Remyelination After Cuprizone-Induced Demyelination Is Accelerated in Juvenile Mice

Sabine Pfeifenbring, MD, Stefan Nessler, MD, Christiane Wegner, MD, DPhil, Christine Stadelmann, MD, and Wolfgang Brück, MD

Abstract

Remyelination capacity decreases with age in adult mice, but data comparing remyelination capacity after toxic demyelination in developing mice versus adult mice are not available. We treated 3-week-old and adult C57BL/6 mice with cuprizone for 1 to 5 weeks and studied demyelination/remyelination and cellular reactions in the corpus callosum and motor cortex by histology, immunohistochemistry, and electron microscopy. We compared results between the 2 treated groups and age-matched controls. In juvenile mice, significant demyelination was detectable in the corpus callosum on Week 2 and in the motor cortex on Week 5. Oligodendrocyte loss, microglial activation, and acute axonal damage peaked on Week 2. Increased numbers of oligodendrocyte precursor cells were evident on Week 1, and remyelination was detectable on Week 3. Juvenile mice showed more rapid demyelination than adult mice, which may be related to greater vulnerability of oligodendrocytes, lower myelin content, or dose-dependent cuprizone effects. Earlier activation of microglia and proliferation of oligodendrocyte precursor cells probably contributed to accelerated remyelination and less pronounced axonal damage. Our data indicate that oligodendroglial regeneration and remyelination are enhanced in the maturing rodent brain compared with the young-adult rodent brain.

Key Words: Cuprizone, Demyelination, Juvenile mice, Pediatric multiple sclerosis, Remyelination.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) in which white matter lesions show demyelination, inflammation, gliosis, and relative axonal preservation (1). Studies have demonstrated that demyelination also affects gray matter (2–7). Multiple sclerosis lesions can show substantial remyelination, particularly in the early stages of the disease (8, 9), and the cortex may show even more abundant remyelination than the white matter (10, 11).

Although MS is most often diagnosed in young adults, it can occur at any age. Three percent to 10% of all patients with MS had disease onset before age 18 years (pediatric MS) (12). Pediatric MS often presents and progresses differently from adult MS. Time to recovery from initial relapse is shorter and degree of recovery is more often complete among patients with pediatric MS than among patients with adult MS (13–18). Better clinical improvement in children with MS may be the result of more efficient and faster remyelination, leading to less axonal damage in the long term (19, 20), better resolution of inflammation, and higher degree of neuronal plasticity in the developing brain (21, 22).

An age-associated decline in remyelination capacity during adolescence and early adulthood was reported using magnetization transfer ratio (MTR) magnetic resonance imaging, a histopathologically validated imaging technique in which changes in myelin content can be quantified (23). A reduction of MTR values is thought to indicate tissue destruction by axonal loss and demyelination and is typical of newly formed MS lesions. Recovery of MTR values corresponds most likely to tissue repair by remyelination (23, 24). Lesional MTR recovery in patients with pediatric MS decreased with increasing age at disease onset and with increasing age at the time of magnetic resonance imaging scan, suggesting a greater capacity for remyelination in younger adolescents (23). Interestingly, prolonged visual evoked potentials in children with acute demyelinating optic neuritis recover faster than prolonged visual evoked potentials in adult patients. The recovery (measured by the latency of visual evoked potentials) is associated with remyelination of demyelinated axons. Thus, this difference in regeneration might be explained by a more rapid remyelination of demyelinated axons in children compared with adults (20). Extensive histopathologic studies of remyelination capacity in patients with pediatric MS have so far been lacking.

That aging affects remyelination is also supported by findings from animal experiments demonstrating that remyelination after toxin-induced experimental demyelination is complete but slower in old-adult animals compared with young-adult animals (19, 25–30). Recruitment of oligodendrocyte precursor
cells (OPCs) and their differentiation into remyelinating oligodendrocytes become less efficient with age (19, 25). Almost all of these experimental studies were performed in adult animals. Data on the regenerative capacity of the developing and maturing CNS are limited (31–33).

