

# Oligodendrocytes and Progenitors Become Progressively Depleted within Chronically Demyelinated Lesions

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**To understand mechanisms that may underlie the progression of a demyelinated lesion to a chronic state, we have used the cuprizone model of chronic demyelination. In this study, we investigated the fate of oligodendrocytes during the progression of a demyelinating lesion to a chronic state and determined whether transplanted adult oligodendrocyte progenitors could remyelinate the chronically demyelinated axons. Although there is rapid regeneration of the oligodendrocyte population following an acute lesion, most of these newly regenerated cells undergo apoptosis if mice remain on a cuprizone diet. Furthermore, the oligodendrocyte progenitors also become progressively depleted within the lesion, which appears to contribute to the chronic demyelination. Interestingly, even if the mice are returned to a normal diet following 12 weeks of exposure to cuprizone, remyelination and oligodendrocyte regeneration does not occur. However, if adult O4<sup>+</sup> progenitors are transplanted into the chronically demyelinated lesion of mice treated with cuprizone for 12 weeks, mature oligodendrocyte regeneration and remyelination occurs after the mice are returned to a normal diet. Thus, the formation of chronically demyelinated lesions induced by cuprizone appears to be the result of oligodendrocyte depletion within the lesion and not due to the inability of the chronically demyelinated axons to be remyelinated. (*Am J Pathol* 2004, 164:1673–1682)**

Although most acutely demyelinated lesions in multiple sclerosis (MS) are remyelinated,<sup>1</sup> the lesions eventually progress to a state of chronic demyelination, characterized by sparse remyelination, few oligodendrocytes, and axon degeneration.<sup>2–5</sup> Although the central nervous system (CNS) has the ability to regenerate new oligodendrocytes that remyelinate demyelinated axons following acute demyelination,<sup>6–9</sup> it has been suggested that mature oligodendrocytes are not regenerated within chronically demyelinated lesions due to: 1) the depletion of the oligodendrocyte progenitors;<sup>4</sup> 2) the inability of the progenitors to proliferate and differentiate within the lesion due to aging<sup>10</sup> or a non-conducive environment;<sup>11–14</sup> and/or 3) axon damage<sup>3,15,16</sup> or the inability of chronically demyelinated axons to be remyelinated.<sup>17</sup>

To test these hypotheses, we used the cuprizone model of chronic demyelination.<sup>18</sup> Previously, we reported acute demyelination in C57BL/6 mice<sup>18,19</sup> and the apoptotic death of mature oligodendrocytes within the corpus callosum in mice exposed to cuprizone for a short duration.<sup>6</sup> Once cuprizone is removed from the diet, the mature oligodendrocytes begin to repopulate the lesion and remyelinate the demyelinated axons. This regeneration of the mature oligodendrocyte population is presumably derived from the differentiation of accumulating progenitors within the demyelinating lesion.<sup>6</sup> However, if mice are maintained on the cuprizone diet for a prolonged period of time, remyelination eventually fails.<sup>18</sup> In addition, we report here that the newly regenerated mature oligodendrocytes, along with the resident progenitors, become progressively depleted within the chronically demyelinated corpus callosum. However, adult O4<sup>+</sup> oligodendrocyte progenitors transplanted into the chronic lesions differentiate into mature oligodendrocytes and remyelinate a large number of the demyelinated axons if the mice are returned to a normal diet following 12 weeks of cuprizone intoxication. Thus, the formation of chronically demyelinated lesions induced by long-term

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cuprizone toxicity is the result of oligodendrocyte depletion within the lesion and not due to a non-conductive environment or the inability of the chronically demyelinated axons to be remyelinated.

## Materials and Methods

### Induction of Acute and Chronic Demyelination

Adult male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). At 8 weeks of age, the mice were fed a diet of milled Purina mouse chow containing 0.2% cuprizone (Sigma Chemical Co., St. Louis, MO) by weight for up to 16 weeks to induce chronic demyelination.<sup>18</sup> Additional mice were fed cuprizone for 6 weeks and then returned to a normal diet to allow remyelination.<sup>6,19</sup> Some mice were anesthetized and perfused through the heart with 4% paraformaldehyde (PFA). The brains were removed and cut mid-sagittally. Half of the brain was then fixed overnight in 4% PFA at 4°C and then embedded in paraffin. 5- $\mu$ m sections were cut with a microtome at the fornix region of the corpus callosum, mounted onto gelatin-coated slides (Fisher Scientific Corp., Fairlawn, NJ) and stored at room temperature until use. The other half of the brain was fixed in 4% PFA for 1 hour at 4°C and then placed into a 20% sucrose for 48 hours at 4°C and then snap-frozen in isopentane. Transverse sections, 10  $\mu$ m thick, were cut with a cryostat at the fornix region of the corpus callosum, mounted onto gelatin-coated slides, and stored at -70°C until staining. All comparative analyses were focused at the medial region of the corpus callosum that was above the fornix and between the ventricles (corresponding to sections 220–260 of the mouse brain atlas<sup>20</sup>). The forebrains from another set of mice were removed for RNA analysis. Mid-sagittal sections of the forebrain were frozen immediately on dry ice. All animal procedures were conducted in accordance with guidelines approved by the IACUC and both the University of North Carolina and Columbia University Division of Laboratory Animal Medicine.

### Glutathione-S-Transferase, Pi Isoform Immunohistochemistry, and Apoptosis Assay

To identify apoptotic mature oligodendrocytes within the brain, the NeuroTACS assay kit (Trevigen, Gaithersburg, MD) was used to identify apoptotic cells and mature oligodendrocytes were identified by the immunohistochemical detection of the pi isoform of glutathione-S-transferase (GST-pi),<sup>21</sup> which does not detect immature NG2<sup>+</sup> oligodendrocyte (unpublished observations). Paraffin-embedded sections were rehydrated in a graded series of alcohol washes, blocked with 5% normal goat serum (NGS)/0.1% Triton for 20 minutes, incubated with 0.1% trypsin/0.5 mol/L Tris for 15 minutes at 37°C, and then incubated with the rabbit polyclonal anti-GST-pi antibody (1:1000; Biotrin, Newton, MA) overnight at 4°C. The tissue sections were then incubated with 1X labeling buffer (Trevigen) for 5 minutes and then incubated with a labeling reaction mixture of 1X labeling buffer, biotin-

labeled dNTPs (Trevigen), and Klenow enzyme (Trevigen) for 1.5 hours at 37°C. The reaction was stopped by placing the tissue sections into a 1X stop reaction buffer (Trevigen) for 5 minutes. The tissue sections were then incubated with a fluorescein-conjugated goat anti-rabbit secondary antibody diluted 1:75 in 5% NGS/0.2% Triton/2% streptavidin-Texas red complex (Vector Laboratories, Burlingame, CA) for 1 hour, counterstained with DAPI (Molecular Probes, Eugene, OR), coverslipped, and examined using an Olympus BX60 microscope equipped with epifluorescence optics.

