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Research report

Abnormal expression of the proliferating cell nuclear antigen (PCNA) in the spinal cord of the hypomyelinated Jimpy mutant mice

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Abstract

In the present study, assessment of the expression of the proliferating cell nuclear antigen (PCNA), a nuclear acidic protein necessary for DNA replication that is expressed through the cell cycle, was used to investigate the proliferative capability of glial cells in the hypomyelinated Jimpy mutant mice. Spinal cords from 10–12 and 20–22 day Jimpy and normal animals were used for quantitative microscopic image analysis. Simultaneous demonstration of cycling cells and oligodendroglia, astroglia or microglia was achieved through the sequential combination of PCNA immunostaining and selective markers for these glial cells. Our results revealed that the density of PCNA-positive cells was higher in Jimpy than in normal spinal cords, this difference being more pronounced at 20–22 days than at 10–12 days and more so in white than in gray matter. In addition, Jimpy glial cells exhibited an abnormal PCNA expression, as demonstrated by quantification of the intensity of nuclear immunostaining. In comparison to normal animals, the percentage of PCNA-positive cells showing intensely stained nuclei was higher in Jimpy. About 50% of PCNA-positive cells in the Jimpy white matter were identified as cells from the oligodendrocyte line, 30% were microglial cells and 20% were astrocytes. The expression of PCNA in relation to the proliferative capability and possible cell cycle abnormalities of the different glial cell types in Jimpy is discussed.

Keywords: Cell cycle; Proliferation; Olidodendroglia; Microglia; Astroglia; Neuroglia; Image analysis

1. Introduction

The Jimpy mouse is a sex-linked recessive mutant that has a point mutation in the gene coding for proteolipid protein (PLP) [12,43,44]. The affected hemizygous males die within 4 weeks of age and are characterized by a severe hypomyelination throughout the central nervous system (CNS) associated with diverse and complex alterations in glial cell populations.

Alterations in oligodendroglial cells include premature oligodendroglial cell death [32,55,61,62], a reduction in the numbers of well-differentiated oligodendrocytes [17,53], and the presence of increased numbers of immature cells [17,21]. In addition, large numbers of cells incorporating tritiated thymidine and identified as oligodendroblasts on the basis of their ultrastructural morphological features are observed through the Jimpy white matter [47,54]. An intrinsic cell cycle defect has also been described in relation to proliferating oligodendroblasts [33]. Astrocytes in Jimpy exhibit a pronounced gliosis with the occurrence of numerous hypertrophied astrocytes [46,53] showing an increased GFAP immunoreactivity [6,15] and GFAP mRNA expression [36]. In addition, it has been reported that Jimpy astrocytes show an abnormal metabolic response to K^+-induced stimulation [28,31], increased intracellular pH [34] and lengthening of their cell cycle [35]. Concerning the microglial cell population, an intense microglial cell reaction has been described in the Jimpy spinal cord [60]. The number of microglial cells was largely increased in white and gray matter and reactive cells were morphologically characterized by a shortening and coarsening of the cell processes, swelling of the cell body and accumulation of lipid inclusions. Recently, a specific relation between microglial cells and apoptotic oligodendrocytes has also been described [61]. All together, these abnormalities affecting differentiation, cell cycle and numbers of glial cells strongly suggest a disturbance in the development and kinetics of glial cells in Jimpy.

In recent years, immunocytochemical detection of cellular proteins that are involved in the control of cell prolifer-

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ation has become a tool for investigating cell kinetics. This is the case of an antigen associated with the cell cycle, the proliferating cell nuclear antigen (PCNA). PCNA is identical to the nuclear protein cyclin [39] and is an auxiliary protein of DNA polymerase delta whose expression is necessary for DNA synthesis at the replication fork [9,45]. Exposure of cells to antisense oligonucleotides to PCNA results in complete cessation of DNA synthesis and proliferation [30]. PCNA is expressed beginning in late G_1-phase, with a 2- to 3-fold increase in S-phase and a decrease at the S/G_2 transition and during $G_2 + M$ [9,58]. Thus, the use of anti-PCNA antibodies provides the means for the estimation of the whole fraction of cycling or proliferative cells present in tissue sections, whereas [^{3}H]thymidine labeling and the non-radioactive method using bromodeoxyuridine are currently standard methods for detection of S-phase cells [8,37,63].

The present study was undertaken to determine the expression of PCNA in Jimpy spinal cord and to define more precisely the proliferative capability of each glial cell type. PCNA expression was assessed by quantitative microscopic image analysis and identification of individual cycling cells was achieved through double labeling with selective markers for oligodendrocytes, astrocytes and microglia.

