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Biochemical Characterization of the Axolemmal Mitogen for Cultured Schwann Cells

Mark A. DeCoster
madneuron1@gmail.com

School of Basic Health Sciences
Virginia Commonwealth University

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[redacted]
Director of Thesis

[redacted]
Committee Member

[redacted]
Committee Member

[redacted]
Committee Member

[redacted]

[redacted]

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BIOCHEMICAL CHARACTERIZATION OF THE AXOLEMMAL MITOGEN FOR CULTURED SCHWANN CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

Mark A. DeCoster

Bachelor of Science,

The College of William and Mary, 1985

Director: Dr. George H. DeVries

Professor, Department of Biochemistry

and Molecular Biophysics

Virginia Commonwealth University

Richmond, Virginia

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS:

AAT: adenine, aminopterin, thymidine

ABTS: 2,2' azino-di-3-ethylbenzothiazoline sulfonic acid

Ara C: cytosine arabinoside

ATCC: American Type Culture Collection

bFGF: basic fibroblast growth factor

BSA: bovine serum albumin

cAMP: 3',5'-cylcic adenylate

CFA: complete Freund's adjuvant

cGMP: cyclic guanosine monophospate

CNS: central nervous system

Con A: concanavalin A

DME: Dulbecco's modified Eagle medium

EDTA: ethylenediaminetetraacetate

EGF: epidermal growth factor

ELISA: enzyme linked immunosorbent assay

FITC: fluorescein isothiocyanate

FGF: fibroblast growth factor

GGF: glial growth factor

GMF: glia maturation factor

HE: DME + HEPES

HF: DME + 10% fetal calf serum

HEPES: N-2-Hydroxymethyl-piperazine-N'-2-ethanesulfonic acid

IBMX: 3-isobutyl-L-methyl-xanthine

IFA: incomplete Freund's adjuvant

IP: intraperitoneally

IV: intravenously

kDa: kilodalton

NGF: nerve growth factor

PBS: phosphate buffered saline

PDGF: platelet-derived growth factor

PHA: phytohaemagglutinin

PNS: peripheral nervous system

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TES: N-tris-hydroxy-methyl(methyl-2-aminoethane-sulfonic acid)

Tris: Tris(hydroxymethyl)aminomethane

TRITC: tetramethyl rhodamine isothiocyanate

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ABSTRACT

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Mark A. DeCoster

Virginia Commonwealth University

Dr. George H. DeVries

The molecule(s) involved at the axon plasma membrane (axolemma) which causes Schwann cells to proliferate has been investigated by three biochemical techniques: 1) Alkaline extraction of axolemma resulted in recovery of 95% of the mitogenic activity and 50% of the protein in the membrane-bound portion of the axolemma. 2) The axonal mitogen for Schwann cells may be associated with heparan sulfate proteoglycans at the axonal surface. Treatment of axolemma with heparitinase, which cleaves the glycosidic bonds of sulfated glycosaminoglycans, allowed mitogenic activity to be solubilized. 3) Treatment of axolemma with heparin, a highly sulfated glycosaminoglycan analogous to heparan sulfate, resulted in a soluble mitogenic extract which had a higher specific mitogenic activity than the starting material. The results of these biochemical treatments support the model of an axonal mitogen for Schwann cells which is positively charged and bound to the negatively charged portion of heparan sulfate proteoglycans.

A monoclonal antibody (1A5-2G3) was raised against the soluble mitogenic heparin extract of axolemma. The monoclonal antibody inhibited the mitogenicity of heparin extract as well as the mitogenicity of the starting axolemmal membrane. Non-specific monoclonal antibodies did not inhibit mitogenicity to as great an extent as 1A5-2G3. Mitogenic heparin extract was incubated with 1A5-2G3-coupled or non-specific antibody-coupled Sepharose. Sepharose coupled with 1A5-2G3 removed significantly more mitogenic activity from the heparin extract than did non-specific antibodies. Using immunoaffinity techniques with the monoclonal antibody and the soluble heparin extract should permit separation of the axolemmal mitogen from other components of the axon plasma membrane.

INTRODUCTION

The characteristic morphology of Schwann cells was first described by Theodor Schwann 150 years ago (Schwann, 1839). We now know that the Schwann cell is responsible for ensheathing and myelinating axons of the peripheral nervous system (PNS), allowing for rapid transmission of neuronal signals in the form of saltatory conduction. The Schwann cell also demonstrates a dramatic ability to remyelinate axons after peripheral nerve damage. Recruitment of the necessary Schwann cells needed during development and peripheral nerve repair is largely accomplished by proliferation of Schwann cells.

Current studies in our laboratory investigate the proliferative signals which the Schwann cell receives from the nervous system. In this introduction I will describe the proliferation of Schwann cells both in vivo and in vitro. The relevance of two different membrane mitogens (axolemma and myelin) for glial cells will be discussed. In addition, one of the few model systems for how membrane mitogens might work outside of the nervous system will be presented. This model system describes the mitogenic effect of bacterial membrane molecules on lymphocytes. Finally, the previous understanding of the axon as a

mitogen to Schwann cells at the molecular level will be described as a preface to the presentation of our work, which characterizes the molecule(s) involved in the axon plasma membrane in signalling Schwann cell proliferation.

PROLIFERATION OF THE SCHWANN CELL IN VIVO

The Schwann cell and neuron form a most intimate relationship in the peripheral nervous system: the main function of the Schwann cell being the support of the neuron by cytoplasmic ensheathment. This ensheathment is most extensive in the case of large axons; Schwann cells surround these axons with compacted layers of their specialized plasma membrane called myelin. The insulating properties of myelin allow for rapid transmission of neuronal signals in larger axons via saltatory conduction.

During embryogenesis the Schwann cell along with sensory and autonomic neurons derives from the neural crest. This origin of Schwann cells was supported by the early work of Harrison (1924) as well as the more recent work of Weston (1970) and LeDouarin (1982). Neural crest precursors migrate during the development of the peripheral nervous system. Tennyson (1965) demonstrated the presence of Schwann cells among the developing nerve cells of cranial and peripheral ganglia. As the peripheral nerve trunks form, Schwann cells migrate outward along axonal fasciculi. During this migration and subsequent to it,

Schwann cells proliferate (Asbury, 1967). Studies by Aguayo et al. (1976) showed that reducing the number of developing neurons in peripheral tissues also reduced Schwann cell number. This result indicated that the neuron may be a primary stimulus for Schwann cell proliferation during axonal outgrowth.

Initially, the limited number of Schwann cells surround many axons (Peters and Muir, 1959; Martin and Webster, 1973; Gamble and Breathnach, 1965). In the presence of these unensheathed axons, Schwann cell numbers increase greatly (Terry et al., 1974; Asbury, 1967). These proliferating Schwann cells segregate the axons (Peters and Muir, 1959; Cravioto, 1965), establishing a 1:1 relationship with the largest axons (those which will be myelinated). Schwann cell proliferation terminates as the cells become committed to myelin formation and the connective tissue of the peripheral nerve develops more fully.

The proliferation of Schwann cells during development of the peripheral nervous system is one of two types observed in vivo; Schwann cells also proliferate during Wallerian degeneration of a peripheral nerve (Abercrombie and Johnson, 1946; Abercrombie and Santler, 1957; Joseph, 1950; Bradley and Asbury, 1970). Earlier studies had shown that the progression of Schwann cell response to crush injury of a peripheral nerve included an increase in the number of Schwann cell nuclei distal to the site of injury

after 25 days, with a slight decline in number thereafter (Bradley and Asbury, 1970). The increase in Schwann cell number in response to injury of a peripheral nerve was found to be most extensive in myelinated nerves and least in thinly myelinated or unmyelinated nerves (Thomas, 1948; Joseph, 1950). More recently it has been shown that the increase in thymidine labelling of Schwann cell nuclei during Wallerian degeneration is greater in sciatic nerve (Bradley and Asbury, 1970; Friede and Johnstone, 1967) than in the unmyelinated cervical sympathetic trunk (Romine et al., 1976).

Pellegrino et al. (1980, 1985) have presented evidence that axonal stimulation of Schwann cell division is important during nerve regeneration. These experiments (Pellegrino and Spencer, 1985) demonstrated that the arrival of a regenerating axon in vivo caused Schwann cells to undergo mitosis, presumably in a similar fashion to the Schwann cell contact with neurites regenerating from explanted dorsal root ganglia (Salzer et al., 1980). The experimental model used by Pellegrino and Spencer (1985) showed that the appearance of myelin formation in the regenerating nerve could be delayed by use of Mitomycin C, an antimitotic drug known to arrest Schwann cell division (Hall and Gregson, 1977; Lown, 1979). The effect of Mitomycin C appeared to be reversible however, since after long enough periods of time, myelin formation would appear in treated nerves. These results suggested that Schwann

cell proliferation may be necessary for myelination to occur. This interpretation may be more complicated, however, since Pellegrino and Spencer (1985) observed poor axonal regeneration in Mitomycin C-treated nerves.

From this discussion it can be seen that there are two situations in which Schwann cell proliferation has been observed *in vivo*. In the first case, proliferation occurs in association with the axon during development of the nervous system. Secondly, in cases of Wallerian degeneration in the peripheral nervous system, the breakdown of myelinated nerves signals the Schwann cell to proliferate. This process is followed by introduction of regenerating axons to Schwann cells as damaged peripheral nerves extend processes. Schwann cell contact with regenerating axons also causes Schwann cell proliferation, allowing for the completion of nerve repair, including the production of myelin.

It is the *in vivo* observations reviewed above which allowed a general description of the proliferative stages of Schwann cells. However, specific biochemical characterization of the proliferative response of Schwann cells to axons and to myelin has required the development of tissue culture techniques. I will review these techniques below, and indicate how they have brought us to the point of isolating and characterizing the axolemmal mitogen for Schwann cells.

PROLIFERATION OF THE SCHWANN CELL IN VITRO

The ability to identify Schwann cell mitogens using an *in vitro* system is possible in part because Schwann cells in culture proliferate at very low levels until stimulated with mitogens. Even in serum containing media, rat Schwann cells in culture are quiescent when isolated (Wood, 1976; Brockes et al., 1979; Salzer et al., 1980).

As *in vivo*, when Schwann cells in culture are in contact with neurons they proliferate (Wood, 1976; Salzer et al., 1980, 1980a). Membrane fragments from the axonal plasma membrane (axolemma) derived from cultured neurons are mitogenic to Schwann cells *in vitro* (Salzer et al., 1980a, 1980b), indicating that a viable neuron is not necessary for axolemmal mitogenicity to be communicated to the Schwann cell. The studies of Salzer et al. (1980a) further showed that both trypsin and heat treatment could destroy the mitogenicity of neurite membranes to Schwann cells, implicating the protein nature of the neurite mitogen. In addition, axolemmal fragments from PC12 pheochromocytoma cells (Ratner et al., 1984), brain (DeVries et al., 1982; Cassel et al., 1982; Sobue et al., 1983), and cerebellar granule cells (Mason et al., 1989) are mitogenic to Schwann cells, indicating that targeted mitogens for Schwann cells are not restricted to the peripheral nervous system (PNS).

Schwann cells will also proliferate in culture in response to myelin fragments (Cassel et al., 1982; DeVries et al., 1982; Yoshino et al., 1984). Whereas the axolemmal mitogenic signal is relevant during development, myelin may serve as a mitogenic signal during Wallerian degeneration.

A number of soluble factors have been identified as mitogenic to cultured Schwann cells. The protein glial growth factor (GGF), obtained from brain and pituitary (Raff et al., 1978; Brockes et al., 1980; Lemke and Brockes, 1984) causes Schwann cell proliferation. The activities of GGF from both bovine brain and pituitary are recovered from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 31,000 daltons and have been shown to be basic in nature (Brockes, 1985). Glia maturation factor (GMF) (Bosch et al., 1984; Lim et al., 1985), another protein purified from brain, is weakly mitogenic to Schwann cells. GMF has been purified from bovine brains to apparent homogeneity with a molecular weight of 14,000 daltons and an isoelectric point of pH 5.2 (Lim et al., 1985). It is also known that agents which increase intracellular 3',5'-cyclic adenylate (cAMP) such as cholera toxin, 3-isobutyl-L-methyl-xanthine (IBMX), dibutyryl cyclic AMP and forskolin, are mitogenic to Schwann cells (Raff et al., 1978, 1978a; Porter et al., 1986). Finally, extracellular components such as laminin (McGarvey et al., 1984) and fibronectin (Baron-Van Evercooren et al., 1982) promote Schwann cell

proliferation. Porter et al. (1987) showed that the effect of laminin and fibronectin could be due to an autocrine growth factor. Bunge has recently proposed that an autocrine growth factor made by Schwann cells could bind to the surface of axons, which would then present a suitable proliferative substrate for the Schwann cells (Bunge, 1987).

Although the above discussion of mitogens to Schwann cells may give the impression that many factors stimulate Schwann cell proliferation this is not the case; many known mitogens for other cell types fail to cause Schwann cell proliferation. Concanavalin A, phytohemagglutinin, nerve growth factor (NGF), epidermal growth factor (EGF), acetylcholine, norepinephrine, insulin, proinsulin, dexamethasone, prostaglandin $PGF_{2\alpha}$, the calcium ionophore A23187, arachidonic acid, ouabain, tubulin and trypsin were tested by Salzer and Bunge (1980) for mitogenicity toward Schwann cells. None of these substances were mitogenic to Schwann cells. Raff et al. (1978) tested luteotropic hormone, follicle-stimulating hormone, thyroid-stimulating hormone, growth hormone, adrenocorticotropic hormone, vasopressin, prolactin, luteinizing hormone, prostaglandin E_1, and isoproterenol at concentrations up to 50 μg/ml and found no mitogenic effect on Schwann cells. DeVries et al. (1983) added gangliosides (mixed, from bovine brain), heparin, cyclic guanosine monophosphate (cGMP), myelin basic protein, and rat liver microsomes to Schwann cells

and found none of these substances mitogenic. Salzer et al. (1980b) found that the particulate fractions of fibroblasts (3T3 cells), and several neuroblastomas were not mitogenic to Schwann cells.

Recently, Ratner et al. (1988) have shown that embryonic rat brain membranes are mitogenic to Schwann cells. Ratner et al. also showed that basic fibroblast growth factor (bFGF) is mitogenic to Schwann cells in contrast to the studies of Salzer and Bunge (1980), who found that fibroblast growth factor (FGF) was not mitogenic to Schwann cells. Ratner et al. (1988) found that bFGF caused approximately 10 times less maximal proliferation of Schwann cells than a neuronal source, using a maximal dose of bFGF at 80 ng/ml. Salzer and Bunge (1980) however, found that FGF at doses as high as 10 μg/ml caused no Schwann cell proliferation.

The above discussion demonstrates that the in vitro studies of Schwann cell mitogens support observations made in vivo. Schwann cells undergo proliferation during two general situations in the peripheral nervous system: 1) during development in response to axonal contact and 2) during Wallerian degeneration and the events consequent to it (release of myelin fragments, which are mitogenic to Schwann cells in vitro and stimulus from the regrowing axon). In addition, tissue culture studies have revealed the mitogenic effect of soluble growth factors such as GGF and GMF on Schwann cells; the role of these growth factors

during normal development of the nervous system or in cases of injury is not yet clear.

RELEVANCE OF MEMBRANE MITOGENS FOR GLIAL CELLS

Mitogens for Schwann cells are isolated from two sources in our laboratory: myelin and axolemma. Although the work in this thesis describes isolation and characterization of the axolemmal mitogen for Schwann cells, I will review our current understanding of the myelin mitogen for Schwann cells, and why it appears distinct from the axolemmal mitogen.

As described above, myelin is a relevant mitogen for Schwann cells during degenerative states such as Wallerian degeneration. Since myelin is formed by the Schwann cell, it serves as a mitogenic signal after development is complete, when fully myelinated axons are injured.