### NG2 Immunohistochemistry

To identify oligodendrocyte progenitors, frozen tissue sections were dried, rehydrated, blocked with 5% NGS/0.2% Triton, and then incubated with rabbit anti-NG2 (gift from Dr. William Stallcup, San Diego, CA) diluted 1:400 in 5% NGS/0.2% Triton overnight at 4°C. The sections were then incubated with a fluorescein-conjugated goat anti-rabbit secondary antibody (1:75; Vector Laboratories) for 1 hour, counterstained with DAPI, coverslipped, and examined using an Olympus BX60 microscope equipped with epifluorescence optics.

### RNA Analysis

Total RNA from entire half of a forebrain sectioned mid-sagittally along midline was isolated, separated, and transferred to a Zeta-probe nylon membrane as described previously.<sup>22</sup> Membranes were hybridized with <sup>32</sup>P-labeled cDNA probes, synthesized using double-stranded polymerase chain reaction (PCR) methodology<sup>23</sup> for amplification of cDNA for myelin-associated glycoprotein (MAG).<sup>24</sup> Filters were washed and radioactivity in RNA was quantitated using a Packard Instant Imager electronic autoradiography system. To control for variability in sampling handling, values obtained were normalized to the amount of rRNA in each lane as described previously.<sup>22</sup>

Additional RNA was reverse transcribed into cDNA and then amplified by PCR as described previously.<sup>6</sup> The primer sets for paranodin were 5'-ACAAGGGAGTTACTGCCATGAAC (sense) and 3'-GCACGGATTGGAGG-GAAACG (antisense). The primer sets for G3PDH have been published elsewhere.<sup>25</sup> Templates were denatured at 95°C for 5 minutes, followed by 30 cycles of denaturation (95°C for 1 minute) primer annealing (62°C for 1.5 minutes) and extension (72°C for 1 minute) with a final extension step at 72°C for 15 minutes. The amplified cDNA mixture of each sample was separated on a 2.5% agarose gel containing ethidium bromide and photographed. The amplified products for paranodin and G3PDH were 696 and 983 bp, respectively. Amplification of the housekeeping gene, G3PDH, confirmed RNA amplification and equal loading of each sample. The mRNA profiles were semiquantitated using a densitometer to normalize all samples for comparison.

### Lectin Histochemistry

Microglia/macrophages were identified by binding biotin-conjugated *Ricinus communis* agglutinin-1 (RCA-1; Sigma Chemical Co.) to paraffin-embedded sections and then visualized by the 3'3'-diaminobenzidine methodology (Vector Laboratories) as described previously.<sup>19</sup>

### Isolation of O4<sup>+</sup> Progenitors

Transgenic C57BL6 mice, in which green fluorescent protein expression is driven by the chicken  $\beta$ -actin promoter (GFP tg mice), were purchased from Jackson Laboratories. Progenitors were isolated from the corpus callosum in 8-week-old GFP tg mice as described previously.<sup>26,27</sup> Briefly, the mice were anesthetized and the corpus callosum was carefully dissected and then mechanically and enzymatically dissociated with 0.125% trypsin (Sigma Chemical Co.) and 0.02% collagenase (Worthington Biochemical Co., Lakewood, NJ). The cellular suspension was filtered through sterile 70- $\mu$ m mesh and the trypsin neutralized with 10% heat-inactivated serum. Cells were collected by centrifugation and then resuspended in 0.9 mol/L sucrose/MEM (Gibco, Carlsbad, CA). This cellular suspension was centrifuged and the progenitors (bottom layer) were collected and resuspended in serum-free medium (see below). Approximately  $5 \times 10^5$  cells were isolated from the corpus callosum of each adult mouse with the cells being pooled from three corpora collosa.

O4<sup>+</sup> progenitors were then purified from the total progenitors using immunopanning methods described previously.<sup>26</sup> Briefly, immunopan plates were created by incubating goat anti-mouse IgM antibody (20  $\mu$ g/ml; Sigma Chemical Co.), diluted in 50 mmol/L Tris, in Falcon 1007 petri dishes (Fisher) overnight at 37°C. The plates were then washed, incubated with O4 hybridoma supernatant (undiluted) at 37°C for 3 hours, and then blocked with 10% normal goat serum (NGS)/10% bovine serum albumin (BSA) at 37°C for 30 minutes.  $5 \times 10^5$  cells were then incubated in each O4 immunoplate for 1 hour at 37°C. The plates were then washed and coated with trypsin/EDTA for 10 minutes at 37°C. The detached cells were collected in an equal volume of 10% heat-inactivated serum to neutralize the trypsin, centrifuged, and then resuspended in serum-free medium ( $1 \times 10^4$  O4<sup>+</sup> cells/ $\mu$ l). Approximately  $3.1 \times 10^5$  O4<sup>+</sup> cells were isolated from the three corpora collosa.

### Medium for Isolated Progenitors

Serum-free medium was composed of *d*-biotin (10 ng/ml; Sigma Chemical Co.), insulin (5  $\mu$ g/ml; Sigma Chemical Co.), progesterone (20 nM; Sigma Chemical Co.), putrescine (100  $\mu$ mol/L; Sigma Chemical Co.), selenium (5 ng/ml; Sigma Chemical Co.), transferrin (50  $\mu$ g/ml; Sigma Chemical Co.), HEPES buffer (15 mmol/L; Sigma Chemical Co.), 3,3,5-triiodo-L-thyronine (15 nM; Sigma Chemical Co.), penicillin/streptomycin (100U/100  $\mu$ g/ml; Gibco)

and BSA (1 mg/ml; Sigma Chemical Co.) in DMEM/F12 (Gibco).

