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Expression of myelin proteins in the adult heterozygous Trembler mouse

Abstract The Trembler mouse (Tr) suffers from a dominantly inherited autosomal mutation (glycine to aspartic acid, G150D) affecting the PMP22 gene, which results in an abnormal myelination of the peripheral nervous system. The Tr mouse represents an animal model for the human hereditary neuropathy, Charcot-Marie-Tooth disease. We compared the expression of PMP22, P0, myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) in nerve biopsies from a 1-year-old heterozygous Tr mouse (Tr/+) with that of a control mouse of the same age. Quantitative and qualitative ultrastructural immunocytochemical analysis showed a significant decrease in PMP22, P0 and MBP and an abnormal location of the MAG in the sciatic nerve of the Tr/+ mouse. We also noted a marked reduction in number of myelinated fibers associated with the presence of two types of myelinated axons: a population of abnormally thin myelin sheaths with lower PMP22 labeling than that observed in myelinated fibers from the control mouse, and some others fibers with normal sheaths for axons of comparable diameter to those of normal mouse with no difference in the PMP22 immunolabeling. This pointed to an involvement of PMP22 in the structure of myelin sheaths.

Key words Genetic neuropathies · Myelin proteins · Peripheral nerve · Trembler mouse

Introduction

The Trembler (Tr) mouse suffers from a dominantly inherited autosomal mutation affecting Schwann cell activities, which results in an abnormal myelination of the peripheral nervous system. The mutation affects the PMP22 gene and leads to the replacement of a glycine by an aspartic acid residue at position 150 in the transmembrane domain of the protein. This mutant is considered to represent an animal model for certain human inherited neuropathies, including Charcot-Marie-Tooth disease (CMT 1).

Immunological and biochemical studies have shown that most of the myelin proteins are affected by this Tr/+ mutation [3, 5, 6]. Nevertheless, the results obtained with these techniques may result from a reduction in the overall amount of myelin like that found in many hypomyelinating or demyelinating neuropathies but with different mechanisms.

In the present study, we employed an immunocytochemical technique to assess quantitatively the expression of myelin proteins at the ultrastructural level in the 1-year-old Tr/+ mouse. The method also provided a qualitative evaluation of alterations in expression, not readily determined by other immunological and biochemical methods.

Materials and methods

Routine electron microscopy

Fragments of sciatic nerves were immediately fixed in 5% glutaraldehyde and post-fixed in 1% osmium tetroxide. Epon-embedded semithin and ultrathin sections were prepared for light and electron microscopy examination.

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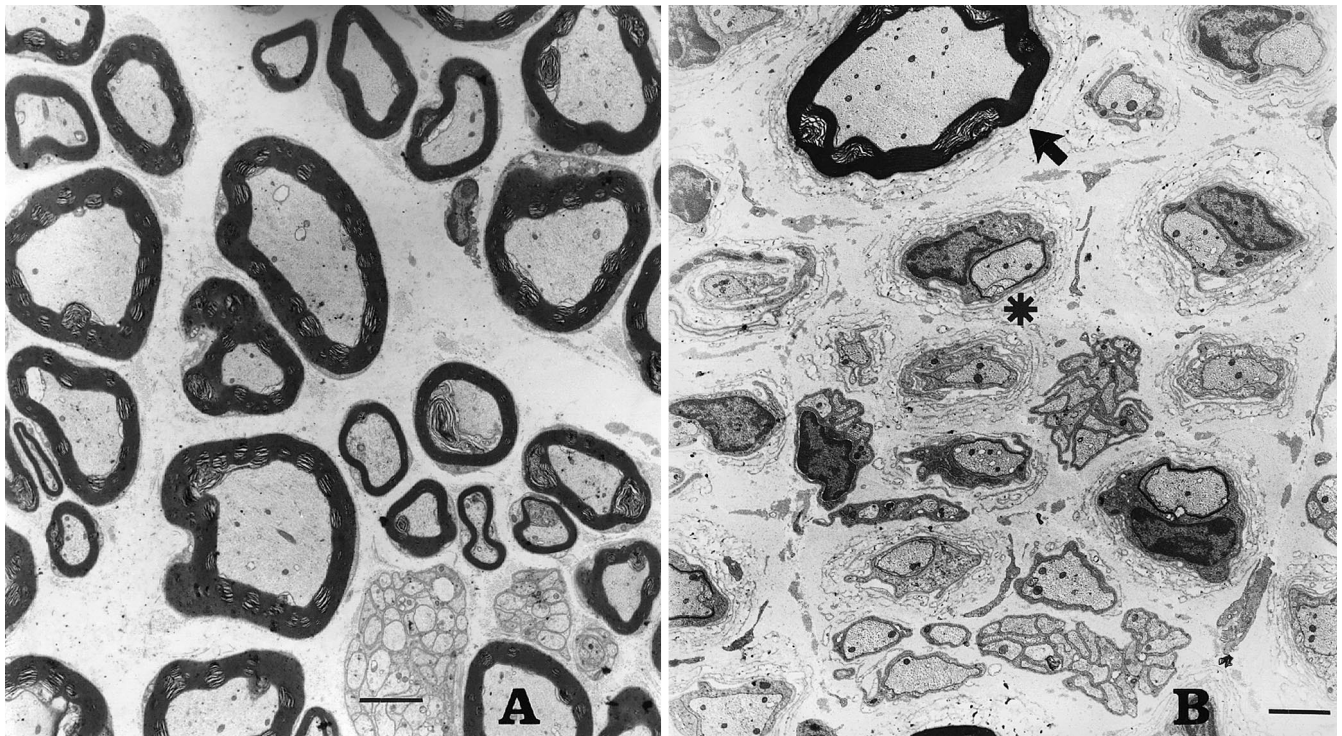


