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A Therapeutic Vaccine Approach to Stimulate Axon Regeneration in the Adult Mammalian Spinal Cord

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Summary

Axon growth inhibitors associated with myelin play an important role in the failure of axon regeneration in the adult mammalian central nervous system (CNS). Several inhibitors are present in the mature CNS. We now present a novel therapeutic vaccine approach in which the animals' own immune system is stimulated to produce polyclonal antibodies that block myelin-associated inhibitors without producing any detrimental cellular inflammatory responses. Adult mice immunized in this manner showed extensive regeneration of large numbers of axons of the corticospinal tracts after dorsal hemisection of the spinal cord. The anatomical regeneration led to recovery of certain hind limb motor functions. Furthermore, antisera from immunized mice were able to block myelin-derived inhibitors and promote neurite growth on myelin in vitro.

Introduction

The failure of injured axons to regenerate long distances in the adult mammalian CNS leads to permanent paralysis and other functional deficits such as those seen after spinal cord injuries. Although axons do not regenerate through adult CNS tissue, they retain the ability to regrow for long distances if provided with an appropriate cellular environment, e.g., a peripheral nerve graft (David and Aguayo, 1981). Work by Schwab and his colleagues led to the discovery that the failure of axons to regenerate through CNS tissue was likely to be due in large part to the influence of axon growth inhibitory activity associated with myelin (Caroni and Schwab, 1988; reviewed by Schwab et al., 1993). A monoclonal antibody (IN-1) was shown to recognize a 250/35 kDa glycoprotein in myelin which has axon growth inhibitory properties (Schwab et al., 1993). Although blocking this inhibitor with the IN-1 monoclonal antibody stimulates axon regeneration in long fiber tracts in the adult rat spinal cord (Schnell and Schwab, 1990; Bregman et al., 1995), the number of axons that regenerate is small and likely reflects the presence of other inhibitors (reviewed by David, 1998). In addition to the already identified inhibitors associated with CNS myelin (Caroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994), other inhibitory activities that have yet to be characterized have also been detected in myelin (McKerracher et al., 1994; Li et al., 1996). The important influence of axon growth inhibitors associated with myelin is supported by the strong correlation that exists between the loss of the capacity for axon regeneration and the onset of myelination (Savio and Schwab, 1990; Keirstead et al., 1995). Furthermore, delaying the onset of myelination extends the period of regenerative growth in the developing rat and avian spinal cord (Savio and Schwab, 1990; Keirstead et al., 1995). Axon growth inhibitors associated with the scar at the injury site also appear to be important contributors to regeneration failure (McKeon et al., 1991; Pindozolla et al., 1993; Davies et al., 1997; Wang et al., 1997). For example, proteoglycans expressed by astrocytes at the site of lesion have strong neurite growth inhibitory activity (McKeon et al., 1991).

Achievement of a substantial measure of axonal regeneration in the CNS will require blocking several of these axon growth inhibitors. Here, we present a simple and effective therapeutic vaccine approach to stimulate the animals' own immune system to generate polyclonal antibodies that block the inhibitory properties of the CNS and thus permit profuse long-distance regeneration of corticospinal tract fibers in the adult mouse spinal cord. Furthermore, the immunization protocol employed was such that it did not result in the production of a cellular inflammatory response.

Results

Regeneration of Corticospinal Fibers Assessed by Anterograde Labeling

Adult female BALB/c mice (8–10 weeks old) were immunized twice weekly with a homogenate of mouse spinal cord (which is rich in myelin and contains some inhibitory proteoglycans; Pindozolla et al., 1993; Wang et al., 1997) in incomplete Freund's adjuvant (IFA). Control mice were injected with IFA alone. As discussed later, the use of IFA for the immunizations avoids the production of unwanted inflammatory demyelinating lesions. Three weeks later, the spinal cords were hemisected dorsally to lesion both corticospinal tracts at the lower thoracic cord (T9) level. Care was taken to assure consistency of the depth of the lesion as described in the Experimental Procedures. After a further 3 weeks, during which the animals continued to receive twice weekly immunizations, regeneration of the corticospinal tracts was assessed using the anterograde neuronal tracer wheatgerm agglutinin–conjugated horseradish peroxidase (WGA-HRP) injected into the sensory–motor cortex (Li et al., 1996). About

