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SYMPOSIUM: Pathobiology of the Oligodendrocyte

The Neurotoxicant, Cuprizone, as a Model to Study Demyelination and Remyelination in the Central Nervous System

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Myelin of the adult CNS is vulnerable to a variety of metabolic, toxic, and autoimmune insults. That remyelination can ensue, following demyelinating insult, has been well demonstrated. Details of the process of remyelination are, however difficult to ascertain since in most experimental models of demyelination/remyelination the severity, localization of lesion site, or time course of the pathophysiology is variable from animal to animal. In contrast, an experimental model in which massive demyelination can be reproducibly induced in large areas of mouse brain is exposure to the copper chelator, cuprizone, in the diet. We review work from several laboratories over the past 3 decades, with emphasis on our own recent studies, which suggest an overall picture of cellular events involved in demyelination/remyelination. When 8 week old C57BL/6 mice are fed 0.2% cuprizone in the diet, mature olidgodendroglia are specifically insulted (cannot fulfill the metabolic demand of support of vast amounts of myelin) and go through apoptosis. This is closely followed by recruitment of microglia and phagoctytosis of myelin. Studies of myelin gene expression, coordinated with morphological studies, indicate that even in the face of continued metabolic challenge, oligodendroglial progenitor cells proliferate and invade demyelinated areas. If the cuprizone challenge is terminated, an almost complete remyelination takes place in a matter of weeks. Communication between different cell types by soluble factors may be inferred. This material is presented in the context of a model compatible with present data — and which can be tested more rigorously with the cuprizone model. The reproducibility of the model indicates that it may allow for testing of manipulations (*e.g.* available knockouts or transgenics on the common genetic background, or pharmacological treatments) which may accelerate or repress the process of demyelination and or remyelination.

Introduction

Feeding of cuprizone (bis–cyclohexanone-oxaldihydrazone) to young adult mice induces a synchronous consistent demyelination. This occurs in lesion sites large enough to allow detection of changes in the relevant cellular, molecular, biochemical and morphologic parameters. Furthermore, removal of cuprizone from the diet of mice permits the study of remyelination, (part of our own work has been to determine that these stages of demyelination and remyelination are not mutually exclusive: remyelination occurs simultaneously with demyelination).

The intent of this review is to describe how this model system may be used to define issues important to understanding of the pathophysiology of demyelination, and to gain understanding of the mechanisms involved in remyelination. Long term relevance of this research is to define steps in demyelination that might be blocked, or steps in remyelination, which might be promoted, by possibly clinically relevant *in vivo* manipulation. In this context, studies concerning the roles played by various cytokines and growth factors may be particularly relevant.

Mechanism of Action of Cuprizone

Copper is an essential trace element for a number of metalloenzymes (44) including copper-zinc superoxide dismutase and ceruloplasmin (45). Cuprizone is a copper chelator produced for use in clinical chemistry, and in animals dosed with this substance, it is assumed

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although not proven that the binding of copper results in the observed pathology. Feeding of cuprizone produces copper deficiency and CNS demyelination (2, 4, 19, 25, 35, 40). This demyelination is an early observable event, without damage to other cell types in the central nervous system (CNS) other than oligodendrocytes (4, 9, 15, 21, 25). Slightly more severe challenge, however, leads to formation of megamitochondria (enlargement or clustering) in the liver. This alteration in liver mitochondria may well be a consequence of deficiency in activity of a crucial component of oxidative phosphorylation, the copper requiring electron carrier, cytochrome oxidase (39, 40). In fact, cuprizone administration leads to a reduction in brain activity of cytochrome oxidase as well as another mitochondrial enzyme, monoamine oxidase (43). Brain copper levels are also reduced. This and other abnormalities did not take place if the cuprizone was copper chelated prior to administration to animal. Thus, it is assumed that in animals dosed with this substance it is binding of copper which results in the observed pathology. We note, however, a challenge to this hypothesis, since administration of copper failed to reduce cuprizone-induced toxicity (12).