Fig.1 Ultrathin sections of sciatic nerves of a 1-year-old *Tr/+* mouse (**B**) and its control (**A**). The thickness of myelin sheaths of some fibers is normal (*arrow* in **B**), while in others it is abnormally thin (*asterisk* in **B**); both sheaths present a normal compaction of the myelin lamellae. Note the significant reduction in number of large myelin fibers in the *Tr/+* mouse (**B**) compared with the control (**A**) (*Tr* Trembler). Bars **A** 3.16 μm , **B** 1.78 μm

Immunoelectron microscopy

Other fragments were fixed and stored overnight at 4°C in a 0.08 M phosphate-buffer solution containing 2.5% glutaraldehyde and 4% paraformaldehyde, pH 7.4. The nerves were processed for ultracyromicrotomy as described by Tokuyasu [16]. Sections of 95 nm were cut on an ultracyromicrotome and processed for ultracyrocytochemistry.

Immunogold procedure

Several antibodies were employed: P0-7 monoclonal antibody (mAb) of the IgG1 class at 1:50,000 dilution (kindly provided by Dr. Archelos, Würzburg, Germany), PMP22 mAb at 1:100 dilution (kindly provided by Dr. Windebank, Rochester, USA) myelin basic protein (MBP) mAb at 1:200 dilution (from Boehringer) and myelin-associated glycoprotein (MAG) mAb (B11F7) of the IgG2b class at 1:100 dilution (kindly provided by Dr. Quarles). These antibodies were revealed by IgG-coated colloidal gold particles (12 nm in diameter at 1:25 dilution). P0-7 mAb was characterized by ELISA, immunohistochemistry and Western blotting; it reacted with whole native rat and human P0 and recognized the extracellular domain of the P0 protein (P0-ED). PMP22 antibody was raised against sequence 118–133 of the human PMP22 protein, and MBP recognized the human sequence 130–137. The lack of labeling of specific structures with several anti-connexin 32 antibodies was attributed to the fixation procedure.

