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Encephalitogenicity of myelin-associated oligodendrocytic basic protein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase for BALB/c and SJL mice

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SUMMARY

In search of new encephalitogenic myelin antigens, the 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) and 19 000 MW isoform of myelin-associated oligodendrocytic basic protein (MOBP) were obtained as recombinant proteins by the baculovirus expression system in *Spodoptera frugiperda* cells and purified to homogeneity by immobilized metal chelate affinity chromatography (IMAC). The purified MOBP was soluble in water and showed retarded migration on sodium dodecyl sulphate–polyacrylamide gel electrophoresis similar to myelin basic protein (MBP). MOBP induced experimental autoimmune encephalomyelitis (EAE) in nine of 15 susceptible SJL OlaHsd mice, causing death in two animals, whereas three of 14 BALB/c mice showed mild symptoms of EAE, manifested as transient weakness of hind limbs. In both mouse strains, periventricular infiltrates of mononuclear cells were observed. In addition, both 46 000 MW and 48 000 MW CNP isoforms were shown to be non-encephalitogenic for both mouse strains.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disorder of the central nervous system (CNS), which can be induced in rodents through a single immunization with CNS tissue homogenate or purified myelin antigens in complete Freund's adjuvant (CFA).[1] EAE has been widely used as a model for the human disease multiple sclerosis (MS). The encephalitogenicity of myelin basic protein (MBP),[2,3] proteolipid protein (PLP) and its DM-20 isoform[4–6] and myelin oligodendrocytic glycoprotein (MOG)[7] has been characterized in different mouse strains. In this study we have evaluated the encephalitogenic potential of the 19 000 MW isoform of rat myelin-associated oligodendrocytic basic protein (MOBP; rOPRP1)[8,9] (named MOBP-19 hereafter) and mouse 2′,3′-cyclic nucleotide 3′-phosphodiesterases (CNP) in susceptible SJL OlaHsd and genetically resistant BALB/c AnNHsd mice. Mouse MBP including all its isoforms[10] was used as a reference antigen.

MOBP is expressed in mammalian brain and spinal cord and at least five distinct isoforms are created by alternative splicing mechanisms.[8,9] In all these isoforms, the first 68 amino acids are identical but the C-terminal parts vary in length.[8,9] Expression of MOBP is developmentally regulated and possibly connected to the formation of compact myelin.[8,9,11]

CNP exists as two isoforms (CNP1, 46 000 MW; CNP2, 48 000 MW) formed similarly by alternative splicing leading to two mRNA differing only at their 5′-terminus.[12,13] These two enzymes are expressed at high levels in the CNS during active myelination[14] and the expression is restricted to oligodendrocytes.[15] However, low levels of these enzymes are found also in the peripheral lymphoid tissues.[12]

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Abbreviations: CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37); CNS, central nervous system; DM-20, 20 000 MW isoform of proteolipoprotein; EAE, experimental autoimmune encephalomyelitis; ECL, enhanced chemiluminescence; IMAC, immobilized metal chelate affinity chromatography; MBP, myelin basic protein; MOBP, myelin-associated oligodendrocytic basic protein; MOG, myelin oligodendrocytic glycoprotein; MS, multiple sclerosis; p.i., postimmunization; PLP, proteolipoprotein.

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MATERIALS AND METHODS

Reverse transcription–polymerase chain reaction (RT-PCR)

For preparation of complementary DNA (cDNA) for rat MOBP-19 (rOPRP1), see Yamamoto *et al.*[8] The cDNA of mouse CNP1 and CNP2 mRNA were synthesized using an oligonucleotide specific for the 3′-terminus of both the CNP mRNA. Total RNA was isolated from mouse brain at the second postnatal day by Ultraspec reagent (Biotecx inc., Houston, TX)[16] and subjected subsequently to reverse transcription. Briefly, the reaction mixture (40 μl) containing 5 μg total RNA, 1·2 mM dNTP, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 50 mM Tris–HCl, pH 8·3, 75 mM KCl, and 50 pmol primer specific to the 3′-terminus of both CNP mRNA

(Table 1) was heated at 60° for 10 min, chilled on ice and 200 U Moloney murine leukaemia virus (MMLV)-RT (Promega, Madison, WI) were added. After 1 hr incubation at 37° the synthesis was stopped by heating for 5 min at 95°.

