Axon regeneration: **Vaccinating against spinal cord injury**

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Myelin is a potent inhibitor of axon regeneration, but has been viewed as just one of many factors that prevent regeneration after injury. So it comes as a surprise that immunization against myelin has been found to allow extensive axon regeneration after injury, without apparent autoimmune-induced demyelination.

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The inability of the adult mammalian central nervous system (CNS) to regenerate after injury has confounded clinicians and scientists alike for centuries. In contrast to the adult CNS, young neurons will readily extend axons and successfully regenerate after injury [1,2]. The suggestion has been that, with development and age, CNS neurons somehow lose the intrinsic capacity to regrow. It came then as a pleasant surprise when Aguayo and colleagues [3,4] showed that with a favorable environment

very long axons into the implanted tissue. But growth stopped, or was very limited, when the regenerating axons again encountered host tissue.

These observations implied that something in the adult CNS environment was actively inhibiting axonal regeneration. Later work implicated myelin as one important component of the adult CNS environment that acts to prevent axon regeneration — but it was thought to be just one of a number of inhibitory factors. Surprisingly, it has now been reported [5] that the immunization of an animal against myelin can permit extensive axon regeneration after injury, even in the absence of any additional treatment to block other factors that influence regeneration, and without any apparent autoimmune-induced demyelination.

As noted by Ramon y Cajal [6] almost a century ago, at a lesion site in the CNS a scar-like formation appears, which Cajal speculated physically prevented growth. We now know that the scar at the lesion site is composed of glial cells that have undergone both a morphological change — the extension of interdigitating processes — and a biochemical change — up-regulation of a number of chondroitin sulfate proteoglycans [7]. The first of these transformations poses a physical barrier to regrowth, while the second presents a non-permissive environment for regeneration. Undoubtedly, the glial scar can directly block regeneration, but it takes weeks to be fully formed, even though the up-regulation of chondroitin sulfate proteoglycans begins within hours to days after injury.

The question, then, is why axons do not start to grow immediately after injury, before the glial scar has time to form? The most likely answer is that the delay is a consequence of a third factor — inhibitors of axonal regeneration found in myelin [8,9]. About a decade ago, a number of groups simultaneously demonstrated that myelin, or white matter in general, was non-permissive for axonal regeneration. If neurons were cultured on tissue sections of spinal cord, for example, those neurons that fell on the gray matter extended long axons, while those that fell on white matter did not [10–12]. Consistent with this finding is the observation that, when purified myelin was used as a substrate, neurons extended very short or no neurites at all [13–15].

The conclusion was that something specific to, or greatly enriched in, CNS myelin actively prevents regeneration. In contrast, but consistent with their ability to regenerate

young axons can regenerate *in vivo* is extended if the onset of myelination is delayed [17]. It appears, then, that two developmental events occur that result in loss of regenerative capacity: the environment changes (myelin is formed) and the intrinsic axonal response to that environment changes (axonal outgrowth becomes inhibited by myelin). Injury to the CNS damages not only axons but also myelin, and the damaged axon is thus prevented from immediate regeneration by exposure of, most likely, both secreted and membrane-associated inhibitory components of myelin. The glial scar then forms and seals the fate of damaged axons to no regeneration.

So how can we encourage axons to regrow immediately after injury and continue to do so until they reach their target? There are two general approaches that could be taken. One would be to induce the neuron to revert to a ‘young’ state such that an adult environment does not inhibit it. The second would be to block the myelin inhibitors of regeneration. A recent study has suggested that the intrinsic growth capacity of adult CNS axons can indeed be changed *in vivo*. Neumann and Woolf [18] showed that, for dorsal root ganglion neurons that have both axons that extend into the peripheral nervous system (PNS) and axons that extend into the CNS, if a lesion is created in the PNS branch one or two weeks before a

subsequent lesion of the CNS branch, the CNS axons will regrow through what is normally a highly non-permissive environment for those axons. It would seem that the peripheral-branch-conditioning lesion has changed the growth capacity of dorsal root ganglion neurons such that inhibitors in the environment no longer block growth of the CNS axons. The molecular mechanism responsible for the change in these neurons remains to be determined. However, recent studies have shown that, when the endogenous neuronal cAMP levels are elevated, axonal growth is no longer inhibited by myelin [19]. It is possible that the conditioning lesion in some way alters the cAMP levels in dorsal root ganglion neurons, which then allows them to grow through myelin [20].