2. Materials and methods

2.1. Experimental animals

Mice C57BL/6J used in this study were males raised in the UAB animal center by mating Jimpy carrier females (jp/+) obtained from the Institut Pasteur with normal males (+/Y). Jimpy males (jp/Y) were distinguished from normal control animals (+/Y) by their characteristic tremors and drastic myelin reduction. Five Jimpy mice of 10–12 and five of 20–22 days, together with the same number of normal male littermates of each age, were used in these experiments.

2.2. Preparation of sections

Animals were anesthetized with sodium pentobarbital (100 mg/kg body weight) and sacrificed by intracardiac perfusion with a 4% paraformaldehyde solution in 0.1 M phosphate buffer, pH 7.2, for 5 min. The cervical segment of the spinal cord was carefully removed and immersed in the same fixative for 2 h at 4°C. Samples were then rinsed in phosphate buffer for 4 h, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin. Spinal cords were coronally sliced with a microtome to obtain 6 μm thick sections that were mounted on glass slides pretreated with 1:10 poly-L-lysine solution (Sigma). Finally, sections were dewaxed, hydrated and immunocytochemically processed.

2.3. Immunocytochemical reaction for PCNA

Sections were rinsed 3×5 min in TBS (0.05 M Trizma base containing 150 mM of NaCl), pH 7.4, treated for 10 min with 2% H_2O_2 in 100% methanol to block endogenous peroxidase and rinsed again 3×5 min in TBS with 0.1% Triton X-100. Sections were then placed for 30 min at room temperature (RT) in TBS containing 1% bovine serum albumin (blocking buffer; BB) and then incubated overnight at 4°C with the primary mouse monoclonal anti-human PCNA antibody (clone PC10; Dakopatts) diluted 1:70 in BB. After rinsing 3×5 min in TBS with 0.1% Triton X-100, sections were incubated for 60 min at RT with anti-mouse IgG biotinylated antibody (Amersham) in a 1:200 dilution in BB, rinsed 3×5 min in TBS with 0.1% Triton X-100, and incubated for 60 min at RT with a 1:600 dilution of avidin-peroxidase (Sigma) in BB. Sections were rinsed again 3×5 min in TBS and the peroxidase reaction was visualized by transferring the sections for 5 min to 100 ml of 0.05 M Trizma base (TB), pH 7.4, containing 50 mg 3,3′-diaminobenzidine (DAB; Sigma), 4 ml of 1% ammonium nickel sulfate, 5 ml of 1% cobalt chloride and 66 μl H_2O_2. Finally, sections were rinsed, dehydrated in graded ethanol, cleared in xylene, and coverslipped in DPX. As a control for the immunocytochemical staining, the primary antibody was omitted.

2.4. Double labeling technique

Simultaneous demonstration of PCNA and oligodendroglia, astroglia or microglia was achieved through the sequential combination of PCNA immunostaining and selective markers for these glial cells. Oligodendroglial cells were demonstrated by immunocytochemical detection of myelin basic protein (MBP) [56], astrocytes by immunodetection of glial fibrillary acidic protein (GFAP) [7], and microglial cells by tomato lectin histochemistry [1].

2.4.1. Immunocytochemical reaction for MBP and GFAP

PCNA immunoreacted sections were rinsed 3×5 min in TBS, treated for 10 min with 2% H_2O_2 in 100% methanol to block peroxidase and rinsed again 3×5 min in TBS including 0.1% Triton X-100. Afterwards, sections were placed in BB for 30 min to reduce unspecific staining and then incubated overnight at 4°C either with the rabbit anti-MBP antibody (Dakopatts) diluted 1:500 in BB or with the rabbit anti-GFAP antibody (Dakopatts) diluted 1:200 in BB. After rinsing 3×5 min in TBS with 0.1% Triton X-100, the sections were incubated for 60 min at RT with a secondary anti-rabbit IgG biotinylated antibody (Sigma) in a 1:800 dilution in BB and rinsed again 3×5 min in TBS with 0.1% Triton X-100. Sections were then incubated for 60 min at RT with avidin-peroxidase (Sigma) in a 1:600 dilution in BB and rinsed 3×5 min in TBS with 0.1% Triton X-100. The peroxidase reaction was visualized by incubating the sections in 100 ml of TB

containing 50 mg DAB (Sigma) and 66 μl H_2O_2 for 5 min. Finally, sections were rinsed, dehydrated in ethanol, cleared in xylene, and coverslipped in DPX. As a control for the immunocytochemical staining, the primary antibody was omitted.