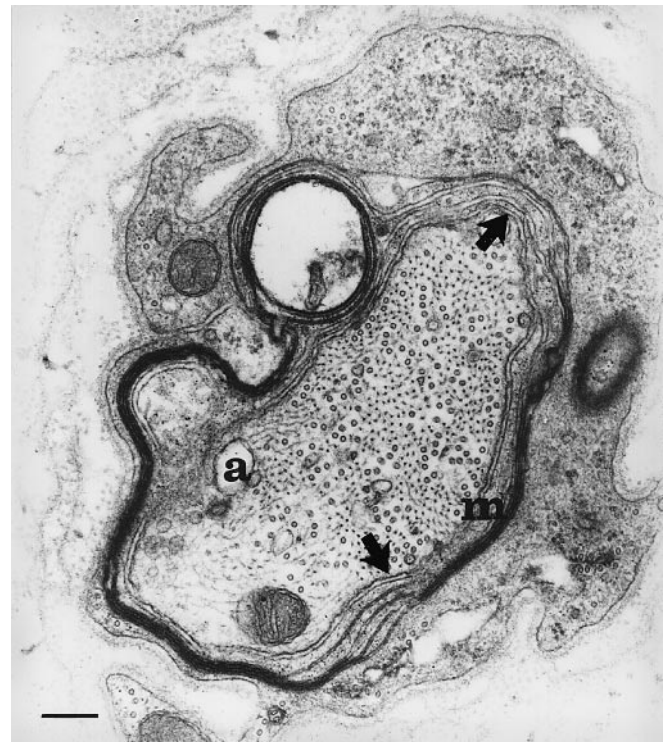


Fig.2 Ultrathin section of a thin myelin sheath of a 1-year-old *Tr/+* mouse. Note the lack of compaction of myelin lamellae (*arrows*) (*a* axon, *m* myelin) Bar 2.17 μm

Quantitative ultrastructural analysis

PMP22, P0-7, MBP and MAG immunoreactivity was observed under a CM 10 Philips transmission electron microscope (at $\times 28,500$ or $\times 39,000$). More than twenty photomicrographs were taken for

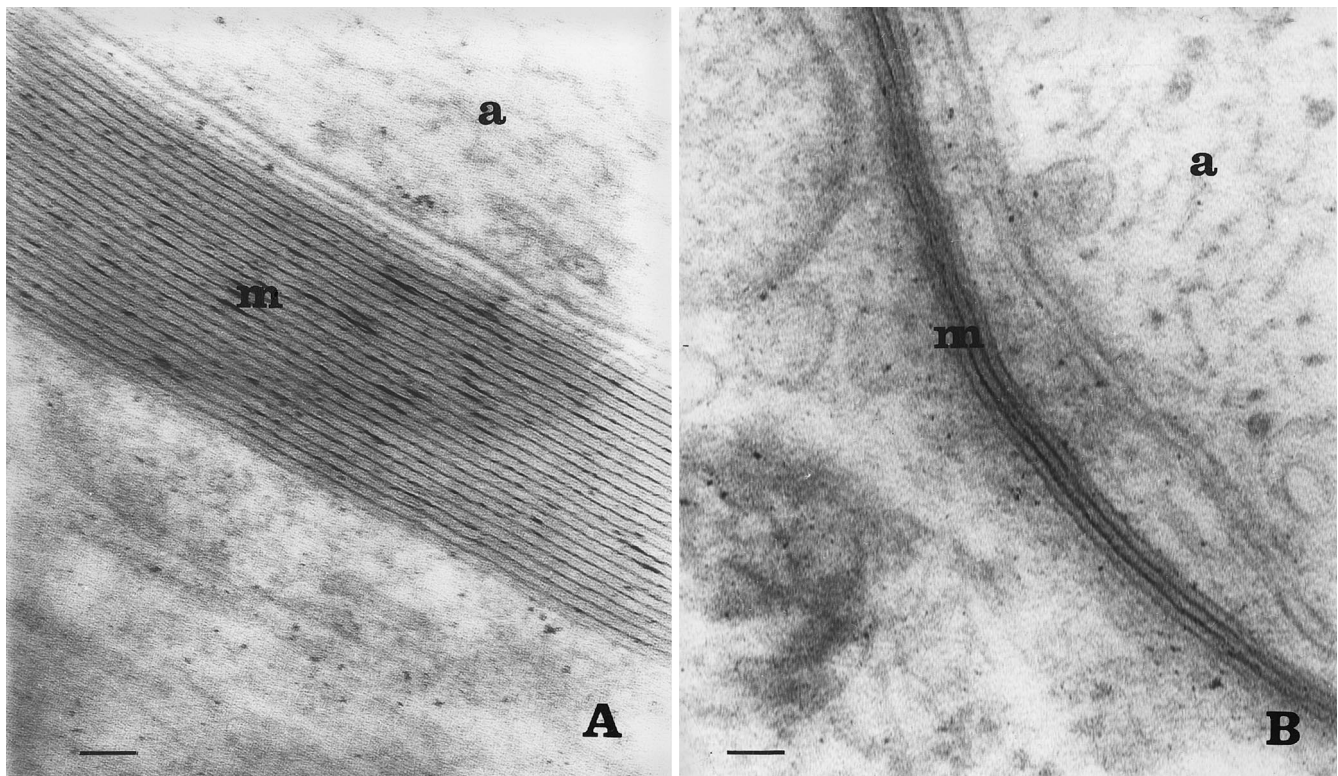


Fig. 3 Ultrathin sections at high magnification of a normal myelin sheath (**A**) and a thin one (**B**) of a 1-year-old Tr/+ mouse. No abnormality in the periodicity of the myelin lamellae is seen (*a* axon, *m* myelin). Bars 0.05 μm