The work of Beuche and Friede (1984) demonstrated the importance of macrophages in removing myelin debris from sites of nerve degeneration. The work of Yoshino et al. (1984) showed that the mitogenicity of axolemma and myelin to cultured Schwann cells was distinct: the shape of the dose response curve for [^{3}H]thymidine incorporation by Schwann cells in response to axolemma and myelin was different, and the lysosomal inhibitor ammonium chloride blocked the mitogenicity of myelin, while not affecting the mitogenicity of axolemma to Schwann cells.

Bigbee et al. (1987) found that macrophages were present in Schwann cell cultures isolated by the method of Brockes et al. (1979). Baichwal et al. (1988) demonstrated that conditioned medium from cultured peritoneal macrophages which had phagocytosed myelin was mitogenic to Schwann cells. Conditioned media from macrophage cultures given axolemma was not mitogenic to Schwann cells. Recent results (Baichwal and DeVries, Science, submitted) indicate that the mitogenicity of myelin processed by macrophages may be related to myelin basic protein.

The mitogenicity of myelin to Schwann cells seems to be unique in a number of respects. First, as a product of the glial cell, myelin will serve as a proliferative signal after development is complete and axons are already fully ensheathed; the primary role of the axonal mitogen seems to be during development. Second, the mitogenicity of myelin to Schwann cells is mediated by macrophages, and may be due to a polypeptide derived from the degradation of myelin basic protein; the mitogenicity of axolemma to Schwann cells is not mediated by macrophages.

It is of further interest to note that whereas axolemma can stimulate oligodendrocytes to proliferate (Chen and DeVries, 1989) as well as Schwann cells, myelin membranes do not cause oligodendrocytes to proliferate (Chen and DeVries, 1989). The differential response of oligodendrocytes and Schwann cells to myelin may be due to the absence and presence of macrophages in the respective

cultures; this may also correlate with the observations in vivo: glial proliferation and repair in response to nerve damage in the central nervous system (CNS) is much less extensive than is the case for the peripheral nervous system.

A recent observation of Schwann cell proliferation in culture indicates that these cells may make an autocrine growth factor (Porter et al., 1987). Bunge proposes (Bunge, 1987) that the axon surface may serve as a substrate to which an autocrine growth factor may bind. The axon may be an essential substrate for the autocrine growth factor to transmit its mitogenic signal to the Schwann cell. If the idea of the axon as a substrate for an autocrine growth factor is true, similar arguments could be made to explain why axolemma is mitogenic to oligodendrocytes. Another possibility is that an autocrine growth factor for glial cells might be related to myelin basic protein. As described above, recent results from our laboratory indicate that the mitogenicity of myelin to Schwann cells mediated by macrophages may be due to the presence of myelin basic protein. Since myelin is a product of glial cells, similarities between myelin proteins and an autocrine factor may exist. The myelin mitogen and autocrine factor are thus distinct from an axolemmal mitogen: myelin and autocrine factors are products of the glial cell; an axolemmal mitogen for glial cells would presumably be produced by the neuron, which

would then be an appropriate signal for glial proliferation.

Having discussed the relevance of other membrane mitogens for glial cells, I will now concentrate on the mitogen studied in this thesis: the axonal surface as a mitogen for glial cells. As already mentioned, the axon surface provides a mitogenic signal to Schwann cells during development, and may also do so during nerve regeneration. A similar situation most likely exists for oligodendrocytes, at least in vitro. We now know that direct contact with axons is required for oligodendrocytes to proliferate in culture (Wood and Bunge, 1986), and that axolemma fragments are also mitogenic to these cells (Chen and DeVries, 1989). Why axonal damage leads to Schwann cell proliferation and remyelination but not a similar response from oligodendrocytes is not yet clear.

The relevance of an axonal plasma membrane mitogen for glial cells is clear. During ensheathment of axons by glia during development, too few cells exist to organize all of the axons. Axonal stimulation of glial cell proliferation might then be a signal of recruitment for glial cells to complete the organizational ensheathment of axons.

In the peripheral nervous system, commitment of one Schwann cell to one axon leads to differentiation (myelin formation) with the concomitant cessation of Schwann cell proliferation. One or more axons may be ensheathed by Schwann cells not forming myelin, but this too may be a

commitment to differentiation and thus cessation of cell proliferation.

A similar concept of the axon as a stimulus for glial proliferation in the CNS is complicated by the fact that oligodendrocytes myelinate many axons (Bunge, 1968; Peters et al., 1976). The axonal surface may present a stimulus for recruiting more oligodendrocytes to help organize the central nervous system. However, commitment of an oligodendrocyte to myelination of one axon may not prevent it from proliferating (Skoff et al., 1976). Furthermore, the importance of an axonal signal for oligodendrocyte proliferation in regulating CNS organization may be mediated by astrocytes. Astrocytic processes subdivide neurite bundles, and may serve as a supportive framework for oligodendroglial differentiation (Webster et al., 1981). Astrocytes also express bFGF (Ferrara et al., 1988) which is mitogenic to oligodendrocytes (Eccleston and Silberber, 1985); thus, astrocyte-derived bFGF could play an important role in the development and differentiation of oligodendrocytes, and in the synthesis of the myelin sheath.

Another complexity in the ability for the axon to stimulate glial cell proliferation lies in its apparent lack of specificity between the CNS and PNS. Axonal sources from the PNS (DeVries et al., 1982) and CNS (DeVries et al., 1982; Ratner et al., 1987) both cause Schwann cell proliferation. Both CNS (Chen and DeVries,

1989) and PNS (Wood and Williams, 1984; Wood and Bunge, 1986) neuronal sources are mitogenic to oligodendrocytes. Further, granule cell neurons from the cerebellum cause Schwann cells to proliferate but not oligodendrocytes (Mason et al., 1989a). Granule cells are not ensheathed by any glia once development is complete, yet the type of mitogenic signal that they provide to Schwann cells appears similar to that of myelinated neurons (Mason et al., 1989).

Less well understood is the role that the surface of the axon may have in causing glial proliferation in regenerative situations. It can easily be envisioned that the bare axonal surface of regenerating axons may provide a mitogenic signal to Schwann cells similar to the signal encountered during development. Pellegrino and Spencer (1985) propose that regrowing axons do indeed provide a mitogenic signal to Schwann cells. The work of Brockes (1985) suggests that Schwann cells may require axonal signals to proliferate in blastema formation of amphibian limb regeneration. The blastema is a growth cone composed of undifferentiated cells which arises at the site of amputation. The amputated limb is regenerated by proliferation and morphogenesis of the blastemal cells. In amphibian limb regeneration, peripheral nerves appear to stimulate the division of blastemal cells. The work of Brockes (1985) indicates that this peripheral nerve supply may provide a source of cells for the blastema from the Schwann cells of the nerve sheath.

In light of our limited understanding of nerve regeneration we can only make general statements about the observations that appear to hold true. The peripheral nervous system responds to injury with a substantial degree of remyelination by the Schwann cell; there appear to be few circumstances where deficiency of Schwann cells contributes to the failure of remyelination after a peripheral demyelinating event (Bunge, 1982). In contrast to the PNS, the CNS, particularly as characterized in multiple sclerosis, fails to remyelinate sufficiently for full recovery of function. What prevents remyelination after injury in the CNS remains unclear.

It is clear from the above discussion that the surface of the axon provides a proliferative signal for glial cells which support neurons. This proliferative signal is developmentally relevant in that it recruits the necessary glial cells for ensheathment and organization of axons. The bare surface of the axon may also present a proliferative signal to glial cells during regenerative events.

The molecular identity of the axonal mitogen for glial cells is not known. Considering the close apposition between glial cells and axons, the mitogenic signals communicated to glia may be novel in character. As an introduction to the possible molecular characteristics of the axonal membrane-bound mitogen for glia, I will review

the few model systems that exist for membrane mitogens outside the nervous system.

MODEL SYSTEMS FOR MEMBRANE MITOGENS (NON-NEURONAL)

Mitogenic signaling via the close apposition of glial cell and axon is one type of mitogenic membrane system already described. One of the few cases of membrane mitogens outside of the nervous system exists for lymphocytes. A review of membrane mitogens for lymphocytes will show that even for these cells, little is known about the molecular identity or mechanism of action of membrane molecules causing proliferation.

Concanavalin A (Con A) is a well known plant lectin that is mitogenic to lymphocytes. Many investigators use this lectin in the soluble form to study lymphocyte proliferation. Studies by Kazura et al. (1979) showed however, that Con A-pretreated human monocytes would bind human lymphocytes and cause the mitogenic transformation of these lymphocytes in the absence of soluble Con A. This result implied a similar role for lymphocyte-monocyte interaction as the work of Rosenstreich et al. (1976) who showed that a purified guinea-pig T-cell population proliferates in response to phytohaemagglutinin (PHA) or Con A only when reconstituted with macrophages. These results added to the increasing evidence that the monocyte-macrophage is necessary for optimal antigen- and mitogen-

induced lymphocyte transformation (Cline and Swett, 1968; Hedfors et al., 1975; Lohrmann et al., 1974; Seeger and Oppenheim, 1970). The monocyte thus serves as a substrate for mitogens which are then presented to lymphocytes, causing the lymphocytes to proliferate. The monocyte as a substrate for mitogenic PHA and Con A which then is presented as a mitogenic signal to the lymphocyte could be looked upon as a model similar to that outlined by Bunge (1987) for the axon and glial cell: an autocrine growth factor released by the Schwann cell requires presentation by the axon for the mitogenic signal to be effectively transmitted to the Schwann cell.

Takada et al. (1981) demonstrated that cytoplasmic membranes of Staphylococcus aureus L-forms were mitogenic to human lymphocytes. The membrane mitogen(s) was characterized as resistant to trypsin, although it was partially solubilized by this treatment. Mitogenic activity of the Staphylococcus aureus membranes to human lymphocytes was not extracted with chloroform-methanol, though the chloroform-methanol soluble fraction was strongly mitogenic to murine splenocytes. Previous work by Takada et al. (1980) had shown that the mitogenicity of membranes from the same source as above for B-cells of mice and guinea pigs was resistant to heating at 100^{o} C for 10 min. These results indicated that the mitogenic activity of Staphylococcus aureus membranes may be due to a lipid or carbohydrate molecule. Takada et al. (1981) theorized that

the membrane mitogen(s) they characterized was similar to the mycoplasma mitogens acting on B-cells of mice and rats described by Naot et al. (1977, 1978, 1979). The molecular identity of these mitogens is not known.

A membrane mitogen from Escherichia coli which causes human B lymphocyte proliferation has recently been described (Ponniah et al., 1989) in greater molecular detail than the mitogens discussed above. Escherichia coli is known to produce a mannose-sensitive adhesin molecule that has been shown to be associated with type 1 fimbriae expressed at the surface of the bacteria (Salit and Gotschlich, 1977; Ofek and Beachey, 1978). These adhesin-bearing fimbriae mediate specific attachment of E. coli to mannose-containing receptors on the surface of human epithelial cells, macrophages, and polymorphonuclear cells (Ofek et al., 1977; Bar-Shavit et al., 1977). The work of Ponniah et al. (1989) suggests that the adhesin molecule stimulates a mitogenic response in B cells, and that other structural components of the fimbriae are responsible for evoking a mitogenic response in T cells. Various reports suggest that the adhesin may consist of a combination of two proteins: the 29-kiloDalton (kDa) product of the fimH gene and the 14-kDa product of the fimG gene (Klemm and Christen, 1987; Abraham et al., 1987; Maurer and Orndorff, 1985). More recent results indicate however, that the adhesive function might be due solely to the 29-kDa protein

(Maurer and Orndorff, 1987; Hanson and Brinton, 1988; Abraham et al., 1988).

From the above discussion we can gain an appreciation of the state of understanding about membrane mitogens outside the nervous system: activities have been observed for a number of membranes on different cells, but molecular identities and mechanisms for these mitogenic actions are yet to be elucidated. At least for the case of adhesins in E. coli, and their mitogenic activity to lymphocytes, molecular understanding is clearer: specific gene products have been isolated as likely mediators in the mitogenic signal. It is of interest to note that a general theme appears important when considering membrane-bound mitogens: recognition is essential for proliferation. The binding of bacterial membranes to lymphocytes, possibly by the adhesin molecules described above, appears to be required for recognition of these membranes as mitogenic to lymphocytes. Binding of axolemma membranes to Schwann cells also correlates with the mitotic response to these membranes (Sobue and Pleasure, 1985). Once again, recognition leads to proliferation. Thus, understanding the identity and mode of action of membrane mitogens outside the nervous system may help us elucidate signals transduced by glial-axcn association. Below I will review the molecular understanding of axolemma as a membrane mitogen for glia which led to the work in this thesis.

RATIONALE OF THE PROJECT

Although Schwann cells normally function in the peripheral nervous system, an abundant source of mitogenic activity for Schwann cells has been found in adult rat brain axolemma (Yoshino et al., 1984). The molecular identity of the axolemmal membrane mitogen for Schwann cells is not known. To further understand the interaction between Schwann cell and axon we thus continued previous studies in our laboratory using this axolemmal membrane.

DeVries et al. (1982) showed that the mitogenic effect of axolemma is heat and trypsin labile, suggesting that the mitogen may be a protein. The work of Cassel et al. (1982) supported the idea that the axolemmal mitogen for Schwann cells is a protein, and this work indicated that the protein may be integrated in the axonal membrane; attempts to dissociate the mitogenic activity from the membrane were variable. Salzer et al. (1980a) showed that after treating neurites in culture with trypsin, loss of mitogenicity to Schwann cells due to the enzymatic treatment could be recovered with time. This indicated that the neurites were able to regenerate the mitogenic signal after trypsin treatment; the process appeared to require 1-2 d. The work of Sobue and Pleasure (1985) demonstrated that the adhesion of axolemmal fragments to Schwann cells was closely linked to axolemmal induction of Schwann cell mitosis. This result showed that axolemmal fragments, like neurites

(Salzer et al., 1980a), require contact with the Schwann cell to transmit a mitogenic signal. Studies in our laboratory (Meador-Woodruff et al., 1985) further supported the idea that the mitogenicity of axolemma may be transduced at the Schwann cell surface.

The molecular architecture of the neuronal surface necessary for mitogenicity to Schwann cells began to be investigated by Ratner et al. (1985). Ratner et al. (1985) demonstrated that blocking proteoglycan synthesis in neurites destroys the mitogenic effect of neurites towards Schwann cells. Treatment of neurites with heparitinase, an enzyme which cleaves the glycosidic bonds of sulfated glycosaminoglycans, also depleted the mitogenicity of the treated neurites. In addition, membranes prepared from neurons treated as above were also depleted in mitogenic activity to Schwann cells. However, Ratner et al. (1986) showed that castanospermine, which specifically blocks essential steps in the processing of asparagine-linked oligosaccharides, does not affect the ability of axons to stimulate Schwann cell proliferation. These results suggested that the neuronal mitogen may be associated with sulfated proteoglycans at the membrane surface of the axon; presentation of the mitogenic signal to Schwann cells required this proteoglycan architecture. Recent results by Ratner et al. (1988) demonstrated that mitogenic activity for Schwann cells could be extracted from embryonic brain tissue, and that this mitogenic activity bound to heparin.

The molecular identity of this mitogenic factor is not yet known, but it does not appear to be bFGF (Ratner et al., 1988).

SUMMARY

The mitogenic signal which the axon transmits to the Schwann cell is relevant *in vivo* during development and during the regeneration of peripheral nerves. Mitogenic signals from the axon to the Schwann cell recruit the necessary glia needed for ensheathment and myelination of nerves during the early organization of the nervous system. The regrowth of unensheathed axons after peripheral nerve damage presumably provides similar proliferative signals that permit the extraordinary regenerative capacity of Schwann cells.

Tissue culture techniques have allowed initial biochemical characterization of the proliferative signal contained on the axon for glial cells. The relevance of a mitogenic signal on the axonal surface would seem to exist for both PNS and CNS glia; *in vitro* experiments have begun to investigate proliferation in both systems.

The intimate relationship that requires glia to ensheath or myelinate axons may utilize novel proliferative signals. Many of the mitogens characterized to date are soluble in nature. Of the few cases of membranous mitogens

studied outside the nervous system, molecular identities and mechanisms of action are still unclear.

