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Insulin-like growth factor I gene expression is induced in astrocytes during experimental demyelination

(cuprizone/remyelination/oligodendroglia/myelin basic protein/insulin-like growth factor I receptor)

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ABSTRACT **To investigate insulin-like growth factor I (IGF-I) and IGF-I receptor gene expression during experimental demyelination and myelin regeneration, young mice were fed cuprizone [bis(cyclohexanone) oxaldihydrazone]. This copper-chelating agent produces demyelination in the corpus callosum and superior cerebellar peduncles, and when treatment is stopped, there is rapid remyelination. At intervals during cuprizone treatment and recovery, brain sections were hybridized with specific probes and immunostained with antibodies to determine the localization and relative amounts of IGF-I and IGF-I receptor mRNAs and peptides. In untreated littermates, IGF-I and IGF-I receptor mRNAs and peptides were not detected in white matter. In cuprizone-treated mice, high levels of both IGF-I mRNA and peptide were expressed by astrocytes in areas of myelin breakdown. Astrocyte IGF-I expression decreased rapidly during recovery and oligodendroglial expression of myelin-related genes increased. In severely demyelinated areas, immature oligodendroglia exhibited a transient increase in IGF-I receptor mRNA and peptide immunoreactivity during early recovery. This highly specific pattern of IGF-I induction in astrocytes during demyelination and the expression of the IGF-I receptor in regenerating oligodendrocytes during recovery suggest that IGF-I functions in the regulation of oligodendrocyte and myelin metabolism *in vivo*.**

Insulin-like growth factor I (IGF-I), a member of the insulin gene family (1, 2), has been shown to have a number of potent effects on cultured neural and glial cells. It promotes the mitosis of sympathetic neuroblasts, the survival of fetal neurons, and the stimulation of neurite outgrowth in motor and sensory neurons, and it induces oligodendrocyte differentiation and myelin synthesis (3–10). These effects are mediated by the type I IGF receptor, which is a membrane-bound tyrosine kinase with significant homology to the insulin receptor (11, 12).

IGF-I gene expression is abundant in the developing nervous system (13–18). IGF-I mRNA is selectively concentrated in the large principal neurons of functionally related sensory and cerebellar relay systems during a late predominantly postnatal phase of neural development (18). The specific timing and cellular sites of neural IGF-I synthesis suggest a role for IGF-I in the synaptic maturation or the myelination of these particular systems. Evidence that IGF-I has a role in myelination is the finding that transgenic mice overexpressing IGF-I (19) have increased brain myelin content (20). Also, several studies have shown that IGFs have potent and highly specific effects on oligodendrocytes *in vitro* (7–10). However, except for the olfactory system, very little IGF-I is normally detected in the mature rodent brain (16–18).

To investigate whether IGF-I is important in the regeneration of central nervous system myelin *in vivo*, demyelinating lesions were produced in the corpus callosum, superior cerebellar peduncles, and anterior commissures by adding the copper-chelating agent bis(cyclohexanone) oxaldihydrazone (cuprizone) to the diet of mice (21–23). In this model of primary demyelination, there are early severe changes in oligodendroglia and myelin sheaths in specific brain areas (21–23). Axons are relatively well preserved (21–23) and the blood–brain barrier remains impermeable to plasma proteins (24). There are no perivascular lymphocytic infiltrates (21, 22) and the cessation of cuprizone treatment is followed by substantial myelin regeneration (21, 22). When we examined IGF-I and type I IGF receptor expression at both the genetic and peptide levels, we found that the IGF-I gene was activated in astrocytes located in white matter areas demyelinated by cuprizone treatment. The production of IGF-I in astrocytes localized next to immature oligodendrocytes expressing IGF-I receptor early in recovery suggests that IGF-I produced by astrocytes may play a direct role in stimulating myelin regeneration by oligodendrocytes.

MATERIALS AND METHODS

Animals and Experimental Design. Demyelination was induced in male weanling Swiss–Webster mice by feeding them a diet containing 0.6% cuprizone (G. F. Smith Chemical, Columbus, OH) for 8 weeks, after which they were given a normal diet to allow remyelination.

