Immunocytochemical localization of rat peripheral nervous system myelin proteins: P2 protein is not a component of all peripheral nervous system myelin sheaths

(P2 protein/P1 protein/Schmidt–Lanterman incisures/peroxidase-antiperoxidase)

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ABSTRACT Specific antibodies have been developed against P1, P2, and P0 myelin proteins and were used to study the localization of these proteins in the rat peripheral nervous system. Both peripheral and central nervous system myelin sheaths contain P1 protein. P2 and P3 proteins are found exclusively in peripheral nervous system myelin sheaths. Antiserum to P1 and P0 proteins stain all peripheral nervous system myelin sheaths uniformly. P2 protein is not a component of all peripheral nervous system myelin sheaths. In sheaths that do contain P2 protein, it is concentrated in the area of the Schmidt–Lanterman incisures.

Myelin isolated from mammalian peripheral nervous system (PNS) contains three major proteins (1). These proteins include a glycoprotein, P0, with a molecular weight (Mr) of 30,000 and two basic proteins, P1 and P2, with Mr of 18,500 and 13,500, respectively. The P0 and P2 proteins are unique to PNS myelin whereas the P1 protein appears to be identical to the large central nervous system (CNS) myelin basic protein (2). The P0 protein represents approximately 50% of total PNS myelin protein whereas the quantities of P1 and P2 and the ratio of P1/P2 varies from species to species (1). The ratio of P1/P2 also varies within different PNS fiber tracts obtained from the same animals (1). Myelin purification provides a heterogeneous population of myelin membranes. By virtue of the myelin isolation procedure, anatomical specificity of myelin proteins within individual myelinated fibers or myelin internodes is lost prior to biochemical analysis. Indirect evidence has localized the P0 protein at the intraperiod line (3–5) and the basic proteins at the major dense line (5–7) of myelin. These studies have not defined the anatomical distribution of the major PNS myelin proteins within myelin sheaths of PNS fiber tracts.

In the present study we present light microscopic immunocytochemical evidence that in the rat P2 protein is not a component of all PNS myelin sheaths, but in fact is a component of only some myelin sheaths. In addition, when antiserum to P2 stains a myelin sheath, Schmidt–Lanterman clefts stain more intensely than compact portions of the internode. P0 and P1 proteins are located in all PNS myelin sheaths thus far analyzed, and antiserum to them stain all sheaths uniformly.

MATERIALS AND METHODS

Seven-day-old, 25-day-old, and adult (200–225 g) Sprague–Dawley rats were anesthetized with ether and fixed by intracardiac perfusion with a solution containing 76 ml of saturated HgCl2 and 20 ml of 37% (vol/vol) formaldehyde. Trigeminal ganglia, sciatic nerves, and spinal cords with attached dorsal and ventral roots were dissected and fixed for an additional hour. Tissues were sectioned at a thickness of 20 µm on a vibrating microtome. All sections were stained immunocytochemically by the peroxidase-antiperoxidase method as described (8). After the staining procedure the sections were infiltrated with glycerol, mounted on glass slides, and examined with a Zeiss differential-interference contrast microscope.

The P0 and P2 proteins were isolated from bovine spinal cord by the procedure of Kitamura et al. (9) except that the buffer for the Sephadex G-200 column was 10 mM Tris-HCl, pH 7.5/0.5% sodium dodecyl sulfate (NaDodSO4)/5 mM EDTA/1 mM dithiothreitol/0.2% sodium azide. The purities of P2 and

Abbreviations: PNS, peripheral nervous system; CNS, central nervous system; NaDodSO4, sodium dodecyl sulfate.
FIG. 2. (A–C) Cross sections of 7-day-old spinal cord at low magnification. (A) P1 antiseraum (1:500 dilution) stains all PNS myelin sheaths in the dorsal root (upper arrow); staining of CNS myelin sheaths located in the root entry zone (lower arrow) and spinal cord is more intense. (B) Antiserum to P2 (1:250 dilution) stains some PNS myelin sheaths (arrows); CNS sheaths are unstained. (C) There is very intense staining of all PNS myelin sheaths by P0 antiseraum (1:500 dilution). (D–F) Transverse sections of 25-day-old trigeminal ganglion. P1 (D) and P0 (F) antisera stain all myelin sheaths uniformly. A relatively small proportion of myelin sheaths are stained by P2 (E) antiseraum and the density of this staining is not uniform. Minor variations in the density of fibers stained by P1 and P0 are due to differences in the plane of focus. At other levels of focus individual fibers can appear somewhat lighter or darker. (A–C, scale bar = 100 μm; D–F, scale bar = 10 μm.)
P₀ were determined by electrophoresis on 12.5% polyacrylamide gels made by the method of Laemmli (10). The P₂ and P₀ antisera were prepared in rabbits by multiple injections of 1-mg quantities of each protein in complete Freund's adjuvant. Immunodiffusion was performed on 1.5% agarose plates (Meloy, Springfield, VA). P₂ and P₀ were used at a concentration of 1 μg/μl and were dissolved in 0.05% NaDodSO₄. Diffusion was carried out for 24–72 hr at 4°C. The P₁ protein was isolated from rabbit CNS and antiserum against P₁ was produced in goat. The isolation procedure and methods for P₁ antibody production were those of Cohen and Guarnieri (11). A half milliliter of a 1:100,000 dilution of P₁ antiserum bound 500 pg of iodinated rabbit P₁ protein, as determined by radioimmunoassay. Controls for the specificity of staining consisted of incubating sections with preimmune or absorbed antisera in which P₁, P₂, and P₀ antibodies were removed by precipitation with their respective purified antigens.