The above observations lead to the hypothesis that cuprizone-induced copper deficit might be detrimental to mitochondrial function in brain (43), as well as in liver, and that it is a disturbance of energy metabolism in oligodendroglia and cell function that leads to demyelination (10, 15, 21, 32). A deficit in activity of other copper containing enzymes might be involved also. Why oligodendroglia should be preferentially susceptible to copper deficit is not known, although an obvious hypothesis is that the perikaryon of this cell type has to maintain a vast expanse of myelin and this extraordinary metabolic demand places it in jeopardy if the demand cannot be met. Lack of detailed knowledge of the molecular details of the point of pathological action targeted by copper deficiency remains frustrating, as is our lack of knowledge as to the reason for preferential susceptibility of oligodendroglia. An encouraging note is the possibility that these questions may be studied in cultured oligodendroglia (8), a system greatly simplified relative to the complications of *in vivo* studies.

The Animal Model

Although it is well known that cuprizone induces damage to myelin, much depends on the specifics of the animal model used. Rats and guinea pigs (13), as well as mice (40) exposed to cuprizone have been reported to show spongiform encephalopathy. It is possible that mice are uniquely suitable for study of cuprizone induced demyelination. Wistar weanling male rats treated with 0.5-2% cuprizone showed oligodendrocyte perturbation and intramyelinic edema but did not develop demyelination (23). These features were largely reversed after the cuprizone was removed from the diet. There was, however, some peripheral nerve degeneration; myelinated axons of the sciatic nerve were affected whereas unmyelinated axons were spared.

In contrast to rats, mice challenged with cuprizone exhibit frank demyelination. A well-studied model is that of Swiss or ICI mice fed 0.5 or 0.6% cuprizone in the diet (2, 25, 40). Within some weeks, depending on several factors, there is extensive demyelination of at least several well myelinated tracts. This dose level is, however quite severe and results also in significant weight loss and death of many animals. There is discussion of variation in the response of mice to cuprizone (14, 36). Removal of cuprizone from the diet allows for significant remyelination, (3, 27). An elegant example of morphometric analysis of relevant ultrastructural data is that of Blakemore (3). Much of the earlier literature is reviewed by Blakemore, 1984 (6), and Ludwin, 1994 (24). This earlier work established the basic model. Feeding of cuprizone induces demyelination, in a time frame of weeks; the exact time course is dependent on dose of cuprizone and age of application. Assuming demyelination has not progressed too far, such as in chronic demyelination, the animals can be rescued by removal of cuprizone from the diet (27). Within four weeks time, there is then substantial remyelination.

It had been rumored that other strains of mice are not susceptible to cuprizone-induced demyelination. In fact, this is not the case, as shown by our work with C57BL/6 mice (below). We note also that the assertion that female mice do not undergo demyelination when exposed to cuprizone (25) may hold under some restricted conditions, but in our unpublished studies, we do not note differential susceptibility to cuprizone between male and female mice.

We describe and reference the dosing and species model we use in some detail, but acknowledge the large pre-existing literature alluded to above and apologize in advance for not referencing every detail which influenced our decisions. We were interested in using a model of demyelination that permits consistent, detectable and easily scored demyelination and remyelination. This requirement is not well met by the extensively studied models of experimental allergic encephalomyelitis (EAE) or virally-induced demyelination; these are characterized by sporadic, asynchronous

and scattered lesions as mentioned previously (25). Lack of anatomical reproducibility of lesions between animals greatly hampers accurate and reliable assessments of changes in cell populations, quantification of demyelination, and biochemical analyses. Correlation of changes with time of these variables becomes extremely complicated. Thus, we adapted the cuprizone intoxication model, in large part to take advantage of the synchronous and anatomically reproducible course of disease.

Another consideration influencing our choice of the cuprizone model is that interpretation of data may be somewhat simpler than in other models of demyelination. Again, comparison of cuprizone-induced demyelination and subsequent remyelination can be contrasted to the EAE model. Results obtained with the EAE model must be considered with respect to the immune system. T cells are present and direct many complex immunological events, *e.g.*, promoting production of antibodies which may prevent remyelination (7), (for review see Miller *et al*., 31). Also, the blood-brain barrier is breached, allowing for other external influences to affect the course of demyelination and remyelination. These complexities of the EAE model are, in fact, an important reason why it is so extensively studied; many aspects of this autoimmune disease mimic important human disorders. In this context, the cuprizone model is an adjunct because it helps tease out the events most directly related to demyelination and remyelination, bypassing some of the considerable complexities of the immune system. T cells are almost completely absent during cuprizone-induced demyelination (Hiremath, submitted). Also, in preliminary experiments we used RAG-1$^{-/-}$ mice (devoid of T and B cells) and noted no marked difference in onset of cuprizone-induced demyelination. In general, the only immune cells present in the demyelinated areas appear to be microglia/macrophages.