### Transplantation of GFP<sup>+</sup>/O4<sup>+</sup> Progenitors into the Chronically Demyelinated Corpus Callosum

At 8 weeks of age, wild-type male recipient mice (Jackson Laboratories) were fed a diet of milled Purina mouse chow containing 0.2% cuprizone by weight for 12 weeks to induce chronic demyelination within the corpus callosum. The mice were then anesthetized with ketamine (80 mg/kg body weight; Henry Schein Pharmaceutical, Port Washington, NY) and xylazine (10 mg/kg body weight; Henry Schein Pharmaceutical) and positioned in an immobilization apparatus designed for stereotactic surgery (David Kopf Instruments, Tujunga, CA). The scalp was opened and the O4<sup>+</sup> oligodendrocyte progenitors isolated from the corpus callosum in GFP tg donor mice were injected ( $1 \times 10^4$  O4<sup>+</sup> cells in 1  $\mu$ l of serum-free medium) unilaterally into the chronically demyelinated corpus callosum in the wild-type recipient mice with a 10- $\mu$ l Hamilton syringe using stereotactic coordinates of 0.7 mm posterior and 0.3 mm lateral to bregma at a depth of 1.7 mm.<sup>28</sup> Additional control recipient mice were stereotactically injected with serum-free medium containing no progenitors. The opening in the skull was then filled with Gelfoam, the area swabbed with penicillin and streptomycin (Gibco) and the wound was sutured. Immediately after transplantation, the recipient mice were returned to a normal diet for 4 weeks allowing recovery. The mice were then anesthetized, perfused through the heart with either 4% PFA (light microscopy) or 2.5% glutaraldehyde (electron microscopy) and processed for analysis as described previously.<sup>6,18</sup> All animal procedures were conducted in accordance with guidelines approved by the IACUC and the Columbia University Division of Laboratory Animal Medicine.

For light microscopy, frozen tissue sections were dried, rehydrated, blocked with 5% NGS/0.2% Triton and then incubated with the rabbit polyclonal anti-GST-pi antibody (1:1000; Biotrin) and the mouse monoclonal anti-GFP antibody (1:500; Chemicon, Temecula, CA) overnight at 4°C. The sections were then incubated with a biotin-conjugated goat anti-mouse secondary antibody (1:100; Vector Laboratories) and a rhodamine-conjugated goat anti-rabbit antibody (1:100; Southern Biotechnology Associates) for 1 hour. The sections were then incubated with a 2% streptavidin-fluorescein complex (Vector) in 5% NGS/0.2% Triton, counterstained with DAPI, coverslipped, and examined using an Olympus BX60 microscope equipped with epifluorescence optics.

For electron microscopy, glutaraldehyde-fixed brain samples were prepared as described previously.<sup>18</sup> Coronal sections (1  $\mu$ m) were stained with toluidine blue and the medial region of the corpus callosum was identified by light microscopy. The tissue was then trimmed and reoriented for thin sectioning so that cross sections of the corpus callosum could be examined by electron microscopy. Thin sections were cut, stained with uranyl acetate and lead citrate, photographed, and then the electron



micrographs were analyzed as described previously.<sup>18</sup> One thousand fibers ( $\geq 0.3 \mu\text{m}$  in diameter) from five different random sections of the corpus callosum were examined from each mouse (three mice at each time point).

### Cell Number Quantification

Immunopositive cells were obtained by counting only those cells with an identified nucleus, observable by DAPI staining, within the medial region of the corpus callosum. Individual cell counts were conducted on both sides of midline corresponding to an area of  $0.033 \text{ mm}^2$ . The cell counts from both areas were averaged to give a total for each tissue section. The cell counts (number of cells/ $\text{mm}^2$ ) are presented in the text as the mean and SEM from at least three mice for each time point.

### Statistical Analysis

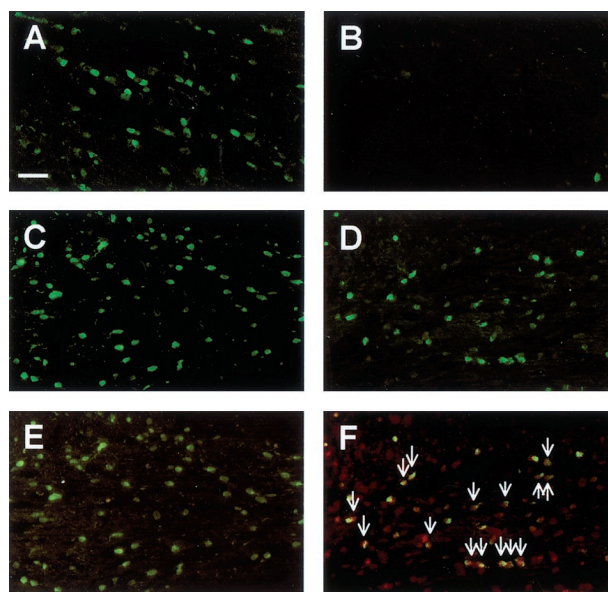
Statistical comparisons were made using a one-factor between-subjects analysis of variance. Multiple comparisons between groups were made using Tukey's test.

## Results

### *The Mature Oligodendrocyte Population Is Inadequately Regenerated within Chronically Demyelinated Lesion*

Cuprizone toxicity markedly depleted the mature oligodendrocyte population, as detected by immunocytochemistry using the anti-GST-pi antibody (Figures 1, A and B, and 2A).<sup>6</sup> When mice were returned to a normal diet after 6 weeks of cuprizone feeding, the oligodendrocyte population returned to control numbers (Figures 1C and 2B).<sup>6</sup> In contrast, in mice that were maintained on a cuprizone diet for up to 12 weeks, a large number of oligodendrocytes reappeared (Figure 1D), although numbers never fully returned to control (Figure 2A). Despite the presence of oligodendrocytes, however, there was little remyelination.<sup>18</sup>