RESULTS

The purified P₀ and P₂ proteins were compared to whole bovine myelin by analytical NaDodSO₄ gel electrophoresis (Fig. 1 A–C). These purified proteins were used to prepare P₀ and P₂ antisera and were used for antibody absorption and immunodiffusion tests. P₀ and P₂ proteins reacted with their respective antisera in immunodiffusion (Fig. 1 D and E). No crossreactivity was found between P₂ and P₀ antisera, and P₀ and P₂ did not react with antisera to P₁ or myelin-associated glycoprotein (12) or with their respective preimmunization sera.

The specificity of P₁, P₂, and P₀ antisera for PNS and CNS myelin sheaths is demonstrated by staining patterns observed in sections of 7-day cervical spinal cord with attached dorsal roots. The P₁ antisemurum (Fig. 2A) stained CNS and PNS myelin sheaths. The intensity of staining was greater in the CNS than in the PNS. P₀ (Fig. 2B) and P₂ (Fig. 2C) staining was restricted to PNS myelin sheaths. P₁, P₂, and P₀ staining of individual myelin sheaths is shown in cross sections of trigeminal ganglia from 25-day-old rats. P₁ (Fig. 2D) and P₀ (Fig. 2F) antisera uniformly stained all myelin sheaths within the ganglion. The staining pattern of P₂ antisemurum (Fig. 2E) was different from that found for P₁ and P₂. P₀ antisemurum did not stain all myelin sheaths, and the intensity of staining in those fibers that did stain was not uniform. A similar pattern of staining for P₀ antisemurum was found in sciatic nerves from adult rats. P₂ antisemurum stained a portion of the myelin sheaths (Fig. 3B) and the intensity of staining varied in those sheaths that did stain. P₁ (Fig. 3A) and P₀ (Fig. 3C) antisera uniformly stained all sciatic nerve myelin sheaths. The proportion of myelin sheaths containing P₀ protein varied with the age of the animal and the fiber population sampled. For example, in a section of the trigeminal ganglion from a 25-day-old rat, 5–10% of the myelinated fibers clustered between neurons (Fig. 2E) were stained by P₀ antisemurum. However, in a nearby root fascicle, P₂ was present in approximately 30% of the myelin sheaths. Ganglion cells, Schwann cells, unmymelinated axons, and satellite cells were not stained by P₂, P₀, or P₁ antisera. Prior to immunostaining, all sections were treated with 2% osmium tetroxide. This treatment enhances immunostaining of compact myelin but inhibits staining of Schwann cells.

In longitudinal sections of 7-day dorsal root, P₂ antisemurum stained few myelin sheaths. When fibers did stain, staining was relatively uniform along the length of the myelin internode and then was more intense in paranodal areas (Fig. 3D). This concentration of P₂ staining was also present in PNS hemi-nodes located at the PNS/CNS root junctions (Fig. 3E). When longitudinal sections of adult dorsal root were stained with P₂ antisera, only some of the myelin sheaths were stained (Fig. 3F). Variation in staining intensity occurred along the length of myelin segments stained by P₂ antisera. P₂ staining in adult dorsal roots was concentrated at the Schmidt–Lanterman incisures (Fig. 3F). The unusual staining pattern of P₂ was also observed in teased fiber preparations of adult cauda equina (Fig. 3G). Again, only some of the teased fibers were stained and those that did stain exhibited concentrated staining at the Schmidt–Lanterman incisures. In longitudinal sections and teased fiber preparations, P₁ and P₀ antisera stained all myelin sheaths uniformly.