Animals were sacrificed after 7, 8, 14, 21, or 56 days of cuprizone administration and 1, 3, 7, 21, or 36 days after cessation of treatment. Four mice were used for *in situ* hybridization and two or three animals were used for immunocytochemistry at each time point. Sex- and age-matched untreated animals were used as controls. For *in situ* hybridization, mice were killed by decapitation. The brains were quickly removed and frozen in powdered dry ice and kept at −70°C until used. Sections 12 μm thick were cut at −14°C at the levels of the anterior commissure in the forebrain and the superior cerebellar peduncles in the brain stem with a Reichert–Jung Frigocut model 2800 cryostat. Sections were mounted on gelatin-coated glass slides and stored at −70°C until used. For immunocytochemistry, mice were anesthetized and perfused with an aldehyde fixative; after removal, the brains were cryoprotected and frozen, and sections were cut as described above and elsewhere (25).

Nucleotide Probes and *in Situ* Hybridization Procedures. RNA probes for IGF-I and IGF-I receptor were prepared and *in situ* hybridization was performed as described (26). Control hybridizations in the form of parallel sections hybridized

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Abbreviations: IGF-I, insulin-like growth factor I; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein.

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with IGF-I and IGF-I receptor antisense probes after pretreatment with RNase A or, alternatively, hybridization with an IGF-I receptor sense probe showed minimal nonspecific signal. Oligonucleotides specific for myelin basic protein (MBP), proteolipid protein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase were prepared on an Applied Biosystems synthesizer and gel-purified prior to labeling. The MBP probe corresponds to exon 1 and will, therefore, hybridize to all forms of MBP (27). The synthetic oligonucleotides (48-mers) were labeled with [α-^{35}S]dATP (Amersham International) using terminal deoxynucleotidyltransferase (Stratagene).

The technique of Young *et al.* (28) was used for *in situ* hybridization with DNA probes. The freshly prepared hybridization mixtures (29) contained 10^6 cpm of probe, 1 μl of 5 M dithiothreitol, and 50 μl of hybridization buffer. Hybridization mixture (20 μl/cm^2 of slide area) was applied to the sections, which were incubated overnight at 37°C.

Autoradiography and Quantitative Comparisons of Autoradiograms. Hybridized sections were exposed to Hyperfilm-βmax (Amersham International) for 3–5 days. To minimize errors in detecting and comparing levels of radioactivity, we followed the protocol recommended by Davenport and Nunez (30). The resulting autoradiograms were analyzed by using a computer-assisted image analysis system and Image software version 1.4 (31). Mean densities (average gray level of the pixels within the given area) were measured in arbitrary units in comparable areas of the superior cerebellar peduncles. For microscopic analysis, the sections were dipped in Kodak NTB3 emulsion and developed after exposure for 10–14 days.

Immunochemicals and Immunocytochemical Procedures. Sheep anti-IGF-I was purchased from Chemicon, rabbit affinity-purified polyclonal IGF-I receptor IgG was from Upstate Biotechnology (Lake Placid, NY), and anti-glial fibrillary acidic protein (GFAP) antibodies were from Boehringer Mannheim and Advanced Immunochemicals Services (Long Beach, CA). Biotinylated secondary antibodies, avidin–biotin horseradish peroxidase complex, fluorescein- and Texas red-labeled secondary antibodies, and avidin were purchased from Vector Laboratories. Single immunolabelings were carried out as described (25). Double immunolabelings were done by combining the indirect immunofluorescent and the biotin–avidin-Texas red detection methods.