The PCR reaction mixture (50 µl) consisted of a sample of 5 µl of the CNP reverse transcription reaction mixture or 100 ng of MOBP-19 cDNA, 2 U TAQ polymerase (HyTest, Finland), 1·2 mM $MgCl_2$, 0·25 mM dNTP, 50 mM KCl, 0·1% Triton-X-100, 10 mM Tris–HCl pH 8·8, and 50 pmol of the specific primers (Table 1). The PCR reaction included 42 cycles. The first PCR cycle included 2 min denaturation (95°), 2 min annealing (50°) and 2 min elongation (72°) steps. Cycles 2–6 had 60 seconds denaturation (95°), two separate annealing steps, one at 70° and a subsequent step at 40° (both 40 seconds) due to differences in primer length, and a 60-second elongation step (72°). During cycles 7–42 the latter annealing temperature was raised to 62°. The final elongation step lasted 5 min. The PCR products were first cloned in pGEM-T vector (Promega) and their intactness was confirmed by sequencing.[17] For all genes a nucleotide sequence encoding a histidine hexamer was included in the 3′-terminal oligonucleotides (Table 1) to facilitate the purification by immobilized metal chelate affinity chromatography (IMAC).[18]

Preparation of expression vectors

The CNP- and MOBP-19-encoding constructs were cloned in p503-9 vector, a Fastbac (Gibco, Gaithersburg, MD) derivative obtained from Dr Keinänen, VTT Research Laboratory, Espoo, Finland. The p503-9 contains a Flag-peptide (DYKDDDDK)[19] encoding sequence, which was positioned in frame with the 5′-terminus of the CNP and MOBP cDNA. The N-terminal Flag-peptide allows recombinant protein detection by the M1 monoclonal antibody (Kodak, Rochester, NY).[19] These constructs were used to transform DH10Bac *Escherichia coli* cells.[20] Colonies containing recombinant baculovirus genomes (bacmids) were screened using blue/white selection and a Southern hybridization technique with gene-specific probes. The presence of insert of correct size was ascertained by PCR using oligonucleotides specific for polyhedrin promoter in the transfer vector and kanamycin-resistance gene in the viral DNA.

Expression of recombinant proteins

The recombinant baculoviral DNAs were transfected into Sf9 insect cells on six-well culture plates by Lipofectin (Gibco) according to the manufacturer's instructions. Cells were analysed for recombinant protein production by Western blot analysis 48 hr after transfection. Primary culture supernatants containing recombinant baculoviruses were used to infect monolayer cultures of Sf9 cells and were passaged once. Stock virus obtained from these cultures was used to infect suspension culture at cell densities of $1{\cdot}3 \times 10^6 - 2{\cdot}0 \times 10^6$ cells/ml in 400 ml of TMN-FH medium supplemented with 10% fetal calf serum (FCS) and gentamycin (50 µg/ml) and the suspension was rotated at 50 r.p.m. in 500 ml spinner flasks (Bellco, USA). After 72 hr producing maximal protein amount, the cells were pelleted and stored at −70°.

Purification of recombinant proteins

Frozen insect cells were homogenized with a tissue homogenizer (Janke Kunkel) in 20 ml of ice-cold distilled water containing 1 mM protease inhibitors phenylmethyl sulphonium fluoride and aminoethyl isothiouronium bromide (both from Sigma, St Louis, MO). The homogenates were sonicated thereafter on ice for 1 min by a needle sonicator. According to Western analysis, in all cases the recombinant proteins were located in the water-insoluble membrane-containing fraction obtained by centrifugation at +4° with 35 000 ***g*** for 15 min using a Sorvall SA-600 rotor. The pellet was suspended in 20 ml of lysis buffer (6 M guanidine hydrochloride, 0·5 M NaCl, 20 mM sodium phosphate, pH 7·8) and clarified by centrifugation as above. Supernatants were run through a column containing 7 ml of $NiCl_2$-regenerated Chelating Sepharose Fast Flow resin (Pharmacia Biotech, Uppsala, Sweden) at a flow of 10 ml/hr. The column was washed with 20 ml of lysis buffer (pH 7·8), and then subsequently with 30 ml of the same buffer pH adjusted at 6·0, 5·0 and 4·7. The proteins were eluted by adding 50 mM ethylene diaminetetraacetic acid (EDTA) to the pH 4·7 buffer and 2 ml fractions were collected. Protein-containing fractions were pooled and dialysed extensively against distilled water at +4°.