2.4.2. Histochemical reaction for tomato lectin binding

PCNA immunoreacted sections were rinsed 3×5 min in TBS and peroxidase activity was blocked with 2% H_2O_2 in 100% methanol for 10 min at RT. Following rinsing 3×5 min in TBS containing 0.1% Triton X-100 the sections were incubated overnight at 4°C with the biotinylated tomato lectin (*Lycopersicon esculentum*; Sigma) diluted to 15 μg/ml in TBS. Sections were then rinsed 3×10 min in TBS, incubated for 60 min at RT with a 1:600 dilution of avidin-peroxidase (Sigma) in TBS, and rinsed again 3×5 min in TBS. Subsequent peroxidase demonstration was carried out using the same procedure described above for immunocytochemistry. Finally, sections were dehydrated and coverslipped in DPX. As a control for the histochemical staining, lectin was omitted.

2.5. Quantification of PCNA-positive cells

The number of PCNA-positive cells was obtained by counting PCNA immunostained nuclei within randomly selected fields, from similar areas of the white and gray matter, using a $40\times$ objective. Fields in white and gray matter were digitized by a videocamera connected to a Leitz microscope and interfaced to a Macintosh computer. National Institute of Health Image software (NIH 1.52) was used to quantify the number of glial cells showing nuclear PCNA immunostaining. Five sections per animal, amounting a total of 25 Jimpy and 25 control spinal cord sections per age were used. Four fields of 17 424 μm^2 (gray matter) and five fields ranging from 10 000 μm^2 to 17 424 μm^2 (white matter) per section were analyzed. Values of density of PCNA-positive cells were then calculated.

As expression of PCNA is not homogeneous through the cell cycle [9,42], a quantitative microscopic image analysis method including two detection thresholds has been applied in order to establish the unambiguous cut-off between positive and negative cells and analyze possible differences in the intensity of staining. We used the computer grayscale (white = 0; black = 255) to measure the maximum background staining (MBS) and the maximum nuclear staining (MNS) in each animal analyzed. All positive cells were comprised between these two limits. The first threshold ($T_{100\%}$) considered all positive cells, since all cells showing staining values higher than MBS were quantified. The second threshold ($T_{25\%}$) only considered positive cells whose staining values were included within the interval accounting for the 25% of the more intensely stained cells.

In order to determine statistical differences in the density of PCNA-positive cells, analysis of variance (ANOVA) and Scheffé test were performed at the 95% significance level, taking into account the effect of the following variables: type of mouse (control or Jimpy), age (10–12 or 20–22 days), and location (gray or white matter).

Finally, 10 Jimpy spinal cord sections per age, processed for the different double labeling techniques, were used to estimate the percentage of PCNA-positive cells simultaneously expressing either the oligodendrocytic, or the astrocytic or the microglial marker.

3. Results

3.1. Expression of PCNA

Microscopical examination of spinal cord sections processed for PCNA immunocytochemistry revealed that PCNA-positive cells were present in the gray and white matter of both the 10–12 and 20–22 day Jimpy and normal animals (Fig. 1). Immunostaining was exclusively located in the nucleus of some cells. Most PCNA-positive cells were identified as glial cells on the basis of their location and nuclear size, although some positive cells resembling neurons and showing a weak immunostaining were also observed in gray matter. The intensity of immunostaining was not homogeneous in all PCNA-positive glial cells, but a gradient in the intensity of immunostaining was seen.

There were remarkable differences in the density of PCNA-positive cells depending on the type of mouse, the age of the animal and the location. These differences were

Table 1
Estimation of density of PCNA-positive cells in the spinal cord of normal and Jimpy mice

	Normal				Jimpy			
	10–12 days		20–22 days		10–12 days		20–22 days	
	Gray matter	White matter	Gray matter	White matter	Gray matter	White matter	Gray matter	White matter
$T_{100\%}$	509 ± 42	1313 ± 92	140 ± 12	268 ± 25	632 ± 40	1332 ± 69	491 ± 31	1092 ± 47
$T_{25\%}$	41 ± 8	103 ± 20	13 ± 4	21 ± 5	94 ± 10	227 ± 19	61 ± 7	191 ± 15
Ratio T_{25}/T_{100}	0.08	0.078	0.093	0.078	0.149	0.17	0.124	0.175

Density is expressed in cells/mm^2. Values are means ± S.E.M.

