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IMMUNOCHEMICAL STUDIES OF MYELIN BASIC PROTEIN IN SHIVERER MOUSE DEVOID OF MAJOR DENSE LINE OF MYELIN

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SUMMARY

The myelin-deficient mutant Shiverer (*Shi/Shi*) lacks basic protein (MBP) in the myelin of its central nervous system (CNS). Less than 3% of the normal content in MBP is present in a brain extract of *Shi/Shi* as determined by radioimmunoassay. Indirect immunofluorescence is negative when using specific anti-MBP serum. The importance of *Shi/Shi* (as compared to other hypomelinating mutants) stems from the specificity of this genetic lesion, i.e. the lack of basic protein.

The inner surfaces of the unit membrane of oligodendrocyte (OD) processes involved in myelin formation fuse to form a single major dense line (MDL) while the intraperiod line results from close apposition of the outer leaflets of the same unit membrane. The electron densities in myelin period represent protein, and the clear spaces lipid [17]. Myelin basic protein (MBP) is inaccessible to anti-MBP antibody in intact myelin and becomes accessible only when the membrane is disrupted [9,10,14,23,24]. Also, techniques which involve labelling of the external surface proteins do not reveal MBP unless the nerves have been mechanically disrupted, thereby exposing the internal cytoplasmic surface of myelin [8,19]. These indirect approaches have led to the conclusion that MBP is located on the inner surface of the membrane bilayer corresponding to the major period region of the myelin sheath. On the other hand, it was found [5] that the lack of MBP under conditions which removed only MBP from myelin, led to the collapse of the structure with disappearance of the intraperiod line. This result was interpreted as evidence of the location of this protein in the intraperiod line. The Shiverer dysmyelinating mutant (*Shi/*

Shi) [2] contains a myelin devoid of its MDL [13,21].

C3H/SWV mice and *Shi/Shi* mutants of the same strain were kindly provided by Prof. T. Bird (Department of Neurology and Genetics, Veterans Administration Hospital, Seattle, U.S.A.); Jimpy mutants were procured from the Jackson Laboratory. These strains were raised in our department (INSERM U. 134). 3-month-old mice were used for biochemical analysis and immunochemistry. Lipid analysis was performed as described [20]. MBP was prepared after delipidation overnight of human white matter by homogenization in chloroform/methanol, 2 : 1 (v/v), 6.6 ml/g fresh wt. After centrifugation 30 min at 48,000 *g*, the pellet was acetone-washed (2 ml/g initial fresh wt.) and dried. Acid extract was made in 0.1 N HCl (pH 1.7) (20 ml/g initial fresh wt.) overnight at 4°C and centrifuged 40 min at 16,000 *g*. The supernatant was dialysed 4 h against distilled water and lyophilized. Purification was performed on sulphoethyl Sephadex C-50 [11]. The protein purity was checked by polyacrylamide gel electrophoresis [16]. A single band was obtained. Specific antibodies were raised in the rabbit by using a procedure involving double emulsion of MBP. The emulsion consisted of 2.5 mg/ml of MBP in aqueous solution (1 vol.) and incomplete Freund adjuvant (3 vols.) After vortex mixing, 1 vol. of this emulsion was distributed under magnetic stirring over 4 vols. of a 10% solution of Tween 80. The dispersion was controlled by phase contrast microscopy. Each animal received a total of 3 mg of MBP. The immune sera were obtained and checked by a complement fixation test [7]. Soluble proteins were extracted from forebrain with a barbital buffer of pH 8.6 (2 ml/g wet wt.) by using a Potter-Elvehjem homogenizer. The supernatant was collected after centrifugation at 100,000 *g* and used for determination of MBP with the radioimmunoassay. Specific activity of the labelled MBP prepared according to ref. 3 was about 5 μCi/μg. Briefly, 100 μl of several dilutions of the extracts (or of the human MBP standard solution) and 50 μl of a 1/300 dilution of the antiserum were added to 350 μl of 0.05 M phosphate buffer 0.025 M containing calf thymus histone (pH 7.3). After 15—18 h at 4°C, 50 μl (about 3.000 cpm) of MBP tracer solution was added. The free and bound fractions were separated on charcoal-dextran after an additional 4 h incubation at 4°C. As the competition curves obtained with the dilutions of the extracts were parallel to the human standard curve, the mouse MBP content could be measured. For the immunohistochemical investigations, brains were fixed in 3% formalin in phosphate buffered solution (PBS, pH 7.2), 0.12 M, overnight at 4°C washed for two days in several baths of the buffer solution containing 8% glucose at 4°C, then dehydrated and embedded in parafin. 4 μm coronal sections were placed on gelatin-coated glass slides. After drying, removal of paraffin and rehydration, sections were incubated first with normal sheep antiserum (1/20) during 20 min and treated at room temperature by an indirect immunofluorescence technique [4]. We used a fluorescein-labelled anti-rabbit serum (Institut Pasteur, France) diluted to 1/100. The slides were examined under an epi-illumination UV microscope equipped with an interference primary filter (KP 500 and LP 455) and a HBO 200 excitation lamp. Anti-MBP sera were