The above noted near absence of T cells may be related to the presence of an intact blood-brain barrier (1, 22). This was demonstrated directly by subjecting Swiss mice to a 9 week period of cuprizone exposure which induced massive demyelination; the integrity of the barrier stood firm to penetration by horse radish peroxidase (22). Similarly, Bakker and Ludwin saw no breakdown of the blood-brain barrier in mice exposed to cuprizone intoxication (1). A question arises as to whether the functional integrity of the blood-brain barrier reflects on the ability of circulating monocytes to enter the brain and add in number to the population of recruited phagocytic cells involved in clearance of myelin debris (discussed in a subsequent section). The accumulation of microglia/macrophages into lesions and phagocytosis of myelin during cuprizone-induced demyelination may require intense proliferation of local microglia. However, no mitotic microglia were reported in previous ultrastructural studies (2, 4). Thus, the source of the large number of microglia recruited into the area of demyelination is unclear. It is possible that microglia may be entering from other regions of the brain and/or the peripheral macrophage may be contributing to the population, and that the relative contribution of these processes might vary during the course of the demyelination/remyelination events.

An important choice to be made for studies of cuprizone-induced demyelination concerns the mouse strain to be used. We opted to use the C57BL/6 mouse strain to facilitate future gene function studies by making possible use of the numerous knockout and transgenic mice available on this background (17). Any change in mouse strain requires recalibration of dose regimens. Indeed, titration of cuprizone showed C57BL/6 mice could only tolerate a 0.2% dose of cuprizone without significant weight loss or hepatocyte toxicity (17). This dose produces demyelination in the brains of C57BL/6 mice. The regional pattern of demyelination we observe falls within the range described in other mice (2, 9). The corpus callosum as well as the superior cerebellar peduncle showed complete and consistent demyelination (17, 32).

Age at time of presentation of cuprizone is a variable with respect to induction of demyelination. Some studies have been done with weanling mice in which demyelination was observed within four weeks, or older mice where demyelination was not observed until the eighth week (4, 9, 25). Our initial experience with mutant mice on the C57BL/6 background showed weanling survival highly variable even at the 0.2% dose of cuprizone primarily due to the varying birth weights and growth rates (personal observations). At 8-10 weeks of age, the weight of the mutant mice and the C57BL/6 animals were similar and consequently provided a more reproducible demyelination and minimized detrimental systemic effects such as liver toxicity. Similar comments on dose and weight of animals to induce adequate and survivable demyelination were mentioned for other mouse strains (5, 25).

Finally, the age of the animal and period of time of exposure to cuprizone are critical considerations. As noted above, we placed 8 week old C57BL/6 mice on a diet of milled chow containing 0.2% by weight of cuprizone. This dosage permitted study of an "acute" model so that animals could be maintained for 6 weeks (at

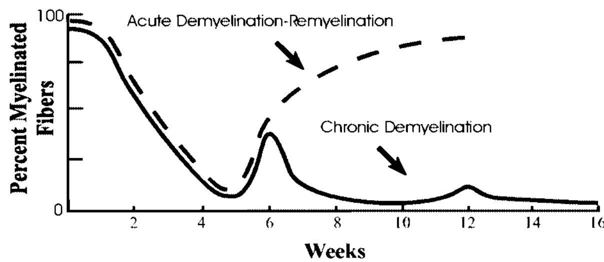

Figure 1. Percent of myelinated fibers in the corpus callosum above the fornix during acute and chronic exposure to cuprizone. Dashed lines indicate exposure to cuprizone for 6 weeks followed by a return to normal diet for the remaining 6 weeks. The solid line shows episodic demyelination and remyelination with continued exposure to cuprizone over a 16 week period.

which time demyelination was profound), but still allowed for essentially complete recovery when mice were then transferred to a cuprizone-free diet. We also studied "chronic" demyelination, which consisted of maintaining the mice on cuprizone for 16 weeks, allowing study of adaptation to continued metabolic insult. Almost all animals survived such a 12-week exposure, but by 14 weeks systemic effects were observable and animals started to die at week 16. This is in contrast to other strains of mice which can tolerate continued exposure for at least 6-7 months (27).