The inability of the oligodendrocyte population to regenerate adequately and remyelinate the axons during long-term cuprizone treatment could be a consequence of the newly regenerated cells undergoing continued apoptosis as observed with the oligodendrocytes in acute lesions (Figure 2A).<sup>6</sup> To address this issue, we used immunohistochemistry to determine whether apoptotic (TUNEL assay) GST-pi<sup>+</sup> oligodendrocytes were present within the chronically demyelinated corpus callosum. Indeed, a large number of apoptotic GST-pi<sup>+</sup> oligodendrocytes were identified in the corpus callosum in mice maintained on the prolonged cuprizone diet (Figures 1F and 2A). In contrast, no apoptotic GST-pi<sup>+</sup> oligodendrocytes were observed in the remyelinating corpus callosum in mice returned to a normal diet after 6 weeks of cuprizone feeding (Figures 1E and 2B). Thus, there appears to be a number of newly generated GST-

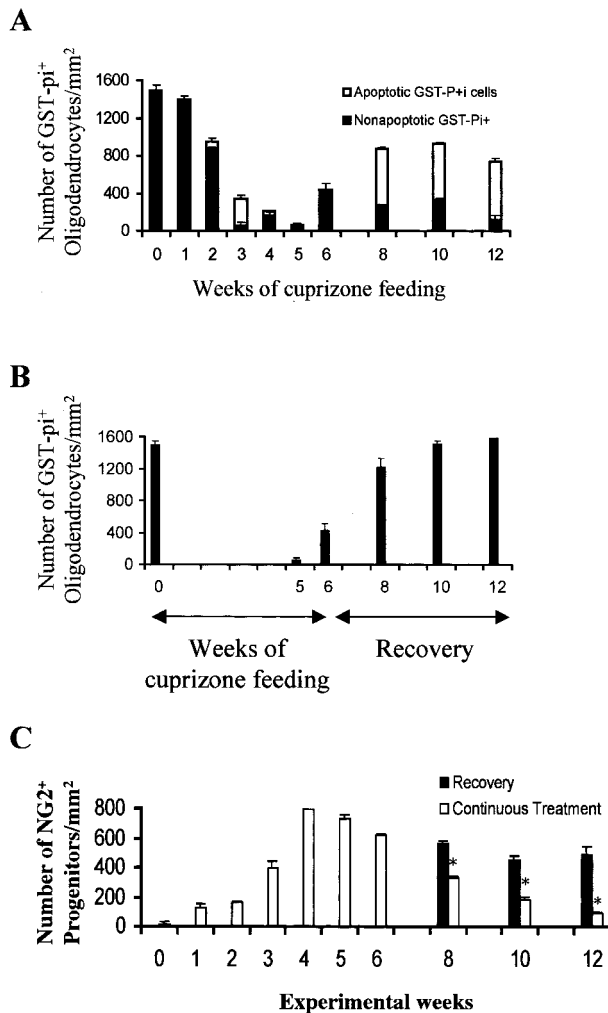


**Figure 1.** The long-term feeding of cuprizone has a profound effect on the mature oligodendrocyte population. Paraffin-embedded sections were immunostained with anti-GST-pi (green cells; **A-F**), while some sections were also examined for the presence of apoptotic cells (red nuclei; **E-F**). **A:** A large number of GST-pi<sup>+</sup> oligodendrocytes were observed within the corpus callosum in untreated control mice. **B:** The GST-pi<sup>+</sup> oligodendrocytes were almost completely absent at 5 weeks. The GST-pi<sup>+</sup> oligodendrocytes reappeared 4 weeks after 6 weeks of cuprizone feeding (**C**) and after 12 weeks of continuous cuprizone feeding (**D**). No apoptotic GST-pi<sup>+</sup> oligodendrocytes were identified 4 weeks after the mice were returned to a normal diet following 6 weeks of cuprizone feeding (**E**). A large number of apoptotic nuclei were co-localized with GST-pi<sup>+</sup> oligodendrocytes after 10 weeks of cuprizone feeding (**F**). Scale bar, 25  $\mu\text{m}$ .

pi<sup>+</sup> oligodendrocytes undergoing cell death during long-term cuprizone toxicity.

### *Oligodendrocyte Progenitors Become Progressively Depleted during the Formation of Chronically Demyelinated Lesions*

The large number of newly regenerated mature oligodendrocytes in the corpus callosum in mice fed cuprizone for 8 to 12 weeks (Figure 2A), many of which are undergoing apoptosis, suggests that these cells are being replenished by differentiating progenitors. Few NG2<sup>+</sup> cells were observed in the corpus callosum in untreated control mice (Figures 2C and 3A). However, during the initial cuprizone-induced demyelination, NG2<sup>+</sup> progenitors accumulated within the demyelinating corpus callosum (Figures 1C and 3B).<sup>6</sup> A large number of these cells remained within the remyelinating corpus callosum after the mice were returned to a normal diet (Figures 1C and 3C). In contrast, there was a progressive loss of NG2<sup>+</sup> progenitors within the chronically demyelinated corpus callosum in mice maintained on a cuprizone diet for up to 12 weeks (Figures 1C and 3D). This progressive depletion of progenitors in the face of increased death of newly generated oligodendrocytes may be a major reason for the failure of remyelination (see Discussion). Under our conditions, NG2 staining did not co-localize with F4/80<sup>+</sup>