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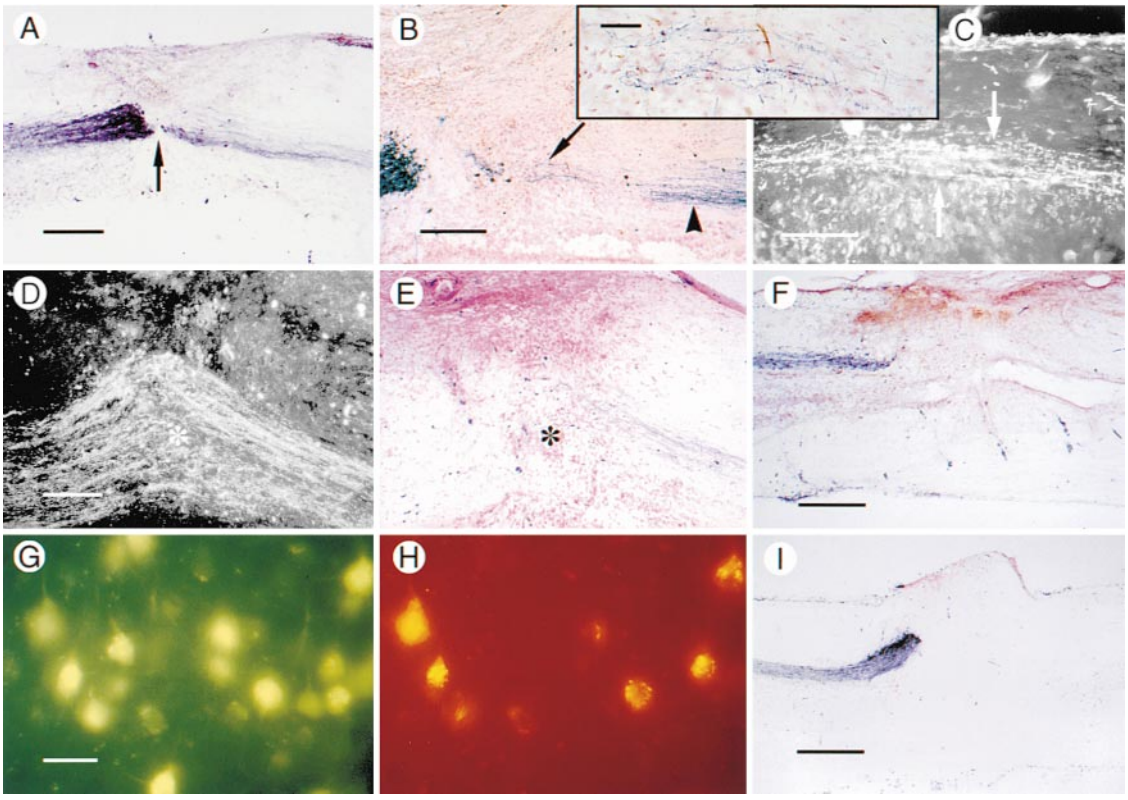

Figure 1. Regeneration of Corticospinal Tract Axons

In (A) through (F), (I), and (J), rostral is located toward the left side and caudal is located toward the right side.

(A) Micrograph of a longitudinal section of the lesioned corticospinal tract in a mouse immunized with spinal cord homogenate in IFA. WGA-HRP labels the tract rostral and caudal to the lesion (arrow). Many regenerated axons can be seen caudally.

(B) Higher magnification of an adjacent section showing the scar at the site of lesion. WGA-HRP-labeled axons can be seen coursing through the scar (arrow; the same area is shown at higher magnification in the inset). A large number of HRP-labeled fibers that have regenerated caudal to the lesion (arrowhead) have bypassed the scar.

(C) Dark-field micrograph showing a bundle of regenerated axons (between arrows) 7.5 mm caudal to the lesion in an immunized mouse.

(D) Montage of two adjacent longitudinal tissue sections from another immunized animal in which most of the WGA-HRP-labeled fibers in the tract course through the area of lesion (asterisk), making a kink-like displacement. Caudal is located to the right side of the lesion.

(E) A bright-field image of one of the sections from which the montage in (D) was made. Note the lesion at the center (asterisk), which is seen as the area of high cellularity. In contrast, the dorsal columns away from the lesion, which are seen toward the right and left margins of the micrograph, have markedly fewer cells and a paler appearance.

(F and I) Longitudinal sections through the spinal cord of a control mouse injected with IFA (F) and a control mouse injected with liver homogenate in IFA (I). WGA-HRP-labeled axons of the corticospinal tract stop at the site of lesion in both cases. Note that the scar at the site of lesion is minimal in the liver control (I).

(G) Fluorogold-labeled neurons in the motor cortex in a mouse immunized with bovine myelin in IFA.

(H) The same field as in (G) showing neurons double labeled with Fluororuby.

(J) Longitudinal section through the spinal cord of a mouse immunized with mouse myelin that showed double-labeled cortical motor neurons. This section shows that the two fluorescent markers in the spinal cord remain widely separated from each other. The Fluorogold labeling spread over a greater distance than Fluororuby because it was injected directly into the lesion at the time of hemisectioning. However, the amount of spreading was similar in immunized and control mice. The signal for Fluororuby appears weak in this micrograph because it was photographed at a very low magnification.

Scale bars: 300 μm (A, F, and I); 200 μm (B, D, and E); 100 μm (C); 50 μm (G, H, and inset); and 1 mm (J).