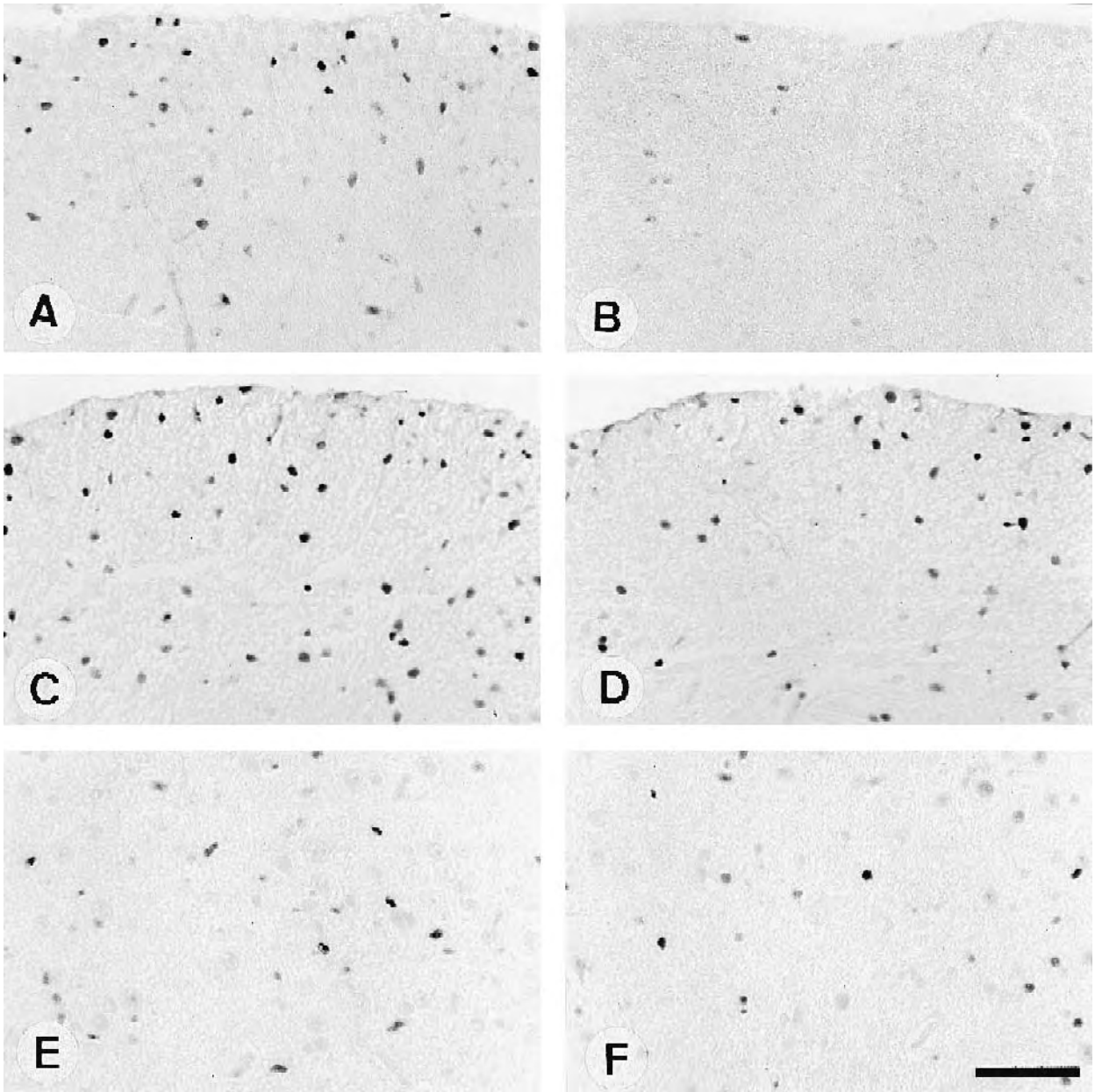

Fig. 1. Photomicrographs showing PCNA-positive cells in spinal cord sections from normal (A, B) and Jimpy (C–F) mice. Note that PCNA-positive cells in normal animals were abundant at 10–12 days (A), whereas few PCNA-positive cells were observed at 20–22 days (B). PCNA-positive cells were numerous in the white (C, D) and gray (E, F) matter of 10–12- (C, E) and 20–22-day-old (D, F) Jimpy mice. Bar = 50 μm.