The molecular architecture required for axonal presentation of a mitogenic signal to glial cells has just begun to be investigated; the work of Ratner et al. (1985) has demonstrated the importance of proteoglycans in presentation of this signal to Schwann cells. Although the molecular identity of the axonal mitogen for Schwann cells is not known, previous studies had shown that the mitogen was heat and trypsin labile (DeVries et al., 1982) suggesting the protein nature of the mitogen. Bunge (1987) had suggested that the axonal surface might serve as a substrate to which a Schwann cell autocrine factor might bind; once bound to the axon, the autocrine factor would then present a mitogenic signal to Schwann cells. The most recent work of Ratner et al. (1988) suggested that an embryonic brain neuronal mitogen for Schwann cells was peripheral rather than intrinsic to the membrane, since mitogenic activity to Schwann cells could be bound to heparin. These studies have not provided an answer to what molecule(s) on the axon cause Schwann cell proliferation. The work in this thesis presents biochemical clues which unmask the identity of the axonal membrane mitogen for Schwann cells.

MATERIALS AND METHODS

--Preparation of Schwann Cell Culture:

Schwann cells were isolated from rat sciatic nerves by a modification of the method described by Brockes et al. (1979). The nerves were obtained from Sprague-Dawley rats aged 1-3 days. Rat pups were placed in an air-tight jar containing ethyl ether (for anesthesia). Each rat pup was anesthetized for approximately 4 minutes. Anesthetized rat pups were removed from the jar and decapitated. Rat pup bodies were pinned to a Styrofoam board in a horizontal laminar flow hood. Rat pup bodies were sprayed with 95% ethanol to remove surface bacteria. Dissections were carried out in the hood using an American Optical dissection microscope. As sciatic nerves were removed, they were placed in a sterile beaker containing approximately 10 ml of HE, and the nerves in the beaker were kept at room temperature in the hood during the dissection procedure. Dissection lasted no longer than 90 minutes. HE medium consisted of 90 parts Dulbecco's modified Eagle medium (DME), 10 parts sterile distilled water, 2 parts 25 mM N-2-Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer, and 0.1 parts Pen-

Strep. Careful efforts were made to remove sciatic nerves free of attached muscle.

Typically, 40-80 sciatic nerves were dissected per preparation. The pooled nerves were transferred by pasteur pipette to a 50 ml sterile flask. HE medium was removed from the flask by pasteur pipette without taking up nerves; 2.5 ml of HE was then added to the flask along with 300 µl of Serva collagenase (0.3%) which had been dissolved in HE. Collagenase treatment of the nerves was carried out in the flask in a water bath at 37° C while shaking for 15 min. Medium was removed from the flask by pasteur pipette, avoiding taking up intact sciatic nerves. HE and collagenase were added back to the flask for a second treatment as described above. Medium was again removed from the flask, avoiding taking up intact pieces of sciatic nerve. At this point small, dissociated pieces of nerve were removed with the medium, as these often consisted of muscle and connective tissue not associated with intact nerves.

HF medium was added to the flask of nerves in the amount of 3.5 ml. HF medium consisted of 90 parts DME, 10 parts fetal calf serum, 1.4 parts 7.5% $NaHCO_3$, and 0.1 parts Pen-Strep. Nerves were then triturated by pasteur pipette for 5-10 min, until HF medium became cloudy with released cells. An additional 3.5 ml of HF medium was added to the flask, and the above trituration step repeated. Triturated nerves were then passed through Nitex

209 polyamide nylon fiber to remove nerve pieces; medium containing cells was collected into a 15 ml centrifuge tube and centrifuged at 200 x g. HF medium was removed from the pelleted cells, and then 1 ml of HF added back to the centrifuge tube. Pelleted cells were resuspended in the 1 ml of HF by vortexing. Vortexing was continued until the cell pellet was uniformly dispersed.

Ten µl of the resuspended cells was used on a hemacytometer to calculate the cell density obtained. Cells were then plated onto 100 mm diameter pyrex petri dishes in 10 ml of HF at a cell density of approximately 3 x 10^6 cells/dish.

The cells were maintained in HF at 37° C in the presence of 10% CO_2. One day after plating, cytosine arabinoside (AraC), was added to the cell culture at a concentration of 1 x 10^{-5} M in order to reduce the fibroblast contamination. After three days of treatment, AraC was removed and 10 ml of HF added to the cultures.

Three days later, the last traces of fibroblast contamination were eliminated by complement mediated lysis using anti-Thy 1.1. The hybridoma clone TIB-103 was obtained by our lab from the American Type Culture Collection, and was grown in HF to produce anti-Thy 1.1 supernatant. Thy 1.1 has been shown to be an antigenic marker for fibroblasts, and can thus be used for complement mediated lysis of these cells.

Schwann cell cultures were treated with 10 ml of TIB-103 supernatant + 200 µl of Cappel Rabbit complement per dish. Rabbit complement was reconstituted from the lyophilized powder with 1 ml of sterile water/bottle; each lot of complement had been tested by manufacturer for the ability to lyse red blood cells. Reconstituted complement was stored at -20° C and reused only once to avoid inactivation by freezing-thawing. TIB-103 supernatant and complement remained on Schwann cell cultures for approximately 1 hour at 37° C in a 10% CO_2 incubator. The complement containing medium was then removed from treated cultures and each petri dish of Schwann cells treated with 5 ml of Saline I + 250 µl of 0.4% ethylenediaminetetraacetate (EDTA) to remove cells from the dish. Petri dishes were swirled for 5-10 min or until cells had lifted from the plate as observed by microscope. Cells were removed from each petri dish and put in a separate 15 ml centrifuge tube for each dish. Each dish was rinsed with 5 ml of HF and this medium added to the corresponding centrifuge tube for that dish. Each petri dish was examined by microscope to ensure cells were removed. Cells were then centrifuged in each tube at 200 x g for 5 min.

The supernatant from each tube was removed by pasteur pipette, with care being taken not to disturb the cell pellet. One ml of HF was added back to each centrifuge tube and the cell pellets resuspended via vortexing. Cell

densities were determined be counting 10 µl of the cell suspension from each tube in a hemacytometer. The cells in each tube were then diluted to 80,000 cells/ml by addition of HF to each tube. The cells from each tube were then aliquoted into separate 96 well microtiter plates. Each well received 100 µl (8,000 cells). Cells were maintained in 10% CO_2 incubators at 37° C for at least 1 day before the addition of mitogens.

--Isolation of Axolemma- and Myelin-Enriched Fractions:

Axolemma- and myelin-enriched fractions were prepared as described by Yoshino et al. (1984). Adult rat brainstem white matter was removed from decapitated Sprague Dawley rats. After removal from the brain, each brainstem was placed in a beaker of homogenizing medium on ice. Homogenizing medium consisted of 1 M sucrose, 150 mM NaCl, and 0.02% sodium azide in 10 mM N-tris-hydroxy-methyl(methyl-2-aminoethane-sulfonic acid) (TES), pH 7.4. After collecting all brainstems, homogenizing medium was drained, and the wet weight of brainstems determined. Brainstems were segregated into 1 gram portions. Each portion of brainstems was minced until homogeneous with a 75 mm microtome blade on a mincing plate on ice.

Each 1 gram portion of minced brainstem was transferred to a Dounce homogenizer with 37 ml of homogenizing medium. Tissue was homogenized by making 6

passes with the loose pestle (A), followed by 4 passes with the tight pestle (B). Each homogenate was transferred to a polyallomer centrifuge tube and using the SW27 rotor, centrifuged at 25,000 rpm (82,000 x g) for 20 min at 4° C. This procedure generated a floating pad of myelinated axons, a supernatant, and a pellet.

The floating pad was removed from the centrifuge tube by breaking the interface between tube and tissue by squeezing the tube gently and flipping the pad into a Dounce homogenizer with a prewetted spatula. Floating pads from up to 6 tubes were consolidated into one homogenizer and homogenized in 35 ml of 10 mM TES, pH 7.4, by making 10 passes with the tight pestle. This procedure was done to disrupt the myelinated axons osmotically and mechanically.

Sucrose gradients were prepared using 10% sucrose (w/w), 0.02% sodium azide in 10 mM TES, pH 7.4 and 40% sucrose (w/w), 0.02% sodium azide in 10 mM TES, pH 7.4. Continuous gradients were prepared using 34 ml of 10% sucrose and 17 ml of 40% sucrose for each gradient; gradients were collected into 38.5 ml cellulose tubes. One gradient was prepared for each gram of brainstem tissue isolated. Gradients were stored upright on ice during preparation of membranes.

Floating pad homogenates were layered evenly on top of each sucrose gradient. Approximately 3 ml of homogenate was applied to each gradient. The liquid level of each gradient tube was brought to about 2 ml from full by the

addition of 10 mM TES. Sucrose gradient tubes were loaded onto a SW27 rotor and centrifuged at 82,000 x g overnight at 4° C.

Two ml fractions were collected by puncturing the bottom of the gradient tubes. The sucrose density of each fraction was determined by refractometer. Axolemma fractions were assayed for acetylcholinesterase activity. Typically, axolemma was collected at sucrose densities of 28-32% and myelin collected at densities of 16-19%. Fractions pooled as axolemma were found to have the highest specific activity for acetylcholinesterase.

Pooled fractions were transferred to sterile Ti 35 centrifuge tubes. Centrifuge tubes were sterilized by spraying with 95% ethanol; the tubes were then allowed to dry in a sterile hood until all the ethanol had evaporated. No more than half of each tube was filled with a particular fraction; when a given fraction was greater than half the volume of the tube, the fraction was distributed to another centrifuge tube. Each centrifuge tube was then filled to the shoulder with sterile isotonic saline and inverted several times to mix the fraction and saline. This step served as a method for washing each fraction and removing azide. Ti 35 tubes were centrifuged for 1 hour at 70,000 x g at 4° C to obtain membrane pellets.

Supernatants were decanted from membrane pellets in a sterile hood using sterile pipets. Pellets were resuspended in sterile isotonic saline by trituration until

homogeneous, in as concentrated a form as possible. Resuspended pellets were aliquoted into sterile microfuge tubes, 500 µl of membrane added to each tube. Tubes were stored at -70° C and thawed before use. Membranes were not refrozen after thawing. Protein concentration of the isolated membranes was determined by the Bio-Rad method (Bradford, 1976). Typically, isolated axolemma fractions were resuspended at 2-10 mg/ml protein concentration.

Axolemma from bovine white matter was isolated as described by Detskey et al. (1988). Membranes were stored at -70° C until used. Membranes were not refrozen after thawing.

--Measurement of Proliferative Response:

The [^{3}H]-thymidine incorporation assay was carried out as described by Yoshino et al. (1984). After the Schwann cell cultures were treated with anti-Thy 1.1, (see above), the cells were plated in 96-well dishes at a density of 8,000 cells per well in 100 µl of HF. Membranes or chemical pretreatments were added to the Schwann cells 48 hr later to bring the total volume in each well to 100 µl. One day later 0.3 µCi of [^{3}H]-thymidine (15-16 Ci/mmole, NEN Research Products) in 25 µl of HF was added to each well.

The cells were harvested 48 hr after addition of the label. Media containing [^{3}H]-thymidine was removed from

the cells, and cells dissociated from the plate using 0.05% trypsin and 0.04% EDTA. Cells were collected on Skatron filtermats using a Titertek cell harvester. Two filters stacked on top of each other were used/per 96 well plate. Each set of 12 wells to be harvested was prewetted with 7 seconds of washing followed by 7 seconds of aspiration. Wash media for the cell harvester consisted of 1 part distilled water and 1 part isotonic saline. After the first set of filters for twelve wells was wetted, the cell harvester head was moved to the first twelve wells to be harvested; cells were transferred to filters by a 7 second wash followed by 7 seconds of aspiration. Subsequent wells were harvested in a similar manner. Filters were placed in 5 ml of complete counting cocktail in 5 ml liquid scintillation vials. The radioactivity on the filters was determined by use of a Beckman LS 5801 scintillation counter, using preset channel #1 (4 minutes/vial).

--Treatment of the Axolemmal Membrane:

Mitogenic activities of all treated and untreated axolemma preparations were calculated at 4 or more concentrations on the linear portion of the dose response curve to obtain a specific mitogenic activity, which was used to determine units of mitogenicity as well as enrichment of mitogenic activity.

Alkaline extraction: sodium carbonate/bicarbonate buffer (0.5 M, pH 9) was added to 1-2 mg of axolemma membrane to make the final solution 0.27 M in buffer. The mixture was incubated overnight at 4° C while stirring using a magnetic stir bar and subsequently centrifuged at 82,500 x g for 1 hr. The supernatant was removed from the tube and the pellet was resuspended in isotonic saline (0.9%) as a wash step to remove residual pH 9 buffer. The membrane was centrifuged as above, and supernatant discarded. The washed membrane pellet was resuspended in isotonic saline to a volume identical to the starting membrane volume. Membranes were stored at -70° C until used. Once thawed, membranes were not refrozen. pH 9 supernatants of axolemma were washed with isotonic saline using Amicon Centricon-10 microconcentrators. The pH 9 extract along with isotonic saline was centrifuged in the microconcentrators at 3000 x g for 30 min. at 4° C. Material retained above the filter (>10 kDa) was mixed with isotonic saline and centrifuged as above; this process was repeated 2 more times. Washed pH 9 extract was stored at 4° C and was not frozen. Typical protein concentration of the pH 9 extract was 1-5 mg/ml.

Heparitinase treatment: One unit of heparitinase (Sigma, St. Louis, MO; 0.1 units/µl) was added to axolemma (2-6 mg/ml) for 4 hr. All incubations were carried out in a final volume of 400 µl at 37° C while shaking, and incubations were terminated by centrifugation at 135,000 x

g in a Beckman airfuge for 1 hr to obtain a soluble fraction and an insoluble pellet.

Heparin treatment: Heparin (Sigma, from porcine intestinal mucosa) was added to axolemma for the times and concentrations indicated. Heparin was dissolved in sterile water and added in a volume of 100 µl . All incubations were carried out at 37° C in a volume of 400 µl while shaking. Incubations were terminated by centrifugation at 135,000 x g for 1 hr as above to obtain a soluble fraction and an insoluble pellet. Heparin-solubilized mitogenic extracts were heat inactivated by boiling at 100° C for 10 min.

--Gel Electrophoresis:

Sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 10% acrylamide gel by the method of Laemmli (1970) or in 11-23% Amersham precast gradient gels as indicated. Samples were loaded onto gels in volumes of 10-90 µl. Ten percent gels were run at constant current using 15 milliamps for 1.5 hours followed by 40 milliamps for an additional 1.5 hours or until tracking dye reached the gel front. Gradient gels were run at constant current using 15 milliamps for 20 minutes followed by 40 milliamps for 4 hours or until tracking dye reached the gel front. Proteins in the gel were visualized with a silver stain kit (Bio-Rad).

--Production of Monoclonal Antibodies:

Antibody-producing murine hybridomas were developed using the methods of Taggart and Samloff (1983) and monoclonals were isolated using limiting dilutions. One male RBF/Dn mouse was injected intraperitoneally (IP) with 60 μg (300 μl) of mitogenic soluble heparin extract emulsified in a 1:1 ratio (300 μl) of complete Freund's adjuvant (CFA). Nine days later the same mouse was injected (IP) with another 20 μg (100 μl) of heparin extract in CFA. After another 9 days the same mouse was injected (IP) with 28 μg of heparin extract (140 μl) in incomplete Freund's adjuvant (IFA). Twelve days later the mouse was injected (IP) with 30 μg (160 μl) of heparin extract in IFA. Twenty days later the mouse was injected intravenously (IV) with 20 μg (100 μl) of heparin extract in isotonic saline.