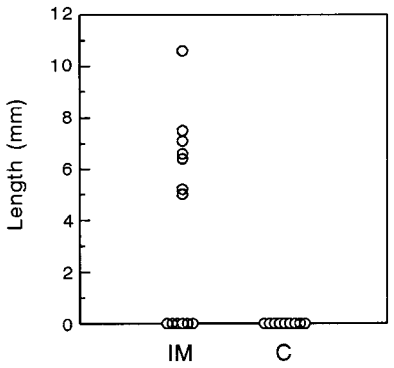

Figure 2. Maximum Length of Regenerating Axons

The maximum distance that injured corticospinal tract axons regenerated caudal to the site of lesion in mice immunized with mouse spinal cord homogenate in IFA ("IM") and controls injected with IFA alone ("C"). This distance was estimated from serial sections and by measuring the maximum distance to which WGA-HRP-labeled axons extended caudally. Each point represents one animal.

54% percent of the immunized mice (n = 13) showed abundant WGA-HRP labeling of corticospinal tract axons across the lesion and for long distances caudally past the lesion (Figures 1A–1E and 2). The maximum distance these labeled axons extended varied in different animals and ranged from 5 mm to about 11 mm from the lesion (Figure 2). A large number of these axons extended about 6 mm and a few reached about 11 mm. Regenerated axons were located in the myelinated dorsal columns caudal to the lesion. Some of these axons appeared to be displaced more dorsal to their normal position but along the boundary with the gray matter (data not shown). Although axons appeared to bypass the scar in some animals, in others many axons extended through the scar at the lesion site (Figures 1A–1E). The presence of the lesion and the scar was easily detected by the cellular nature of the scar as revealed by the neutral red staining (Figures 1B and 1E). The lesioned axons made a kink-like displacement upwards as they passed through the region of the scar. The lesions of the corticospinal tract were judged to be complete in all treated animals, based on these criteria. Furthermore, the variability in the maximum length the WGA-HRP-labeled axons extended caudal to the lesion indicates that these are indeed regenerated axons (Figure 2). Uncut, spared axons would be expected to extend throughout the spinal cord caudal to the injury. Two of the six immunized mice in which regeneration failed had cavitations at the lesion site and two mice had well-formed scars. In contrast to the immunized mice, no WGA-HRP-labeled axons were seen caudal to the lesion in nine control mice injected only with IFA. The depth of the lesion and the characteristics of the scar as judged by the neutral red staining was similar in both the immunized and control groups. Of all the animals used for anterograde tracing, only one control mouse had an incomplete lesion. In this mouse, a small number of axons in the ventralmost portion of the tract were not lesioned and could be seen extending straight across, just ventral to the lesion without making a kink-like displacement. This mouse was therefore not used for any further analysis. The percentage of control mice showing moderate to severe scarring was similar to that seen in immunized mice (~60%). Only one of the control mice showed lesion-induced cavitations. As an additional control, mice were immunized with mouse liver homogenate in IFA (n = 4). Three weeks after spinal cord dorsal hemisection, WGA-HRP injections into the sensory–motor cortex of these mice did not result in any labeling of corticospinal tract axons caudal to the lesion (Figure 1I), indicating the specificity of the myelin-rich spinal cord homogenate in stimulating axon regeneration. To further confirm that the WGA-HRP-labeled axons that crossed the lesion in the immunized mice were indeed lesioned axons that had regenerated and not ones that might have been spared, retrograde double labeling studies were carried out on a separate group of animals.

Regeneration of Corticospinal Fibers Assessed by Retrograde Labeling

Since myelin-associated inhibitors have been reported to play a crucial role in the failure of CNS regeneration, we compared the effects of spinal cord homogenate (n = 6) with myelin purified from either mouse (n = 12) or bovine (n = 4) CNS. The same immunization and lesioning protocol as that described above was used in these experiments, and the retrograde tracer Fluorogold (Schmued and Fallon, 1986; Novikova et al., 1997) was injected at the site of lesion to label cut corticospinal tract axons. Our studies with the unlesioned corticospinal tract (n = 2) as well as previous work (Schmued and Fallon, 1986) indicate that following injection of this tracer into the white matter it is not taken up by axons of passage. Fluorogold can be taken up by synaptic terminals but injection of this tracer into the ventral gray matter at T9 without damage to the corticospinal tract resulted in only about 20% of the cortical motor neurons being labeled as compared to the labeling seen after lesion of the tract at the same level. Importantly, very few of the axons that have terminals at the T9 level project 6 mm caudally (see below). The neuronal labeling after injection into the terminal field was also much weaker than after injection into the lesioned tract. Taken together, these data indicate that Fluorogold reliably labels axotomized corticospinal neurons. Two weeks after dorsal hemisection, a second tracer (Fluororuby [Novikova et al., 1997] or cholera toxin subunit B [Tavares et al., 1996]) was injected directly into the corticospinal tract about 6 mm caudal to the lesion. These injections would thus cause damage to the fibers in the corticospinal tract. Animals were sacrificed 1 week later (i.e., total survival time of 3 weeks post lesion) and cryostat sections of the motor cortex examined for the presence of double-labeled neurons. As with the WGA-HRP labeling studies, 55% of the mice immunized with spinal cord homogenate or purified myelin showed good evidence of regeneration of corticospinal tract axons as revealed by the presence of double-labeled neurons in the sensory–motor cortex (Figures 1G, 1H, and 3). In mice that demonstrated strong evidence of axon regeneration, 50% ± 16% of the motor neurons were double labeled (i.e., regenerated neurons). In some mice, as high as 75% of the neurons were double labeled (Figure