statistically significant at the two detection thresholds. The mean densities of PCNA-positive cells obtained from the quantitative analysis are shown in Table 1. In normal animals, PCNA-positive cells were abundant at 10–12 days, particularly in white matter, whereas few PCNA-positive cells were observed at 20–22 days. In Jimpy, PCNA-positive cells were numerous throughout the spinal cord at both ages, but the mean density of PCNA-positive cells was higher in white than in gray matter. When Jimpy and normal animals were compared, the mutant spinal cord showed higher density of PCNA-positive cells than the normal one, this difference being more pronounced at 20–22 than at 10–12 days and in white than in gray matter. At 10–12 days, the differences in density of PCNA-positive cells between Jimpy and normal spinal cords were only higher than 2-fold at $T_{25\%}$, both in white and gray matter. At 20–22 days, the density of PCNA-positive cells in Jimpy white matter was 4- ($T_{100\%}$) to 9-fold ($T_{25\%}$) higher than in normal animals, whereas in gray matter the differential ranged from 3.5- ($T_{100\%}$) to 4.7-fold ($T_{25\%}$).

Differences not only in the numbers of PCNA-positive cells but also in the intensity of PCNA immunostaining were found when Jimpy and control animals were com-

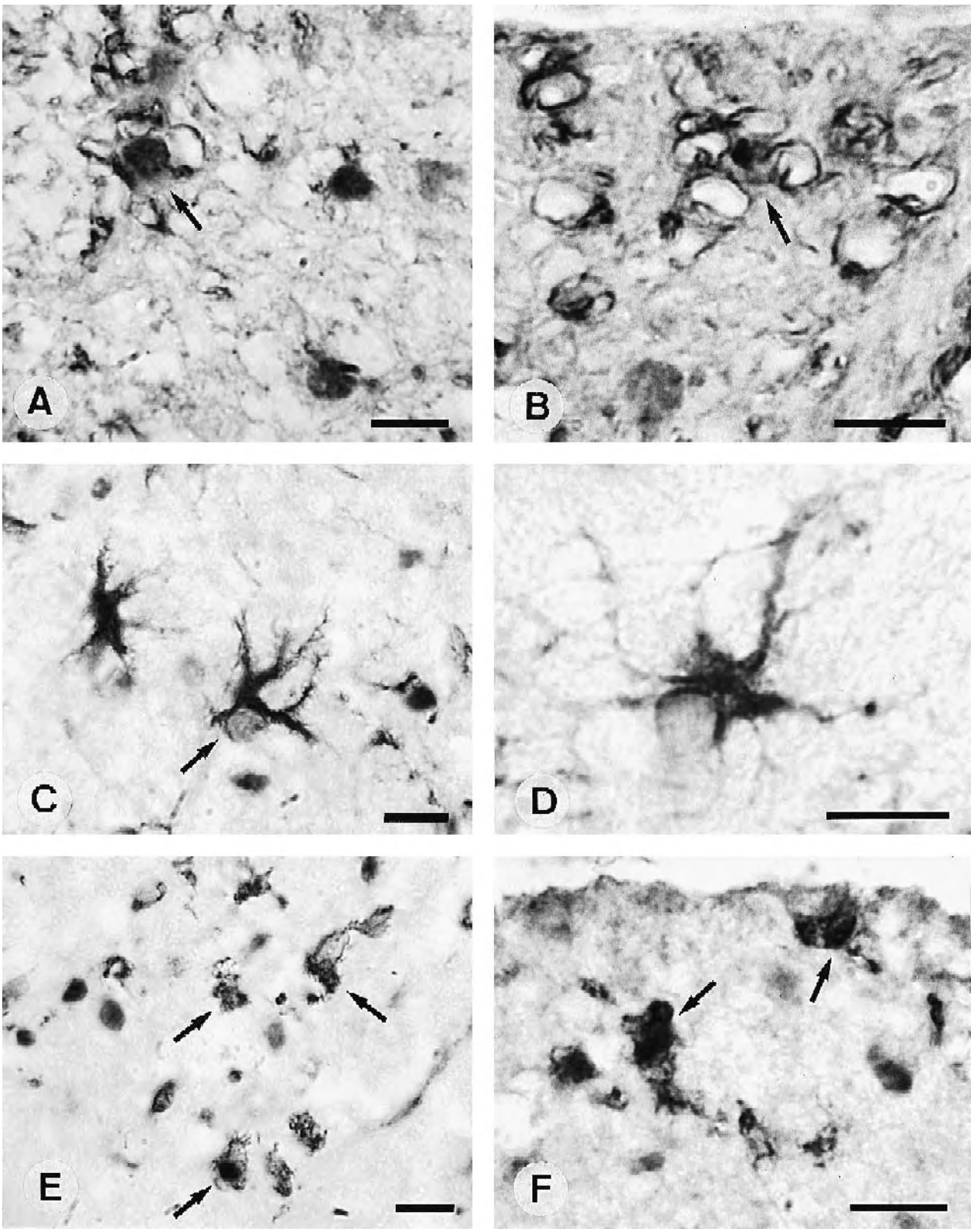

Fig. 2. Simultaneous demonstration of PCNA-positive cells and oligodendroglia (A, B), astroglia (C, D) and microglia (E, F) in the white matter of the Jimpy spinal cord. Double labeling was easily distinguished under the microscope since nuclear PCNA immunostaining was black and staining of glial cells was brown. Oligodendroglial and astroglial cells were demonstrated by immunocytochemical detection of MBP and GFAP, respectively, and microglial cells by tomato lectin histochemistry. In all photomicrographs, arrows point to double labeled cells. Note that PCNA immunoreactivity in relation to astrocytes was weak, whereas a moderate to strong staining was found in relation to the nuclei of oligodendroglial and microglial cells. Bar = 10 μm.

Table 2
Classification of the different cycling glial cells in the Jimpy spinal cord

	10–12 days		20–22 days	
	Gray matter	White matter	Gray matter	White matter
Astroglia (GFAP-positive)	8.6 ± 1.9	17.2 ± 1.1	14.8 ± 1.7	23.3 ± 2.1
Oligodendroglia (MBP-positive)	24.1 ± 3.8	51.1 ± 1.7	30.6 ± 4.7	46.1 ± 2.2
Microglia (tomato lectin-positive)	19.2 ± 2.3	27.2 ± 1.6	21.4 ± 1.9	26.7 ± 1.2

Percentages of PCNA-positive glial cells simultaneously expressing different glial cell markers.
Values are mean percentages ± S.E.M.
Nearly 100% of PCNA-positive glial cells were unambiguously classified in the Jimpy white matter, but a 33% (20–22 days) to 48% (10–12 days) of the PCNA-positive glial cells could not be positively classified in the gray matter due to the low expression of the glial markers.