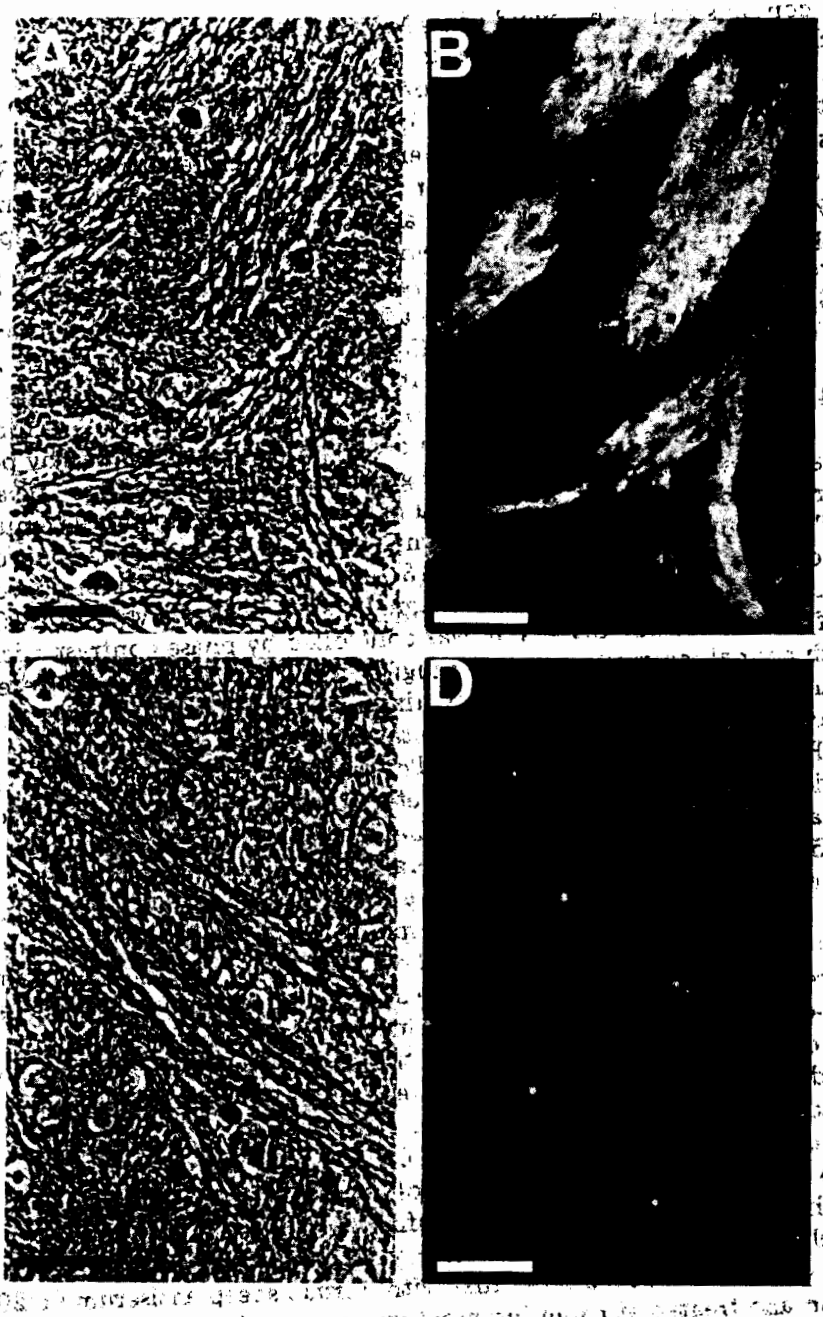

Fig. 1. A: phase contrast photomicrograph taken in the thalamic region of normal mouse brain (coronal section 280 of ref. 22). Bar = 30 μm. B: the same nerve fiber bundles as in A visualized in immunofluorescence by an anti-MBP serum. Bar = 30 μm. C: phase contrast photomicrograph taken in the same conditions and in the same region as A but in *Shi/Shi* mutant brain. Bar = 30 μm. D: the nerve fibre bundles shown in C cannot be visualized with an anti-MBP serum. Bar = 30 μm.