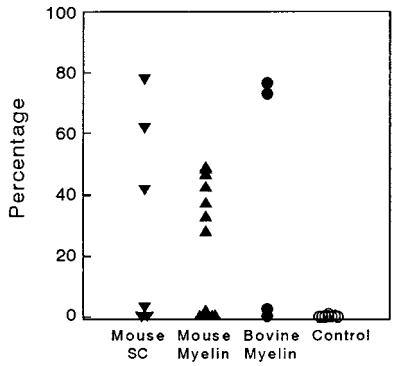

Figure 3. Percentage of Double-Labeled Neurons in the Motor Cortex Retrogradely Labeled with Fluorogold and Fluororuby

Data from four groups are shown. These include mice immunized with mouse spinal cord ("Mouse SC"), mouse myelin, and bovine myelin and controls injected with IFA. These data are an indication of the proportion of lesioned corticospinal tract neurons that regenerated their axons.

3). Previous studies have shown that injection of two fluorescent tracers into the terminal field of the corticospinal tract (ventral gray) in rats at about the same two spinal cord levels as that used here (without damage to the tract) results in only 1.6% of the cortical motor neurons being double labeled (Akinsegun and Buxton, 1992). We also obtained a low percentage of double-labeled neurons after similar injections in the mouse at T9 and 6 mm caudally (5%, n = 2). Therefore, if double labeling were to occur in the lesioned spinal cord of immunized mice via collaterals of uninjured axons, the numbers are not likely to be greater than 5%. The average of 50% and high of 75% of double-labeled neurons seen in the immunized mice, therefore, indicates a substantial degree of axon regeneration. Only 6% of the motor neurons in immunized mice were labeled with the second marker alone (i.e., unlesioned or spared neurons). In the majority of these animals (60%), less than 1% of the neurons were spared. Double-labeled neurons were rarely seen (0.19%) in control animals with spinal cord hemisection (n = 9) (Figure 3). There was no evidence that double labeling could have occurred by spreading and mixing of the two fluorescent tracers as confirmed by the wide separation of the two labels in longitudinal sections of the spinal cord (Figure 1J). The extent of diffusion of the two labels was also the same in immunized and control mice. These results provide strong evidence that injured corticospinal tract axons can regenerate in mice immunized with a homogenate of tissue rich in myelin or with purified CNS myelin in IFA.

Functional Recovery in Immunized Mice

Recovery of motor control of the hind limbs was assessed 3 weeks after lesioning in a group of mice immunized with mouse myelin or controls (injected with IFA only) using the contact placing test. Mice that showed anatomical evidence of axon regeneration also showed improvement in function. Contact placing of the hind limbs in response to light touch of the dorsal surface of the hind limbs, without joint displacement, was detected in 58% of the immunized mice but in none of the control mice. The contact placing response is dependent upon the integrity of the corticospinal tract (Kunkel-Bagden et al., 1993) and is widely used to assess motor control

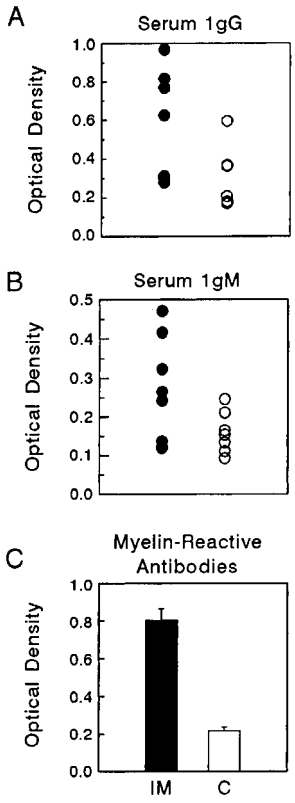

Figure 4. Serum Immunoglobulin

(A and B) Enzyme-linked immunosorbent assays show that the levels of IgG (A) and IgM (B) in sera of mice immunized with mouse spinal cord homogenate ("IM") is higher than in controls ("C").

(C) Sera from immunized mice also show strong myelin reactivity as compared to sera from controls.

of the hind limbs. These results indicate that anatomical regeneration led to functional recovery.

Myelin-Reactive Antibodies in the Serum and Spinal Cord of Immunized Mice

Higher levels of IgG and IgM were present in the serum of immunized mice than in controls (Figures 4A and 4B). Serum immunoglobulin in immunized mice reacted with myelin and spinal cord proteins as detected by ELISA (Figure 4C) and Western blotting (Figure 5A). Immunoglobulin was also detected by immunofluorescence in the lesioned spinal cord of both immunized and control mice. However, the staining in immunized mice was much stronger and extended for far longer distances (5 mm) on either side of the lesion compared to controls when examined 3 days after hemisection. Furthermore, immunoglobulin staining in immunized mice was especially prominent along the dorsal white matter, where it appeared to be localized to myelin (Figures 5B and 5C). The immunization protocol that we used resulted in the production of antibodies without stimulating inflammatory changes in the CNS. There was no evidence of infiltration of inflammatory cells or demyelination similar to that seen in experimental allergic encephalomyelitis (EAE) in Epon-embedded tissue sections of the spinal cord of immunized mice (Figures 5D and 5E).