each mouse and analyzed automatically using the Comptex program (specially developed by Klein Industrie, France) and the Inspector program for morphological analysis. Twenty photomicrographs were selected at random from each grid. The number of gold particles within several areas of the compact myelin were counted automatically and the software calculated the mean particle density per μm^2 of tissue after subtracting background values (calculated within the collagen in the endoneurial space). The ratio between two nerves was calculated by dividing the specific density for each nerve by values obtained from the control nerve. The gold particle density per μm^2 myelin for the Tr mouse was compared to that obtained for the control mouse (normal nerves) using the Mann-Whitney test.

Results

Routine electron microscopy

Apart from the marked reduction in number of myelinated fibers [10], we observed two types of myelinated axons in the 1-year old Tr/+ mouse; fibers with abnormally thin myelin sheaths and some with more or less normal sheaths for axons of comparable diameter to those from the normal mouse. Out of 100 fibers we found 6.5% with normal sheaths and 93.5% with abnormally thin sheaths (Fig. 1).

Nevertheless, in a significant number of fibers, especially those with thin sheaths, we observed a lack of compaction of myelin lamellae. This involved all or just some of the lamellae at the periphery of a given fiber (Fig. 2,

Table 1 Expression of myelin proteins in a 1-year-old heterozygous Trembler mouse. Values represent ratios calculated from the gold particle densities per μm^2 compared to a normal nerve and take into account the relative contribution of heavily myelinated fibers (6.5%) and thinly myelinated fibers (93.5%). The values obtained for PMP22 correlate with those of molecular biological results (*italics*); values of PO and MBP are unexpected reduced. The values were obtained with an automatic quantification and analyzed with the Mann-Whitney test

		Antibodies		
		PMP22	PO-7	MBP
1 year	Trembler mouse (heterozygous)	<i>(0.58)</i> ***	<i>(0.43)</i> ***	<i>(0.30)</i> ***

*** $P < 0.001$

and see Fig. 6A–C). We did not observe any other abnormality of myelin compaction (Fig. 3).

Immunoelectron microscopy

We measured the density of gold particles, per μm^2 myelin, on the fibers with both thin and normal sheaths in a proportion of 14 thin to 1 normal. This was in line with the proportion of 93.5% thin sheaths to 6.5% normal. In practice, for the normal sheaths we determined protein labeling over 5 sheaths since, in view of the reduction in fiber density and the size of the sample, we did not often find 5 normal fibers on a single ultrathin section. This number of 5 normal fibers resulted from the random sampling procedure. Each of these 5 values were compared

Fig. 4 Ultrathin sections of myelin sheaths in 1-year-old Tr/+ mouse immunostained with the PMP22 monoclonal antibody and revealed with colloidal gold particles. Automatic quantitation revealed a significantly reduced expression of the PMP22 protein in the heterozygous Tr mouse for the thin sheaths (**B**) compared with the normal ones (**A**) (*a* axon, *m* myelin). Bars 0.23 μ m