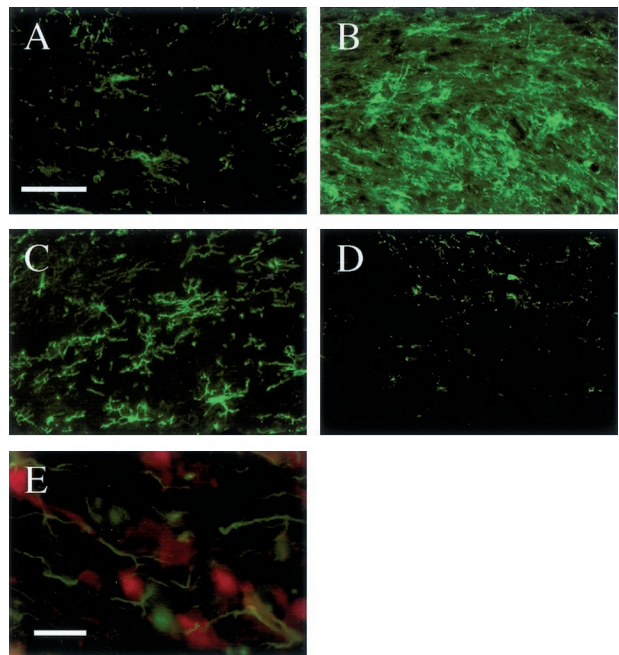


**Figure 2.** Cuprizone induces the apoptotic death of mature oligodendrocytes and the progressive depletion of the oligodendrocyte progenitor population. The total number of mature oligodendrocytes, the number of nonapoptotic mature oligodendrocytes (black bars) and the number apoptotic mature oligodendrocytes (white bars) were quantitated and plotted as mean  $\pm$  SEM for triplicate determinations (A, B). The number of GST-pi<sup>+</sup> and apoptotic GST-pi<sup>+</sup> cells/mm<sup>2</sup> in mice fed cuprizone continuously for 12 weeks (A) and in mice fed cuprizone for 6 weeks and then returned to a normal diet for 6 weeks (B) are shown. C: The number of NG2<sup>+</sup> oligodendrocyte progenitors within the medial region of the corpus callosum were also quantitated and plotted as mean  $\pm$  SEM for triplicate determinations in mice fed cuprizone continuously for 12 weeks (white bars) and in mice fed cuprizone for 6 weeks and then returned to a normal diet for 6 weeks (black bars). \**P* < 0.005.

microglia/macrophages as reported previously (Figure 3E).<sup>29</sup>

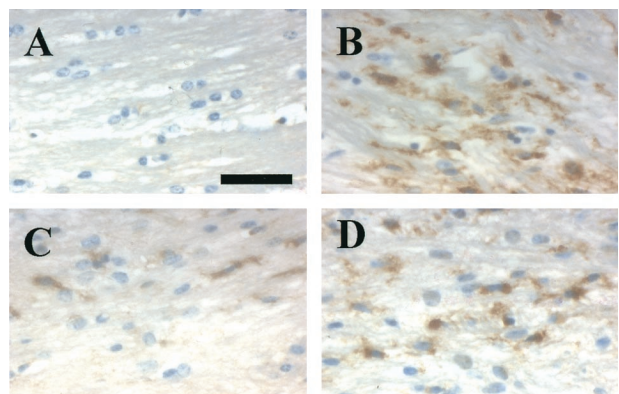
### The Presence of Microglia/Macrophages Coincides with Demyelination and Oligodendrocyte Death

Since microglia/macrophage-derived factors have been implicated in oligodendrocyte death *in vitro*<sup>30–35</sup> and *in vivo*,<sup>36</sup> we examined whether these cells were present within lesions containing apoptotic oligodendrocytes. Few RCA-1<sup>+</sup> microglia/macrophages were present within the corpus callosum in untreated adult mice (Figures 4A

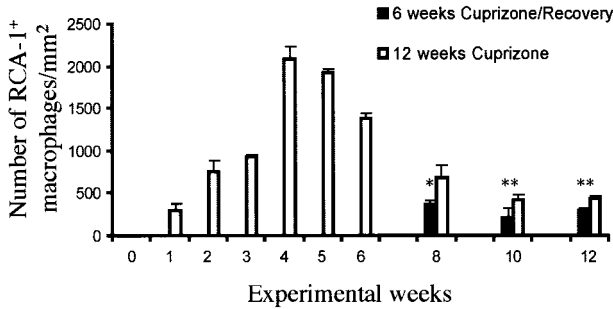


**Figure 3.** The long-term feeding of cuprizone has a profound effect on the oligodendrocyte progenitor population. Frozen brain sections were immunostained with anti-NG2 (green cells). A: Very few NG2<sup>+</sup> progenitors were present in the corpus callosum in untreated control mice. B: At 5 weeks, a large number of NG2<sup>+</sup> progenitors accumulated within the demyelinated corpus callosum. C: A large number of NG2<sup>+</sup> progenitors remained in a remyelinating corpus callosum 6 weeks after the mice were returned to a normal diet. D: Very few NG2<sup>+</sup> progenitors were observed within a chronically demyelinated corpus callosum in mice fed cuprizone for 12 weeks. E: The NG2<sup>+</sup> cells (green) that accumulated within the demyelinating corpus callosum at 3 weeks did not express the microglia/macrophage marker F4/80 (red). Scale bar, 25  $\mu$ m for A–D and 10  $\mu$ m for E.

and 5). However, a large number of microglia/macrophages began to accumulate within the demyelinating corpus callosum by 4 weeks (Figures 4B and 5) and remained through 6 weeks (Figure 5) of cuprizone feeding. Thereafter, microglia/macrophage numbers (Figures



**Figure 4.** The histological detection of microglia/macrophages within acute and chronically demyelinated lesions. Paraffin-embedded sections were stained with the RCA-1 lectin (brown) to detect the presence of microglia/macrophages. A: Few or no RCA-1<sup>+</sup> microglia/macrophages were observed within the corpus callosum in untreated control mice. B: A large number of RCA-1<sup>+</sup> microglia/macrophages were observed in a demyelinated corpus callosum at 4 weeks. A few RCA-1<sup>+</sup> microglia/macrophages were observed in the remyelinated corpus callosum, 6 weeks after the mice were returned to a normal diet following 6 weeks of cuprizone feeding (C) or in the chronically demyelinated corpus callosum after 12 weeks of cuprizone feeding (D). Scale bar, 30  $\mu$ m.

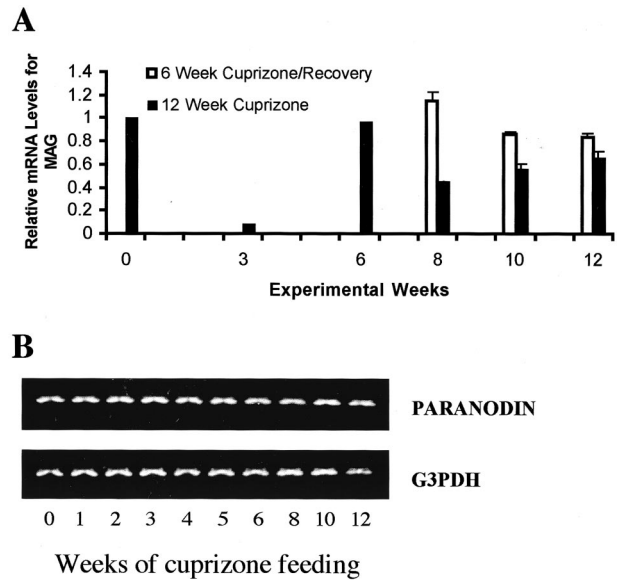


**Figure 5.** The presence of RCA-1<sup>+</sup> microglia/macrophages during acute and chronic demyelination. The total number of microglia/macrophages in the corpus callosum in cuprizone-fed mice (black bars) and mice returned to a normal diet following 6 weeks of cuprizone feeding (white bars) were quantitated and plotted as mean ± SEM for triplicate determinations. \**P* < 0.01, \*\**P* < 0.05.