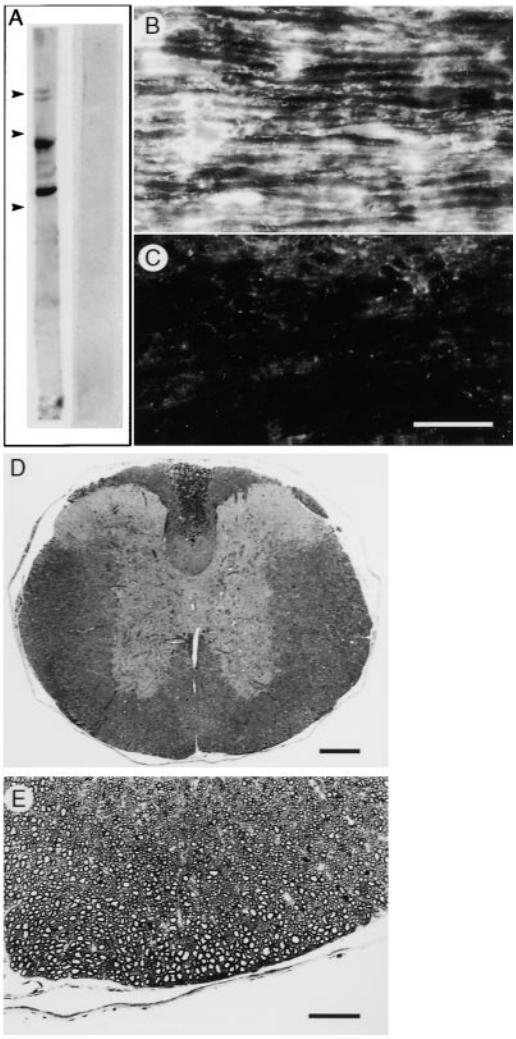

Figure 5. Binding of Antibodies to Spinal Cord and Lack of Inflammatory Cell Responses

(A) Western blot of spinal cord proteins showing an example of the binding of the sera from an immunized mouse (left lane) and a control mouse (right lane), both of which were sacrificed 3 weeks after spinal cord hemisection. The antibodies in the sera of immunized mice bound variably to multiple bands (not yet identified) as compared to controls. MW markers (arrowheads): 103, 76, and 49 kDa.

(B) Staining for mouse immunoglobulin in tissue sections of the spinal cord from a mouse immunized with mouse spinal cord homogenate in IFA and sacrificed 3 days after hemisection. Micrograph taken about 2 mm caudal to the lesion from an area through the dorsal column shows strong labeling of myelin.

(C) A similar area through the spinal cord of a control mouse taken 3 days after hemisection.

(D) Toluidine blue–stained Epon-embedded section of the cervical spinal cord of an immunized mouse shows no evidence of demyelination or infiltration of immune cells into the CNS or submeningeal space. Axonal degeneration is only seen in the fasciculus gracilis whose fibers were severed by the lower thoracic cord hemisection. This appears as an area of darker staining (due to the disruption of the myelin) in the midline of the dorsal columns.

(E) Higher magnification of ventral white matter shows no evidence of inflammatory changes.

Scale bars: 50 μm (B and C); 200 μm (D); and 100 μm (E).

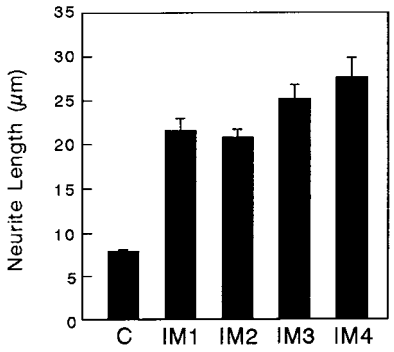

Figure 6. Antisera from Immunized Mice Neutralize Myelin-Associated Inhibition of Neurite Growth

(A) Antisera from immunized mice block the neurite growth inhibitory effects of CNS myelin. Neurite growth on myelin is significantly longer in wells treated with sera from immunized mice ("IM1" through "IM4") compared to control mice ("C"). Data represent the mean ± SEM from two separate experiments. The data from three control mice were pooled. The mean values for all immunized mice are significantly greater than for controls ($p \leq 0.05$); the difference between the mean values for "IM2" and "IM4" is also statistically significant ($p \leq 0.05$).