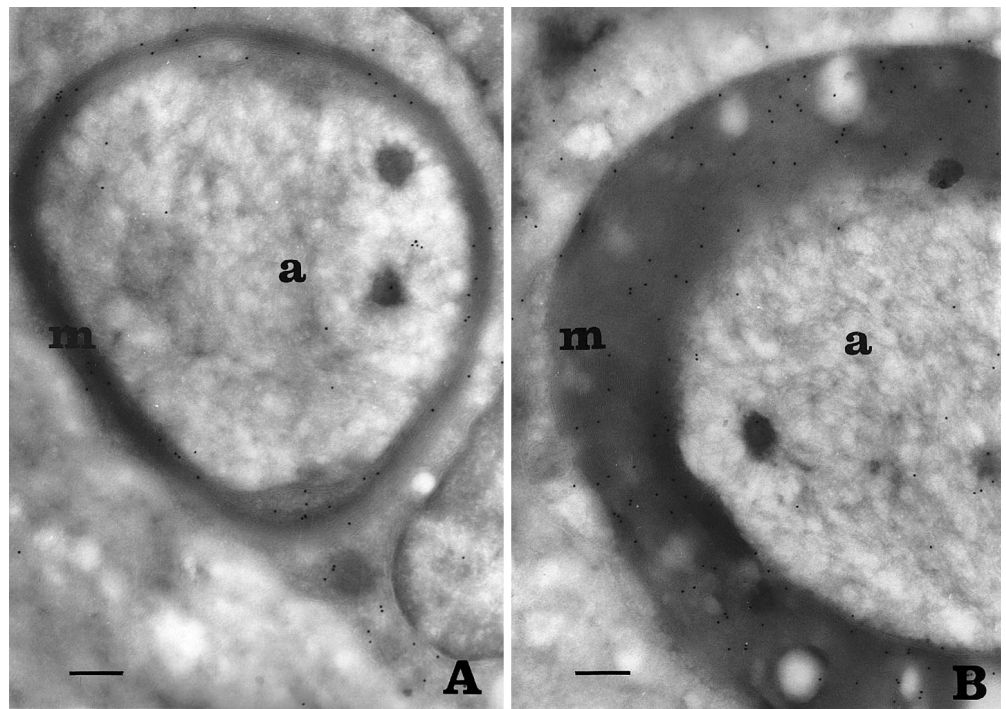
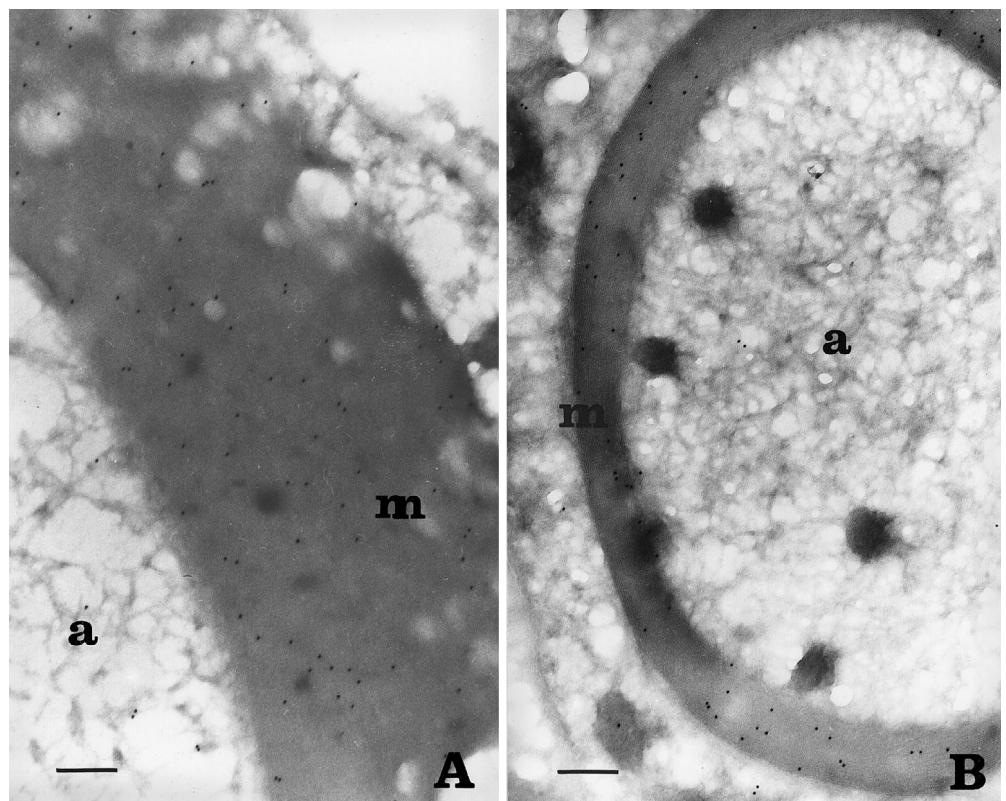


Fig. 5 Ultrathin sections of myelin sheaths in 1-year-old Tr/+ mouse immunostained with the MBP monoclonal antibody and revealed with colloidal gold particles. Automatic quantitation revealed a significantly reduced expression of the MBP protein in the heterozygous Tr mouse (**B**) compared with the control mouse (**A**) (*MBP* myelin basic protein, *a* axon, *m* myelin). Bars 0.22 μ m



with the 14 obtained for the thin sheaths. This was carried out to rule out variability stemming from differences in the labeling of the same type of fiber (in this case the fibers with normal sheaths). After checking that there was no variation in density for the normal fibers, we compared the densities observed in the Tr/+ mouse with those from a control using the Mann-Whitney test.