Quantitation of Myelin During Demyelination/Remyelination

The term "demyelination" has been used in the above sections with perhaps inappropriate casualness. It needs to be defined in terms of the assay used to determine "demyelination" and, whenever possible, in a quantitative manner. Myelin itself is primarily defined morphologically, in terms of the multilamellar structure of defined repeat distance that surrounds axons. Thus, ultrastructure is the defining assay for "demyelination." An obvious complication is the need to very tightly define small brain region to be studied. In such a defined location it is possible to quantitatively determine various parameters related to presence of myelin. These include the number of axons per unit area and the distribution of their sizes, how many are myelinated, the thickness of the myelin sheath, and the relationship of thickness of the myelin sheath in relation to size of the axon. Even a very limited study of this type is enormously labor intensive. Such investigations were undertaken in defined areas of superior cerebellar peduncle to study myelin status during cuprizone challenge (3, 25, 27).

Our own studies focus on corpus callosum of C57BL/6 mice placed on a 0.2% w/w cuprizone in chow diet at 8 weeks of age (17, 32). As assessed by ultrastructure, substantial demyelination was present starting at 3 weeks of age and by 4 –5 weeks of age more than 90% of axons were demyelinated (Figure 1, unpublished data and illustrated in Figure 2). By 6 weeks of age, and in the face of continued exposure to cuprizone, there was recovery so that about 50% of the axons were again myelinated. If cuprizone was removed from the diet at this time, recovery continued, so that by 10 weeks 90% of the axons were remyelinated (Figure 1, 2). If, in contrast the cuprizone challenge was retained the demyelinating process again dominated so that few myelinated fibers remained. Most surprising, however, was that there was another attempt at remyelination, so that at 12 weeks there was significant remyelination (these data of a collaborator, Jeff Mason, are being prepared for publication). Note that fraction of axons remyelinated is not the same measure as bulk recovery of myelin; the scoring for myelinated/unmyelinated does not take into consideration the variable number of wraps of myelin around an axon.

Although only ultrastructure is definitive in the morphological assay, much information can be gained from histology with a Luxol Fast Blue-periodic acid Schiff stain. This method allows for semi-quantitative estimates of myelination status by subjective (but observer blinded) 4 stage scale. A limitation is that lipid debris from degraded myelin still gives a positive signal for this lipid stain — thus this assay may overestimate the degree of organized myelination present. Histology, however, allows semi-quantitative evaluation of large numbers of sections. Although such screening by electron microscopy is not feasible, conclusions made from histology usually require validation by ultrastructural examination at appropriate selected experimental points.

A factor in consideration of morphological assays of myelination status is that a discrete brain region must be chosen for study. We selected the region of the corpus callosum above the fornix, a well-defined tract easy to locate reproducibly. The potential utility of biochemical assays in study of demyelination/remyelination suggests a need, also, for a simple quantitative assay of myelination status of large brain areas. Although it is possible to isolate myelin, the yield is far from quantitative, is difficult to do on a small scale, and is of unknown reliability in a situation where degrading myelin may be present. Perhaps more reliable is biochemical assay for myelin specific components. We have chosen for study the content of the myelin specific lipid, cerebroside (galactosylceramide). This component can be assayed

with high sensitivity by standard derivatization and HPLC procedures. During normal mouse development the accumulation of cerebroside correlates with myelination (33). Preliminary results suggest that cerebroside is also a useful marker for myelination status during cuprizone-induced demyelination and subsequent remyelination. Exposure of mice to dietary cuprizone for 6 weeks under our protocol induces a 15% loss of cerebroside relative to control. If the animals are allowed to recover on a regular diet, the cerebroside loss is completely compensated in another 6 weeks. If, instead, exposure to cuprizone is continued, there is further demyelination and by 12 weeks about a third of the cerebroside is lost relative to control (unpublished data Morell and Matsushima). Related observations were made previously by Carey and Freeman (11). Note that the episodic nature of the demyelination/remyelination elucidated by ultrastructural study of the central portion of the corpus callosum (summarized in Figure 1) is lost when assessing a marker for whole brain — averaging together all regions of the brain.