A useful experimental approach to investigating remyelination is the cuprizone model, a widely used animal model for studying MS-related myelin pathology (19, 31, 34, 35). In this model, animals are fed the copper chelator cuprizone (oxalic acid bis[cyclohexylidene hydrazide]), which leads to reproducible toxic demyelination of distinct brain regions via oligodendroglial death (34–36). The exact mechanisms of cuprizone-induced oligodendrocyte damage are not fully understood (34, 35, 37). The corpus callosum (CC) is the most frequently studied white matter tract in this animal model (34). An almost complete demyelination of the CC in adult C57BL/6 wild-type mice can be observed after 5 to 6 weeks of cuprizone feeding (34, 37). Several gray matter areas, such as the neocortex and the hippocampus, are also affected (37–39). Termination of cuprizone administration leads to spontaneous remyelination; however, partial remyelination occurs simultaneously with ongoing demyelination in adult mice (34, 35, 40, 41).

In the present study, we investigated the extent and kinetics of cuprizone-induced demyelination and remyelination in the maturing brain by assessing cuprizone-induced pathology in juvenile and adult mice in relation to age-matched untreated controls.

MATERIALS AND METHODS

Mice and Cuprizone Treatment

C57BL/6 J wild-type mice were obtained from Charles River (Sulzfeld, Germany). Animal experiments were approved by the Review Board for the Care of Animal Subjects of the district government (Lower Saxony, Germany) and performed according to international guidelines on the use of laboratory animals. The mice were housed in a temperature- and humidity-controlled animal facility with a 12-hour light-dark cycle. C57BL/6 J mice were fed cuprizone (Sigma-Aldrich Inc, St Louis, MO) mixed in powdered standard rodent chow to investigate demyelination. At the beginning of cuprizone treatment, juvenile mice were 3 weeks old—an age at which the CC is myelinated (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A762). Three-week-old mice were fed a diet containing 0.15% cuprizone (the dose found to be most suitable for juvenile mice) ad libitum (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A763). Cuprizone diet was maintained for 1 week (n = 3), 2 weeks, 3 weeks, 4 weeks, or 5 weeks (n = 7 mice per group) to study the course of acute demyelination. Eight-week-old mice received 0.2% cuprizone ad libitum for 1 week (n = 3) and 5 weeks (n = 7) to compare findings in juvenile mice with findings in adult mice. Food containing cuprizone was changed every 2 days. The body weight of mice was determined every 2 days. Three- and eight-week-old C57BL/6 J mice (n = 3 mice per group) fed normal chow throughout were used as controls.

Histology and Immunohistochemistry

At the end of the experiments, cuprizone-treated mice and control mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were postfixied in 4% paraformaldehyde in PBS for 72 hours at 4°C, embedded in paraffin, and cut into 4-μm-thick sections. Serial paraffin sections between bregma 1.18 and bregma −0.82—according to the mouse atlas by Franklin and Paxinos (42)—were analyzed. Sections were stained with hematoxylin and cosin, Luxol fast blue/periodic acid Schiff (PAS), and Bielschowsky silver impregnation. Immunohistochemical stainings were performed using the avidin-biotin method with antibodies against the following targets: myelin proteolipid protein (PLP; Biozol Diagnostica, Eching, Germany); 2,3-cyclic nucleotide 3-phosphodiesterase (CNPase; Covance Inc, Princeton, NJ); myelin oligodendrocyte glycoprotein (MOG; Abcam, Cambridge, United Kingdom); mature oligodendrocytes (Nogo-A, mAb 11C7 [43]); Prof. M.E. Schwab, Brain Research Institute, Laboratory of Neural Regeneration and Repair, University and ETH Zürich, Zürich, Switzerland); oligodendroglial lineage cells (Olig2; Immuno-Biological Laboratories Co Ltd, Gunma, Japan); activated microglia (Mac3; Pharmingen, San Diego, CA); microglia (Iba1; Wako Chemicals, Richmond, VA); acutely damaged axons (amyloid precursor protein [APP]; EMD Millipore, Darmstadt, Germany); astrocytes (gliarial fibrillary acidic protein [GFAP]; Dako, Glostrup, Denmark); and Ki67 (DCS Diagnostics, Hamburg, Germany). Slides were analyzed with an Olympus BX 51 light microscope (Olympus, Hamburg, Germany). Cell F and cellSens (Olympus) were used as imaging software. Digital microscopic images were taken with an Olympus XM10 camera.