The approach of blocking inhibitory components of myelin to encourage regeneration has been pioneered in Schwab's laboratory. Schwab and Caroni [13] identified an inhibitory activity enriched in myelin protein fractions of 250 kDa and 35 kDa. A monoclonal antibody raised to the 250 kDa fraction, termed IN-1, was shown to allow axons to grow, not only on myelin in culture [21] but, more importantly, *in vivo* [22,23]. When hybridoma cells secreting the IN-1 antibody were implanted at the same time as a spinal cord lesion was created, a number of axons were shown to grow relatively long distances and, in some instances, functional recovery occurred. But although some long-distance growth was achieved, at most only 5–10% of axons regrew, suggesting that myelin is likely to contain other inhibitors, in addition to IN-1, that contribute to the lack of regeneration. A novel protein, termed Nogo, has recently been shown to be recognized by the IN-1 antibody [24]. When presented to a growing axon, Nogo induces growth-cone collapse. Another inhibitory component of myelin is the well-characterized myelin-associated glycoprotein (MAG). MAG is a very potent inhibitor of axonal growth *in vitro* [14,15] and an inhibitory proteolytic fragment of MAG, released from damaged myelin [25], is likely to play an important role in preventing regeneration immediately after injury.

In addition to MAG and Nogo, there are likely to be many other inhibitors of axon regeneration in myelin. A number of repulsive guidance cues, which act during development, have also recently been identified in the adult nervous system [26–29] and a proteoglycan associated with myelin has recently been shown to be inhibitory for axonal growth [30]. The task of individually identifying and neutralizing all the inhibitors of axonal regeneration in the adult CNS is thus not as simple or straightforward as at first believed. It is likely that the effects of these inhibitors are not additive, and that the presence of any one will effectively inhibit growth. What is the alternative to blocking each individually? The answer may be to devise a means to block them all simultaneously. That is exactly what David and colleagues have achieved in their recent study [5].

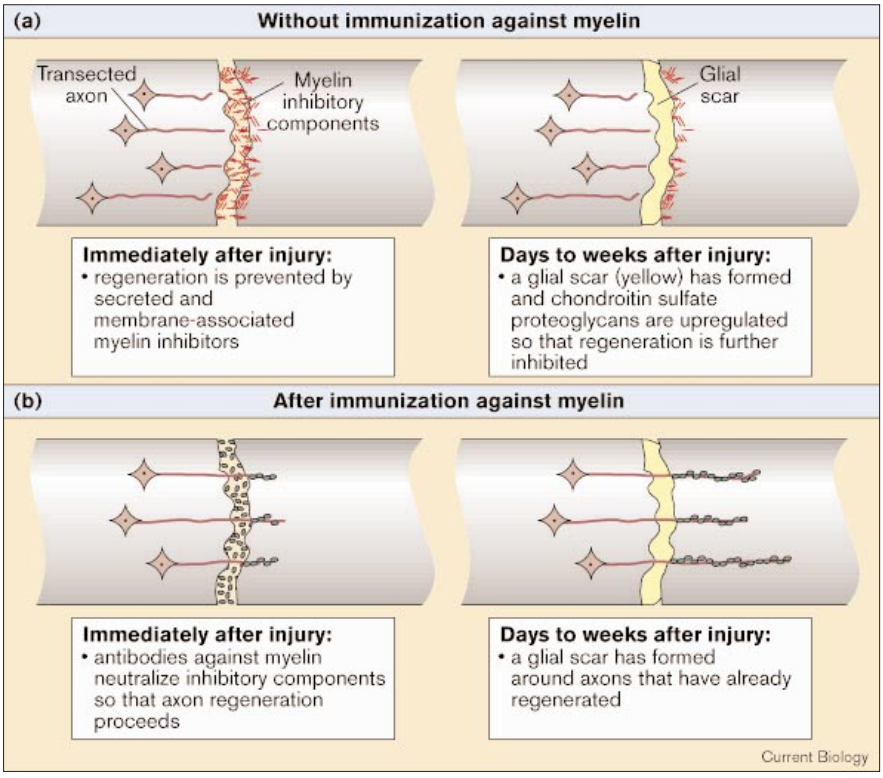
Previous attempts to encourage regeneration focused, logically, on treatment after the injury has occurred. What is different and novel about the approach taken by Huang *et al.* [5] is that they 'vaccinated' mice against myelin inhibitors of regeneration before inflicting the injury. Using a protocol developed by Rodriguez *et al.* [31] to produce antibodies that promote remyelination, mice were immunized for three weeks before injury with either isolated myelin or preparations of spinal cord that were enriched in myelin but also contained some proteoglycans. When examined three weeks after spinal cord injury, numerous axons were seen to have regenerated over long distances in more than 50% of the immunized mice, while no regeneration was seen in control injected animals (Figure 1).