4C and 5) declined to control levels if mice were returned to a normal diet. In contrast, microglia/macrophage numbers within the chronically demyelinated corpus callosum remained elevated in mice fed cuprizone for up to 12 weeks (Figures 4D and 5). Thus, the presence of microglia/macrophages coincided with oligodendrocyte pathology within both acute and chronic lesions. However, it is unclear whether the microglia/macrophages have a direct role in the apoptotic death of the oligodendrocytes.

### Adult O4<sup>+</sup> Progenitors Transplanted into the Chronic Lesions Remyelinate the Chronically Demyelinated Axons

We have previously shown that a dramatic reduction (>90%) in the mRNA levels for the myelin-specific gene, MAG,<sup>22</sup> precedes acute demyelination and oligodendrocyte pathology during cuprizone intoxication (Figure 6A).<sup>6,19</sup> During recovery after a 6-week exposure to cuprizone, there is sustained levels of MAG mRNA that coincides with remyelination. However, during continuous exposure to cuprizone, MAG mRNA diminishes at 8 weeks, far below MAG mRNA levels from mice recovering for 2 through 6 weeks from acute demyelination (Figure 6A). The perturbation in the oligodendrocyte-associated mRNA is in contrast to the lack of alteration in the steady-state mRNA (Figure 6B) and protein (Mason J, Dupree J, Girault J-A, Popko B, Matsushima G, unpublished data) levels for the neuron-associated protein, paranodin, during cuprizone intoxication. These findings suggest that neurons are unaffected by chronic cuprizone intoxication; yet, oligodendrocyte progenitors (Figure 1C) and GST-pi<sup>+</sup> mature oligodendrocytes are diminished in number (Figure 2A). However, even though axon degeneration is also not observed within cuprizone-induced lesions,<sup>18</sup> it is plausible that the eventual failure of remyelination during continuous cuprizone treatment is due to some sort of axon injury that prevents oligodendrocyte survival and/or interaction with demyelinated axons. To determine whether the chronically demyelinated axons retained the capacity to be remyelinated, we isolated O4<sup>+</sup> progenitors from the corpus callosum in untreated adult GFP tg mice and transplanted them into the



**Figure 6.** Cuprizone-induced changes in mRNA levels for the myelin-specific protein, MAG, and the neuronal-specific protein, paranodin. Mice were fed cuprizone for 12 weeks (black bars) or they were returned to a normal diet following 6 weeks of cuprizone feeding (white bars). **A:** Steady-state mRNA levels were determined for MAG and the mean and SEM bars were plotted for triplicate determinations. **B:** RNA samples were analyzed for paranodin mRNA levels using RT-PCR. G3PDH was analyzed to assure that equal amounts of RNA and cDNA were amplified in each reaction.

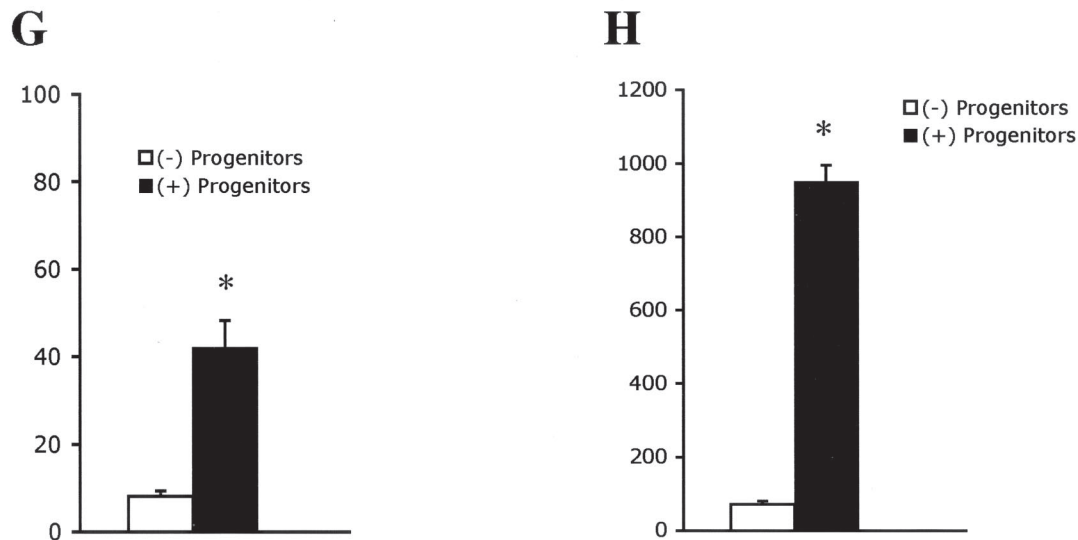
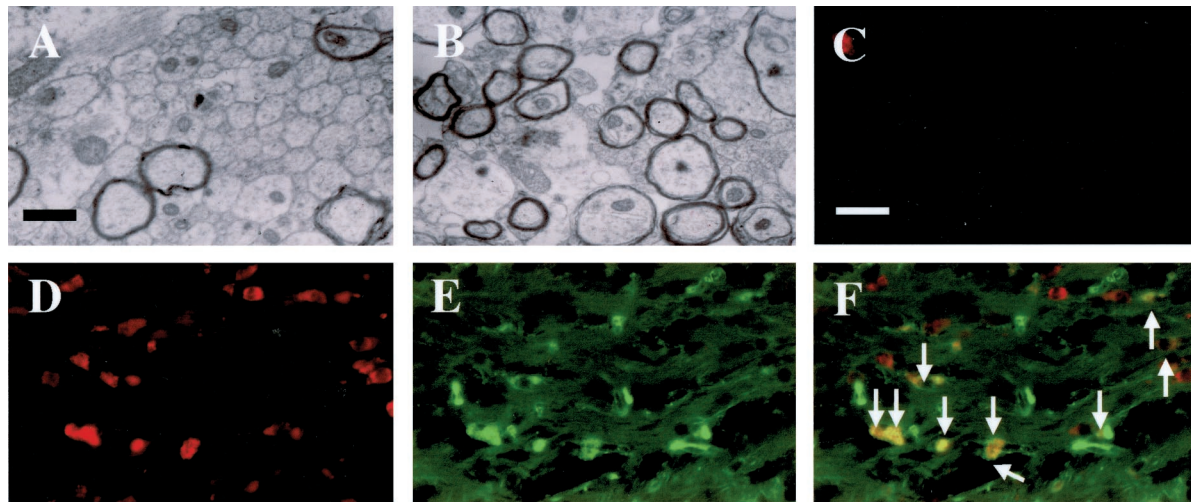
demyelinated corpus callosum in wild-type mice treated with cuprizone for 12 weeks. Control mice were injected with serum-free medium containing no progenitors. The mice were then returned to a normal diet for 4 weeks, allowing recovery. Only 8.1 ± 1.2% of the axons were myelinated within the corpus callosum in mice that were not transplanted (Figure 7, A and G). In contrast, there was a significant increase (*P* < 0.001) in the percentage of axons that were myelinated (42.2 ± 6.2%) within the corpus callosum in mice in to which progenitors were transplanted (Figure 7, B and G).