Combined *in Situ* Hybridization and Immunocytochemistry. Unfixed frozen cryostat sections were postfixed for 1 h at room temperature in a solution containing 4% (wt/vol) paraformaldehyde and 15% (vol/vol) saturated picric acid in phosphate-buffered saline (PBS). Nonspecific protein binding sites were blocked with 20% (vol/vol) normal horse serum for 30 min. GFAP monoclonal antibody was used in dilutions of 1:1 to 1:10 in PBS containing 1% bovine serum albumin and 0.1% sodium azide for 60 min. Anti-mouse biotinylated horse IgG was used at a 1:200 dilution for 30 min. The Vector horseradish peroxidase Elite ABC standard kit was used as recommended and the color reaction was developed with diaminobenzidine. Aqueous solutions were prepared by using diethyl pyrocarbonate/H_2O, and all pro-

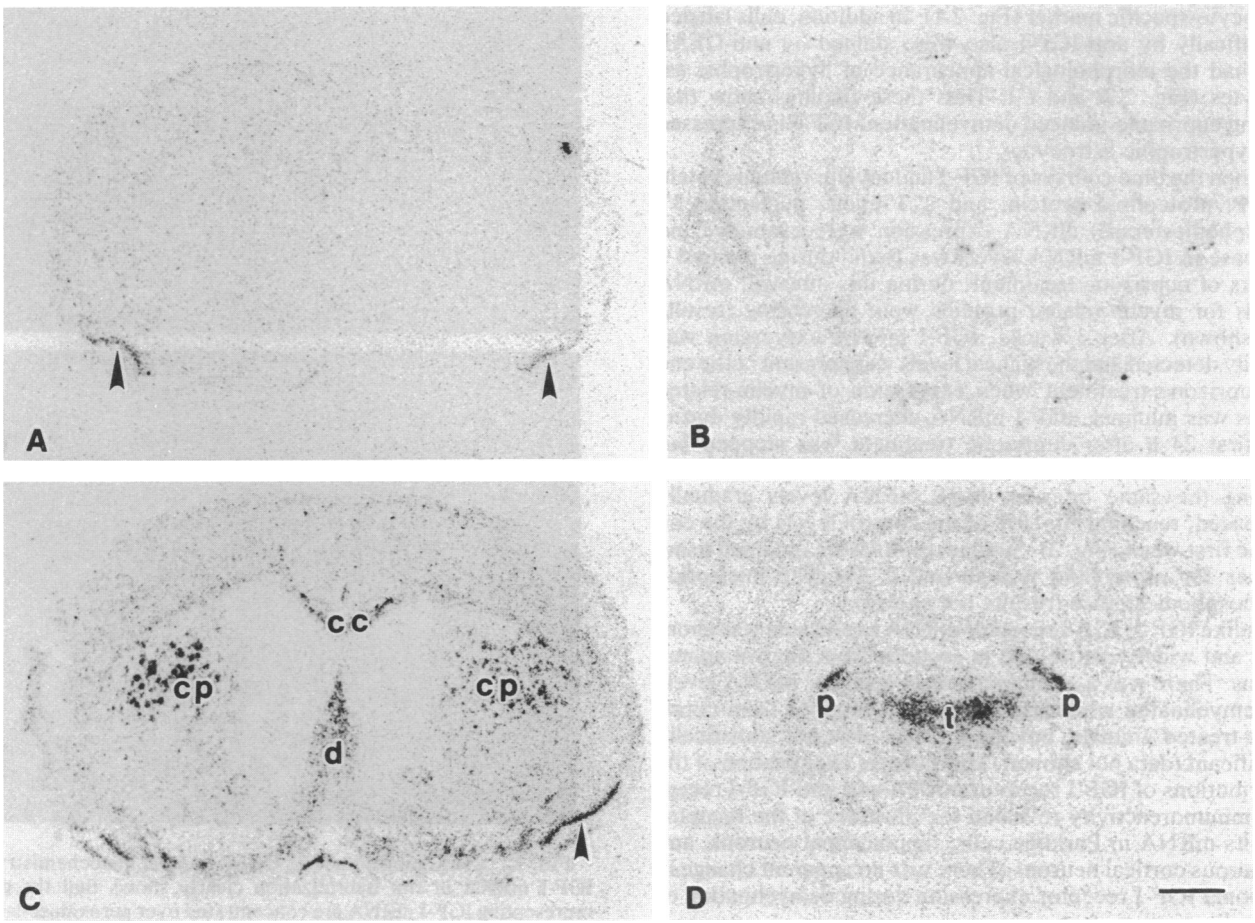

FIG. 1. Autoradiographs of IGF-I mRNA expression obtained on Hyperfilm-βmax. In sections from control animals, IGF-I mRNA level was generally low in the forebrain (*A*) and in the cerebellum and brain stem (*B*). In *A*, arrowheads point to the pyriform cortex, the only structure expressing high levels of IGF-I in controls. (*C* and *D*) Autoradiographs of similar sections after 8 weeks of cuprizone treatment. Very high levels of IGF-I mRNA are present in affected white matter regions both in the forebrain (*C*) and brain stem (*D*). cc, Corpus callosum; cp, caudate–putamen; d, diagonal band; p, superior cerebellar peduncle; t, tegmental tracts. (Bar = 1 mm.)

teinacious solutions also contained RNasin at 160 units/ml. Diethyl pyrocarbonate/H_2O was not used in the diaminobenzidine reaction solutions. After immunocytochemical staining, the sections were pretreated with proteinase K at 1 μg/ml for 30 min at 37°C and preincubated in hybridization buffer for 2 h before *in situ* hybridization (26).

RESULTS

In control brain sections, specific IGF-I mRNA hybridization was confined to neurons in the pyriform cortex and was not detected in white matter (Fig. 1 *A* and *B*). In cuprizone-treated animals, there was a strong IGF-I mRNA hybridization signal in the corpus callosum, caudate–putamen, diagonal bands, anterior commissures, superior cerebellar peduncles, and tegmental tracts (Fig. 1 *C* and *D*). In histologically stained serial sections, high IGF-I mRNA levels were found in areas containing demyelinated axons and abnormal oligodendrocytes, effects known to be caused by cuprizone (21–23). IGF-I mRNA was undetectable in other white matter regions that did not show abnormal oligodendroglia and myelin loss, such as the base of the pons and the optic nerves (Fig. 1 *C* and *D*).