Double immunofluorescence staining was performed to determine the number of proliferating oligodendroglial lineage cells and the number of proliferating microglia in juvenile and adult cuprizone-treated mice and in age-matched controls. For double staining with rabbit anti-Ki67 antibody and either rabbit anti-Olig2 or rabbit anti-Iba1 antibody, we used the highly sensitive tyramide signal amplification staining method (TSATM Detection kit; Invitrogen, Darmstadt, Germany). This widely used enzyme-mediated detection method enhances the sensitivity of antigen detection, allowing the use of a lower concentration of the primary antibody (rabbit anti-Ki67 antibody at a dilution of 1:10000). After exposure to secondary biotinylated anti-rabbit antibody and incubation with horseradish peroxidase, signal was visualized using tyramide-coupled Alexa 555. Afterward, the second primary antibody (anti-Olig2 antibody for oligodendroglial lineage cells or anti-Iba1 antibody for microglia) was added and incubated with anti-rabbit IgG conjugated with Alexa 488.

Morphometry

To assess demyelination in cuprizone-treated and control mice, we stained coronal sections with Luxol fast blue/PAS, PLP, CNPase, and MOG. Myelination status was evaluated in predefined brain regions because demyelination by cuprizone is not uniform (34). Demyelination of the medial part of the genu, isthmus, and/or body of the CC and motor cortex (MC; Fig. 11) was scored (0, completely demyelinated; 1, less than one third myelinated; 2, more than one third to two thirds myelinated; 3, more than two thirds myelinated) directly under a light microscope according to previously described scores (38, 40, 44). Cortical demyelination was assessed by immunohistochemistry for PLP, CNPase, and...
MOG because Luxol fast blue/PAS staining was not sensitive enough to detect cortical myelination (38).

The densities of mature oligodendrocytes (Nogo-A-positive), immature OPCs (strong nuclear Olig2 expression (45)), proliferating OPCs (Ki67-immunopositive and Olig2-immunopositive), astrocytes (GFAP-positive), acutely damaged axons (APP-positive), activated microglia (Mac3-positive), proliferating microglia (Ki67-positive and Iba1-positive), and apoptotic cells (hematoxylin and eosin) in the middle CC and in the MC were counted at 400× magnification in at least 10 standardized microscopic fields, each defined by an ocular grid. Only immunohistochemically stained cells with discernible nuclei were counted. Cell density was expressed as cells per square millimeter.

The relative axonal density of Bielschowsky silver impregnated axons in cuprizone-treated mice (at selected time points: 3-week-old mice on Weeks 2 and 5 of cuprizone exposure and 8-week-old mice on Week 5 of cuprizone exposure) and age-matched controls was determined. Counting was performed in 2 microscopic fields (at 1,000× magnification) within the medial part of the CC and in the MC (I). (A, C, E) Luxol fast blue/PAS. (F, I-M) Immunohistochemistry for myelin proteolipid protein (PLP). (B, D, G, N) 2,3-Cyclic nucleotide 3-phosphodiesterase (CNP). (H, O) Myelin oligodendrocyte glycoprotein (MOG). (D, inset) Electron micrograph. ** p < 0.01, * p < 0.05. P21, light-gray bar; P56, dark-gray bar. Scale bars = (A-D) 200 μm; (I-L) 500 μm.
axonal density among cuprizone-treated mice relative to age-matched controls.

Electron Microscopy
To investigate ongoing remyelination during cuprizone treatment, we performed electron microscopy analyses of the CC in cuprizone-treated and control mice. Fragments of the medial part of the body of the CC were fixed in 3% glutaraldehyde in phosphate buffer, postfixed in 1% osmium tetroxide, and stained with uranyl acetate. After dehydration through a graded ethanol series and propylene oxide, samples were embedded in araldite. Sections were oriented in such a way that cross-sections of axons in the CC were obtained. Semithin sections were stained with azure II–methylene blue, in accordance with Richardson et al (46), for light microscopy examination. Ultrathin sections were stained with lead citrate and examined under a Zeiss EM10 electron microscope (Zeiss). Images were taken with an Olympus Soft Imaging Systems Megaview III digital camera (OSIS, Münster, Germany). At least 10 images per mouse were taken at a magnification of 12,500×. AnalySIS image processing software 3.2 was used to calculate axon diameter, myelin thickness, and fiber diameter. G-ratios (axon diameter divided by fiber diameter) were measured in at least 100 myelinated fibers per mouse (n = 3 per group) to calculate the mean g-ratio per mouse. In addition, the percentage of myelinated axons was determined. All data are reported as arithmetic mean ± SE. Remyelinated axons were identified by their characteristically thin myelin sheaths in relation to axon diameter, leading to increased g-ratio. All investigated fibers had an axon diameter greater than 250 nm (47).