The distance of regeneration observed by Huang *et al.* [5] in the immunized mice was 5–11 mm, comparable to distances reported by Schwab and colleagues [22,23] after application of the IN-1 antibody to transected rat spinal cord. But many more axons regenerated in the myelin-immunized mice than those treated with IN-1 antibody. A further difference between the IN-1-antibody-treated and the myelin-immunized animals is the relative distance traversed by the regenerating axons. Regeneration in the myelin-immunized mice was up to about two-thirds of the entire spinal cord; while axons grew similar absolute distances in the IN-1-antibody-treated rats, in this case the regeneration reached only about one-quarter of spinal cord. Importantly, functional recovery accompanied anatomical regeneration in the immunized mice. The sera from these myelin-immunized mice also allowed extensive axonal growth on myelin in culture, and if the sera was depleted of immunoglobulins, the effect was lost.

Given that it has been known for more than a decade that myelin contains inhibitors of regeneration, why was such a procedure, which in retrospect appears quite simple, not carried out earlier? Most likely, this was because immunization of mice with myelin is used to induce experimental allergic encephalophy (EAE), considered the closest animal model of the human demyelinating disease multiple sclerosis. Allowing axonal regeneration, but at the same time inducing multiple sclerosis, would not be a very appealing treatment. But Huang *et al.* [5] took advantage of Rodriguez *et al.*'s finding [31] that, for induction of EAE, mice must be immunized with myelin in complete Freund's adjuvant; with incomplete Freund's adjuvant there was no indication of inflammation or demyelination.

An additional deterrent to immunization with myelin may have been the expectation that the antibodies produced would be unable to penetrate the blood brain barrier to get to the site of injury in the spinal cord. This concern appears to be unfounded, as immunostaining showed that, three days after injury, immunoglobulins are present

Figure 1



Axon regeneration in the spinal cord **(a)** without and **(b)** after immunization against myelin. As recently reported by Huang *et al.* [5], immunization of mice against myelin allows axon regeneration over long distances; in the absence of immunization, no regeneration is seen. Red, myelin inhibitors of regeneration; green, anti-myelin antibodies; yellow, glial scar. (See text for details.)

along the myelin tracts up to 5 mm from the injury site. So the antibodies are obviously reaching their target.

An outstanding issue is whether the antibodies have to precede or accompany the regenerating axon along its entire path, or if they are only needed for the initial stages of growth, across the lesion site and through the exposed myelin inhibitors. Work from Silver's group [32,33] would suggest the latter. By carefully transplanting isolated adult neurons into adult spinal tracts without inducing any damage and no glial scar, Silver and colleagues observed extensive axonal growth from the transplanted neurons, through white matter tracts. Under these conditions, myelin is not damaged and presumably the inhibitors are not exposed. So the axons are growing on the intact myelin surface, which to our knowledge contains no inhibitors. The same situation may occur in the myelin-immunized mice, in that the antibodies may only be required to allow the axons to traverse the site of exposed inhibitors. Once they reach myelin tracts that are undamaged, perhaps the axons too grow on the outer surface, which appears to be permissive for growth.

What happened to the glial scar in these myelin-immunized mice? Because of 'pinching' at the original lesion site, in some mice it would appear that the scar formed after and around the regenerated axons. This is the strongest direct evidence presented to date that inhibitors in myelin prevent regeneration immediately after injury, and that there is a period of time after injury before the glial scar and its associated proteoglycans become impediments to growth. It should be noted, however, that there are differences between the reaction to injury in rats and mice. In rats there is a greater tendency for a fluid-filled cyst to form (cavitation) at the lesion site than there is in mice. In six of the myelin-immunized mice in which regeneration did not occur there were cavitations. On the other hand, perhaps regeneration immediately after injury in some way limits both cavitation and glial scar formation. In myelin-immunized mice that did not regenerate, perhaps the antibody titer was not high enough and the cavitations and scars formed because of the absence of regeneration.

The big question, of course, is whether this vaccination approach is a valid, potential therapy in humans with spinal cord injury. Although the mice studied by Huang *et al.* [5] showed no sign of demyelination, it would seem very risky and impractical to immunize the population as a whole against myelin antigens. The risk of promoting autoimmune demyelinating disease is just too great. Instead, rather than use immunization as a prophylactic treatment, the ideal situation would be to be able to immunize, with the same beneficial outcome, at the same

time as the spinal cord injury occurred. For this to be successful, the production of myelin antibodies would have to be rapid and plentiful.

We have been surprised by both the ability of axons to regrow at all after injury and the ability to 'vaccinate' against myelin inhibitors [5], so it may not be outlandish to hope that immunization simultaneously with injury will allow regeneration. Alternatively, exogenously produced myelin antibodies can be introduced to the lesion site along with immunization. The consequent passive immunity might compensate for the interim period required for endogenous antibody production. What is even more compelling, yet puzzling, is the possibility that these same myelin antibodies also promote remyelination [31]. To complement this immunization strategy, treatments that alter the intrinsic growth capacity of the axon could also be applied simultaneously. Finally, as always with these regeneration studies, once we have encouraged the damaged axons to regrow we have to redirect them back to their original destinations, a problem that may be greater for long-distance rather than short-distance regeneration.

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