To identify the IGF-I mRNA-expressing cells, IGF-I mRNA *in situ* hybridization was combined with immunocytochemical staining using anti-GFAP; double immunostaining experiments also were done with anti-IGF-I and anti-GFAP. Use of these methods showed that the autoradiographic grains representing hybridized IGF-I mRNA were localized over cells intensely stained by anti-GFAP, an astrocyte-specific marker (Fig. 2*A*). In addition, cells labeled specifically by anti-IGF-I also were stained by anti-GFAP and had the morphological appearance of hypertrophic astrocytes (Fig. 2 *B* and *C*). Thus these findings show that during cuprizone-induced demyelination, IGF-I is expressed by hypertrophic astrocytes.

When the time courses of IGF-I and myelin-related protein (MBP, proteolipid protein, and 2′,3′-cyclic nucleotide 3′-phosphodiesterase) mRNA expression were examined, no increase in IGF-I mRNA levels was found during the first 2 weeks of cuprizone treatment; during this interval, mRNA levels for myelin-related proteins were decreasing (results not shown). After 3 weeks, IGF-I mRNA expression was readily detected and the highest levels were present at the end of cuprizone treatment when expression of myelin-related genes was minimal. IGF-I mRNA decreased rapidly during the first 24 h after cuprizone treatment was stopped but remained above control levels until the end of the first week. During the same interval, MBP mRNA levels gradually increased, reaching 70–80% of the control levels by the end of the first week (Fig. 3). Similar results were obtained using probes for proteolipid protein and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (results not shown).

Unlike IGF-I, IGF-I receptor mRNA expression was abundant and widely distributed in sections from control animal brains. There was a small increase in receptor mRNA levels in demyelinated white matter areas in sections from cuprizone-treated animals, but the increase was not statistically significant (data not shown). Microscopic examination of the distributions of IGF-I receptor mRNA and anti-IGF-I receptor immunoreactivity revealed the presence of the receptor and its mRNA in Purkinje cells, hippocampal neurons, and numerous cortical neurons. There was no apparent change in neuronal IGF-I receptor expression during demyelination or remyelination.