Protein analysis

Protein concentrations were determined by Bio-Rad protein assay (Bio Rad Laboratories, Hercules, CA).[21] SDS–PAGE minigels were run according to Laemmli *et al.*[22] For immunoblot the proteins were transferred onto a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany). The filter was incubated with M1-antibody (Kodak)[19] and the recombinant proteins were detected by peroxidase-conjugated rabbit anti-

Table 1. PCR oligonucleotides*

	SphI
CNP-I.1	5′-TTATA GCATGC ATG CCA TGT CAT CCT CAG GAG CAA AGG-3′
	SphI
CNP-II.1	5′-TTATA GCATGC ATG CCA TGA GCA CAA GCT TTG CCC G
	BamHI * H H H H H H
CNP-2	5′-TTATA GGATCC TCA GTG ATG GTG ATG GTG ATG GAT GAT GGT GCAGAT CTG CAT GG-3′
	NotI
MOBP-1	5′-TTAT GCGGCCG TA AGT CAA AAA GTG GCC AAG-3′
	BamHI * H H H H H H
MOBP-2	5′-AAGC GGATCC TTA GTG GTG ATG ATG GTG GTG CCA GAA CCT AGG AGC TCT-3′

*CNP-I.1 and CNP-II.1 oligonucleotides were used to add SphI and NotI restriction sites to the 5′ end of the CNP and MOBP cDNAs to allow positioning of the genes in frame immediately 3′ to the Flag-peptide encoding sequence present on the p503-9 shuttle vector. Polyhistidine-encoding sequence and a stop-codon were included in the 3′-end of the corresponding cDNAs by CNP-2 and MOBP-2 oligonucleotides. The CNP-2 oligonucleotide was used to generate both CNP1 and CNP2 cDNAs.

mouse antibody (Dako, Glostrup, Denmark) and the enhanced chemiluminescence (ECL) reagents from Amersham (Amersham, Little Chalfont, UK) exposing Kodak X-OMAT AR X-ray film.

Induction of EAE

Six- to eight-week-old specific pathogen-free female BALB/c AnNHsd and SJL OlaHsd mice (both from Harlan Laboratories, IN) were used in the experiments. The antigen–adjuvant emulsion was formed using ultrasound, previously shown to lead to enhanced encephalitogenicity for BALB/c mice.[23] Briefly, the neuroantigen in phosphate-buffered saline (PBS) was emulsified 1 : 1 (v/v) with CFA consisting of mineral oil, *Mycobacterium tuberculosis* H37RA (4 mg/ml) and *M. butyricum* (0·5 mg/ml) (both from Difco Laboratories, Detroit, MI) by 5-min sonication in a Cole-Palmer water bath sonicator until the adjuvant was non-separating on water. A total volume of 0·1 ml of the adjuvant emulsion containing 100 μg MOBP-19 or 400 μg of either isoform of CNP or mouse MBP was injected into hind foot pads of the mice (0·05 ml in each foot pad). *Bordetella pertussis* toxin (200 ng) (List Laboratories, Campbell, USA) was given intravenously 24 hr after immunization.

The mice used in this study were treated in accordance with the guidelines of the Turku University Ethics Committee and were housed in the animal facility of the university. Mice were kept in filter-covered cages in an illumination-regulated room and were observed daily for clinical symptoms of EAE, always at the same time. The disease was scored according to the clinical status as follows: 1, tail atony and/or slight weakness of one hind limb; 2, slight paralysis of hind limbs; 3, severe paralysis of hind limbs; 4, moribund; 5, dead. Mice moribund at the time of observation were killed and they obtained the value 4 in the clinical scoring.