Three days later the immunized mouse was anesthetized by ethyl ether and serum was collected from the mouse by tail bleed. The spleen was removed from the mouse and placed in a petri dish containing wash media: (RPMI-1640). Excess fat was removed from the spleen, and the spleen tissue was forced through a stainless steel wire mesh screen with a 5 ml plastic syringe plunger. Dissociated tissue was collected in a 100 mm plastic petri dish containing 5 ml of wash media. Tissue was transferred to a

50 ml centrifuge tube; the petri dish was washed with media to collect residual spleen cells, bringing the total volume of the centrifuge tube to 35 ml. Cells were centrifuged for 10 min at 400 x g. Supernatant was removed from the pelleted cells. Five ml of Gey's solution (Shook and Schwartz, 1986) was added to spleen cells to lyse red blood cells. The cells were then mixed via vortexing. Cells were placed on ice for 5 min, and then centrifuged as above for 5 min. Spleen cells were washed three times with wash media by centrifugation as above. The number of viable spleen cells obtained was determined by trypan blue dye exclusion using a hemacytometer.

Spleen cells were combined with FOX-NY myeloma cells in a 2:1 ratio as described (Shook and Schwartz, 1986). Spleen cells and myeloma cells were fused using polyethylene glycol at 37° C. Fused cells were plated onto 96-well plates in 200 µl of (adenine, aminopterin, thymidine) (AAT)-selection media (Taggart and Samloff, 1983) at a density of 2.6 x 10^5 cells/well. Cells were maintained in 7% CO_2 at 37° C for 10-14 days until screened as described using enzyme linked immunosorbent assay (ELISA) techniques.

A second male RBF/Dn mouse was injected (IP) with 120 µg (600 µl) of mitogenic soluble heparin extract emulsified in complete Freund's adjuvant as above. Seven months later the same mouse was injected (IP) with 50 µg (250 µl) of heparin extract in complete Freund's. Ten days after this,

the mouse was injected (IV) with 20 µg (100 µl) of heparin extract in isotonic saline. Three days later immune serum was collected from the mouse and the spleen cells were fused with myeloma cells as described above.

--Enzyme Linked Immunosorbent Assay:

Enzyme linked immunosorbent assay (ELISA) was carried out using the general techniques outlined by Shook and Schwartz (1986) with modifications. The antigen was diluted to the indicated concentration in phosphate buffered saline (PBS) and delivered in 100 µl/well of a 96-well microelisa plate. The microelisa plate was incubated at 4° overnight. The following day, contents of the plate were emptied by inversion; as much of the liquid was removed as possible by shaking the plate over paper towels. Primary antibody samples were added in the amount of 100 µl/well. When dilution of the primary antibody was necessary, dilution was carried out using 1% bovine serum albumin (BSA) in PBS. After primary antibody was delivered to the wells, all wells were covered with 100 µl of 5% milk in PBS as a block against non-specific binding. Primary antibody remained on the plate for 1-2 hours. Antibody was removed by submerging the ELISA plate in a beaker of running tap water and then inverting the plate to remove liquid. This process was repeated approximately 5 times. Secondary antibody (goat anti-mouse, Boeringer-Mannheim)

conjugated to horseradish peroxidase was diluted 1:600 with 1% BSA in PBS and delivered in the amount of 100 µl to each well of the ELISA plate. To inhibit non-specific binding, 100 µl of 5% milk in PBS was added to each well as above. Secondary antibody remained on the plate for 1-2 hours. Antibody was washed from the plate using running tap water as described above. The extent of antibody reaction was then determined using the peroxidase substrate. Peroxidase substrate consisted of 1 part 40 mM ABTS (2,2' azino-di-3-ethylbenzothiazoline sulfonic acid) in distilled water, 19 parts citrate buffer (0.1 M citric acid + 0.1 M sodium citrate) at pH 4.0, and 20 parts 0.3% H_2O_2. The peroxidase substrate was delivered in the amount of 100 µl/well and allowed to react for 15-30 min. Optical density was then read at 405 nm using a Titertek Multiskan spectrophotometer.

--Pretreatment of Axolemma and Schwann Cells with 1A5-2G3:

1A5-2G3 hybridomas were grown in a defined medium (Chen and DeVries, 1989) consisting of the following components: Dulbecco's modified Eagle medium and Ham's F-12 1:1 with sodium bicarbonate, 1.2 g/L, and 15 mM HEPES buffer (pH 7.2); insulin (5 µg/ml); human transferrin (50 µg/ml); triiodothyronine (15 nM); sodium selinate (30 nM); hydrocortisone (20 µg/ml); dibutyryl cyclic AMP (1 µM); sodium pyruvate (110 µg/ml); and bovine serum albumin (1

mg/ml). Supernatants were collected and stored at -70° C. Dilutions of concentrated antibody were combined with axolemma and shaken using a Bellco shaker at 4° C for at least 1 hour. Antibody-axolemma mixture was added to Schwann cells and proliferative response measured as described above. For pretreatments with nonspecific IgM, identical methods were used with Sigma IgM κ (TEPC 183). IgM levels in both the 1A5-2G3 supernatants and Sigma IgM were compared using ELISA techniques so that the same amount of specific and non-specific IgM was used. In experiments where Schwann cells were pretreated with antibody, cells were exposed to antibody at the indicated concentration for 24 hours in a 10 % CO_2 incubator at 37° C. The media was subsequently removed from the cells and mitogens were added as described above.

--Antibody Affinity Column:

Monoclonal antibody 1A5-2G3 or Sigma IgM (TEPC 183) was conjugated to Pharmacia activated CH-Sepharose 4B as recommended by the product instructions. The Sepharose powder was suspended in 1 mM HCL and was washed for 15 min. with 1 mM HCL on a sintered glass filter; 1 gram of Sepharose powder produced approximately 3 ml of swollen gel. Approximately 200 ml of 1 mM HCL/g of Sepharose powder was used as a wash. Monoclonal antibody was equilibrated in coupling buffer ($NaHCO_3$, 0.1 M, pH 8.0,

containing NaCl, 0.5 M, 5 ml per gram of Sepharose powder) using Centricon 30 microconcentrators. The coupling buffer was exchanged with the media of the IgM supernatants by mixing the antibody with coupling buffer in the Centricon tubes and centrifuging the mixture at 3000 x g for 30 min. at 4° C. The material retained above the filter (>30 kDa) was mixed with coupling buffer and centrifuged as above. This process was carried out a third and final time; material retained above the filter was mixed with coupling buffer up to 5 ml and added to the Sepharose gel in a stoppered vessel. This mixture was gently shaken at 4° C overnight. Excess monoclonal antibody was washed away with coupling buffer and the remaining active groups on the sepharose gel were blocked with Tris(hydroxymethyl)aminomethane (Tris) -HCl buffer (0.1 M, pH 8) for 1 hour. The product was then washed with three cycles of alternating pH. Each cycle consisted of a wash (15 ml of each buffer/wash) at pH 4 (0.1 M acetate buffer containing 0.5 M NaCl) followed by a wash at pH 8 (0.1 M Tris buffer containing 0.5 M NaCl). The product was stored at 4° C in 0.1 M Tris buffer containing 0.5 M NaCl.

An affinity column was prepared as described above using monoclonal antibody 1A5-2G3. The Sepharose gel was packed into a 1 ml syringe with glass wool packed at the tip to prevent leakage of the gel. All samples were loaded in 250 µl volumes, and after allowing the meniscus of the liquid sample to enter completely into the sepharose gel,

the column was placed in a glass test tube and centrifuged at setting 1 on an IEC clinical centrifuge for 1 min at room temperature. After each centrifugation, samples eluted from the column were collected and saved for further analysis. Samples which differed by more than 1 pH unit (as determined by litmus paper) from the wash buffer (0.1 M Tris, 0.5 M NaCl, pH 8) were immediately adjusted to pH 6-8 with solid Tris.

Alternatively, resin coupled with 1A5-2G3, or Sigma IgM (TEPC 183) as a non-specific source, was shaken with mitogenic heparin extract overnight at 4^{o} C in a microfuge tube. The same amount of 1A5-2G3 and Sigma IgM was interacted with the resin as determined by ELISA techniques. After the heparin extract was shaken with the coupled resins, the microfuge tubes were centrifuged at approximately 200 x g for 1 min at room temperature and supernatants removed. Resin was then washed with buffer as above via vortexing. Supernatants were collected and sterile filtered for further analysis.

For both columns and resins in microfuge tubes, proteins bound to the resins were eluted using urea. Urea was made at 6 M concentration and added to resins at full molar strength or in stepwise gradient fashion as indicated. Urea was added to resins for the same time and in the same fashion as described for samples and washes. Eluted fractions using urea were sterile filtered and saved for further analysis.

Dialysis of urea fractions was carried out using ISCO dialysis cups and 3500 MW cutoff Spectra-por dialysis tubing. Urea fractions were dialysed with distilled water at 4^{o} C overnight with frequent changes of the water.

--Concentration of IgM Monoclonal Antibodies:

IgM monoclonal antibodies were concentrated by filtration using Amicon XM300 ultrafilters. The IgM supernatant of hybridomas grown in defined media (see above) was collected and centrifuged at 1,000 rpm to remove cellular debris. After centrifugation, hybridoma supernatant was removed from the cell pellet and stored in sterile tubes at -70^{o} C. To concentrate the IgM antibody, the hybridoma supernatant was thawed and then placed in a 50 ml amicon concentrator. Using XM300 ultrafilters, the hybridoma supernatant was then concentrated using nitrogen gas at a pressure of 55 psi. Concentrations were carried out at 4^{o} C. Use of XM300 ultrafilters allowed the IgM antibody (MW of approximately 900 kDa) to be concentrated, while all other proteins in the defined media passed through the filter. After concentration the IgM antibody was then sterile filtered by passage through a 0.2 micron filter. Sterile concentrated antibody was stored at 4^{o} C. Final protein concentrations after filtration were approximately 30 mg/ml.

--Filtration of Heparin Extract

Mitogenic heparin extract was centrifuged in a Centricon 30 microconcentrator at 3000 x g for 30 min. at 4°. The material retained above the filter (>30 kDa) was saved in a sterile tube for further analysis. The material passing through the filter (<30 kDa) was centrifuged in a Centricon 10 microconcentrator as above. The material retained above the filter (<30 >10 kDa) and passing through the filter (<10 kDa) was saved as above.

RESULTS

--Preliminary Studies:

Previous results in our laboratory had demonstrated that axolemma could be fluorescently labelled with fluoresein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) as a means for studying the physical interaction between Schwann cell and axon (Meador-Woodruff et al., 1985). We extended these studies by developing a fluorescent binding assay which measured the extent of binding of fluorescently labelled axolemma to Schwann cells under different conditions.

--pH 9 Treatment of Axolemmal Membrane:

As we further investigated the properties of FITC labelled axolemma, we realized that to be a valid probe the membrane should not be significantly altered during the labelling process: otherwise, the labelled membrane would not be useful in reporting the effect of normal axolemma added to cultured Schwann cells. A control experiment which we carried out led to a dramatic and surprising result. We treated axolemma membrane with sodium carbonate/bicarbonate buffer (0.27 M pH 9) as we would

during the FITC labelling procedure (see above). However, when this procedure was carried out in the absence of fluor, 50% of the protein was removed into the supernatant during the centrifugation step. The supernatant could not be directly tested for mitogenicity to Schwann cells because of the high pH and molar strength of the buffer which was toxic to Schwann cells. What was surprising was that the membrane pellet contained 50% of the starting protein, and yet contained 95% of the mitogenicity of the starting material. Thus, the pH 9 treated axolemma was enriched 2-fold in mitogenicity compared to untreated axolemma.

Figure 1 shows a dose response curve of pH 9 treated and untreated axolemma added to Schwann cells, measuring the mitogenic effect of these membranes. At all protein concentrations of membrane tested, pH 9 treated axolemma was significantly more mitogenic than untreated axolemma. Thus, while 50% of the starting axolemmal protein was extracted by pH 9 buffer treatment, little or none of this protein appeared to be mitogenic: 95% of the starting mitogenicity remained with the pellet. This result was indirect evidence that the axolemmal mitogen for Schwann cells was still membrane bound after pH 9 extraction; in this respect the mitogen had been characterized as base-stable.

Because the pH 9 extract of axolemma was toxic to Schwann cells when added to them directly, the above

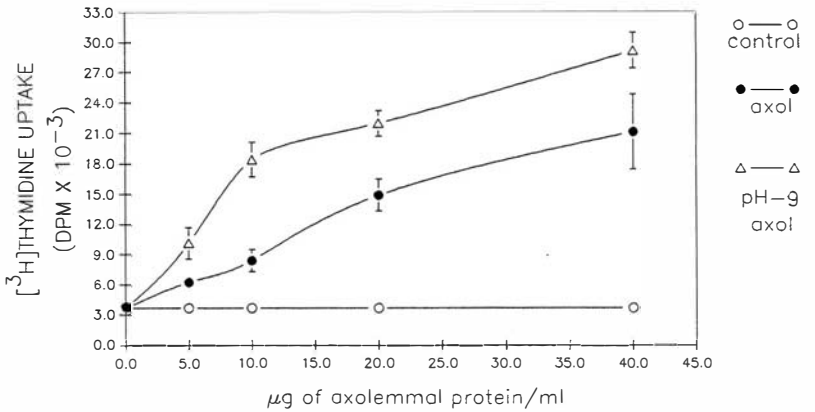

Figure 1. Enrichment of axolemmal mitogen after alkaline treatment. Axolemma was treated with 0.27 M pH 9 buffer as described in Materials and Methods. The pH-9 treated (triangles) or untreated (filled circles) axolemma was added to Schwann cells at the indicated concentrations of protein. Control cells (open circles) received media only. Each point represents five or more determinations and standard deviations are indicated. Where no error bars appear, the standard deviation is less than the range occupied by the symbol.

evidence of 95% of the mitogenic activity remaining with the axolemmal pellet did not exclude the possibility that we had removed some type of inhibitor, and that the alkaline extract of axolemma was also mitogenic. To test this possibility we washed the extract with isotonic saline 4 times using a Centricon 10,000 mw cutoff tube as described in Materials and Methods. After protein was determined for the pH 9 extract, we tested the extract and untreated axolemma for mitogenicity to Schwann cells. Figure 2 shows that compared to untreated axolemma, the pH 9 extract contains low levels of mitogenic activity. This further supports the idea that the majority of mitogenic activity remains associated with the axolemmal membrane after pH 9 extraction, and that little or no mitogen is extracted by pH 9 treatment.

To test whether axolemmal proteins were dissociating from the membrane non-specifically, we treated axolemma with isotonic saline (0.9 %) under the same conditions as the pH 9 extraction. Figure 3 shows that axolemma treated with isotonic saline was not significantly altered in its mitogenicity towards Schwann cells compared to the untreated starting axolemma. Both untreated axolemma and axolemma treated with isotonic saline were significantly mitogenic at the concentrations tested.

In an attempt to determine if more nonmitogenic material could be removed from the axolemma by pH 9 extraction, we treated axolemma with sodium

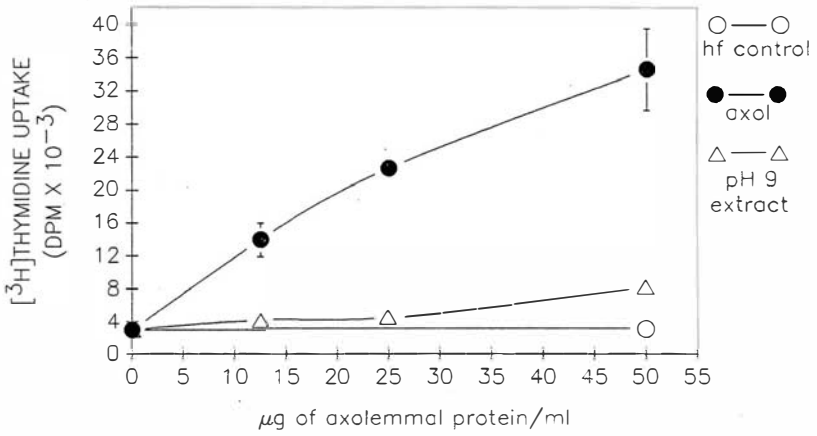

Figure 2. Mitogenic activity of pH 9 extract of axolemma, and untreated axolemma. Axolemma was treated with 0.27 M pH 9 buffer as indicated in Materials and Methods. pH 9 extract of axolemma (open triangles) was washed 4 times with isotonic saline using a Centricon 10,000 mw cutoff tube as described in Materials and Methods and tested for mitogenicity to Schwann cells at the indicated protein concentrations; filled circles indicate mitogenic effect of untreated axolemma; open circles indicate the effect of hf alone on Schwann cells. Each point represents 5 or more determinations and standard deviations are indicated. Where no error bars appear, standard deviation is less than the range occupied by the symbol.