Antisera from Immunized Mice Are Able to Stimulate Neurite Growth on Myelin

Additional experiments were carried out to assess if the myelin-reactive antibodies in the serum of immunized mice were able to block the neurite growth inhibitory activity associated with CNS myelin. Tissue culture substrates coated with CNS myelin were incubated overnight with serum from the mice used for the retrograde double labeling studies described above. Neurite growth on these myelin substrates was assessed using purified neonatal rat cerebellar neurons. The length of the neurites on myelin treated with serum from control mice averaged about 7 μm. In contrast, sera from four mice immunized with mouse CNS myelin were able to significantly increase neurite growth on myelin. The average length of the neurites in wells treated with these four antisera ranged from 20–27 μm. Antisera from two immunized mice that were positive in the double labeling experiments showed the longest neurite growth (IM3 and IM4 in Figure 6). Antisera from the other two immunized mice (IM1 and IM2 in Figure 6), which were negative in the double labeling study, also blocked inhibitors associated with myelin but to a lesser extent. To further confirm that the serum effects were mediated by antibodies, IgGs and IgMs were immunodepleted from the serum (of mouse IM3) with goat anti-mouse IgG+IgM conjugated to agarose beads (Affi-Gel-10, BioRad). The depletion of the samples was effective as determined by ELISA (Figure 7A). The predepleted and depleted serum samples, as well as the antibodies eluted from the agarose beads, were used to incubate myelin-coated substrates prior to plating neurons for neurite growth assays as described above. Immunodepletion of the antibodies from the serum resulted in a loss of the ability of the serum from the immunized mouse to block the neurite growth inhibitory activity associated with myelin (Figure 7B). In addition, this neurite growth inhibitory activity could be blocked by the eluted antibodies (Figure 7B). These results indicate that the antibodies generated in the immunized mice are indeed able to block axon growth inhibitors associated with myelin.

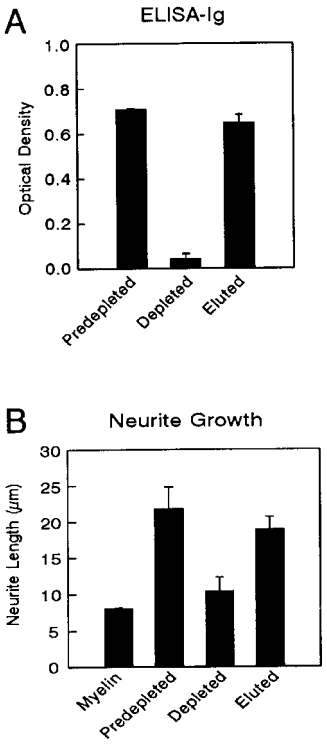

Figure 7. Antibodies Mediate the Blocking Effect of Serum

(A) ELISA showing the level of IgG and IgM in the serum of an immunized mouse ("Predepleted") and after immunodepletion ("Depleted") and the recovery of these antibodies after elution from the agarose beads ("Eluted"), as described in the Experimental Procedures.

(B) Histogram showing the effects of the samples shown in (A) on neurite growth on myelin. Compared to growth on untreated myelin-coated substrates ("Myelin"), the length of neurites is increased by incubating the myelin substrates with serum from the immunized mouse ("Predepleted"). Neurite growth is significantly reduced after depleting the antibodies ("Depleted"). Additionally, the length of neurite growth is increased by treating the myelin substrates with the eluted antibodies ("Eluted"). The differences between the myelin and the predepleted and eluted groups are statistically significant ($p \leq 0.05$). Mean $\pm$ SEM of two experiments.

Discussion

These experiments demonstrate that a simple therapeutic vaccine approach can be used to stimulate extensive regeneration of large numbers of axons of long fiber tracts after spinal cord injuries. The anterograde and retrograde labeling studies clearly demonstrate that injured axons had indeed regenerated. The anatomical evidence of regeneration was accompanied by recovery of hind limb motor control. Furthermore, the immunization protocol led to the production of myelin-reactive antibodies that were shown to be capable of neutralizing axon growth inhibitors present in CNS myelin.

The mice in the present study did not show any evidence of cellular inflammatory changes in the spinal cord similar to those seen in EAE. Induction of EAE in most rodents requires immunization with myelin proteins mixed with complete Freund's adjuvant often along with pertussis toxin (Traugott et al., 1985; Goverman et al., 1997). In fact, pretreatment of mice with myelin or myelin proteins in IFA has been shown to protect animals from EAE (O'Neill et al., 1992; Rivero et al., 1997; Tonegawa, 1997), which is a T cell–mediated disease. Protection by this and related types of treatment is thought to be due to clonal deletion and immune deviation of autoreactive T cells (Gaur et al., 1992; Weiner et al., 1994).

It was shown recently that adult rodent dorsal root ganglion cells microinjected into the adult rat CNS are able to grow axons for long distances along myelinated fiber tracts until they reach the site of a CNS lesion (Davies et al., 1997, 1999). It is therefore possible that myelin-derived inhibitors and astroglial proteoglycans may act primarily at the site of lesion, where rapid myelin breakdown leads to exposure of inhibitors (Tang et al., 1997) and also where astrocytes deposit proteoglycans (McKeon et al., 1991). Work by Schwab's group, however, suggests that some myelin-associated inhibitors act along the entire course of myelinated fiber tracts (Schwab and Schnell, 1991). Since we find that immunization with purified myelin is effective in stimulating regeneration, this effect could be due to blocking of myelin-associated inhibitors leading to axon regeneration across the lesion before the full expression of inhibitory proteoglycans occurs at the lesion site. On the other hand, we have evidence that some astrocyte-derived inhibitors copurify with myelin (unpublished data), and these molecules may also be blocked under our immunization conditions. Clearly, antibodies that were generated in the immunized mice were found to bind to myelin in the white matter after spinal cord hemisection. In addition, there was also much widespread antibody labeling at the lesion site in both the immunized and control mice (due to the direct effect of the lesion on the blood–brain barrier), but it is not known whether these antibodies in the immunized mice bound to specific components of the scar. Importantly, the sera from immunized mice were able to block the neurite growth inhibitory effects of CNS myelin in vitro. In addition, the immunodepletion studies indicate that the blocking effect of the serum was mediated by antibodies.