An extension of the concept of monitoring cerebroside content as a measure of myelination is to assay rate of cerebroside synthesis as a measure of rate of remyelination (33). The methodology involves systemic injection of ^{3}H-water; so that incorporation of label into cerebroside over a 2-4 hour period becomes a measure of the rate of synthesis of cerebroside, and therefore allows for determination of the rate of remyelination at that time. We have shown that this procedure gives the same results as the more tedious methodology of quantitating the amount of a myelin component at many time points, and then calculating the rate of accumulation (18). Preliminary data (unpublished results) indicates this methodology is applicable to measure the rate of synthesis of cerebroside in pieces of brain tissue of 5 mg wet weight. Thus, this methodology is of potential utility as an assay to determine whether, subsequent to cuprizone-induced demyelination, pharmacological or other manipulation promotes or inhibits the rate of remyelination.

Fate of Preexisting Oligodendroglial Cells and Origin of New Oligodendroglial Cells

A primary question concerns the fate of oligodendroglial cells during cuprizone-induced demyelination. Is it myelin that is destroyed first — with the cell body dying back as a consequence? Or is it the cell perikaryon that is initially affected — with consequent failure of the ability to support myelin? The latter suggestion receives support from our data regarding early down

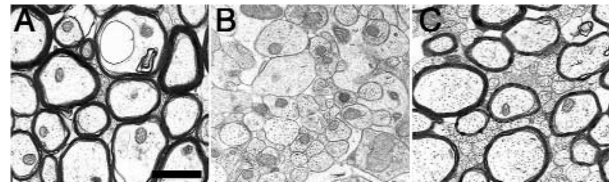

Figure 2. Ultrastructural micrographs showing demyelination and remyelination in the corpus callosum. Panel A is the normal untreated C57BL/6 mouse showing full myelination. Panel B shows near complete demyelination at week 5 and panel C shows remyelination at week 10. Scale bar = 1.2 μm

regulation of expression of myelin specific genes, and from direct demonstration of extensive apoptotic death of oligodendroglia prior to acute demyelination. After only one week of exposure of C57BL/6 mice to cuprizone, steady state levels of mRNA for MBP, MAG and CGT are already dramatically reduced (32) and are summarized in Figure 3. By 3 weeks, when demyelination is first reproducibly evident, expression of myelin genes is maximally depressed by some 80%. This early dramatic decrease in transcripts was also detected for MAG mRNA in Swiss mice treated with cuprizone (15). It is of interest that on a whole brain basis this decrease in myelin-specific message level (32) is considerably greater than the biochemically assayed loss of the myelin specific component, cerebroside (18). This implies that most or all oligodendroglial cells are insulted — not only those destined for demyelination. Thus, we suggest that the oligodendroglia are under a considerable stress (at least to some extent specific for this cell type) during administration of cuprizone.

A clear cut correlate with the whole brain decrement in expression of myelin specific genes is the demonstration that in the corpus callosum (site of particularly profound demyelination) a majority of mature oligodendrocytes were undergoing death by apoptosis (29) and is illustrated in Figure 3. Apoptotic GST-pi$^+$ (glutathione transferase, a marker for mature oligodendrocytes) cells were detected by immunohistochemistry between 2 and 4 weeks, just prior to onset of demyelination. By week 5, nearly all the mature oligodendrocytes were depleted from the corpus callosum. Interestingly, at week 6 when mice were still undergoing exposure to cuprizone, the presence of mature oligodendrocytes was demonstrated and remyelination was detected as described above. This suggestion that events of remyelination follow a preset program, despite the presence of the neurotoxicant, is compatible with previous descriptions of oligodendrocytes remyelinating at week 5 of cuprizone exposure(4).