pared. As shown in Table 1, the fraction of PCNA-positive cells showing intensely stained nuclei (ratio $T_{25\%}/T_{100\%}$) was higher in Jimpy than in normal animals. This was found in gray and white matter at both ages and was demonstrated by the fact that the mean percentage of PCNA-positive cells that remained positive after quantification at $T_{25\%}$ was about 2-fold higher in Jimpy than in normal spinal cords.

3.2. Oligodendroglia, astroglia, microglia and cycling cells

The double staining procedure enabled us to simultaneously visualize PCNA-positive nuclei (black staining) and the different glial cell types (brownish staining). The percentages of PCNA-positive cells expressing the different glial cell markers in Jimpy are shown in Table 2, and photomicrographs showing double labeled glial cells are presented in Fig. 2. In the mutant white matter, about 50% of PCNA-positive were oligodendroglial cells, 30% were microglial cells and 20% were astrocytes. The majority of intensely stained cells corresponded to oligodendrocytes, although microglial cells showing a strong nuclear staining were also found. In contrast, astrocytes showed a weak PCNA immunostaining and GFAP-positive cells showing strong PCNA immunostaining were at no time observed.

Classification of PCNA-positive cells in normal mice could not be carried out due either to the strong MBP immunoreactivity of the densely packed myelinated fibers or to the weak and/or incomplete astrocytic GFAP immunostaining.

4. Discussion

PCNA-positive cells were present in the gray and white matter of the 10–12- and the 20–22-day-old Jimpy and normal mice. In all cases, the PCNA-positive cell population was constituted by a pool of cells showing different intensities of nuclear staining. Differences in PCNA expression between cycling cells reflect the variability in concentration of PCNA related to the cell cycle phase [42]. PCNA synthesis begins in the late G_1-phase of the cell cycle, peaks during the S-phase and decreases at the S/G_2 transition and during $G_2 + M$ [9,58]. There are two intranuclear populations of PCNA, one of which is namely bound PCNA and the other free PCNA [8]. The bound PCNA is only detectable during the S-phase of the cell cycle and is a nuclear matrix-bound form that is associated with replication sites permitting DNA synthesis. The second form, the free PCNA, is detectable during the whole cell cycle, is diffusely dispersed in the nucleoplasm and acts as a reserve pool, presumably inactive, that is not directly involved in DNA replication. Most murine monoclonal antibodies, including PC10 used in this study, recognize both forms of PCNA [48]. Consequently, the whole fraction of cycling cells expressing different levels of bound and free PCNA is labeled after PCNA immunocytochemistry.