Histological staining

Three SJL and three BALB/c mice were anaesthetized by carbon dioxide and perfused with 10% phosphate-buffered formalin. The SJL samples were from a mouse with grade 4 EAE at day 10 postimmunization (p.i.), another animal with grade 2 EAE at day 12 p.i. and a third recovering from grade 3 EAE at day 15 p.i. Two BALB/c samples were from mice both having grade 1 EAE at day 16 p.i. and a third from a mouse recovered from grade 1 EAE at day 23 p.i. Brains and spinal cords were cut into three coronal sections representing cerebrum, cerebellum and medulla oblongata and cervical, thoracic and lumbar spinal cord, respectively. The sections were stored in 10% phosphate-buffered formalin and subsequently embedded in paraffin. Five-millimetre coronal sections were stained with haematoxylin/eosin.

RESULTS

Expression and purification of CNP and MOBP-19

The purity of baculovirus-expressed CNP1, CNP2 and MOBP-19 is demonstrated in Fig. 1. CNP appeared in the crude insoluble fraction of the Sf9 cells and was clearly visible in Coomassie-staining (Fig. 1, lane 1). The ECL-based immunoblot revealed partial degradation of CNP1 in the cell homogenates (Fig. 1, lane 2), whereas the purified CNP1 was homogeneous (Fig. 1, lane 3). However, slight aggregation of

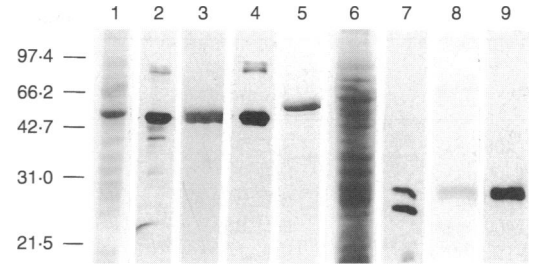

Figure 1. Analysis of recombinant CNS proteins expressed by Sf9 cells. Coomassie-staining: CNP1 crude cell extract, lane 1; purified CNP1 (5 μg), lane 3; purified CNP2 (5 μg), lane 5; MOBP-19 crude cell extract, lane 6; purified MOBP-19 (5 μg), lane 8. Immunostaining: CNP1 crude cell extract, lane 2; purified CNP1 (5 μg), lane 4; MOBP-19 crude cell extract, lane 7; purified MOBP-19 (5 μg), lane 9. Positions of standard proteins of a Promega Mid-Range Molecular Weight Marker run in parallel are indicated. For immunodetection an M1-antibody specific for the N-terminal Flag-peptide was used (see the Materials and Methods).

the protein was seen (Fig. 1, lane 4). Purified CNP2 contained trace amounts of contaminating proteins (Fig. 1, lane 5). The purity of the final protein product was estimated to be >90% for CNP1 and >80% for CNP2 on SDS–PAGE. The final products were insoluble in water and aqueous buffers.

MOBP-19 was initially included in the insoluble membrane fraction similarly to CNP, but the purified protein was soluble in water and appeared as one poorly staining band showing retarded migration on SDS–PAGE (Fig. 1, lane 8). While in the cell homogenate the M1-antibody specific for the N-terminal flag-peptide detected two separate bands (Fig. 1, lane 7), after the IMAC column chromatography only one band was visible in the immunoblot (Fig. 1, lane 9). As the smaller immunoreactive polypeptide visible in crude extracts was not present in the final product, it probably evolved through cleavage of a C-terminal fragment containing the histidine hexamer. The truncated MOBP also showed considerably strong binding to the IMAC resin as it could not be efficiently eluted until pH 4·7 was introduced in the wash buffer (not shown). The high number of cysteine residues at the N-terminal region of the protein probably contributes to the metal binding.[8] Aggregation generally seen in MBP preparations[10] was not observed for purified recombinant MOBP-19, although formation of SDS- and mercaptoethanol-resistant complexes has been suggested for MOBP.[9] However, the apparent molecular weight of the recombinant MOBP-19 was similar to the high-molecular-weight immunoreactive MOBP detected by Holz *et al.*[9] in brain tissue homogenate using SDS–PAGE/immunoblot.

The yields for CNP1, CNP2 and MOBP-19, starting with 300 mg of total cellular protein, were 45 mg, 60 mg and 3 mg, respectively. In cell homogenates CNP activity could be detected using 2′,3′-cyclic NADP as a substrate (not shown).