However, IGF-I receptor immunoreactivity was clearly increased in the affected white matter regions during early recovery. Cells resembling immature oligodendroglia morphologically showed receptor-specific immunostaining (Fig. 4 *A–D* and *F*) and were negative for GFAP. Both the number of the immunolabeled cells and the intensity of the IGF-I receptor immunostaining increased until the end of the third day after the cessation of cuprizone treatment (Fig. 4 *C* and *D*); the numbers of positive cells and the intensity of staining began decreasing after 6 days. IGF-I receptor immunoreac-

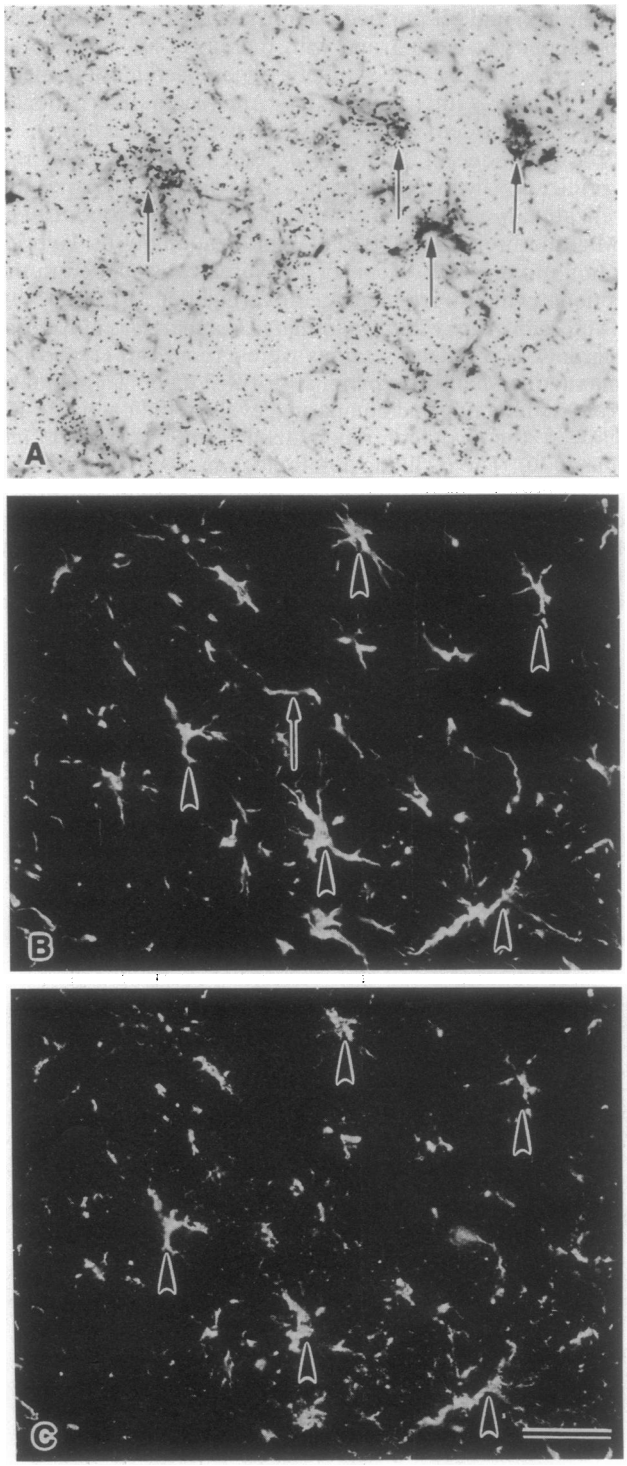

FIG. 2. (*A*) Combined use of GFAP immunohistochemistry and IGF-I mRNA *in situ* hybridization clearly shows that the grains representing IGF-I mRNA are concentrated over peroxidase-labeled GFAP-containing cells (arrows). (*B* and *C*) Double immunolabeling for GFAP (chromogen, Texas red) (*B*) and IGF-I (chromogen, fluorescein isothiocyanate) (*C*) demonstrates that most of the GFAP-immunoreactive cells contain IGF-I immunoreactivity (arrowheads). Note the GFAP-positive astrocytic process (arrow) in *B* that is negative for IGF-I in *C*. (Bar = 20 μm.)