Statistics
All values are expressed as arithmetic mean ± SE. For statistical analysis, nonparametric tests were performed (Kruskal-Wallis test with Dunn multiple comparisons test, Mann-Whitney U-test). All tests were classified as significant if p < 0.05. Statistical analysis was performed using IBM SPSS Statistics Software version 21 (IBM Corp, Armonk, NY) or GraphPad Prism Software version 5 (GraphPad Software Inc, San Diego, CA).

RESULTS
Cuprizone-Induced Demyelination of White Matter and Gray Matter Was Accelerated in Juvenile Mice
In juvenile mice, significant demyelination of the CC was evident after 2 weeks of cuprizone treatment (p = 0.024, Kruskal-Wallis test; Fig. 1E). The extent of demyelination of the CC was similar in juvenile mice treated with cuprizone for 2 weeks and adult mice fed cuprizone for 5 weeks. Expression of the myelin proteins PLP, CNPase, and MOG slightly increased in juvenile mice on Weeks 3 and 4 and decreased again on Week 5 of cuprizone exposure. In the MC of juvenile mice, the density of PLP, CNPase, and MOG continuously decreased between Week 2 and Week 5 of cuprizone treatment (p < 0.001, Kruskal-Wallis test; Fig. 1). Complete cortical demyelination was detectable on Week 5. After 5 weeks of cuprizone treatment, the extent of cortical demyelination was significantly greater in juvenile mice than in adult mice (p < 0.05).

Recently Matured Oligodendrocytes in Juvenile Mice Were Highly Vulnerable to Cuprizone
In juvenile cuprizone-treated mice, the highest numbers of apoptotic cells in the CC and MC were detected during the first 2 weeks of cuprizone treatment (p < 0.0005, Kruskal-Wallis test; Fig. 2). During the course of cuprizone treatment in juvenile mice, the density of apoptotic cells continuously decreased and reached the lowest levels on the fourth and fifth weeks. After a 1-week cuprizone treatment, the increase in the numbers of apoptotic cells was greater in juvenile mice (10.5-fold increase [CC] and 7.5-fold increase [MC] compared with 3-week-old control mice) than in adult mice (4.6-fold increase [CC] and 3.7-fold increase [MC] compared with 8-week-old control mice). In juvenile mice, the density of mature Nogo-A–expressing oligodendrocytes in the CC was greatly reduced after 1 week of cuprizone feeding and that in the MC was greatly reduced after 2 weeks of cuprizone feeding, compared with 3-week-old control mice (Fig. 2). During the course of cuprizone treatment, the number of mature Nogo-A–expressing oligodendrocytes was stable at low levels. The loss of Nogo-A–expressing oligodendrocytes in the CC after a 1-week cuprizone exposure, compared with age-matched controls, was greater in juvenile mice (−44.3% than in adult mice (−30.1%), whereas the reduction of Nogo-A–expressing oligodendrocytes in the MC was similar in juvenile mice (−6.8%) and adult mice (−8.2%) (Fig. 2).

Remyelination of the CC Is Accelerated in Juvenile Mice
In juvenile mice, a continuous increase in g-ratio was observed starting 3 weeks after the initiation of cuprizone treatment (Fig. 3). The mean ± SE g-ratio for juvenile mice given cuprizone for 2 weeks (0.7198 ± 0.004; 33.9% ± 11.7% myelinated axons) was similar to that for 3-week-old control mice (0.728 ± 0.003; 63.7% ± 4.5% myelinated axons) and adult 8-week-old control mice (0.7159 ± 0.004; 85.9% ± 11.7% myelinated axons). The mean ± SE g-ratio for juvenile mice treated with cuprizone for 3 weeks (0.7474 ± 0.004; 44.2% ± 14.2% myelinated axons), 4 weeks (0.7858 ± 0.004; 63% ± 21.2% myelinated axons), and 5 weeks (0.8035 ± 0.004; 44.8% ± 9.7% myelinated axons) compared with 3-week-old control mice (p < 0.001). A mean ± SE g-ratio of 0.7459 ± 0.004 was measured in adult mice fed cuprizone for 5 weeks (49.8% ± 6.6% myelinated axons). The g-ratio was significantly higher in juvenile mice on Weeks 4 and 5 of cuprizone exposure than in adult mice on Week 5 of cuprizone exposure (p < 0.001). This indicates that remyelination is accelerated in juvenile mice compared with adult mice.