Examination of the mature oligodendrocyte population showed that only a few GST-pi<sup>+</sup> mature oligodendrocytes (71 ± 9/mm<sup>2</sup>) were present within the chronically demyelinated corpus callosum in mice that were not transplanted (Figure 7, C and H), while a significantly (*P* < 0.001) larger number of GST-pi<sup>+</sup> oligodendrocytes (950 ± 45/mm<sup>2</sup>) were present within the remyelinated corpus callosum in the mice into which progenitors were transplanted (Figure 7, D and H). Further immunohistochemical analysis showed that approximately 68.2 ± 6.4% of the GST-pi<sup>+</sup> oligodendrocytes were also GFP<sup>+</sup> (Figure 7, D–F), demonstrating that a large percentage of the newly regenerated oligodendrocytes were derived from the transplanted progenitors.

### Discussion

The long-term feeding of cuprizone induces a series of demyelinating/remyelinating episodes leading to the formation of chronic lesions within the corpus callosum in C57BL/6J mice.<sup>18</sup> During the first demyelinating episode,





**Figure 7.** Adult O4<sup>+</sup> progenitors transplanted into the chronically demyelinated corpus callosum differentiate into mature oligodendrocytes and remyelinate the demyelinated axons. DMEM/F12 media containing either no progenitors (**A, C**) or O4<sup>+</sup> progenitors isolated from the corpus callosum in adult GFP tg mice (**B, D, E**) were transplanted into the chronically demyelinated corpus callosum in mice treated with cuprizone for 12 weeks, at which time the mice were returned to a normal diet for 4 weeks to allow for recovery. Electron micrographs are of the corpus callosum in mice that did not have progenitors transplanted (**A**) or in mice that did have progenitors transplanted (**B**). Tissue sections from mice in which progenitors were either not transplanted (**C**) or were transplanted (**D, E**) were stained for the presence of GST-pi<sup>+</sup> mature oligodendrocytes (red; **C, D**) and GFP<sup>+</sup> cells (green; **E**). **Arrows** indicate the GFP<sup>+</sup> cells that express GST-pi (**F**). The percentage of myelinated axons (**G**) and total number of GST-pi<sup>+</sup> mature oligodendrocytes (**H**) were quantitated and plotted as mean  $\pm$  SEM for triplicate determinations from mice that did not have progenitors transplanted (**white bars**) or from mice that did have progenitors transplanted (**black bars**). Scale bars: 1  $\mu$ m in **A** and **B** and 10  $\mu$ m in **C-F**. \* $P < 0.001$

oligodendrocytes become depleted and then oligodendrocyte progenitors accumulate within the lesion and differentiate into mature oligodendrocytes leading to remyelination.<sup>6</sup> In contrast, when a demyelinating insult persists, both oligodendrocytes and progenitors become progressively depleted, leading to the formation of chronically demyelinated lesions (Figure 2). The ability of transplanted adult O4<sup>+</sup> progenitors to remyelinate the chronic lesions suggests that the axons therein retain the capacity to be remyelinated. In addition, the environment within these lesions is conducive for the survival and differentiation of oligodendrocyte progenitors once cuprizone toxicity is removed.

### *Oligodendrocytes Become Depleted within Demyelinating Lesions Progressing to a Chronic State*

Our present work showing the depletion of mature oligodendrocytes within chronically demyelinated lesions in adult mice exposed to cuprizone for a prolonged period of time is consistent with chronic lesions in MS,<sup>37</sup> experimental autoimmune encephalomyelitis (EAE),<sup>38</sup> and the superior cerebellar peduncles in cuprizone intoxication.<sup>39</sup> During cuprizone toxicity, mature oligodendrocytes become depleted, presumably via apoptosis, dur-

ing acute demyelination.<sup>6</sup> Similarly, most of the mature oligodendrocytes regenerated during recovery from acute demyelination also undergo apoptosis if the pathological insult is not removed, eventually leading to the depletion of these cells within the chronically demyelinated lesions (Figure 2, A and B). In contrast to the cuprizone model of demyelination, chronic demyelination and oligodendrocyte depletion is not observed within lesions that have undergone several demyelinating/remyelinating episodes if each regenerative response is allowed to occur in the absence of a demyelinating stimulus.<sup>40</sup> Thus, the depletion of oligodendrocytes during cuprizone intoxication may be the result of a continuous insult on these cells in which they are not able to recover. This depletion of oligodendrocytes is primarily responsible for the inability of the chronic lesions to recover, even after the insult is removed.