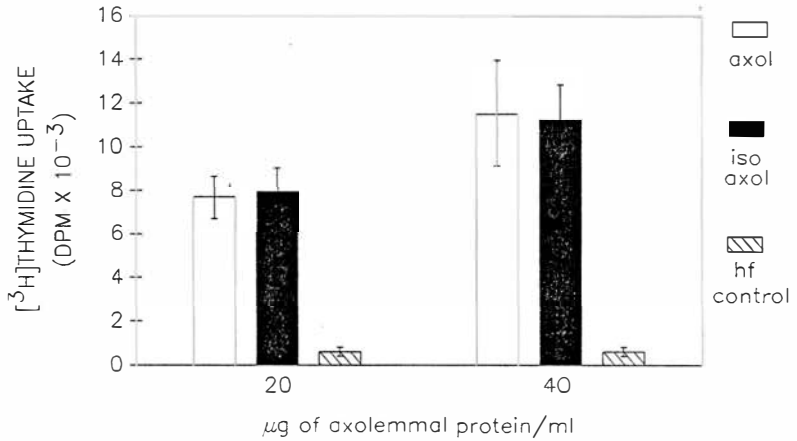

Figure 3. Treatment of axolemma with isotonic saline. Axolemma was treated under identical conditions as pH 9-treated axolemma, but using isotonic saline instead of pH 9 buffer. Axolemma treated with isotonic saline (filled bars) or untreated axolemma (open bars) was added to Schwann cells at the indicated protein concentrations. Control cells (hatched bars) received media only. Each bar represents five or more determinations and standard deviations are indicated.

carbonate\bicarbonate buffer (pH 9) at 0.4 M strength. This treatment was carried out in the same manner as that using 0.27 M buffer. However, when treated with 0.4 M pH 9 buffer, the axolemma pellet obtained was not significantly mitogenic at any of the protein concentrations tested (fig. 4). In addition, no detectable mitogenic activity was observed in the extracted supernatant. This result indicated that the axolemmal mitogen was being inactivated using pH 9 buffer at 0.4 M strength, whereas at 0.27 M strength the mitogenic activity remained associated with the membrane to the extent of 95% activity.

The ability for the mitogenicity of axolemma to Schwann cells to remain associated with the membranous pellet after 0.27 M pH 9 extraction indicated that the molecule responsible for mitogenicity might be basic in character: while other proteins were extracted from the starting axolemma (50%), those responsible for mitogenicity were not. The pH 9 extraction procedure was specific in that using identical conditions as a pH 9 extraction, neither detectable protein nor mitogenicity was extracted from starting axolemma by treatment with isotonic saline. In an attempt to determine if a greater percentage of nonmitogenic protein could be extracted from axolemma by higher molar strength pH 9 buffer (0.4 M), it was determined that 0.27 M strength was permissive for extraction without inactivating the mitogenic activity.

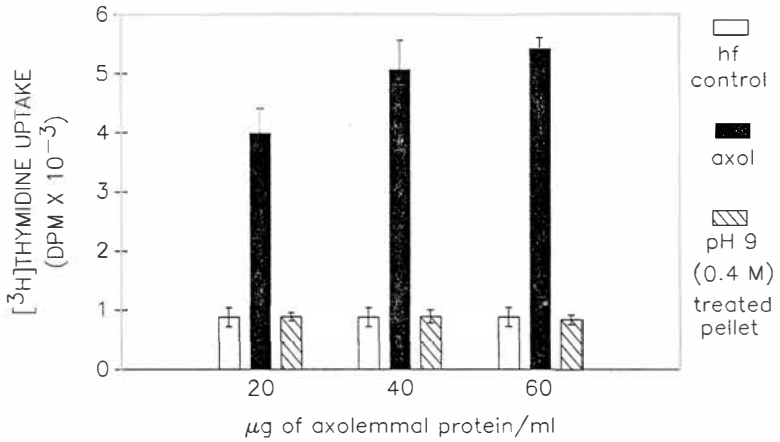

Figure 4. Treatment of axolemma with 0.4 M pH 9 buffer. Axolemma was treated with 0.4 M pH 9 buffer with all other conditions identical to those used for treating axolemma with 0.27 M pH 9 buffer. Axolemma treated with 0.4 M pH 9 buffer (hatched bars) or untreated axolemma (filled bars) was added to Schwann cells at the concentrations of protein indicated. Control cells (open bars) received media only. Each bar represents five or more determinations, and standard deviations are indicated.

--Heparitinase Treatment of Axolemmal Membrane:

The results of 0.27 M pH 9 extraction of axolemma indicated that the mitogenicity was not dissociated from the membrane by this treatment, and thus, that the mitogenic factors in axolemma might be basic in nature themselves, or highly positively charged. The mitogen might thus be associated with negatively charged groups on the axonal surface. This idea had added significance in light of experimental results by Ratner et al. (1985), which indicated that interfering with proteoglycan synthesis of neurons or treatment of neuritic membranes with heparitinase to break down the heparan sulfate glycosaminoglycans, significantly depleted the mitogenicity of these membranes to Schwann cells. The negatively charged heparan sulfate proteoglycans thus seemed important to the mitogenic activity of axons to Schwann cells.

Heparitinase is an enzyme which cleaves the glycosidic bonds of sulfated glycosaminoglycans. We postulated that if heparitinase depleted the mitogenicity of neurite membranes to Schwann cells (Ratner et al., 1985), it might do so by releasing a positively charged mitogen bound to the sulfated heparan sulfate proteoglycans. Since the pH 9 extraction evidence from our work indicated that the axolemmal mitogen might be positively charged, we investigated the effect of heparitinase on axolemma.

Figure 5 shows that when axolemma is treated with heparitinase, a soluble extract can be obtained which is mitogenic to Schwann cells. This result indicated that breaking the glycosidic bonds of sulfated glycosaminoglycans released mitogenic activity from the axolemmal membrane. Such a mechanism may have been responsible for depleting the mitogenicity of neuritic membranes reported by Ratner et al. (1985). In any event, it seemed that sulfated proteoglycans were involved in the mitogenicity of axolemmal membranes, and this result further supported our idea that the axolemmal mitogen was positively charged.

Figure 5 shows that as the dose of heparitinase extract is increased (and thus the amount of heparitinase, which is soluble), Schwann cell proliferation decreases. The heparitinase control contains the amount of heparitinase found in the highest dose of heparitinase soluble extract (assuming that all heparitinase added remains soluble). Compared with cells which received medium only, we can see that heparitinase alone exerts a slight inhibitory effect on Schwann cell proliferation.

In addition to the inhibitory effects which heparitinase has on Schwann cell proliferation, figure 5 shows that the axolemmal membrane pellet which has been treated with heparitinase is still very mitogenic. In fact, only at the lowest doses tested is the heparitinase soluble extract as mitogenic as the recovered (treated)

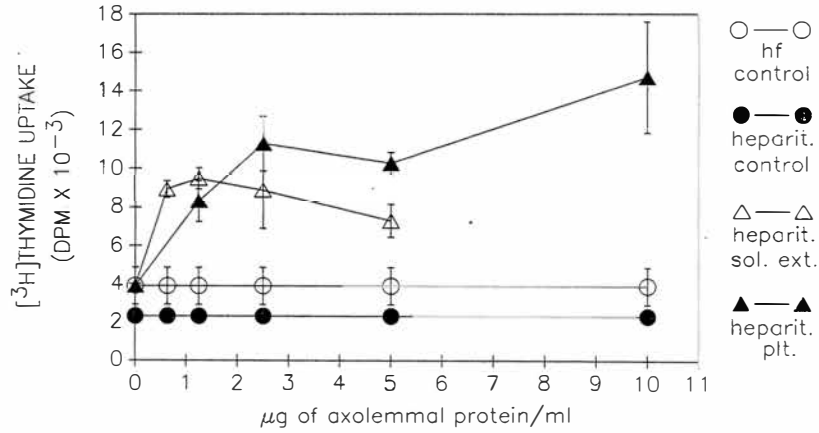

Figure 5. Solubilization of axolemmal mitogen via heparitinase treatment. Heparitinase-treated axolemma was prepared as described in Material and Methods. The soluble extract (open triangles) or recovered (heparitinase-treated) pellet (closed triangles) was added to Schwann cells at the indicated concentrations of protein. Control cells (open circles) received media only. The heparitinase control (filled circles) represents cells treated with heparitinase at the same concentration as would be found in the highest dose of soluble extract. Each point represents five or more determinations, and standard deviations are indicated. Where no error bars appear, the standard deviation is less than the range occupied by the symbol.

pellet. Therefore, heparitinase is not preferentially solubilizing the axolemmal mitogen since the heparitinase extract does not have a higher specific mitogenic activity than the pellet.

--Heparin Treatment of Axolemmal Membrane:

The results of pH 9 extraction of axolemma supported the idea that the axolemmal mitogen is positively charged. Results from heparitinase treatment of axolemma indicated that sulfated glycosaminoglycans (possibly in the form of heparan sulfate proteoglycans) were also important to the mitogenicity of axolemma. We postulated from these two pieces of evidence that it might be possible to dissociate the axolemmal mitogen from the membrane surface using a heparan sulfate analogue such as heparin.

Kraemer (1977) had shown that heparin releases heparan sulfate from the surface of cells, and the work of Kjellen et al. (1980) indicated that the heparan sulfate proteoglycans on cell surfaces could be found in both heparin displaceable and non-displaceable forms. We reasoned that the positively charged nature of the axolemmal mitogen would allow it to associate with the negative charge of the sulfated proteoglycans. Heparin (which is negatively charged) might dissociate the axolemmal mitogen from the membrane surface by dissociating the mitogen from the sulfated proteoglycans, or by

displacing the proteoglycan-mitogen complex from the axolemmal membrane surface.

Figure 6 shows that when we treated axolemmal membrane with a 1 mg/ml solution of heparin, mitogenic activity was preferentially solubilized. At all doses tested, the heparin soluble extract was more mitogenic than the recovered (treated) pellet. In addition, at the highest doses of heparin extract tested (assuming that all heparin added remained soluble) heparin alone had no effect on Schwann cell proliferation.

When compared to the starting axolemma, the heparin treated axolemma pellet was decreased in mitogenicity. As shown in figure 7, the specific mitogenic activity of the heparin extract is increased approximately 6-fold compared to starting material. The lower specific mitogenic activity of the heparin treated pellet indicates that we have not revealed any mitogenic activity. Thus, we have preferentially partitioned the mitogenic activity into the soluble extract: the extract has a higher specific mitogenic activity at the expense of the recovered (heparin treated) pellet.

To investigate whether more concentrated levels of heparin could extract greater amounts of mitogenicity from the axolemmal membrane, we treated axolemma with a 2 mg/ml solution of heparin. Although 70% more protein was solubilized using the more concentrated heparin treatment (Table 4), very little mitogenicity was extracted (see

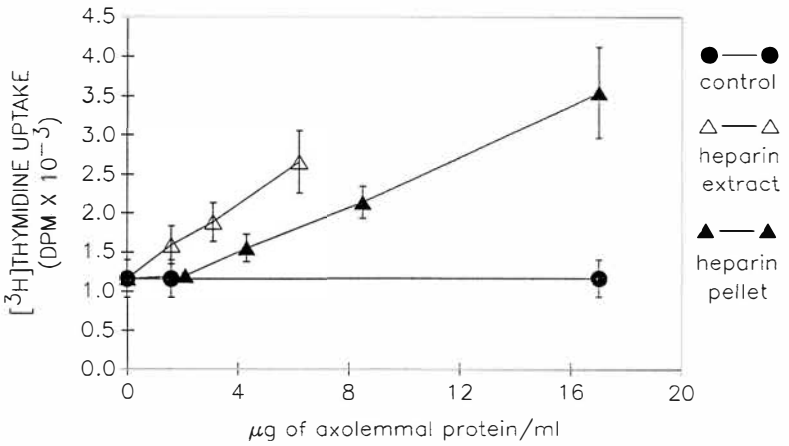

Figure 6. Solubilization of axolemmal mitogen via heparin displacement. Heparin-treated axolemma was prepared as described in Materials and Methods. The soluble extract (open triangles) or the recovered (heparin-treated) pellet (filled triangles) was added to Schwann cells at the indicated protein concentrations. Control cells (filled circles) received media only or heparin at the same concentration as would be found at the highest dose of soluble extract (50 μg/ml of heparin). Cells treated with heparin at 50 μg/ml did not differ in mitotic response from those treated with media only. Each point represents five or more determinations and standard deviations are indicated.

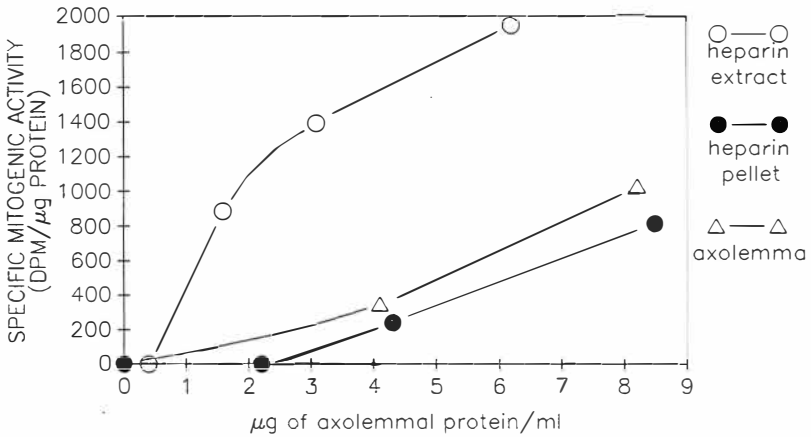

Figure 7. Specific mitogenic activity of heparin-solubilized extract, heparin-treated axolemma, and untreated axolemma. The mitogenic activity of heparin-solubilized extract of axolemma (open circles), untreated axolemma (open triangles), and heparin-treated axolemma pellet (closed circles) was determined at the protein concentrations indicated. Five or more determinations were obtained for each point; determinations were averaged and the background level of proliferation (indicated by cells receiving media only) subtracted from these averages. The proliferative response in the presence of mitogen minus the background response was divided by the amount of mitogen used to obtain the specific mitogenic activity.

Table 1). One possible explanation for the low levels of mitogenicity in the 2 mg/ml heparin extract was the toxic effect of heparin itself. At the highest dose of heparin extract tested (assuming all heparin added remains soluble) heparin does inhibit proliferation of Schwann cells slightly: the heparin control showed approximately half the level of proliferation as cells receiving HF only. However, as Table 1 demonstrates, even diluting the heparin extract (and thus the amount of heparin) did not result in any increase in mitogenic activity. Thus the 2 mg/ml heparin extract was not inhibiting Schwann cell proliferation in the same manner as observed in the heparitinase experiment (where decreasing doses were more mitogenic).

It seems that the effect that heparin exerts on extracting axolemmal protein is a dynamic one. While a greater percentage of protein is extracted using 2 mg/ml of heparin compared to 1 mg/ml, the population of molecules extracted also appears different (see also figure 10). The toxic effect of heparin at higher concentrations does not appear to fully explain why less mitogenic activity is recovered in the heparin extract using 2 mg/ml treatment. As shown in table 1, when we account for the concentration of heparin in the 1 mg/ml and 2 mg/ml heparin extracts (assuming that all heparin added remains soluble), some protein doses for the two different treatments are

TABLE 1: MITOGENIC POTENTIAL OF HEPARIN EXTRACTS[a]

	1 mg/ml		2 mg/ml	
PROTEIN DOSE	HEPARIN EXTRACTION		HEPARIN EXTRACTION	
0 + HF	1451 ± 196	(0)[b]	481 ± 137	(0)
0 + HEPARIN	1164 ± 237	(40)	283 ± 49	(53)
9.5 μg/ml	N. D.[c]		464 ± 42	(53)
7.1 μg/ml	N. D.		447 ± 69	(40)
6.2 μg/ml	2656 ± 399	(40)	N. D.	
4.7 μg/ml	N. D.		411 ± 67	(26)
3.1 μg/ml	1884 ± 245	(20)	N. D.	