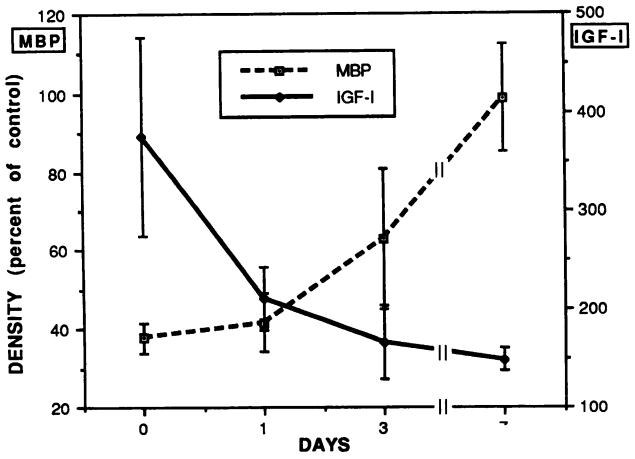

FIG. 3. Time course of MBP and of IGF-I mRNA expression in the superior cerebellar peduncles during remyelination. Densities are given as percentages of values found for age-matched control mice.

tivity was not detected after 21 days. Some of the IGF-I receptor-positive GFAP-negative cells also were stained by an antibody raised against the 04 antigen, a glycolipid expressed by immature differentiating oligodendroglia (Fig. 4 *E* and *F*).

DISCUSSION

This study describes the striking induction of IGF-I mRNA and peptide expression in hypertrophic astrocytes located in white matter areas affected by cuprizone-induced demyelination. Early in recovery, there was an accompanying strong increase in IGF receptor immunostaining in immature oligodendroglia in the same regions. These observations suggest that there may be significant interactions between IGF-producing astrocytes and IGF receptor-expressing oligodendroglia in response to the demyelinating process.

IGF-I-expressing cells in demyelinated regions were identified as hypertrophic astrocytes based on finding IGF-I mRNA transcripts in GFAP-positive cells, on demonstrating that IGF-I-positive cells also were intensely stained by anti-GFAP, and on their characteristic morphology. IGF-I-receptor-expressing cells were identified as immature oligodendroglia based on their resemblance to MBP-expressing cells during central nervous system development (32) and recovery after cuprizone (33), their failure to stain with anti-GFAP, and in some cases, their intense staining by both anti-IGF-I receptor and anti-04 antigen.

IGF-I mRNA was first detected in affected regions 3 weeks after starting cuprizone treatment, a time at which transcripts encoding myelin-related proteins were drastically reduced and previous studies had demonstrated severe oligodendroglial abnormalities and myelin breakdown (21–23). IGF-I increased at lesion sites until cuprizone was discontinued and the remyelination process began. Levels of IGF-I receptor immunoreactivity were increased from the time cuprizone was discontinued until approximately 1 week later, during the peak time of recovery of injured oligodendroglia, maturation of dividing oligodendroglial precursors (34), and activation of myelin synthesis (33).

The fact that cuprizone-induced IGF-I expression in astrocytes is specifically localized in demyelinated areas and is not detected in unaffected white matter suggests that a factor or factors related to the demyelinating process are responsible for triggering IGF expression by astrocytes. This could be one or more products of oligodendroglial injury or myelin breakdown. A cytokine-mediated response seems unlikely since previous studies have shown that inflammatory cell