Proliferation of OPCs in the CC of Juvenile Cuprizone-Treated Mice Started Early
Olig2 immunostaining was used to study OPCs. Olig2 is expressed in oligodendrogial lineage cells, whereas OPCs show strong nuclear Olig2 staining and mature oligodendrocytes display only weak nuclear Olig2 staining (45). In the CC of juvenile mice, there was a distinct increase in the number of
Olig2-strong-expressing OPCs detectable after 1 week of cuprizone exposure, compared with 3-week-old control mice (Fig. 4). During the course of cuprizone treatment, the densities of OPCs in juvenile mice gradually decreased ($p < 0.03$, Kruskal-Wallis test). However, we observed a 4.6-fold increase in the number of proliferating Ki67-positive/Olig2-positive OPCs on Weeks 1 and 2, compared with 3-week-old control mice. In the CC of adult mice, we did not observe a significant increase in the number of OPCs or in the proliferation of OPCs after 1 week of cuprizone exposure, compared with 8-week-old control mice. Elevated numbers of OPCs (1.6-fold) and proliferating OPCs (26.3-fold) in the CC of adult mice were detectable on Week 5. In summary, oligodendroglial proliferation and regeneration in the CC of juvenile mice started early. In the MC of juvenile mice, the numbers of OPCs decreased during cuprizone treatment (Fig. 4). In adult cuprizone-treated mice, the densities of OPCs in the MC were similar to those in 8-week-old control mice. We did not detect a significant increase in the numbers of proliferating OPCs in the MC of juvenile and adult cuprizone-treated mice (data not shown).

Rapid Activation of Microglia in Juvenile Mice

In the CC and MC of juvenile mice, the numbers of activated Mac3-expressing microglia peaked on Week 2 of cuprizone treatment ($p < 0.009$, Kruskal-Wallis test; Fig. 5). Within the first 2 weeks of cuprizone feeding, the density of proliferating Ki67-positive/Iba1-positive microglia in the CC was higher in juvenile mice (1 week of cuprizone treatment, $11.8 \pm 2.5$; 2 weeks of cuprizone treatment, $15 \pm 7.6$) than in adult mice (1 week of cuprizone treatment, $3.7 \pm 0.4$), but the overall extent of microglial activation was greater in adult mice (Week 5) than in juvenile mice (Week 2). On Week 5 of cuprizone treatment, the densities of activated microglia in the CC and MC were significantly higher in adult mice than in juvenile mice ($p < 0.05$). Proliferating Ki67-positive/Iba1-positive microglia were rarely found in the MC of juvenile and adult cuprizone-treated mice (data not shown).

Similar Extent of Astrogliaosis in Juvenile and Adult Cuprizone-Treated Mice

The numbers of GFAP-expressing astrocytes in the CC and MC of juvenile mice continuously increased during the course of cuprizone treatment (Fig. 6). On Week 5 of cuprizone exposure, the numbers of astrocytes in juvenile mice and adult mice were similar.

Extent of Acute Axonal Damage Is Less in Juvenile Mice Than in Adult Mice

To assess the extent of acute axonal damage in the CC of juvenile and adult cuprizone-treated mice, we counted acutely

Copyright © 2015 by the American Association of Neuropathologists, Inc. Unauthorized reproduction of this article is prohibited.
FIGURE 3. Remyelination is accelerated in the CC of juvenile mice. Remyelination results in thin myelin sheaths and is reflected as increased $g$-ratio (axon diameter divided by fiber diameter). A significantly increased $g$-ratio was detectable in juvenile mice on Week 3 of cuprizone (Cup) treatment versus 3-week-old control mice, and in juvenile mice on Week 4 of Cup treatment versus adult mice fed Cup for 5 weeks (w) (E). In juvenile mice, the percentage of myelinated axons was lowest on Week 2 of Cup treatment (F). (A–D) Electron micrographs. *** $p < 0.001$, * $p < 0.05$. P21, light-gray bar; P56, dark-gray bar. CTRL, untreated control mice. Scale bars = (A–D) 2 μm.
damaged axons, which are characterized by swelling and accumulation of APP (48). In juvenile mice, acute axonal damage was most extensive in the first 2 weeks (w) of Cup exposure in the CC (F, G) of juvenile mice; significant proliferation of OPCs was not present on Week 1 in adult mice (G). The densities of OPCs did not increase significantly in the MC of juvenile Cup-treated mice (C, H) or adult Cup-treated mice (D, H). (A–D) Immunohistochemistry for Olig2. (F) Double immunofluorescence staining with anti-Ki67 antibody (Alexa 555) and anti-Olig2 antibody (Alexa 488). ** p < 0.01, * p < 0.05. P21, light-gray bar; P56, dark-gray. CTRL, untreated control mice. Scale bars = (A–D, F) 50 μm.