4.1. Abnormal PCNA expression in Jimpy

Spinal cords from Jimpy animals showed increased density of PCNA-positive cells than normal cords, the differential being higher in white matter than in gray matter and at 20–22 days than at 10–12 days. This is in agreement with previous in vivo studies reporting a higher percentage of glial cells incorporating tritiated thymidine in the mutant white matter [33,47,54]. Although there is some variability in the reported data, the quantifications made by these authors in the white matter revealed that the number of labeled cells was 2- to 3-fold (10–12 days) and 3- to 8-fold (20–22 days) higher in the mutants. Strictly, we cannot compare our results with previous ones since immunocytochemical assessment of PCNA allows us the detection of the whole fraction of cycling cells, whereas only S-phase cells are labeled after tritiated thymidine incorporation analysis [8,63]. However, our results substantially agree with those obtained from tritiated thymidine incorporation studies if the fraction of cells quantified at $T_{25\%}$ and characterized by showing the strongest PCNA immunostaining is exclusively considered. On the basis of our study we cannot establish a simple correspondence between the fraction accounting for the 25% of the more intensely stained cells and S-phase cells, but we would emphasize here that expression of PCNA reaches the maximum during the S-phase [9,42] and that the PCNA

tightly bound to DNA in S-phase cells account for 25% of the total PCNA [10].

4.1.1. Oligodendroglia

About 50% of PCNA-positive cells in the Jimpy white matter were MBP-positive oligodendroglial cells at both ages. Similarly, at 16–20 days in the mutant spinal cord white matter, at least 50% of the total number of cells incorporating tritiated thymidine were ultrastructurally identified as immature oligodendroglia [54]. The presence of numerous immature oligodendroglial cells keeping proliferative capability after the second postnatal week is one of the most prominent features in Jimpy, but there are also clues suggesting a cell cycle disturbance in relation to Jimpy oligodendrocytes. The percentage of the PCNA-positive cell population that showed intensely stained nuclei (quantified at $T_{25\%}$) was higher in Jimpy than in normal spinal cords. This higher PCNA expression in Jimpy cycling cells, mainly if not exclusively affecting oligodendroglia, could be explained either by a specific cell phase-related overexpression of PCNA or, more likely, by a lengthening of the cell cycle in a phase in which the PCNA expression was high, such as the S- or the G_2-phase. This second possibility is also suggested in a previous study of Knapp and Skoff [33]. These authors observed that many Jimpy oligodendroglial cells incorporate thymidine without undergoing timely cell division and suggested that either the S- or the G_2-phase is lengthened or that there is an arrest in G_2. In fact, there is evidence that cells may become quiescent not only in G_0 or G_1, but also in S and G_2 [2,14].

An abnormal oligodendrocyte development in Jimpy is also evidenced by in vitro studies reporting abnormal maturation and decreased ability of Jimpy oligodendrocytes to elaborate membrane sheets [4]. In vivo, the abnormal oligodendrocyte development is mainly supported by the fact that abundance of proliferating oligodendroblasts is not accompanied by a parallel increase in the number of differentiated oligodendrocytes. On the contrary, the number of well-differentiated oligodendrocytes in Jimpy is severely reduced [17,21,53] as a consequence, probably, of their premature apoptotic death [55,61].

4.1.2. Astroglia

About 20% of PCNA-positive cells in the Jimpy white matter were GFAP-positive astrocytes. Most studies, however, indicate that astroglial hypertrophy in Jimpy is not accompanied by an increase in the number of astrocytes [6,53]. In the same way, autoradiographic studies at the electron microscope level indicated that Jimpy astrocytes did not show thymidine incorporation at 16–20 days [54]. Although we cannot discard the possibility that some astrocytes proliferate, there are several clues suggesting that the expression of PCNA by Jimpy astrocytes may be the result of an intrinsic abnormality in their cell cycle rather than true mitotic activity. Cycling astrocytes, contrarily to oligodendrocytes, always showed a weak PCNA immunostaining, indicating that Jimpy astrocytes do not arrive at the cell cycle phases in which the PCNA expression is higher. As PCNA synthesis begins in the G_1-phase and immediately reaches the maximum during the S-phase, a lengthening or arrest in the G_1-phase is suggested. In a previous study, by using the non-radioactive bromodeoxyuridine incorporation method, Knapp and Skoff [35] found that the cell cycle of cultured Jimpy astrocytes is lengthened and also suggested that the bulk of the difference is due to an increase in the length of the G_1-phase. In fact, several models support the hypothesis that late G_1 events play a major role in the control of cell proliferation and there is evidence that cells may become quiescent in G_1 since some factors are required for progression from G_1 to S [3,20]. In this way, it has been described that synthesis of PCNA is induced by several growth factors [59] and that the presence of certain growth factors may generate an increase in the number of PCNA immunoreactive cells without producing a parallel increase in the number of S-phase cells [38]. It is also known that G_1 arrested cells express PCNA and are weakly stained with anti-PCNA antibodies [20,64]. Thus, a weak PCNA expression is potentially possible in G_1 non-proliferative Jimpy astrocytes either by absence or presence of determinate growth factors or their respective receptors. Finally, there are other instances, such as DNA repair [51] or aneuploidy/polyploidy [11], where PCNA expression does not lead to division, but at the moment we do not have available information to support the plausibility of either of these situations in Jimpy.