The data above highlights a question, if cuprizone

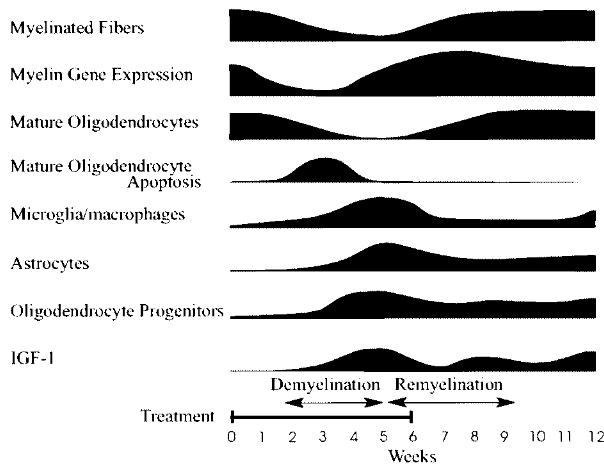

Figure 3. Summary of morphologic, cellular, and biochemical trends during acute demyelination followed by recovery. A number of events associated with remyelination appear to occur in the midst of demyelination and exposure to cuprizone. Diagram was adapted from Mason *et al.* 2000 (29).

causes apoptosis of most oligodendrocytes, what is the source of the new population of mature oligodendrocyte resynthesizing myelin. Possibilities include more myelin put out by the remaining oligodendroglia, dedifferentiation and proliferation of remaining mature oligodendroglia, or proliferation and differentiation of either local or recruited progenitor cells. Our studies strongly suggest the latter is the case. We took advantage of an immunohistochemical marker specific for oligodendroglial progenitors, NG2 (34). In the model we used, corpus callosum of C57BL/6 mice exposed to a 0.2% cuprizone, 3 weeks of exposure was a time of early demyelination. As early as week 1 to 3, bipolar oligodendrocyte precursors were present in substantial numbers in the corpus callosum (29). By week 3, there was transition of many of these to (the more differentiated) star-like progenitors whose appearance peaked at week 4. Our subjective evaluation was that some oligodendrocyte precursors may have been residing within the myelinated tract, while others appear to have been proliferating and migrating from the subventricular zone or surrounding tissue.

By week 6 there was considerable remyelination. This coincided with a drop in the number of these progenitor cells, presumably because of their conversion to GST-pi positive (41) mature olidgodendrocytes which were becoming prominent. If cuprizone challenge was removed, the accumulation of mature oligodendrocytes continued — as did remyelination (29). This analysis of a localized area, the corpus callosum, matched well with Northern blot analysis of increased steady-state level of mRNA for myelin-specific genes MBP and MAG (15, 32). Our results are compatible with earlier morphological studies (2, 26). These investigators observed remyelinating oligodendrocytes after demyelination. They also detected proliferating (^{3}H-thymidine incorporating) oligodendrocytes early during remyelination. Thus, early electron microscopy experiments also suggested that a substantial number of mature oligodendrocytes arise from cells that proliferated during or just after demyelination.

Further evidence that remyelination events (proliferation and differentiation of recruited progenitor cells) take place even in the face of continued challenge with cuprizone was provided by studies of chronic cuprizone intoxication. In this model cuprizone-fed animals are not restored to a normal diet after 6 weeks. Instead, the metabolic challenge is continued. As noted earlier there is remyelination in corpus callosum by 6 weeks followed by demyelination and, even in the face of continued cuprizone challenge, some remyelination at 12 weeks (Figure 1). The remyelination efforts, however, become less successful with time. This is compatible with the work of Ludwin, 1980 who showed that the number of oligodendroglia continued to decrease; this was cited as the cause of failure to remyelinate.