**FIGURE 5.** Microglial activation in juvenile cuprizone (Cup)–treated mice. In juvenile mice, microglial response in the CC (A, E) and in the MC (C, H) peaked on Week (w) 2 of Cup treatment. The density of activated microglia was higher in adult Cup-treated mice (CC [B, E]; MC [D, H]) than in juvenile Cup-treated mice, whereas the numbers of resting microglia were similar in juvenile control mice and adult control mice (data not shown). The proliferation of microglial cells (immunopositive for Ki67 and Iba1) in juvenile Cup-treated mice started early (F, G). Activated microglia contained myelin degradation products (A, bottom-right inset). (A–D) Immunohistochemistry for Mac3. (A, bottom-right inset) Immunohistochemistry for MOG. (F) Double immunofluorescence staining with anti-Ki67 antibody (Alexa 555) and anti-Iba1 antibody (Alexa 488). *** p < 0.001, ** p < 0.01, * p < 0.05. P21, light-gray bar; P56, dark-gray bar. Scale bars = (A–D, F) 50 μm.
of cuprizone feeding ($p < 0.05$), whereas the reduction in axonal density in juvenile and adult cuprizone-treated mice was similar, compared with age-matched controls (juvenile mice on Week 2 [Week 5] of cuprizone treatment, 20.9% [11%] reduction in axonal density compared with 3-week-old controls; adult mice on Week 5 of cuprizone treatment, 20.5% reduction in axonal density compared with 8-week-old controls). Acutely damaged axons were not found in controls.

**DISCUSSION**

In the current study, we investigated the course of cuprizone-induced demyelination and remyelination and accompanying cellular reactions in juvenile mice. This study is of particular interest because data on the extent and kinetics of cuprizone injury (31, 33, 36, 49) and on the regenerative capacity of the maturing CNS (31–33) are limited. To our knowledge, this is the first study to compare remyelination after toxic demyelination in developing animals versus adult animals.

The onset and extent of cuprizone-induced demyelination were accelerated in juvenile mice compared with adult mice. Recently matured oligodendrocytes were highly vulnerable to toxic injury (49), and apoptosis was more extensive in juvenile mice than in adult mice. These differences may be related to developmental effects. Programmed cell death or apoptosis is a common phenomenon of CNS development; elimination of excess cells, especially neurons, is achieved via apoptosis. Furthermore, the susceptibility of the brain to apoptosis after injury is higher in the developing brain than in...
the mature brain (50, 51). Brain mitochondria isolated from immature rats (8–17 days old) have elevated levels of pro-apoptotic Bax proteins and release more cytochrome c than do adult brain mitochondria (51). Furthermore, maturation requires a great amount of energy. In early postnatal life, the immature brain has to be supplied with energy for functioning and for the synthesis of proteins, phospholipids, and lipids necessary for myelin formation and maintenance (52). Much of this energy is generated by mitochondria (organelles that show structural changes and functional disturbances during cuprizone treatment) (34). This may contribute to the higher vulnerability of oligodendrocytes to toxic injury in a developing organism; however, the kinetics of toxic demyelination is also influenced by myelin content, and dose-dependent cuprizone effects cannot be excluded (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A763). Difference in myelination status between juvenile mice and adult mice was most obvious in the gray matter (Figure, Supplemental Digital Content 1, http://links.lww.com/NEN/A762). Hence, less myelin has to be removed in juvenile mice.

Our study shows that remyelination is accelerated in juvenile mice compared with adult mice. This is in line with previous studies that reported an age-associated decrease in remyelination efficiency after toxic demyelination (19, 25–30), which resulted from impaired OPC recruitment and differentiation (19, 25, 29).