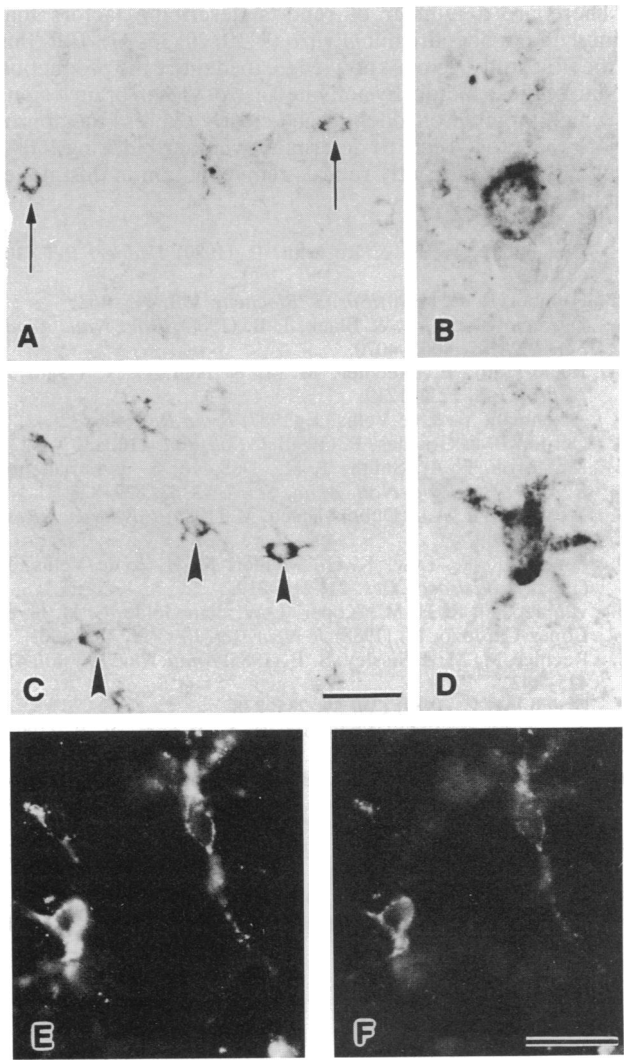

FIG. 4. (*A*) One day after stopping cuprizone treatment, oligodendroglia (arrows) were stained by anti-IGF-I receptor. (*B*) Higher magnification of another IGF-I-receptor-positive oligodendrocyte in the tegmental tract. (*C*) Three days after stopping cuprizone treatment, the IGF-I receptor antibody labeled more cells in the same region and the staining intensity was greater. (*D*) Higher magnification shows that IGF-I receptor immunoreactivity is also found on oligodendroglial processes. (*E* and *F*) Double immunolabeling experiments demonstrating colocalization of IGF-I receptor and 04 antigen in the same oligodendroglia. (*E*) Anti-04 antigen (Texas red). (*F*) Anti-IGF-I receptor (fluorescein isothiocyanate). (Bars: *A* and *C*, 50 μm; *B*, *D*, *E*, and *F*, 20 μm.)

infiltrates are rarely seen in cuprizone-induced demyelination (21–23) and macrophages arrive in the lesion after IGF-I gene expression is first detected (21–23).

The fact that the time course of IGF-I receptor immunostaining in oligodendroglia parallels the return of myelin-related proteins as shown by levels of transcripts in the present study, by the presence of myelin proteins in oligodendroglia (33), and by myelin regeneration in electron micrographs (21, 22, 34) suggests that IGF action in this setting may stimulate functions of differentiated oligodendroglia, as demonstrated *in vitro* (7–10, 20). Thus, we conclude from the present findings that oligodendroglial injury and/or myelin breakdown trigger astroglial IGF-I production and that IGF-I released from astrocytes may diffuse in a paracrine mode to act on IGF-I receptors on nearby oligodendroglia and stimulate oligodendroglial functions that produce myelin regeneration.

There are a number of reports describing factors that stimulate oligodendroglia *in vitro* (7–10, 20, 35–37). But, this is the first study, to our knowledge, to identify the production of one of these factors by activated astrocytes in brain lesions in which targeted oligodendroglia clearly express the appropriate receptor. Tests to determine whether blocking IGF-I prevents or significantly retards remyelination in this model should be done.

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