Encephalitogenicity of CNPs and MOBP-19

Table 2 summarizes the incidence of EAE in CNP-, MBP-and MOBP-immunized mice. Nine out of 15 SJL mice immunized with 100 μg of MOBP-19 showed clinical signs of EAE. The symptoms, including tremor, stiffness, forced position of head, ataxia and fur ruffing, became apparent in six mice at day

Table 2. Clinical EAE in BALB/c AnNHsd and SJL OlaHsd mice immunized with myelin antigens*

Antigen	Mouse strain	No. of diseased mice	No. of dead mice	Mean day of disease onset†	Mean disease severity†
CNP1 (400 μg)	SJL	0/6	n.a.	n.a.	n.a.
CNP2 (400 μg)	SJL	0/6	n.a.	n.a.	n.a.
CNP1 (400 μg)	BALB/c	0/10	n.a.	n.a.	n.a.
CNP2 (400 μg)	BALB/c	0/10	n.a.	n.a.	n.a.
MOBP-19 (100 μg)	SJL	9/15	2/15	11·0±0·87	3±1·32
MOBP-19 (100 μg)	BALB/c	3/14	0/14	11·7±0·57	1±0
MBP (400 μg)	SJL	4/4	1/4	13·0±0	3·25±1·09
MBP (400 μg)	BALB/c	0/10	0/10	n.a.	n.a.

*For immunization protocol and protein production see Materials and Methods
†Mean±SD.

11 p.i. At day 12 p.i. two mice showed paraparesis and weakness of the forelimbs similar to symptoms of EAE induced by MBP in SJL mice. One of these severely affected mice died and another was killed at a moribund stage at day 10 p.i. In the histological samples, mainly lymphocytic rim-like inflammatory infiltrates were seen in periventricular and perivenular locations (Fig. 2a,b); less intense but more diffuse infiltrates of same composition could be seen in brain parenchyma (Fig. 2a,b). Perivascular and subarachnoidal infiltrates were detected also in the spinal cord and medulla oblongata (not shown). Similar, but less intense, infiltrates were present also in the CNS of a mouse with grade 2 EAE at day 12 p.i. Symptoms of EAE in the surviving mice were transient and the animals recovered until 15 days p.i. and only slight subarachnoidal infiltrates were present in the cerebrum of a recovering mouse but not in the spinal cord (not shown). No relapses were observed during the 30-day follow-up period. Three out of 14 MOBP-immunized BALB/c mice showed mild symptoms of EAE as indicated by hind limb weakness. In one BALB/c mouse sampled at day 16 p.i. a small scattered mononuclear cell infiltrate was detected in the left cerebral ventricle (Fig. 2c).

