenged by cord homogenate, the lesions were fewer and milder than the animals given MBP, and also severity of the lesions were increased relative to the severity of the disease and the amount of protein injected.

The location of lesions were related to the type and severity of the disease. In the cases that lesions in both brain and cord were seen, spastic paralysis was observed, and in the cases that more lesions in the brain, but no lesion in the cord were seen, flaccid paralysis occurred.[27]

The type of immunological cells in plaques were different compared to the age of the lesions. Since most of the tissues were taken after death, T-reactive cells were the dominant cells in the inflammatory lesions. The results were similar to the previous reports.[19,28]

The results of DTH were as presented (Table V & Fig. 5). It shows that the injection of protein could induce the cell-mediated immunological responses, so the protein is a target of the cellular immune system.[4,15,16]

Table V. Diameter of the induration in DTH (mm) in black-eye guinea pigs.

Diameter of induration (mm) / Animals	Induced by	
	Myelin basic protein	Saline
Control group	0	0
Injected group by tissue homogenate	3-10	0
Injected by MBP		
Diseased	1-5	0
Not diseased	1-5	0

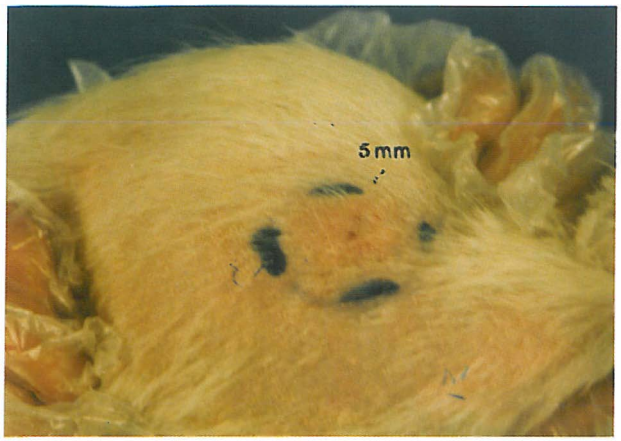


Fig. 5. DTH test in injected animals with tissue homogenate, the diameter of induration is 5 mm.

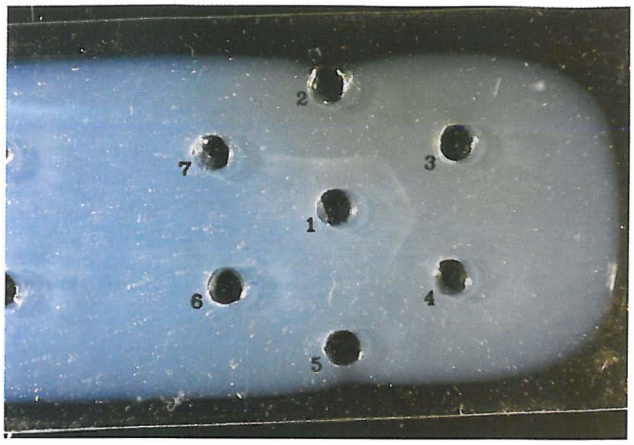


Fig. 6. Ouchterlony immunodiffusion analysis of anti - MBP antibodies. (Well 1 :isolated protein, Wells 2 to 7: double serial dilutions of serum of injected animals).

Table VI. Mean titer of anti-MBP antibodies in the serum of test groups.

Titer of serum / Groups	1	1/ 2	1/ 4	1/8	1/16	1/32
Control	+	-	-	-	-	-
Challenged by MBP (Red-eye) (Not diseased)	+	-	-	-	-	-
Challenged by MBP (Black-eye) (Diseased)	+	+	+	-	-	-
Challenged by tissue	+	+	+	+	-	-

In all of the injected and not injected animals, anti - MBP antibodies were found by double diffusion precipitation, but the titer of antibodies in the animals not challenged with MBP was less than in the animals injected by MBP or cord homogenate (Table VI & Fig 6). So, it could be concluded that the protein isolated consisted of antibody-reacting peptides. The presence in normal sera of antibodies that inhibit binding of a variety of autoantibodies have been demonstrated. These binding or blocking antibodies are considered to play a role in humoral self tolerance.[29,30]

The anti-MBP antibodies have also been shown previously in demyelinating diseases.[23,29]

These results show that the protein isolated from bo-

vine cord can be myelin basic protein or at least it may contain the encephalitogenic peptides.[31-33] Based on the clinical signs of disease and histological lesions in the CNS and compared to the previous studies,[7] the disease was EAE. It has been reported that guinea pig is the most sensitive animal to all of the MBPs isolated from CNSs of different animals,[19,24] but MBP of bovine had not been used until now as an experimental antigen to induce EAE. Because of the availability of bovine cord, it can be introduced as a good and economical source for purification of encephalitogenic protein to induce EAE and also the related investigations.

Since EAE and CREAE are the best models of human demyelinating diseases, especially MS, this method offers an easy and safe way to investigate different aspects of demyelinating diseases and the effect of innovated drugs.

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