The results (Table 1) showed that the Tr/+ mouse exhibited a significantly reduced expression of the myelin proteins PMP22, P0-7 and MBP. Ratios (mean immunolabeling density in the Tr mouse divided by that of the control) were 0.58 ($P = 0.001$), 0.43 ($P = 0.003$) and 0.30 ($P = 0.005$) for PMP22 (Fig. 4), P0 and MBP (Fig. 5), respectively.

Fig. 6 Lack of compaction in the 1-year-old Tr/+ mouse (*asterisk*) on electron microscopic examination (**A**). **B**, **C** Ultrastructural immunocytochemistry using an MAG polyclonal antibody revealed with colloidal gold particles: note the abnormal distribution of MAG in the uncompacted myelin lamellae (*asterisk*) in the Tr/+ mouse (**C**) compared to the control mouse (*arrows* in **B**) (MAG myelin-associated glycoprotein, *a* axon, *m* myelin). Bars **A** 0.16 μ m, **B**, **C** 0.12 μ m

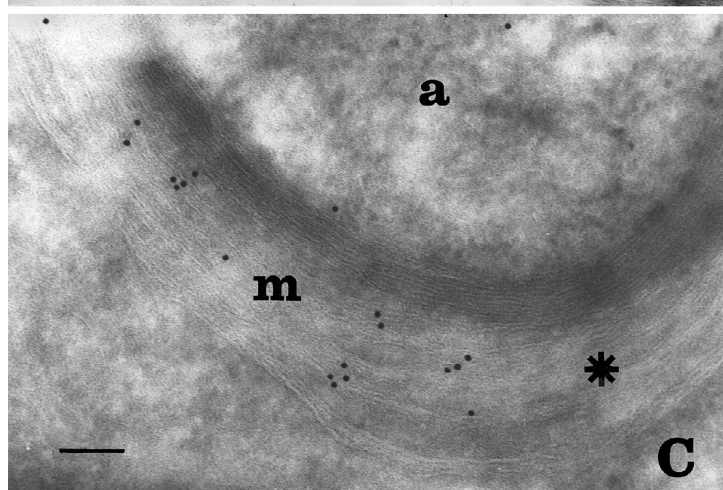
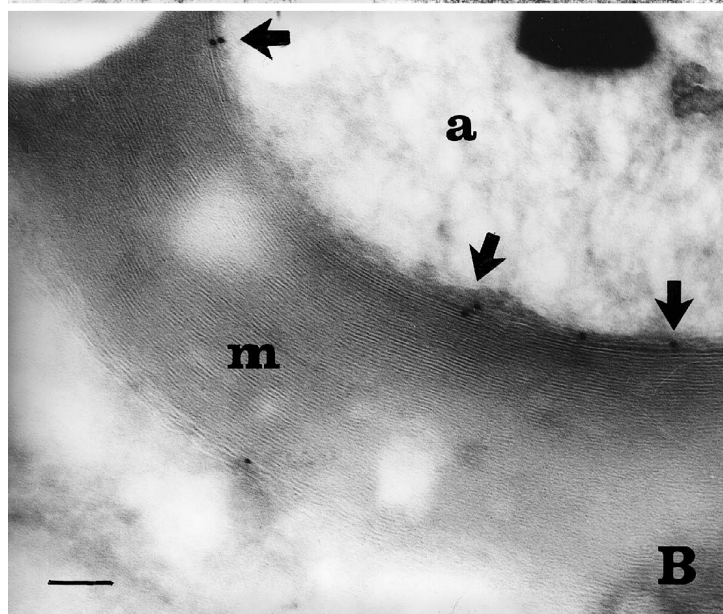
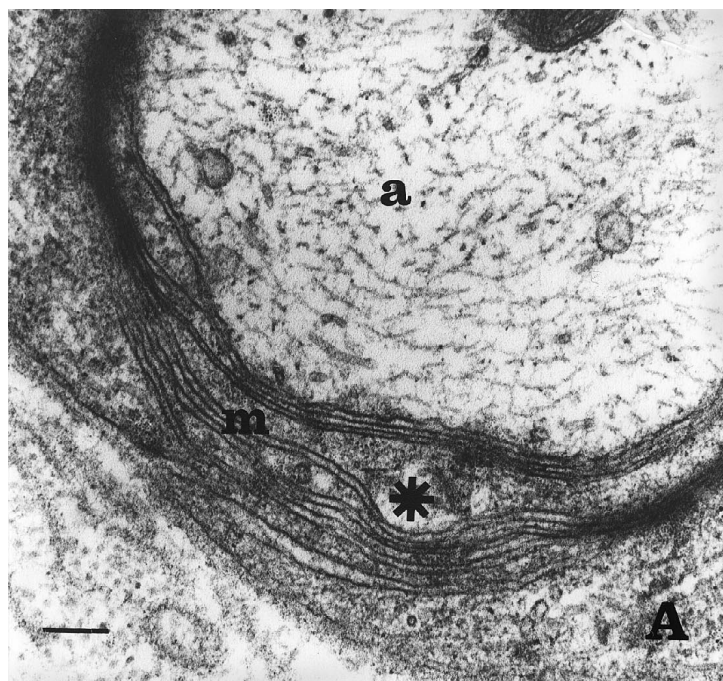