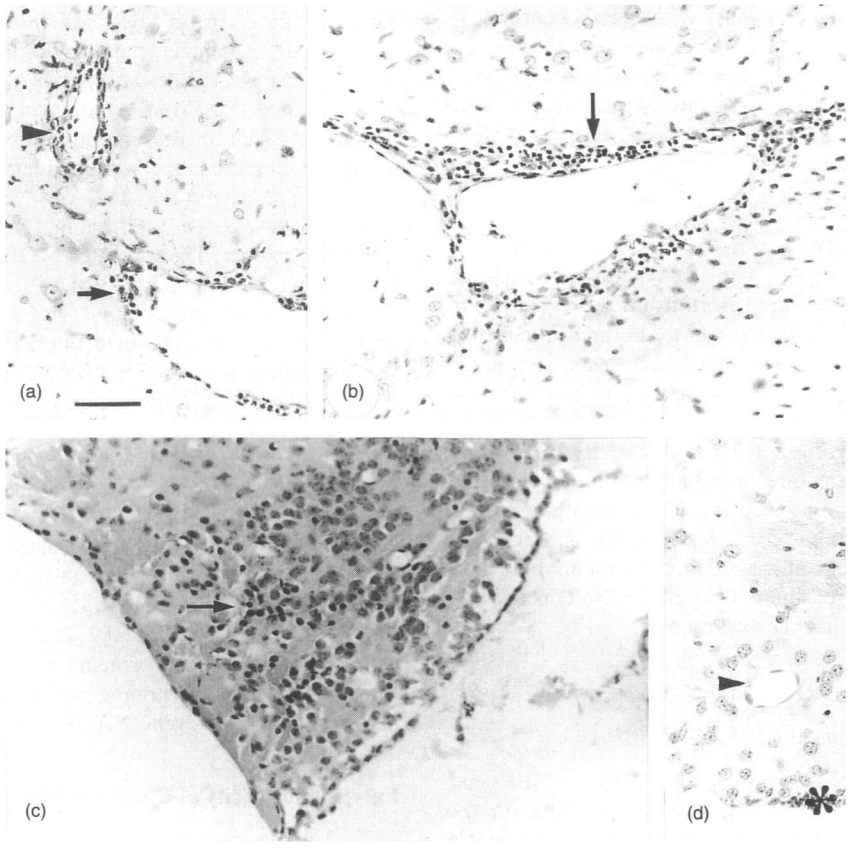

Figure 2. Histological analysis of mouse brains. (a) Perivenular (arrowhead) and part of periventricular (arrow) cellular infiltrate in the cerebrum of a SJL mouse with grade 4 EAE 10. (b) Large periventricular infiltrate in the cerebrum of the same SJL mouse. (c) Scattered periventricular mononuclear cells (arrow) in cerebrum of affected BALB/c mouse. (d) Normal venule (arrowhead) and surface of ventricle (asterisk). Scale bar in (a) represents 52 μm for all figures.

None of the mice immunized with CNP in sonicated CFA showed symptoms of EAE during the 30 days observation period after immunization suggesting that this myelin enzyme is non-encephalitogenic as also shown for CNP1 peptide 153–164 in Lewis rats.[24] Mouse MBP used as a control autoantigen was non-encephalitogenic for the BALB/c mice, but caused fulminant EAE in SJL mice (not shown).

DISCUSSION

The change in the water-solubility of MOBP might be due to an initial association of the protein with cell membranes and later dissociation during the purification process as observed for p69 islet cell antigen (our unpublished result). The relatively low yield of MOBP-19 might in part be attributed to the instability of MOBP-19 indicated by the truncated form of the protein in cell homogenates. Moreover, the expression of MOBP-19 appeared to interfere with the growth of Sf9 cells as ≈50% of the cells died within 48 hr after infection thus reducing the protein yield, whereas expression of CNP did not affect the viability of the cells.

The diffuse, poorly staining appearance on SDS–PAGE of purified MOBP-19 (Fig. 1, lane 8) suggests post-translational modifications, e.g. glycosylation of the protein.[8,9] The deduced molecular weight of the recombinant MOBP-19 including the flag-peptide and the C-terminal histidine hexamer is ≈20 500 MW. However, on SDS–PAGE MOBP-19 appeared at the level of carbonic anhydrase (31 000 MW) showing similarly anomalous behaviour as previously observed for MBP.[10,25] This might be due to the high number of cationic residues rendering MOBP-19 highly cationic with an isoelectric point (pI) of 11·5,[10] and/or is due to glycosylation of the protein which could markedly alter the molecular weight of the protein.[26]

CNP is probably expressed at low levels also in mouse thymus, as has been previously shown for the rat.[12] Low thymic expression might be sufficient to induce tolerance to these autoantigens. This hypothesis is in part supported also by the previous data of Khoury *et al.*[27] who showed that intrathymic injection of MBP renders Lewis rats resistant to EAE induction. Moreover, the control antigen mouse MBP was able to induce severe EAE in the SJL mice, while available rat MOBP was used in the experiments and it is possible that the encephalitogenicity of MOBP may have increased relative to the mouse counterpart. However, as only one methionine to valine substitution has been demonstrated for the 8 000 MW isoform of mouse MOBP compared to the rat protein,[28] one would expect similar homology between the 19 000 MW isoforms.

Expression of MOBP has not been detected in tissues other than brain and spinal cord.[8,9] Given this sequestered expression it is not surprising that the recombinant rat MOBP-19 was found to be encephalitogenic for the susceptible SJL mouse. The amount of MOBP-19 needed for disease development (100 μg) was noticeably lower than that previously observed for other myelin antigens, DM-20, MBP, MOG, and PLP to induce EAE in mice.[2–7] Moreover, mild symptoms of EAE were detected in a minority of the resistant BALB/c mice, suggesting that MOBP has a higher encephalitogenic potential in BALB/c mice than does MBP.

The total unresponsiveness of both SJL and BALB/c mice to CNP raises hopes that thymic expression of autoantigens mediated by suitable expression vehicles could provide a useful means to prevent autoimmune diseases.

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