[a]Mitogenic potential of heparin extracts using 1 mg/ml or 2 mg/ml of heparin are represented at the indicated protein concentrations as dpm of [^{3}H]thymidine incorporated by Schwann cells as determined by cell harvester techniques. Each data point represents 5 or more determinations and standard deviations are indicated.

[b]Figures in parenthesis indicate the amount of heparin (μg/ml) included in the given protein dose, assuming that all heparin used in the extraction remained soluble.

[c]N. D. = not determined.

comparable, and yet the protein extracted by the 2 mg/ml treatment is only marginally mitogenic.

The work of DeVries et al. (1982) showed that Schwann cells cultured from rat sciatic nerve could be stimulated to proliferate by both rat and bovine CNS axolemma membranes. To investigate whether the mitogenic activity of bovine CNS axolemma membranes could be extracted with heparin, bovine white matter was treated with a 0.5 mg/ml concentration of heparin for 60 min., and the soluble extract and extracted pellet were tested for mitogenicity toward Schwann cells. As shown in table 2, although the starting material and extracted pellet were mitogenic, the heparin extracted protein from bovine CNS white matter was not. Under the same conditions, rat CNS axolemma showed mitogenic activity in the heparin soluble extract at protein doses comparable to those extracted from bovine sources.

Although the heparin extract from bovine white matter was not mitogenic (see table 2), the heparin appeared to be extracting more protein with time. As in the work of Kraemer (1977) we too saw a rapid increase in the protein solubilized by heparin after only 5 min. We also observed a gradual increase in the amount of protein solubilized after 30 minutes treatment with heparin (fig. 8).

Table 3 summarizes the mitogenicity of similar doses of rat axolemma heparin extracts obtained under varying conditions, as well as the amount of protein solubilized by

TABLE 2: MITOGENIC ACTIVITIES OF RAT AND BOVINE AXOLEMMAL HEPARIN EXTRACTS, TREATED PELLETS, AND STARTING AXOLEMMA[a]

PROTEIN DOSE	RAT AXOLEMMA	BOVINE AXOLEMMA
0 + HF	1.066 ± 0.337	0.962 ± 0.230
0 + HEPARIN[b]	0.953 ± 0.377	0.780 ± 0.194
	HEPARIN EXTRACTS:	
8.4 μg/ml	3.906 ± 0.716	N. D.[c]
6.8 μg/ml	N. D.	0.994 ± 0.248
5.6 μg/ml	2.893 ± 0.645	N. D.
5.1 μg/ml	N. D.	1.333 ± 0.408
	TREATED PELLETS:	
51.5 μg/ml	14.546 ± 1.599	N. D.
42.3 μg/ml	N. D.	12.354 ± 0.900
	STARTING MEMBRANE:	
75.2 μg/ml	25.124 ± 2.773	N. D.
65.1 μg/ml	N. D.	22.803 ± 5.581

[a]Rat or bovine axolemma was treated with 0.5 mg/ml of heparin for 60 minutes as indicated in Materials and Methods. The mitogenic activities for each protein dose are expressed as dpm x 10^{-3} of [^{3}H]thymidine uptake by Schwann cells as determined by cell harvester. Each data point represents 5 or more determinations and standard deviations are indicated.

[b]Heparin control contained 20 μg/ml of heparin.

[c]N. D. = not determined.

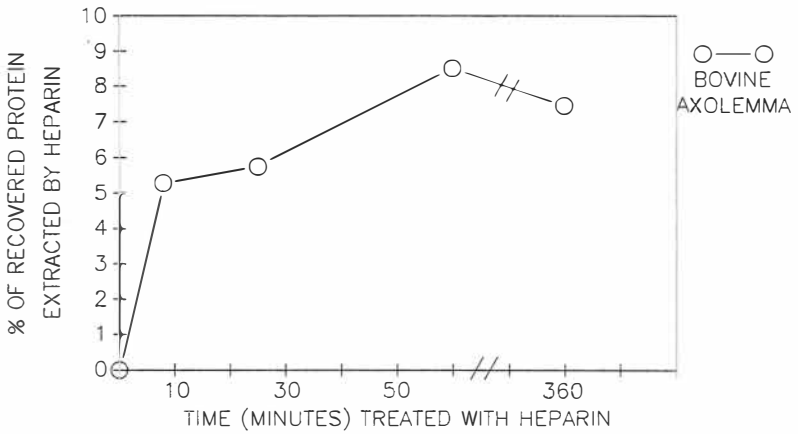

Figure 8. Percent of bovine axolemma protein extracted with time. Bovine axolemma was extracted with 0.5 mg/ml of heparin as indicated in Materials and Methods for the amounts of time indicated. The percent of recovered protein extracted by heparin was determined using the Bio-Rad protein determination.

TABLE 3: PROTEIN AND MITOGENIC ACTIVITY OF RAT AXOLEMMA SOLUBILIZED BY HEPARIN

[^{3}H]THYMIDINE UPTAKE (DPM X 10^{-3})	% OF RECOVERED PROTEIN EXTRACTED BY HEPARIN
TREATMENT 1: 0.5 mg/ml heparin for 60 minutes cells treated with 0.56 µg of extract	
HEPARIN CONTROL: 0.953 ± 0.377	6.8% OF PROTEIN EXTRACTED BY HEPARIN
HEPARIN EXTRACT: 2.893 ± 0.645	
TREATMENT 2: 1 mg/ml heparin for 180 minutes cells treated with 0.62 µg of extract	
HEPARIN CONTROL: 1.164 ± 0.237	7.6 % OF PROTEIN EXTRACTED BY HEPARIN
HEPARIN EXTRACT: 2.656 ± 0.339	
TREATMENT 3: 1 mg/ml heparin for 360 minutes cells treated with 0.55 µg of extract	
HEPARIN CONTROL: 1.800 ± 0.530	7.3 % OF PROTEIN EXTRACTED BY HEPARIN
HEPARIN EXTRACT: 2.212 ± 0.335	
TREATMENT 4: 2 mg/ml heparin for 240 minutes cells treated with 0.47 µg of extract	
HEPARIN CONTROL: 0.283 ± 0.049	11.9 % OF PROTEIN EXTRACTED BY HEPARIN
HEPARIN EXTRACT: 0.411 ± 0.067	

these different treatments. We found that after 60 min. with either 0.5 mg/ml or 1 mg/ml concentration of heparin, mitogenic activity was extracted from axolemma. However, if heparin concentration or treatment time was increased too much, little mitogenic activity was recovered.

As described by Yoshino et al. (1984), both axolemma and myelin-enriched fractions produced a dose-dependent uptake of [^{3}H]thymidine into Schwann cells; however, the shape of the dose response curves was different for the two membranes. As shown in figure 9c, the dose response curve of heparin extract from axolemma appears to most closely resemble the dose response curve of axolemma (9a). The dose response curve for myelin (9b) is hyperbolic in shape and shows an immediate rise in [^{3}H]thymidine uptake at low doses of protein. The dose response curves of heparin extract and of axolemma show an initial lag of [^{3}H]thymidine uptake at low doses of protein, are sigmoidal in shape, and continue to rise at protein doses where myelin membrane curves have plateaued.

It should also be noted that due to a higher specific mitogenic activity, heparin extract is significantly mitogenic at much lower protein doses than the starting axolemma membrane; however, when we represent figures 9a and 9c as Hill plots (9d and 9e respectively), we see that both axolemma and the heparin extract of axolemma have similar slopes. Yoshino et al. (1984) found that axolemma exhibited a Hill coefficient of approximately 2.0 when

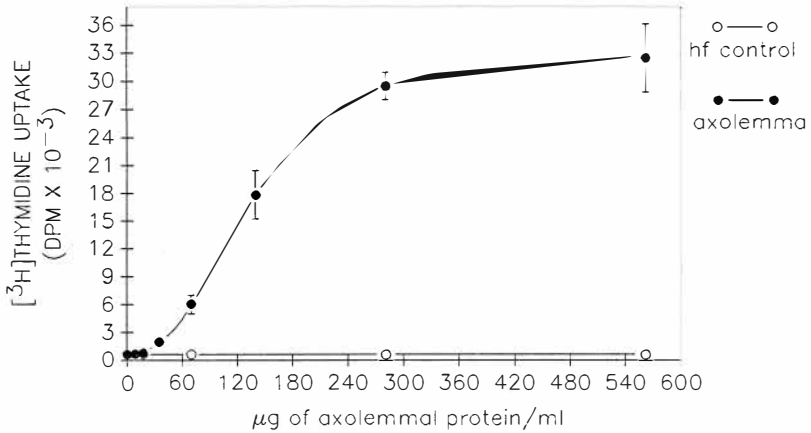

Figure 9a. Mitogenic response of Schwann cells to axolemma. Axolemma (closed circles) was added to Schwann cells at the protein concentrations indicated. Control cells (open circles) received media only. Each point represents five or more determinations and standard deviations are indicated. Where no error bars appear, standard deviations are less than the range occupied by the symbols.

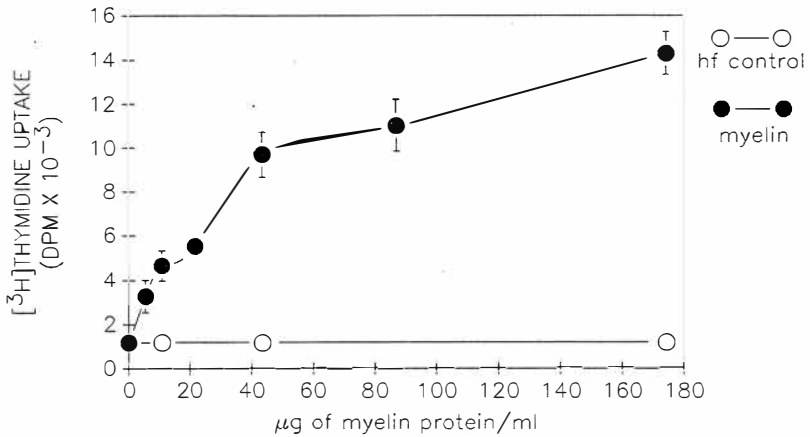

Figure 9b. Mitogenic response of Schwann cells to myelin. Myelin (closed circles) was added to Schwann cells at the indicated protein concentrations. Control cells (open circles) received media only. Each point represents 5 or more determinations and standard deviations are indicated. Where no error bars appear, standard deviation is less than the range occupied by the symbol.

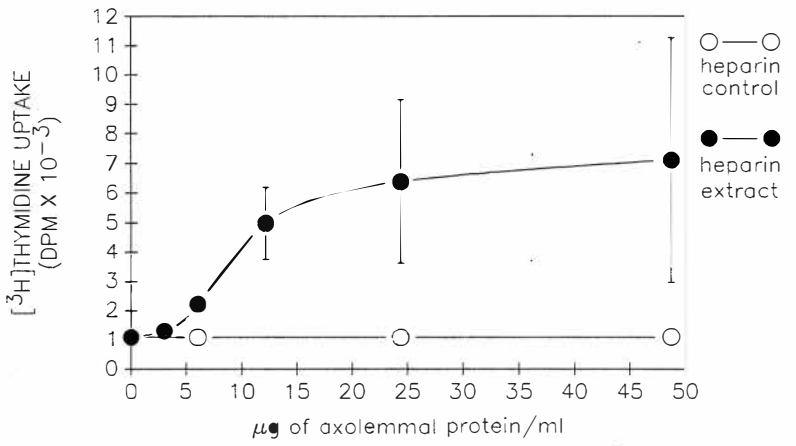

Figure 9c. Mitogenic response of Schwann cells to heparin extract of axolemma. Heparin extract of axolemma (closed circles) was added to Schwann cells at the indicated protein concentrations. Control cells (open circles) received media only. Each point represents five or more determinations and standard deviations are indicated. Where no error bars appear, standard deviation is less than the range occupied by the symbols.

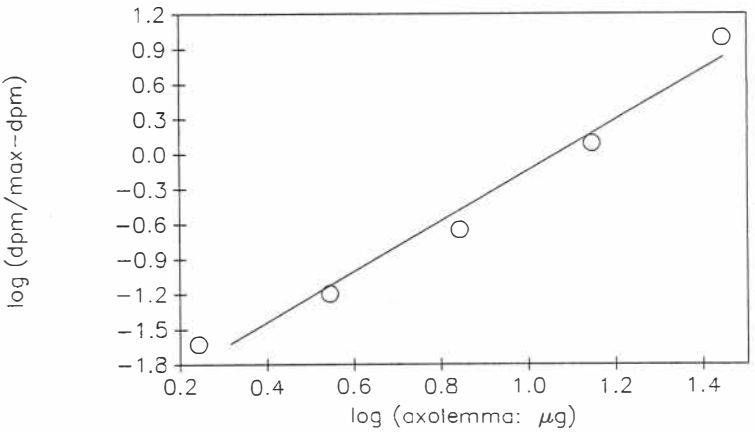

Figure 9d. Hill plot of axolemma mitogenicity to Schwann cells. Data from figure 9a was expressed as a Hill plot to determine the cooperativity of axolemmal stimulation of [^{3}H]thymidine uptake by Schwann cells. Maximum stimulation of [^{3}H]thymidine uptake was chosen as 32,490 dpm. The slope (Hill coefficient) of the plot was determined to be 2.18.

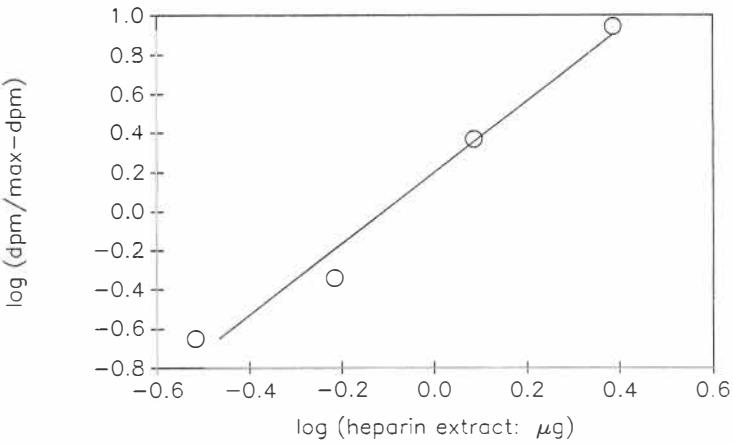

Figure 9e. Hill plot of heparin extract mitogenicity to Schwann cells. Data from figure 9c was represented as a Hill plot to determine the cooperativity of heparin extract stimulation of [^{3}H]thymidine uptake by Schwann cells. Maximum stimulation of [^{3}H]thymidine uptake was chosen as 7115 dpm. The slope (Hill coefficient) of the plot was determined to be 1.90.

plotted as ability to stimulate [^{3}H]thymidine uptake per μg of axolemma. The Hill coefficients of Figures 9d and 9e are both approximately equal to 2.0, thus showing similar cooperativity in the ability to stimulate [^{3}H]thymidine uptake per μg of axolemma or heparin extract protein.