The specificity of P₂ and P₀ antisera was tested by staining adult CNS and PNS tissue with preimmune sera and P₂ (Fig. 3H) and P₀ (Fig. 3I) antisera which were absorbed with their respective purified proteins. In all controls no staining of myelin sheaths or cells was observed.

DISCUSSION

Our immunocytochemical results clearly show that P₀ and P₂ proteins are found only in the rat PNS and are localized in myelin sheaths. P₁ protein, as previously shown, is found in both PNS and CNS myelin sheaths (8). Antiserum to P₁ and P₀ proteins uniformly stained all PNS myelin sheaths. Antiserum to P₂ protein stained only selective myelin sheaths and, when they were stained, the staining was concentrated in the Schmidt–Lanterman incisures (13). This finding was verified in three separate fiber tracts in rats of various ages. The variability in the content of P₂ protein between various species and within different fiber tracts of the same animal is probably due to the number of myelin sheaths that contain P₂ protein. It has been assumed from biochemical investigations that P₂ protein is a minor component of all PNS myelin sheaths (14). It is clear from the present study that P₂ protein is found only in certain myelin sheaths. These myelin sheaths must contain a much higher percentage of P₂ protein than that calculated from biochemical analysis of myelin isolated from whole nerves. The factors governing the occurrence of P₂ protein in certain myelin sheaths remain to be clarified. A critical question is whether the axon triggers the Schwann cell to synthesize and incorporate P₂ into its myelin sheath or if only a select population of Schwann cells is capable of synthesizing P₂ protein regardless of axonal influence. The axon appears to be the trigger for the initiation of myelination by the Schwann cell and all Schwann cells apparently have the potential to form myelin (15). The function of the axon (sensory compared to motor) appears to have no influence on the occurrence of P₂ in myelin sheaths, as both dorsal and ventral spinal roots are stained by P₂ antisera. P₂ staining appears to be localized predominately in large myelin sheaths.

Longitudinal sections (Fig. 3F) and teased fiber preparations (Fig. 3G) clearly demonstrate that the Schmidt–Lanterman incisures are more densely stained by P₂ antisemurum then the remainder of the myelin sheath. This increased staining of the incisures accounts for the variation of staining intensity found in cross sections of various fiber tracts. The absence of concentrated staining of Schmidt–Lanterman incisures in the 7-day-old dorsal root (Figs. 3D and E) is due to the immaturity of the myelin sheaths. The Schmidt–Lanterman incisures do not appear until approximately six spiral turns of compact myelin have been formed (13, 16). In 7-day dorsal roots, paranodal areas are more densely stained than the remainder of the myelin sheaths (Fig. 3D and E). This increased staining intensity of nodal areas can also be found in 25-day and adult animals (Fig. 3F) although it is not as prominent as that found in the 7-day-old rats.

The difference in the immunocytochemical localization of P₁ and P₂ proteins suggests that these two basic proteins are not
FIG. 3. (A–C) Cross sections of adult sciatic nerve. P₁ (A) and P₂ (C) antisera stain all sheaths uniformly. P₂ antiserum (B) stains only some myelin sheaths. Longitudinal sections of 7-day dorsal root demonstrate more intense staining of P₂ at hemi-nodes (arrows) within the root (D) and at the PNS portion of the PNS/CNS junction (E). Longitudinal sections of adult dorsal root (F) and teased intradural spinal root (G) demonstrate more intense staining of Schmidt–Lanterman incisures (arrows). A node of Ranvier is also present (F, arrowhead). Cross sections of 25-day-old trigeminal ganglion stained with P₀-absorbed (H) and P₂-absorbed (I) antisera demonstrate complete absence of staining. Antisera dilutions used for staining were identical to those in Fig. 2. (Scale bar = 10 μm.)
topographically disposed in the same manner and do not serve
the same function within the PNS myelin sheaths. P2 protein
contains a high percentage of hydrophobic residues and few
charged residues (14). This suggests that P2 is strongly bound
to other myelin components via hydrophobic interactions insteadd of electrostatic interactions as is the case with P1 basic
protein. It is apparent that P0 and P1 proteins are structural
proteins of PNS myelin since they uniformly stain all PNS
myelin sheaths. P2 protein may have a more specific role in the
sheaths where it is located. Based on its hydrophobic nature,
P2 may participate in the formation of tight junctions present
in Schmidt–Lanterman incisures (17). Another possible explanation for the intense staining of P2 antiserum at the
Schmidt–Lanterman incisures is that P2 protein is more ac-
cessible in this region. The Schmidt–Lanterman incisures are
formed by the splitting of myelin's major dense lines. If P2 is
located at the major dense line, it may be more accessible to the
immunostaining reagents where the line is split.

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