The reasons for the failure of regeneration in some of the immunized mice are not clear at present, but there are several possible explanations. One factor could be variability in the immune response. Second, two of the negative mice in the HRP labeling study had injury-induced cavitations near the lesion and two had well-formed scars. It is important to note, however, that the percentage of mice showing scarring and cavitation was similar in the immunized and control groups. For reasons that are not fully understood at present, lesion-induced cavitations do not occur often and as extensively after spinal cord lesions in mice (Li et al., 1996) as compared to rats (Neumann and Woolf, 1999). In addition to the presence of inhibitory proteoglycans that can prevent axon growth (McKeon et al., 1991; Davies et al., 1997), some scars produced by such penetrating lesions can contain a leptomeningeal–astroglial interface (glia limitans) (Li and David, 1996), which could provide a physical barrier to axon growth. Therefore, in addition to blocking myelin-associated inhibitors, efforts to control the fibrotic scar, to block inhibitory proteoglycans expressed in these regions, and to prevent tissue necrosis

resulting in cavitations will be required to achieve regeneration in a larger percentage of animals.

We have demonstrated that not only does the immunization procedure lead to increased levels of circulating myelin-reactive antibodies but that these antibodies cross the blood–brain barrier in the hemisected spinal cord and then bind to myelin in the white matter. Previous studies have shown that contusion injury to the adult rat spinal cord results in increased permeability of the blood–spinal cord barrier for about 30 mm on either side of the lesion (Popovich et al., 1996). The same study also showed a secondary increase in blood–spinal cord permeability specifically in the white matter at 14–28 days post injury (Popovich et al., 1996). In addition to the acute breakdown of the blood–brain/spinal cord barrier immediately after injury, at later stages this barrier may be opened by the action of microglia (Popovich et al., 1996; Jensen et al., 1997). Therefore, the myelin-reactive antibodies that are produced by our immunization procedure are likely to enter into the degenerating fiber pathways in the white matter as the blood–brain barrier is opened by the trauma and the action of activated microglia. These antibodies then bind to inhibitors associated with myelin and thus stimulate axon regeneration over long distances. Further evidence that this is likely to occur was provided by the in vitro experiments, which showed that the antisera from the immunized mice were indeed able to block the neurite growth inhibitory effects of CNS myelin.

In the present study, we have tested the effects of this immunization procedure on animals with acute spinal cord injuries. Animals with chronic or long-standing lesions will likely require additional manipulations to remove the scar and to induce the chronically lesioned neurons into a growth mode (Hagg et al., 1988; Tetzlaff et al., 1991; Ye and Houle, 1997). By immunizing with purified myelin that contains axon growth inhibitors we have demonstrated that a simple, noninvasive therapeutic vaccine approach can be used to stimulate axon regeneration after spinal cord injuries in adult mice. Application of such an approach in humans will require further refinement and testing of the immunogen. This will likely involve identification of many of the inhibitors associated with myelin and the scar, and the use of a cocktail of these inhibitors in purified or recombinant form as the vaccine. Alternatively, identifying the blocking antibodies could also lead to the development of passive immunization approaches to stimulate axon regeneration.

Experimental Procedures

Immunization and Spinal Cord Lesioning

Mouse and bovine CNS myelin were prepared as previously described (McKerracher et al., 1994; Li et al., 1996) and resuspended in phosphate buffered saline (PBS). Homogenates of adult BALB/c mouse spinal cord and liver were prepared in PBS. Eight- to ten-week-old female BALB/c mice were immunized twice weekly with 50 μg of either of these preparations emulsified in an equal volume of IFA (Gibco BRL) by subcutaneous injection in the region of the back. After 3 weeks, the mice were anesthetized with Somnitol (1 mg/20 g body weight), and a lower thoracic laminectomy was done (T9). The dorsal half of the spinal cord was then cut with a pair of microscissors to sever the corticospinal tracts. The depth of the lesion, which was about 0.5 mm, was estimated by a mark placed on the tip of the microscissors. After cutting the dorsal part of the spinal cord with the microscissors, the completeness of the lesion was further assured by passing a 0.2 mm thick microdissection knife (Fine Science Tools) across the cut dorsal half of the spinal cord at the same depth. Mice continued to receive twice weekly immunizations for another 3 weeks. After this 3 week survival period post lesion, the mice were anesthetized and a 5% solution (w/v) of WGA-HRP (Sigma) was injected into the sensory–motor cortex as described previously (Li et al., 1996). Forty-eight hours after injection of WGA-HRP, the animals were sacrificed by intracardiac perfusion, and longitudinal cryostat sections of the spinal cord were reacted for HRP histochemistry as described previously (Li et al., 1996).