Presence and Role of Microglia

Demyelination is accompanied by the appearance of microglia and by astrogliosis (2, 4, 25). In our model, utilizing C57BL/6 mice, microglia/macrophages are present within the first two weeks after exposure to cuprizone. At week 3 when significant demyelination is detected, an increased number of microglia/macrophages accumulate within the lesion, the number peaking between 4-6 weeks (17, 32). What is the origin of these cells? The gliosis presumably is related to a preceding proliferation of astrocytes. Early ultrastructural studies suggest that the cuprizone does not induce microglial mitosis (2, 4). With respect to microglia, the increase in their number in the corpus callosum might involve primarily recruitment from other brain regions and/or local proliferation. It appears to us, however, that the whole brain increase in phagocytically active cells (as indicated by the elevation in mRNA for lysozyme — a marker for phagocytosis, 42), is very considerable. We suggest the possibility of active infiltration of circulating monocytes to add to the population of those recruited from other brain regions. The question as to whether this is compatible with an intact blood-brain barrier (discussed earlier) remains to be resolved. We utilize the

Growth Factors & Cytokines	Weeks of Treatment 0	1	2	3	4	5	6	6+1*
FGF	+	+	+	+	+	+	+	+
PDGF	+	+	+	+	+	+	+	+
IGF-1	-	-	-	+/-	++	++	++	++
NT-3	+	+	+	+	+	+	+	+
NGF	+	+	+	+	+	+	+	+
IL-1	-	+/-	+/-	++	++	++	++	+/-
IL-2	-	-	-	-	-	-	-	-
G3PDH	+++	+++	+++	+++	+++	+++	+++	+++

*Six weeks exposure to cuprizone and one week recovery

Table 1. Gene expression of growth factors and cytokines present during active cuprizone-induced demyelination and remyelination.

term microglia to indicate brain localization, without an implied judgement as to origin of these cells.

The recruitment and/or proliferation of microglia raise question as to their role in the pathophysiology of cuprizone-induced demyelination. We assume that microglia/macrophages are functioning, in part, to clear myelin debris as suggested previously (2, 28, 37). Alternatively, or additionally, we note that microglia/macrophages are strategically located in the lesion so as to make it feasible that they aid in the remyelination process. The appearance of microglia/macrophages coincides with the onset of oligodendrocyte precursor numbers and an increase in astrocytic numbers, particularly beginning at week 3 during the exposure to cuprizone (17, 29, 32). A functional relationship between the presence of microglia and events leading to remyelination would presumably involve cell to cell signaling. We have, therefore initiated studies as to presence of certain cytokines and growth factors during the period of cuprizone-induced demyelination and recovery associated with removal of cuprizone from the diet. Only certain cytokine and growth factors accumulate dramatically during this 3 week period between week 3 to week 6, these include IL-1 and IGF-1 (Table 1). Genes for growth factors such as FGF, PDGF, NT-3 and NGF appear to be transcriptionally active; however, their mRNA levels do not change during demyelination or remyelination. Lack of IL-2 suggests minimal T cell participation in the disease process. Thus, we speculate that it is activated microglia/macrophages that are providing components that contribute to the recovery of the demyelinated lesions.

There is elevation of levels of insulin-like growth factor-1 starting at week 3 (29), the same time that recruitment and accumulation of oligodendroglial progenitors becomes prominent and apoptotic death of preexisting oligodendroglia is vigorously ongoing. Immunohistochemistry using double labeling for cell type-specific marker and IGF-1, indicated that both microglia and astrocytes were involved in release of IGF-1. This result is completely compatible with the earlier work of Komoly *et al*. (20) who demonstrated that the brains of mice exposed to cuprizone for 8 weeks contained IGF-1 positive astrocytes. Preliminary studies indicating that interleukin 1 levels are elevated even before IGF-1, suggest that release of this cytokine by microglia might initiate a cascade leading to IGF-1 production and release (see Table 1). Thus, microglia may be intimately involved in the process of remyelination; they may be responsible for jump-starting the repair process. The diagram in Figure 3 illustrates microglia accumulating at a position in time prior to and early in the remyelination process. This discussion concerning the beneficial aspects of involvement of microglia with respect to remyelination echoes points made by others (12, 38).