The migration and differentiation of OPCs into myelinating oligodendrocytes are critical steps in remyelination. Here, we showed that the proliferation of OPCs was accelerated in juvenile mice compared with adult mice. Another important part of this regenerative process is the removal of damaged myelin. Myelin contains proteins that inhibit OPC differentiation (53–55). Hence, rapid clearance of myelin debris by activated microglia enhances the process of remyelination (26, 28, 55, 56).

Microglial activation and proliferation in juvenile mice started early, leading to rapid clearance of damaged myelin (Fig. 5A, right-bottom inset); however, absolute microglial number was greater in adult mice than in juvenile mice. The extent of microglial activation is influenced by the amount of damaged myelin that needs to be removed. Hence, in juvenile mice, fewer activated microglia are required for myelin debris clearance. Furthermore, microglia from young animals are more efficient in myelin removal than microglia from old animals (28, 56, 57). An age-associated increase in microglial response after toxin-induced demyelination has been observed in adult animals (58). It is assumed that most microglial cells have a local origin in the cuprizone model. They are recruited from other brain regions and proliferate locally (37). As it is known for adult mice (37), the blood-brain barrier seems to be closed in juvenile mice (both in controls and in cuprizone-treated animals) (Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A764).

Accelerated remyelination and lesser degree of microglial activation might be responsible for the lesser extent of acute axonal damage in juvenile mice. The extent of acute axonal damage was 2.2-fold higher in adult mice (on Week 5) than in juvenile mice (on Week 2), whereas the reduction in axonal density was similar in juvenile and adult cuprizone-treated mice, compared with age-matched controls. This is in line with the findings of Irvine and Blakemore (58), who detected an age-associated increase in axonal damage in old-adult mice (6–7 months) compared with young-adult mice (8–10 weeks) using the cuprizone model.

Regeneration by remyelination during continuing cuprizone administration was clearly evident in the CC. In contrast, cortical remyelination did not occur in juvenile and adult mice during continuing cuprizone administration. In addition, we did not detect a significant increase in the densities of OPCs and proliferating OPCs in the MC of juvenile and adult cuprizone-treated mice. In adult mice, cortical remyelination starts only after cuprizone is removed from the diet (38, 39). In line with the different time course of remyelination, we observed delayed demyelination of the MC compared with the CC in juvenile and adult mice (37, 40). Hence, the kinetics of damage and repair differs between white matter and gray matter.

Cuprizone-induced pathology was accompanied by astrogliosis. The total numbers of astrocytes in the CC and MC were similar in juvenile and adult mice on Week 5 of cuprizone treatment. However, when the densities of astrocytes in juvenile and adult cuprizone-treated mice were related to those of untreated age-matched controls, we found a greater increase in astrocyte activation in adult mice (7.1-fold increase in the CC and 2.9-fold increase in the MC) than in juvenile mice (2.2-fold increase in the CC and 1.6-fold increase in the MC). Owing to myelination glialesis (59, 60), untreated juvenile control mice showed higher numbers of astrocytes than did untreated adult mice. In the literature, an age-associated increase in astrocyte response was observed in old-adult mice (6–7 months) compared with young-adult mice (8–10 weeks) (58). Furthermore, cell culture experiments with cells from aged rats revealed increased proliferation and activated phenotypes of astrocytes and microglia (61).

In conclusion, demyelination is accelerated in juvenile mice because of the high susceptibility of recently matured oligodendrocytes to toxic injury and the lower myelin content in juvenile mice compared with adult mice. However, dose-dependent toxic effects cannot be ruled out. In juvenile mice, the damage reached its maximum on Weeks 1 and 2 of cuprizone feeding, as determined by microglial activation, loss of oligodendrocytes, numbers of apoptotic cells, and extent of acute axonal damage. The repair process in juvenile mice started early. We observed a rapid activation of microglia and an early proliferation of OPCs, which led to efficient remyelination of the CC and reduced the extent of axonal damage. Thus, regenerative capacity in the maturing CNS is enhanced after toxic oligodendrocyte damage.

ACKNOWLEDGMENT

We would like to thank Brigitte Maruschak, Angela Dettmar, Mareike Gloth, and Jasmin Reichl for excellent technical assistance.

REFERENCES


58. Irvine KA, Blakemore WF. Age increases axon loss associated with primary demyelination in cuprizone-induced demyelination in C57BL/6 mice. J Neuroimmunol 2006;175:69–76