absorbed with MBP coupled to CNBr activated Sepharose 4-B: 30 mg of MBP were fixed on 2 g of Sepharose to absorb 3 ml of antiserum by incubating for 1 h at room temperature. After adsorption, the sera were negative when tested by complement fixation with MBP as antigen.

With 3 anti-MBP sera, intense immunofluorescent staining was seen in myelin of CNS tissue sections in normal C3H/SWV mice (10 animals with sections taken from different parts of the brain). The fluorescence was only present in myelin and visible in coronal and sagittal sections (Figs. 1A, B). The same result was obtained in midbrain, pons and cerebellum. Incubations of sections of the same levels with either preimmune rabbit sera, normal rabbit sera or anti-MBP sera absorbed with MBP, demonstrated a complete absence of reaction with the tissue. Brain sections of *Shi/Shi* incubated with the anti-MBP sera remained unstained whatever the serum or the concentration of the antiserum used (Figs. 1C, D). Radio-immunoassay performed in forebrain extracts of 3-month-old mice showed that, when expressed in relative amount per weight, only 3% of MBP present in the normal brain of the same age was recovered in Shiverer. The same percentage was found for 18-day-old Shiverer mice. It should be noted that in Jimpy of the same age, where dysmyelination is even more pronounced, 20–30% of MBP is present. The results of the quantitative lipid analysis are presented in Table I. The amount of lipid is 73% of normal. Cerebrosides plus sulfatides are largely reduced: 20% of normal.

The present study demonstrates the lack of MBP in the CNS of *Shi/Shi*, whereas a decrease in the myelin content was observed by electron microscopy and confirmed by lipid analysis. Cerebrosides and sulfatides, which are essential components of myelin, were most affected. The amount of these glycolipids is 20% of normal. By comparison, their level is inferior to that found in Quaking mice (38% of normal) [1] and superior to Jimpy's (2–5% of normal) [12,18]. The MBP defect cannot be the mere consequence of a deficit in myelin, since in a case where the deficit is even more pronounced than it is in Jimpy, the MBP can still be detected by radio-immunoassay and immunofluorescence (Dupouey et al., unpublished data). The hypothesis of an antigenic abnormality

TABLE I

COMPOSITION OF BRAIN LIPIDS IN THE SHIVERER MUTANT AND LITTERMATE CONTROL

The results are mean values from at least 3 determinations from 3 separate brains.

mg/g wet wt. of brain	Normal	Shiverer
Total lipid extract	95.6	69.9
Cholesterol	20.0	18.8
Phospholipids	54.1	41.8
Glycolipids (cerebrosides + sulfatides)	12.9	2.7
Gangliosides (NeuAc content)	0.86	0.73

of the MBP cannot be ruled out a priori since both techniques used for the detection of MBP are based on its immunochemical properties. However, we do not favour this interpretation for two reasons. (i) MBP presents several distant antigenic sites along its polypeptide chain [6] and the probability of simultaneous mutations is low. (ii) The parallelism of the competition curves obtained with the dilution of the mouse brain extracts to the human MBP standard curve argues in favour of a good recognition of the MBP in the *Shi/Shi* brain extracts by antibodies raised against human MBP. Thus, we conclude that this mutant is characterized by an almost total absence of MBP. We also demonstrated the almost total absence of the major dense line in CNS myelin [21]. Thus, we propose that the MBP is localized in the cytoplasmic leaflet of the oligodendroglial membrane. From our data, it appears that the initial steps of myelination can occur in absence of MBP. Therefore, the role of this protein as an initiator of myelination, previously proposed [15], should be questioned. Indeed, in *Shi/Shi*, the myelin sheath is manufactured by the oligodendroglia and wraps normally around the axon whereas the MBP is almost absent.

From a genetic point of view, it would be of interest to check the presence of MBP in peripheral myelin of *Shi/Shi*. Shiverer mutation constitutes a model to gain new insight into the relationship of structure and chemical composition of myelin.

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