Retrograde Neuronal Double Labeling

At the time of the spinal cord hemisection in 8- to 10-week-old female BALB/c mice, 0.5 μl of a 5% (w/v) solution of Fluorogold (Molecular Probes) was injected into the site of lesion. Two weeks later, the mice were anesthetized and a laminectomy was done about 6 mm caudal to the site of hemisection. A single injection of 0.1–0.2 μl of 25% (w/v) solution of Fluororuby (10,000 MW, lysine fixable; Molecular Probes) was injected directly into the corticospinal tract at this level with a micropipette in most of the mice. This injection would damage the fibers in the corticospinal tract. In two of the mice immunized with bovine myelin, 0.2 μl of a 1% solution of cholera toxin subunit B (List Biological Labs) was injected instead of Fluororuby. No differences were seen in the results using these two tracers. One week later (i.e., 3 weeks post lesion), animals were anesthetized and perfused with 4% paraformaldehyde. Cryostat sections (30 μm) through the sensory–motor cortex were examined with a Leitz fluorescence microscope. Tissue sections of mice injected with cholera toxin were processed for immunofluorescence detection. Counts of labeled neurons were made from every third tissue section, and only large neuronal profiles through the cell nucleus were counted. On average, about 1000 neurons were counted in each animal. Longitudinal sections of the spinal cord were also cut and examined with a fluorescence microscope to visualize the size and location of Fluorogold and Fluororuby injections.

Functional Testing

Contact placing response was tested by lightly touching the dorsal aspect of the hind limb without causing joint displacement. The ability of the animals to lift the foot and place it onto the support surface was then assessed in three to six repetitions. Greater than a 30% response was scored as positive (Kunkel-Bagden et al., 1993).

ELISA

Serum from immunized and control mice was collected prior to perfusion 3 weeks after hemisection. ELISA for serum IgG and IgM was carried out using a standard protocol with an isotyping kit from Southern Biotechnology Associates (Birmingham, AL). For binding to myelin, 10 μg of mouse myelin was plated onto poly-L-lysine-coated 96-well plates. After blocking the substrate with 3% bovine serum albumin (BSA), the wells were incubated with test sera at 1:40. Antibody binding was detected using a secondary antibody conjugated to alkaline phosphatase. Western blots were done as we have described previously (McKerracher et al., 1994).

Immunohistochemistry

Three days after spinal cord hemisection, immunized and control mice were perfused with 4% paraformaldehyde, and 10 μm thick longitudinal cryostat sections of the spinal cord were picked up on gelatin-coated glass slides. The sections were incubated with a biotinylated goat anti-mouse antibody (1:200) overnight and then with streptavidin-conjugated fluorescein (1:200) for 1 hr, to detect circulating antibodies that would have leaked into the spinal cord tissue due to lesion-induced breakdown of the blood–brain barrier.

Neurite Growth Assay

96-well plates were first coated with solubilized nitrocellulose and preincubated with 5 μg/ml poly-L-lysine (Sigma). Purified bovine CNS myelin (0.2 μg) in a 2 μl drop was placed in the center of these wells and incubated for 4 hr at 37°C. These wells were then incubated

overnight at 4°C with serum (1:50) from either control mice or mice immunized with mouse CNS myelin. The serum was removed, and postnatal day 10 rat cerebellar neurons purified by Percoll density gradient centrifugation were plated at a density of 1×10^5 cells per well (Li et al., 1996). The cells were cultured in serum-free chemically defined medium (Li et al., 1996) for 24 hr, fixed with 4% paraformaldehyde, and stained with Coomassie blue. Neurite length was measured in one or two wells from two separate experiments using a Universal Image I image analysis system. Data were analyzed using the Student-Newman-Keul's test to determine statistically significant differences.

Immunodepletion

Six milligrams of goat anti-mouse IgG+IgM (Jackson Immunoresearch) was conjugated to 2 ml of Affi-Gel-10 beads according to the manufacturer's instructions. IgGs and IgMs from the serum (40 μl diluted 1:25 in PBS) from an immunized mouse were immunodepleted by incubating with 1 ml of the goat anti-mouse antibody-conjugated Affi-Gel-10 beads for 18 hr at 4°C. The beads were centrifuged, and the supernatant consisting of the depleted sample was collected. The beads were loaded onto a column and washed in Tris buffered saline, and the bound antibodies were eluted with a low pH buffer (200 mM glycine [pH 2.5]) and immediately neutralized with Tris buffer (pH 11). The eluted antibodies were then dialyzed and concentrated. ELISAs were carried out on these samples to determine the extent of immunodepletion and recovery of eluted antibodies. These samples were then used to preincubate myelin-coated substrates used for neurite growth assays as described above. Neurite growth was quantified as noted above.

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