--Mitogenic Activity Recoveries of Treated and Untreated Axolemma:

Table 4 presents the quantitative protein and mitogenic activity recoveries of untreated, pH 9-treated, heparitinase-treated, and heparin-treated axolemma. All treatments were carried out on at least two different axolemma preparations, and all treated membranes were added to at least five different sets of Schwann cells. Data from different preparations undergoing the same treatment (for example, different pH 9 preparations) showed variability in the absolute numbers obtained. In addition, theoretically similar membrane such as untreated axolemma shows variability in mitogenic activity as shown in Table 4. This variability is due both to the sensitivity of different Schwann cell preparations to membrane mitogen, as well as the mitogenic potency of different axolemma preparations. For example, the mitogenic activities of untreated axolemma shown in Table 4 for the heparitinase and heparin experiments cannot be compared: different Schwann cell preparations were used to obtain the indicated

TABLE 4: Protein and mitogenic activity recoveries of untreated, pH 9-treated, heparitinase-treated, and heparin-treated axolemma[a]

Experiment	Fraction	ug of protein	mitogenic activity (dpm/ug)[b]	protein (% of untreated axolemma)	units of mitogenic activity ($dpm \times 10^{-4}$)[c]	mitogenic activity (% of untreated axolemma)
pH 9	untreated axolemma	1870	5333	100	997	100
	supernat.	860	N. D.[d]	45.9	N. D.[d]	N. D.[d]
	pellet	851	12,017	45.5	1022	102
	% recov.			91.5		102
Heparitase.[e]	untreated axolemma	93.5	8760	100	81.9	100
	supernat.	7.02	6380	7.51	4.47	5.46
	pellet	78.0	9280	83.4	72.3	88.3
	% recov.			90.9		93.7
Heparin 1 mg/ml	untreated axolemma	714	1015	100	72.4	100
	supernat.	45.0	2406	6.30	10.8	14.9
	pellet	544	804	76.2	43.7	60.4
	% recov.			82.5		75.3
Heparin 2 mg/ml	untreated axolemma	1016	1541	100	156	100
	supernat.	110	230	10.8	2.5	1.63
	pellet	824	1657	81.1	136	87.1
	% recov.			91.9		88.7

[a] The data presented represents a typical result from one of each of the different treatments (pH 9, heparitinase, and heparin); at least two different axolemma preparations were used for each treatment. See results section of text.

[b] The specific mitogenic activity was calculated for the linear portion of dose response curves as described in the text.

[c] Total units of mitogenic activity were calculated by multiplying total ug of protein by the specific mitogenic activity.

[d] Mitogenic activity of the pH 9 supernatant was not determined due to the toxicity of the pH 9 buffer on Schwann cells.

[e] Axolemma was treated with 1 unit of heparitinase at 37 degrees C for 4 hours.

results for different treatments of the axolemma. Despite the variability of mitotic response of different Schwann cell preparations and variable mitogenic activity of different axolemma preparations, given treatments of axolemma always showed the same trends for enrichment of mitogenic activity when compared to the untreated axolemma used in that experiment. Therefore the data shown in Table 4 are typical results from one of each of the different treatments (pH 9, heparitinase, and heparin).

When axolemma obtained from adult rat CNS white matter (brainstem) was treated with 0.27 M sodium carbonate/bicarbonate buffer at pH 9 and then centrifuged, a pellet was recovered which represented 50% $\pm$ 5% of the starting protein and 95% $\pm$ 10% of the starting mitogenic activity. The supernatant from the pH 9-treated axolemma contained approximately 50% of the starting protein; although the mitogenicity of this supernatant could not be directly tested because of the high pH and molar strength of the buffer, which was toxic to cells, after washing the pH 9 supernatant with isotonic saline, only low levels of mitogenic activity were detected. The specific mitogenic activity of the recovered pellet was higher than that of the starting material at all doses of axolemmal protein tested (Fig. 1).

Two chemical treatments of axolemma solubilized mitogenic activity for Schwann cells. The mitogen solubilized by heparitinase represented 7-10% of the

starting protein and 5-10% of the starting mitogenic potential (Table 4). Thus, the heparitinase treatment did not seem to be selectively removing the mitogen. Heparin can displace the axolemmal mitogen for Schwann cells, resulting in a soluble extract which is more mitogenic at given doses of protein than the recovered pellet and than the starting axolemma. Solubilization of the mitogen reached a maximum after 2 hr of treatment with heparin; treating the axolemma membrane for longer time periods did not increase the amount of mitogen solubilized (see Table 3). Solubilization of the axolemmal mitogen via heparin displacement is advantageous in that it results in an extract which has a higher specific mitogenic activity than the starting material. For the result shown in Figure 6, only 6.3% of the starting protein was solubilized by heparin treatment, but this soluble extract represented 14.9% of the starting mitogenic activity (Table 4). Thus the soluble extract was approximately threefold enriched in specific mitogenic activity compared to the recovered (heparin-treated) pellet and 2.4-fold compared to the starting axolemma.

Heparin-solubilized extracts containing 1 mg/ml of heparin (assuming that all heparin added remained soluble) were found to be mitogenic to Schwann cells. After dilution of the extract to obtain the indicated protein concentrations as well as dilution due to proliferative response assay conditions (Yoshino et al., 1984), Schwann

cells would be exposed to 50 μg/ml of heparin at the highest protein dose of soluble extract shown (fig. 6). As a control, Schwann cells were exposed to 50 μg/ml of heparin alone and were found to undergo the same extent of proliferation as cells receiving medium only (Fig. 6).

When axolemma was treated with 2 mg/ml of heparin, a greater percentage of the starting protein (10.8) was solubilized than with the 1 mg/ml treatment of heparin (Table 4). However, the protein solubilized was less mitogenic than that solubilized by the 1 mg/ml treatment, containing only 1.63% of the mitogenic activity (Table 4). This result may be due in part to an inhibitory effect of heparin in the mitogenic assay. Whereas 50 μg/ml of heparin added alone to Schwann cells had no effect (Fig. 6), 100 μg/ml of heparin alone, representing the concentration of heparin found in the highest dose of soluble extract for the 2 mg/ml treatment, inhibited Schwann cell mitosis over twofold compared to cells receiving medium alone.

The mitogenic activity of the starting axolemma membrane appears to be due to the presence of protein using the criteria of heat and trypsin inactivation (DeVries et al., 1982). The mitogenic activity of heparin-solubilized extract isolated from axolemma membrane also appears to be due to the presence of protein, as this mitogenic activity is destroyed by heat treatment. Figure 10 shows the effect

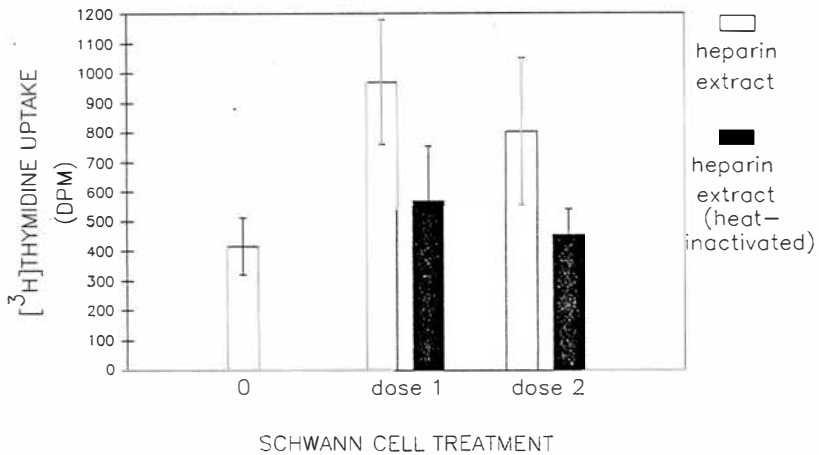

Figure 10. Heat inactivation of the mitogenic activity of heparin extract. Heparin extract was heat inactivated as described in Materials and Methods. Heat inactivated heparin extract (filled bars) or identical volumes of untreated extract (open bars) was added to Schwann cells. The open bar at "0" indicates the effect of hf alone on Schwann cells. Each bar represents 5 or more determinations, and standard deviations are indicated.

of heat inactivation on the mitogenicity of heparin extract to Schwann cells.

Since heat inactivation of the mitogenic activity of heparin extract from axolemma indicated the mitogenic activity might be due to a protein, we separated the extract using SDS-PAGE, and stained for protein. Figure 11 shows the heparin-solubilized mitogenic extract separated on a sodium dodecyl sulfate polyacrylamide gel (10%) in the presence of 2-mercaptoethanol. In Figure 11, lanes 3 and 6 show that heparin treatment of the axolemma results in a soluble extract of select molecules. Many of the proteins apparent in the untreated axolemma (lanes 1 and 4) are not solubilized by heparin. Lane 6 shows that a more diverse population of proteins are displaced from the axolemma with more concentrated (2 mg/ml) treatments of heparin than in lane 3 (1 mg/ml). For the preparation in lane 3, 6.3% of the starting protein was solubilized; this represented 14.9% of the starting mitogenic activity (Table 4). For the preparation in lane 6, 10.8% of the starting protein was solubilized; however, this represented only 1.6% of the starting mitogenic activity. It is interesting to note that in lane 3, one protein band (indicated at molecular weight 50 kDa) appears greatly enriched compared to the recovered pellet (lane 2). Lane 6 does not show this enriched protein band.

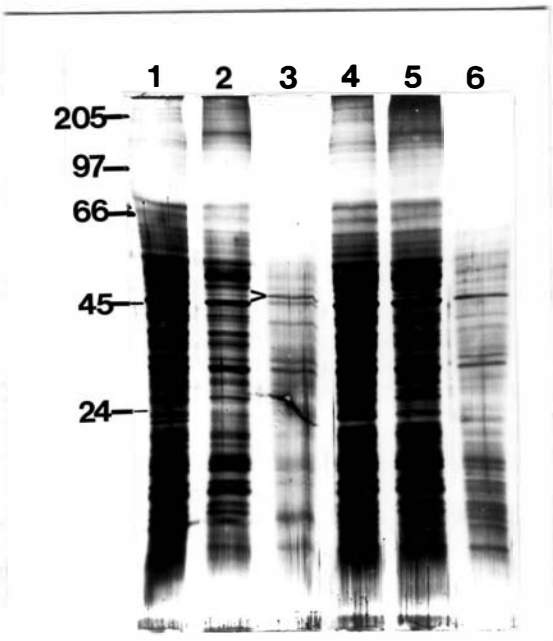

Figure 11. SDS-polyacrylamide gel electrophoresis of heparin-soluble extracts and axolemmal membrane. Gel electrophoresis was carried out in 10% acrylamide and stained using silver stain as described in Materials and Methods. Molecular weights in kilodaltons are indicated at the left of the gel. Lane 1 was loaded with 47 μg of starting axolemma protein; Lane 2 with 27.2 μg of the recovered axolemma protein which had been treated with 1 mg/ml of heparin for 180 min; and Lane 3 with 2.48 μg of soluble protein displaced by the 1 mg/ml heparin treatment. Lane 4 was loaded with 47 μg of starting axolemma protein; Lane 5 with 41.2 μg of the recovered axolemma protein which had been treated with 2 mg/ml of heparin for 240 min; and Lane 6 with 5.68 μg of soluble protein displaced by the 2 mg/ml treatment.

--Isolation of a Monoclonal Antibody which Inhibits the Mitogenicity of Axolemma to Schwann Cells:

As shown in figure 10, the heparin extract of axolemma contains a selected population of the original membrane proteins. Furthermore, as indicated in table 4, the heparin extract has a higher specific mitogenic activity than the original axolemma; thus it is enriched in mitogen. For these reasons we felt it was worthwhile to produce antibodies against the heparin extract in an attempt to obtain monoclonal antibodies against the axonal mitogen for Schwann cells.

Our initial criterion for isolating monoclonal antibodies that might be relevant for studying the axolemmal mitogen was that these antibodies bind to the original starting axolemmal membrane as determined by ELISA techniques. Although the antibodies were raised by using the heparin extract of axolemma, heparin extract was not readily available in large enough quantities of protein to be used for screening by ELISA (as indicated by table 4, the mitogenic heparin extract of axolemma represents only 6.3% of the starting membrane protein). Furthermore, we would be most interested in antibodies that recognized the axolemmal mitogen both in its heparin-solubilized and membrane-bound forms.

Heparin solubilized extract of axolemma which had been tested as mitogenic to Schwann cells was injected into one

RBF\Dn mouse as indicated in Materials and Methods. Fusions were carried out as described in Materials and Methods. The fused cells were plated onto three 96-well plates, and of these 288 wells, 57 contained viable hybridomas. The media supernatants from these mixed hybridomas along with the immune serum from the injected mouse were tested against 10 μg/ml of axolemma by ELISA techniques.

As indicated in figure 12, only 2 of the 57 mixed hybridomas showed any reactivity whatsoever toward axolemma membrane; the immune serum was also reactive, indicating that antibodies had been raised to the injected antigen. The supernatants from the 55 hybridoma wells not shown in figure 12 were found to be identical in reactivity to the blank control. The mixed hybridomas in wells 3F8 and 1A5 were expanded and then cloned by limiting dilution. This cloning procedure resulted in the identification of 44 wells with single cell clones. These monoclonals were screened against 10 μg/ml of axolemma using ELISA techniques.

As shown in figure 13, one monoclonal (1A5-2G3) was isolated which was significantly reactive with axolemma. The 1A5-2G3 monoclonal antibody was subtyped as IgM κ using a Boehringer Mannheim mouse immunoglobulin subtype identification kit. The mixed hybridoma from which this monoclonal was isolated was still reactive; the high reactivity of the mixed hybridoma compared to the

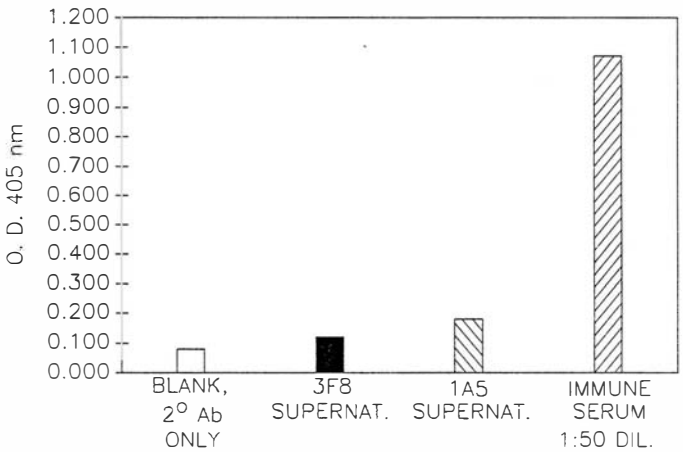

Figure 12. Reactivity of mixed hybridoma supernatants to axolemma. Hybridomas were produced from a mitogenic heparin soluble extract as described in Materials and Methods. The supernatants of mixed hybridomas were tested undiluted against 10 μg/ml of axolemma membrane using ELISA techniques as described in Materials and Methods.

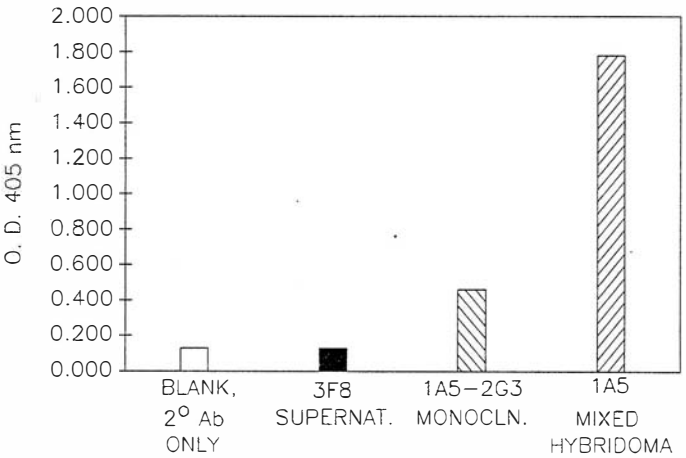

Figure 13. Reactivity of monoclonal and mixed hybridoma supernatants to axolemma. Supernatants from monoclonal (1A5-2G3) or mixed (3F8) hybridomas were tested undiluted for reactivity against 10 µg/ml of axolemma using ELISA techniques. Hybridomas from 3F8 were cloned out as described in Materials and Methods, but none of the clones were reactive to axolemma, and as indicated the original mixed hybridoma of 3F8 (filled bar) was no longer reactive to axolemma at the time of cloning.

monoclonal indicates the limited growth of the monoclonal cells at the time of their isolation. Figure 13 also shows that the minor reactivity of mixed hybridoma 3F8 was insignificant after cloning; none of the clones from 3F8 were reactive to axolemma compared to the blank control, and the 3F8 mixed hybridoma itself was shown to be non-reactive to axolemma at the time that clones were isolated.