The above discussion presupposes that IGF-1 somehow promotes oligodendrocyte differentiation and survival. This hypothesis was tested by an experimental design involving transgenic mice in which IGF-1 is constitutively expressed under the metallothein promoter. The 3-fold elevated levels of IGF-1 protect against cuprizone-induced apoptosis of oligodendroglial cells (30). There is damage to myelin, and demyelination is apparent at week 3 of cuprizone exposure, but injury to oligodendoglial cells seemed restricted to distal processes. Most notably, the recovery after this brief partial demyelination was accelerated dramatically such that by week 5 when wild type mice show near complete demyelination, most of the axons had remyelinated in the IGF-1 transgenic (30). This would suggest that killing of the mature oligodendrocyte and demyelination can be independent events; demyelination does not necessarily mean oligodendrocytes are dying. We note that

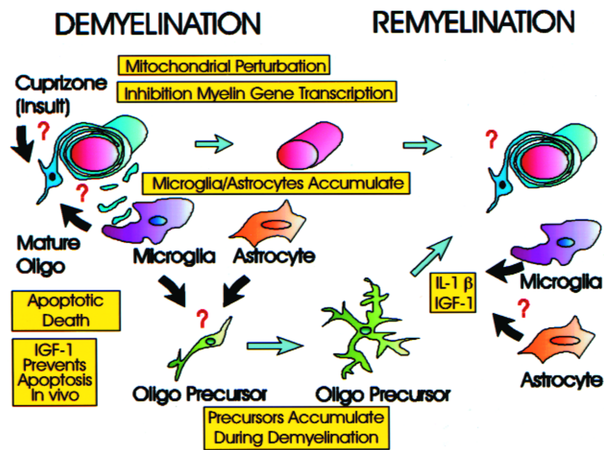

Figure 4. Diagram of the interplay between oligodendrocytes, microglia and astrocytes during insult by cuprizone and recovery from demyelination (see text under Conclusions).

many soluble signals must be involved in the interplay between different cell types as it relates to demyelination and remyelination. For example, it has been shown (16) that transgenic mice expressing low levels of interferon-gamma are resistant to cuprizone intoxication. The effect might be due directly to lowered interferon gamma levels or secondly to the observed increase in level of IGF-1, which might be acting to protect oligodendrocytes from apoptosis.

Conclusions

We offer a framework in which to consider cuprizone-induced demyelination and subsequent remyelination (Figure 4). We readily acknowledge that the scheme is simultaneously very naïve, and yet goes far beyond the presently available data. We present this in the spirit of the goal of this review — to show how the cuprizone model may help to "define issues" in a framework facilitating design of experiments to solve the issues raised. As presented, the presence of cuprizone in the diet inhibits mitochondrial function. We have little data leading to mechanistic insight as to why oligodendroglial cells should be particularly susceptible. A simple line of reasoning is that the extraordinary metabolic demand on oligodendroglial cells renders them preferentially susceptible to interference with energy generating mechanisms (decreased oxidative phosphorylation due to lessened activity of copper requiring cytochrome C). The energy depletion leads to increasing failure to function adequately in metabolic support of myelin; the damage to oligodendroglia is reflected in down regulation of genes specific to synthesis and support of myelin. The damage to mitochondria eventually leads to apoptosis of myelinating oligodendroglia and related collapse of the myelin they support. Some soluble factors released during this process initiate recruitment and proliferation of microglia (and possibly infiltration of macrophages from the circulation). The microglia released factors (perhaps early on including IL-1β) initiate recruitment and proliferation of oligodendrolial precursors and their subsequent differentiation. Microglia might also stimulate astrocytes to release factors which also promote an environment supporting remyelination. Large numbers of progenitors are recruited; these differentiate and continue through to actual remyelination. Surprisingly, this occurs even in the presence of continuing challenge with cuprizone. We suggest this is allowed because increased IGF-1 levels (due to synthesis and release by microglia and astrocytes) protect many of the newly forming mature oligodendroglia against apoptotic death. If the challenge by cuprizone is relieved at this time, recovery and remyelination continue. If, however, cuprizone administration is continued the newly differentiated oligodendroglia die as they start to put out myelin. Eventually, however, the number of mature oligodendroglia is decreased and the progenitor pool available for their resupply is depleted.

Many aspects of the above scheme are testable. Since the model is reproducible with respect to time course and anatomic location of demyelinating areas, temporal studies of appearance of cell types and factors released are possible. This type of investigation may suggest hypotheses — but it usually cannot support an experimental design offering unambiguous proof. The availability of numerous knock-out animals on the required genetic background, however, offers the possibility of design of functional tests for the scheme outlined above and/or those yet to be developed.

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