Table 2 Mean values \pm standard deviation of the gold particle densities for Trembler mouse and control mouse both for large and thin myelin fibers using the PMP 22, P0-7 and MBP antibodies.

Values were compared between large and thin fibers for each mouse using the Mann-Whitney U-test (*NS* no significant difference, **/** significant differences)

Antibodies	Trembler mouse (heterozygous)			Control mouse		
	Large myelin fibers	Thin myelin fibers	U-test	Large myelin fibers	Thin myelin fibers	U-test
PMP 22	12.25 \pm 1.73	3.88 \pm 1.01	*** <i>P</i> = 0.006	8.46 \pm 2.42	8.21 \pm 1.15	<i>NS</i> , <i>P</i> = 0.425
P0-7	31.60 \pm 4.67	13.90 \pm 7.55	** <i>P</i> = 0.02	33.92 \pm 1.83	40.03 \pm 9.95	<i>NS</i> , <i>P</i> = 0.296
MBP	13.04 \pm 2.22	2.67 \pm 0.83	** <i>P</i> = 0.01	13.85 \pm 3.95	13.83 \pm 1.93	<i>NS</i> , <i>P</i> = 0.344

We also found, with all antibodies used here, a significant decrease in mean gold particle density for the thin myelin sheaths and no significant difference in mean particle density for the large myelin sheaths in the Tr/+ mice compared with controls (Table 2). In all cases, the thin fibers were less immunolabeled than the large myelin fibers in the Tr/+ mouse ($P = 0.006$, $P = 0.02$, $P = 0.01$ for PMP22, P0-7 and MBP antibodies, respectively), whereas in the control mouse there was no significant difference in labeling density between the two types of fibers.

The anti-MAG antibody demonstrated that MAG was strongly expressed in the loose myelin lamellae (Fig. 6A–C). In the control nerves, MAG was normally present only in inner myelin sheaths (Fig. 6B).

Discussion

Several authors have noted a marked reduction in mRNA and proteins (PMP22, P0, MBP) of compact myelin in the Tr mouse [3, 6, 8]. The extent of the loss of these proteins may indicate the severity of the hypomyelination. However, these findings may be secondary to a reduction in amount of myelin as found in many hypomyelinating or demyelinating neuropathies, irrespective of the exact mechanism. However, the method we employ here enables comparison of the amounts of protein (PMP22, MBP, P0) expressed in compact myelin for identical surface areas between a 1-year-old Tr/+ mouse and a normal mouse of the same age. The true reduction in amounts of the proteins thus appeared to be directly linked to the mutation of the PMP22 gene and not to simple demyelination. We have previously observed variations in the amount of PMP22 in a case of CMT 1A and HNPP, which were not observed in a patient with severe chronic inflammatory demyelinating neuropathy, an acquired condition [18].

A significant reduction in P0 in compact myelin of the adult Tr/+ mouse has been reported [5]. Using our quantitative immunocytochemical technique, we have shown that, as with PMP22, this reduction was directly related to the PMP22 mutation. Even after careful scrutiny at high magnification under the electron microscope, we did not find any evidence of an abnormality of myelin membrane packing, as has been observed in sural nerve biopsy specimens from patients with P0 gene mutations [9]. These lesions may stem from a mutation of the P0 gene rather than

from a loss of this protein in compact myelin. Furthermore, the age and hence the course of the condition in our 1-year-old mice is much less than that of patients with manifestations of a mutation of the P0 gene (CMT-1B).