4.1.3. Microglia

About 30% of PCNA-positive cells in the Jimpy white matter were microglial cells. The presence of proliferating microglial cells correlates well with a previous study in which we described a marked increase in the numbers of microglial cells populating the Jimpy spinal cord [60]. Thus, present results suggest that the proliferation of intrinsic microglial cells, but not the extravasation of blood monocytes, is the main source of additional microglia in Jimpy. This view is also supported by the fact that Jimpy is a model in which microglial reaction results from an intrinsic defect, without apparent affectation of the vascular wall integrity. In non-invasive injuries, such as Wallerian degeneration of the optic nerve, there is no significant monocyte recruitment and most phagocytes derive from proliferation of intrinsic microglia, extrinsic monocytes accounting for only a small fraction (less than 3%) of the [^{3}H]thymidine labeled cells [52]. In the same way, lethal and non-lethal neuronal injuries induced by toxic ricin injection into the facial nerve [57] and by facial nerve axotomy [26], respectively, lead to a rapid proliferation of

intrinsic microglia in the facial nucleus without significant participation of blood-borne monocytes.

The exact significance of microglial proliferation in Jimpy remains to be determined. Nevertheless, proliferation and subsequent accumulation of microglia may be important to improve the efficiency in the removal of cell debris and to enhance the microglial production of cytokines. The phagocytic role of microglia has already been reported in Jimpy, and as we have shown in a previous ultrastructural study, phagocytic microglia in the mutant is characterized by intracytoplasmatic accumulation of lipid inclusions and dense and heterogeneous bodies [60]. Reactive microglia in Jimpy seem to be directly involved in the removal of defective myelin sheaths and degenerative cells, as suggested by the observation that microglial cells were closely attached to, even engulfing, poorly myelinated or unmyelinated axons and pyknotic cells [60]. Interestingly, we have recently demonstrated through sequential combination of in situ labeling of nuclear DNA fragmentation and specific glial cell markers, that microglial cells are physically related to apoptotic oligodendrocytes [61]. In this study, a role for microglia in the elimination of defective oligodendrocytes is further suggested by the observation that most, if not all, apoptotic oligodendrocytes were partially or completely surrounded by microglial cell processes.

Although signaling mechanisms leading to division of microglia are poorly known, it is likely that the presence of dying oligodendrocytes was ultimately responsible for the reactive proliferation of microglial cells in Jimpy. Del Rio-Hortega [13] first suggested that dead cells and cell debris are among the most potent signals to elicit reactive microgliosis. Soluble proteins stimulating proliferation of microglia have also been isolated from damaged brain [23]. These molecules are produced in the injured and developing mammalian brain and are selective mitogens for microglia. Certain classes of colony-stimulating factors (CSFs), originally identified as growth factors for hematopoietic precursor cells, are also able to induce microglial proliferation. In particular, granulocyte-macrophage CSF (GM-CSF) and multipotential-CSF (multi-CSF; also referred to as interleukin-3) are potent microglial mitogens [18,22,50]. Some of these mentioned mitogens are produced by astroglia and it is known that reactive microglia secrete a variety of cytokines, including interleukin-1 and tumor necrosis factor-alpha, that promote reactive astrogliosis [19,24,40]. In fact, several findings point to the existence of a regulatory network involving a variety of soluble factors that influence the growth and differentiation of glial cells [5,25,29,49]. There are studies supporting the existence of an astroglia-microglia [5,19,25,40,41], microglia-oligodendroglia [27,40] and astroglia-oligodendroglia [16] interaction not only in injured CNS, but also during development and myelinogenesis. Therefore, the study of cytokine production and expression of cytokine receptors is of vital importance for the understanding of glial relations in Jimpy.

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