Interestingly, microglia/macrophages persist within lesions progressing to a chronic state either during a prolonged demyelinating insult (Figures 4 and 5) or within lesions that are unable to recover from acute demyelination.<sup>41</sup> It should be noted that only a few microglia/macrophages were present within the lesions being remyelinated by the transplanted O4<sup>+</sup> progenitors in the absence of cuprizone intoxication (data not shown). Although microglia/macrophages secrete factors that induce the apoptotic death of mature oligodendrocytes *in vitro*,<sup>30–35</sup> it is not clear whether these immune cells are responsible for inducing the death and depletion of oligodendrocytes within lesions progressing to a chronic state. However, in addition to microglia/macrophage-derived TNF- $\alpha$  being highly expressed within demyelinating lesions containing apoptotic oligodendrocytes, we have also previously reported that the apoptotic death of oligodendrocytes is delayed within TNF- $\alpha^{-/-}$  mice.<sup>36</sup> Thus, there is supporting evidence to the hypothesis that microglia/macrophages are responsible for oligodendrocyte death during demyelination.

We assume that the ability of the CNS to generate new oligodendrocytes following acute demyelination and also during the progression toward chronic demyelination is due to the differentiation of progenitors within the lesion. Although a large number of progenitors remain within the remyelinating lesion after the mice are returned to a normal diet,<sup>6</sup> the progenitor population decreases dramatically during continuous cuprizone exposure (Figure 2C). This progressive depletion suggests that these cells are either dying, differentiating into oligodendrocytes, or both. However, since some of the progenitors appear to be differentiating into oligodendrocytes during the progression of the demyelinating lesion to a chronic state, it suggests that the depletion of the progenitor population is a consequence of the progenitors differentiating into mature oligodendrocytes. This assumption would suggest that a limited pool of oligodendrocyte progenitors exist within the adult CNS parenchyma and that the depletion of this pool following a long-term pathological insult may contribute to the formation of chronically demyelinated lesions.

In the adult CNS, oligodendrocyte progenitors residing within the parenchyma do not migrate into a demyelinat-

ing lesion in the surrounding area and contribute to the remyelination process.<sup>7,42</sup> In contrast, others have speculated that progenitors residing in the subventricular zone (SVZ) appear to migrate into an acute demyelinating lesion, proliferate, and then differentiate into mature myelinating oligodendrocytes.<sup>6,43</sup> The present work showing the depletion of progenitor cells within the chronically demyelinated lesion demonstrates the inability of progenitors, within or outside the lesion, to accumulate within chronically demyelinated lesions. This lack of accumulation appears to be the consequence of the local progenitor pool becoming depleted. Furthermore, we speculate that progenitors residing outside the lesion may not be able to migrate into the lesion due to either the absence of a chemo attractant signal required to recruit these progenitors into the chronic lesion and/or an inability of the progenitors to migrate within the parenchyma.

### *Chronically Demyelinated Axons Retain the Capacity to Be Remyelinated*

A prolonged or repetitive demyelinating insult can induce a state of chronic demyelination within the adult CNS.<sup>18,38,39,44</sup> It has been speculated that axon degeneration within demyelinating lesions in MS and EAE is due to long-term demyelination and/or cytotoxic factors derived from infiltrating immune cells.<sup>3,16</sup> Although axon degeneration occurs within chronically demyelinated lesions in MS<sup>15</sup> and EAE,<sup>3</sup> this type of pathology does not occur within the chronically demyelinated corpus callosum induced by cuprizone.<sup>18</sup> In addition, while demyelinating lesions induced by cuprizone contain microglia/macrophages and an array of macrophage-derived cytotoxic factors,<sup>36,45,46</sup> these lesions contain few T lymphocytes (unpublished observations). Thus, axon degeneration may be the consequence of a direct attack against axons within demyelinated lesions in EAE mice or MS patients by T-lymphocyte-derived factors that are not present with cuprizone-induced lesions.

Even though oligodendrocyte progenitors have been identified within some chronic lesions in MS patients, it has been suggested that these progenitors are unable to differentiate into mature oligodendrocytes and remyelinate the demyelinated axons.<sup>12–14,17</sup> The progenitors within these lesions may not be able to differentiate due to axon degeneration and/or the absence of an appropriate differentiation signal. The ability of transplanted progenitors to differentiate into mature oligodendrocytes and remyelinate chronically demyelinated axons within cuprizone-induced chronic lesions suggests that the appropriate differentiation signals are present within chronic lesions, at least under the conditions tested here. However, the environment within chronic MS lesions and chronic lesions induced by cuprizone may be dramatically different. Elucidating the environmental differences within MS chronic lesions and cuprizone-induced chronic lesions may provide insight into the signals required for oligodendrocyte progenitor differentiation within demyelinating lesions.



## Adult O4<sup>+</sup> Progenitors Are Able to Differentiate into Mature Oligodendrocytes and Remyelinate Chronically Demyelinated Axons in the Adult CNS

Although endogenous cycling progenitors have the capability to differentiate into mature oligodendrocytes and remyelinate demyelinated axons, the identity of these cells is unknown.<sup>7,42</sup> We have recently isolated a population of O4<sup>+</sup> progenitors from the corpus callosum in adult rodents that differentiates into mature oligodendrocytes *in vitro*.<sup>26</sup> The work presented here further shows that these O4<sup>+</sup> progenitors have the capability to differentiate into mature oligodendrocytes and remyelinate not only demyelinated axons, but chronically demyelinated axons *in vivo*. These O4<sup>+</sup> progenitors do not express NG2 on isolation<sup>26,27</sup> and it is not known whether they express NG2 at any time during their development. Therefore, the O4<sup>+</sup> progenitors used in this study may either be at a different developmental stage compared to the NG2<sup>+</sup> progenitors identified *in situ* or they may constitute a completely separate population of progenitors all together. Interestingly, some of the newly generated mature oligodendrocytes (approximately 30%) did not arise from the transplanted O4<sup>+</sup> progenitors. This finding suggests that some of the transplanted progenitors down-regulate GFP or they may express a factor that either induces the proliferation and differentiation of the few remaining endogenous progenitors, promotes the survival of residual oligodendrocytes or recruits progenitors residing outside the lesion. Thus, it appears that these O4<sup>+</sup> cells may constitute a primary source of oligodendrocyte progenitors within the adult CNS white matter. Further characterization of these cells may provide additional information regarding the factors and/or mechanisms that lead to the survival, migration, proliferation, and differentiation of oligodendrocyte progenitors during pathological conditions *in vivo*.

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