We also noted a marked reduction in MBP. We were surprised that the reductions in both P0 and MBP did not appear to produce abnormalities in compaction of the remaining myelin in the Tr/+ mice. In this respect, Notterpek et al. [12] have suggested that the altered expression of P0 and also MAG may indicate an interaction of PMP22 with MAG and P0, and also with MBP.

The difference in protein contents between the large and small diameter fibers was indicative of a role of PMP22 in the normal development of myelin sheaths. It may be involved in the initialization of myelin spirals, and the regulation of growth and differentiation of Schwann cells [4, 14]. Electron microscopic examination of the nerves from Tr/+ mice did not reveal any accumulation of debris either in Schwann cell cytoplasm or in endoneurial macrophages. Such signs, which would indicate a process of demyelination, have only rarely been described in comparable ultrastructural studies for adult Tr, Tr-J mice or in another recently reported mutant [1, 2, 10, 13, 15]. We failed to detect any abnormally thick myelin (tomacula) in our adult mice, although Notterpek et al. [12] described a low frequency of myelin fragmentation in sciatic nerves of very young (10-day-old) Tr-J/+ mice. The observation in the adult Tr/+ mice of a small number of normal myelinated fibers with no signs of destruction would tend to be indicative of a defect in formation of myelin rather than demyelination. In 1-year-old homozygous Tr mice (unpublished results), very little myelin appears to be formed and there is no evidence of demyelination. PMP22 thus appears to control the thickness of myelin and confers structural and functional stability to it. An abnormal PMP22 may thus lead to the formation of abnormally thin myelin sheaths or a slowing or arrest of maturation of Schwann cells or even cell death.

With respect to MAG, non-morphological studies (determination of mRNA and corresponding protein) have produced rather conflicting results (although it appears that expression of MAG is only slightly affected by the Tr mutation): slightly reduced [3], slightly increased [7], or unaffected in the Tr-J mouse [12]. In an electron microscopic study in the Tr mouse, Ayers and Anderson [1] reported a lack of compaction of myelin lamellae, clearly seen on transverse sections. We confirm these results.

Trapp and Quarles [17] showed on light microscopic examination that these uncompacted myelin lamellae were immunoreactive for MAG. Using our immunocytochemical technique we have shown at the ultrastructural level that the loose myelin lamellae were directly linked to the abnormal presence of MAG (Fig. 6A–C). No MAG staining was observed in compact myelin of control nerves, although it was abundant in mesaxonal membranes especially in the periaxonal zone (Fig. 6B). In view of the frequency of uncompacted myelin lamellae, the increase in expression of MAG in the Tr/+ mice (using biochemical techniques) was not altogether unexpected [7]. Normal compaction of myelin lamellae and formation of the intraperiod line would appear to be related to removal of MAG from uncompacted myelin lamellae, solely in axons with thin myelin sheaths. The abnormal level of uncompacted myelin lamellae in the Tr/+ mouse may be akin to that observed during formation of compact myelin in embryonic life. The abnormal distribution of MAG in the adults may, thus, be a sign of an immaturity in the development of normal compact myelin.

Conclusion

The present quantitative ultrastructural study using immunocytochemistry confirms and extends earlier morphological and non-quantitative immunochemical studies. In the adult Tr/+ mouse, mutation of the PMP22 gene is directly responsible for the alteration in protein content (P0, MBP) of compact myelin. The mutation also leads to a qualitative alteration in MAG content in uncompacted myelin. The morphological abnormalities in the myelin in sciatic nerves of these mice were attributed to a defect or a delay in myelination. This PMP22 mutation may also lead to a slowing or an arrest of maturation of Schwann cells. The Tr mouse thus represents a valuable animal model for a subgroup of demyelinating or hypomyelinating hereditary sensory-motor neuropathies. Indeed, the phenotype of non-duplicated CMT 1 patients, also due to a point mutation in the PMP22 gene, closely resembles that of the Tr mouse [11]. It is of interest that we recently detected by quantitative ultrastructural immunocytochemistry a marked reduction in P0 content in a patient with this condition (unpublished observation).

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