Having isolated a hybridoma secreting a monoclonal antibody directed against axolemma, we tested whether this antibody had any effect on the mitogenicity of axolemma to Schwann cells. As shown in figure 14, monoclonal antibody 1A5-2G3 inhibited the mitogenic effect of axolemma to Schwann cells. The same concentration of antibody did not inhibit the mitogenic effect of myelin to Schwann cells. The inhibition of mitogenicity did not seem to be due to any toxic effect of the antibody alone, as 1A5-2G3 did not inhibit proliferation of Schwann cells by itself.

To investigate the specificity of inhibition of mitogenicity to Schwann cells by the antibody 1A5-2G3, we used another IgM monoclonal antibody, TIB-103, in similar experiments. TIB-103 is directed against Thy 1.1, which is found on the surface of fibroblasts and neurons. We obtained the hybridoma cells from the American Type Culture Collection (ATCC) and collected antibody supernatants from the cultures in a similar fashion as for 1A5-2G3. Figure 15 shows that TIB-103 had no detectable inhibitory effect on the mitogenicity of axolemma to Schwann cells. In

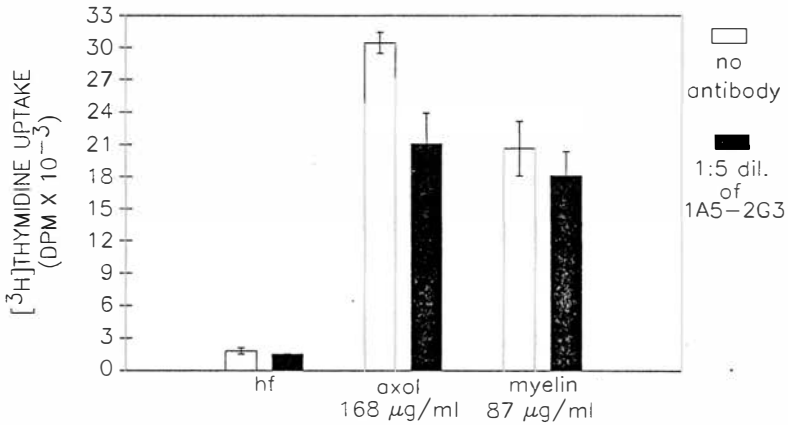

Figure 14. The mitogenic effect of axolemma but not of myelin to Schwann cells is inhibited by 1A5-2G3 antibody. The 1A5-2G3 hybridoma supernatant was diluted 1:5 in fetal calf serum and incubated with axolemma and myelin as indicated in Materials and Methods. The mixture of antibody and either axolemma or myelin was added to Schwann cells at the indicated protein concentrations (filled bars). Other cells of the same culture received identical amounts of axolemma or myelin not treated with antibody (open bars). Control cells (HF) received media only (open bar) or antibody at a 1:5 dilution (filled bar). Each bar represents 5 or more determinations and standard deviations are indicated.

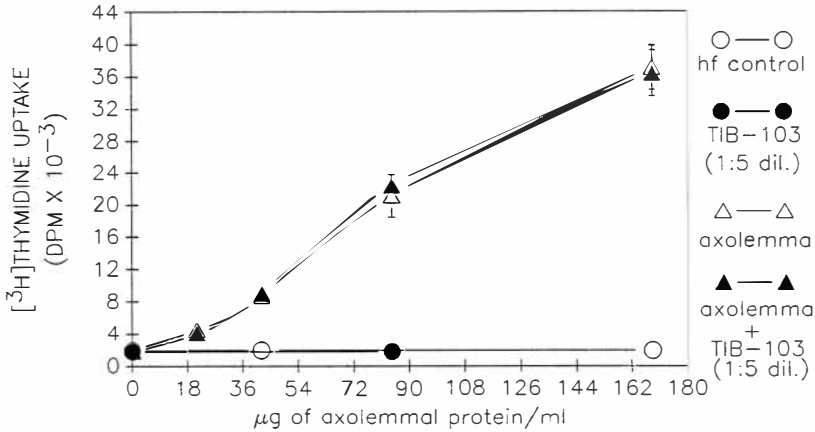

Figure 15. TIB-103 IgM antibody does not inhibit the mitogenicity of axolemma to Schwann cells. TIB-103 hybridoma supernatant diluted 1:5 in HF was incubated with axolemma. The antibody-axolemma mixture (filled triangles) or axolemma alone (open triangles) was added to Schwann cells at the indicated protein concentrations. Control cells received media only (open circles) or antibody diluted 1:5 in HF (closed circles). Each point represents 5 or more determinations and standard deviations are indicated. Where no error bars appear, standard deviation is within the range of the symbols.

figure 16 we show that another IgM monoclonal antibody, 1D3-3H12, raised against rat pup CNS microsomes to produce murine hybridomas, was also unable to inhibit the mitogenic effect of axolemma to Schwann cells. Both TIB-103 and 1D3-3H12 were positive against axolemma using ELISA techniques, yet neither inhibited the mitogenic effect of axolemma to Schwann cells.

Figure 17 shows that 1A5-2G3 can also completely inhibit the mitogenic activity of the soluble heparin extract to Schwann cells at low protein doses. As shown in figure 18, 1A5-2G3 can inhibit the mitogenicity of heparin extract to Schwann cells at higher protein doses.

To ensure that the inhibitory effect of 1A5-2G3 was not due to non-specific binding of the antibody to protein or other components in the axolemma or heparin extract we tested the specificity of binding of 1A5-2G3 by ELISA techniques. Figure 19 shows the reactivity of 1A5-2G3 to rat axolemma, liver membrane, bovine serum albumin, and heparin as determined by ELISA. At the protein doses shown, 1A5-2G3 is specific for rat axolemma. It should be noted, however, that the amount of both antibody and antigen delivered to the ELISA plates is critical: we found that too much of antibody or antigen resulted in non-specific binding. For the result shown in Figure 19, greater than 1 μg/ml of antigen resulted in decreased reactivity of 1A5-2G3 to axolemma and increased reactivity of the antibody to rat liver microsomes and bovine serum

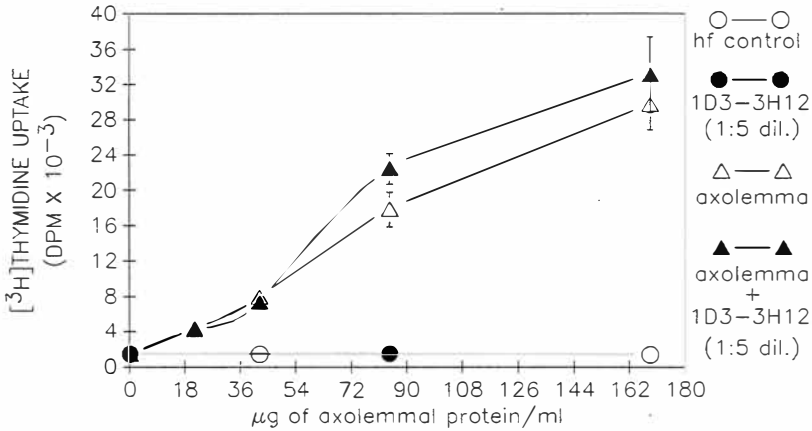

Figure 16. 1D3-3H12 antibody does not inhibit the mitogenicity of axolemma to Schwann cells. Hybridoma supernatant containing antibody 1D3-3H12 was diluted 1:5 in HF and incubated with axolemma. The antibody-axolemma mixture (filled triangles) or axolemma without antibody (open triangles) was added to Schwann cells at the indicated protein concentrations. Control cells received media only (open circles) or antibody at a 1:5 dilution in HF. Each point represents 5 or more determinations and standard deviations are indicated. Where no error bars appear, standard deviation is less than the range occupied by the symbols.

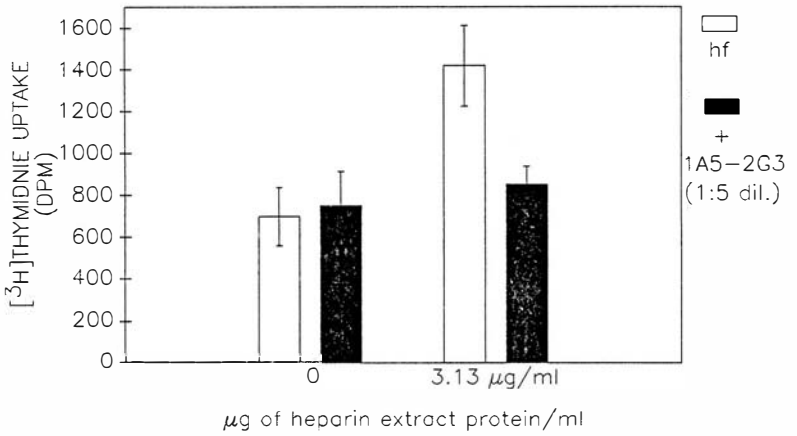

Figure 17. 1A5-2G3 inhibits the mitogenicity of heparin extract to Schwann cells. 1A5-2G3 at a 1:5 dilution was incubated with heparin extract at the indicated protein concentration (filled bar at far right). The open bar at 3.13 μg/ml indicates the mitogenicity of untreated heparin extract. The filled bar at "0" indicates the effect of antibody alone at a 1:5 dilution; the open bar at "0" indicates the effect of hf alone on Schwann cells. Each bar represents 5 or more determinations and standard deviations are indicated.

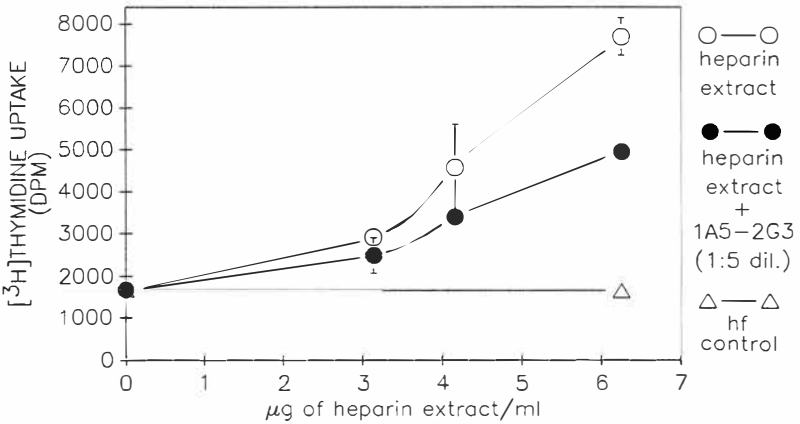

Figure 18. 1A5-2G3 significantly inhibits the mitogenicity of heparin extract: dose response curve. 1A5-2G3 at a 1:5 dilution was incubated with heparin extract at the indicated protein concentrations. Open circles show the mitogenic response of Schwann cells to untreated heparin extract; closed circles show mitogenic response to heparin extract treated with antibody. Open triangles show the effect of hf alone on Schwann cell; antibody alone had an identical mitogenic effect on Schwann cells as hf. Each point represents 5 or more determinations and standard deviations are indicated. Where no error bars appear, standard deviation is less than the range occupied by the symbol.

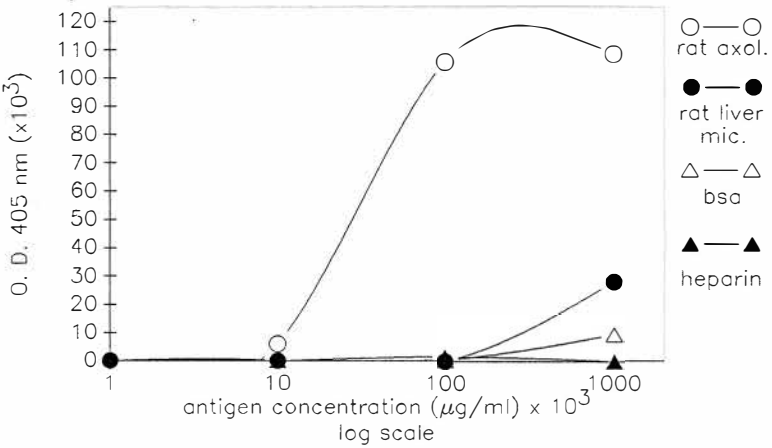

Figure 19. Reactivity of 1A5-2G3 to axolemma, liver microsomes, BSA, and heparin. 1A5-2G3 hybridoma supernatant diluted 1:100 was reacted with the antigens at the indicated concentrations using ELISA techniques as described in Materials and Methods.

albumin. It is of importance to note the lack of binding of 1A5-2G3 to heparin shown in figure 19. Presumably, since heparin extracts used to immunize mice contain large amounts of soluble heparin, isolated antibodies may have recognized glycosaminoglycan epitopes; the ELISA results shown in figure 19 indicate that 1A5-2G3 does not bind to heparin.

Although 1A5-2G3 was able to inhibit the mitogenicity of both untreated axolemma and the soluble heparin extracts, only at the lowest doses of heparin extracts could we achieve total inhibition of the mitogenic effect on Schwann cells. Furthermore, since we were using small dilutions of hybridoma supernatants (1:5 in figures 17 and 18) we could not increase the amount of antibody used without concentrating the supernatants.

We attempted to concentrate 1A5-2G3 by passing hybridoma medium through XM300 amicon filters as described in methods. Our rationale was that the large molecular weight of IgM (>900 kDa) would prevent passage through the filter while other proteins in the hybridoma medium would pass through, thus concentrating the IgM in the retained volume. However, at that time we were growing the 1A5-2G3 hybridoma in HF with 15% fetal calf serum. When this medium was concentrated using the XM300 amicon filter, we found it was mitogenic to Schwann cells in comparison to background levels of proliferation, which were cells receiving HF medium with 10% fetal calf serum. Thus, the

concentrated 1A5-2G3 alone was mitogenic to Schwann cells, confounding the interpretation of its inhibitory effect on the mitogenicity of axolemma to Schwann cells. The most likely explanation for this result was that the fetal calf serum in the hybridoma supernatants contained growth factors for Schwann cells; concentrating the supernatant also increased the concentration of these growth factors, causing the concentrated supernatant to be mitogenic to Schwann cells compared to HF, which contains 10% fetal calf serum.

Since no convenient methods of purifying IgM were available we attempted to grow the 1A5-2G3 hybridoma in a defined medium. Medium with defined components would allow concentration without the mitogenic effect on Schwann cells seen for fetal calf serum (which contains undefined mitogenic factors). Figure 20 shows that when the 1A5-2G3 hybridomas were grown in a defined medium developed by Chen and DeVries (1989), antibody was produced which specifically bound to axolemma compared to rat liver microsomes as determined by ELISA. Figure 21 shows that 1A5-2G3 produced in defined medium and concentrated using the Amicon XM300 filters retains specificity to axolemma. The concentrated antibody shown in figure 21 is reactive to axolemma at dilutions up to 700-fold; the concentrated antibody does not show reactivity to rat liver microsomes until approximately 200-fold dilutions. It is important to note that the specificity of binding to axolemma (at least

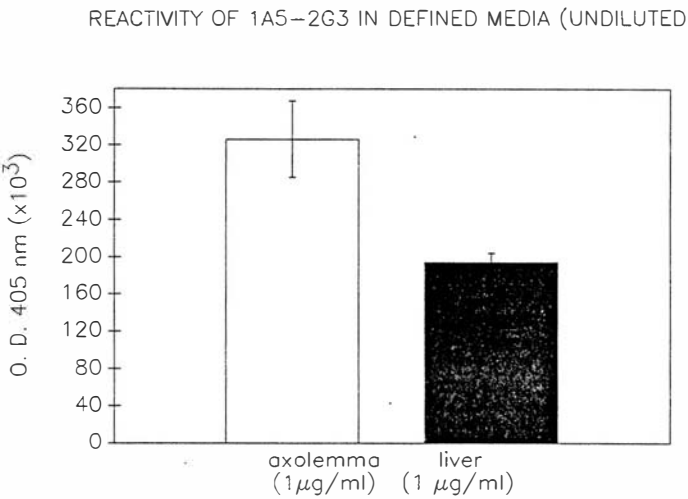

Figure 20. Preferential reactivity of 1A5-2G3 in defined media to axolemma compared to liver microsomes. Undiluted 1A5-2G3 hybridoma supernatant grown in defined media as described in Materials and Methods was reacted with rat axolemma or liver microsomes at 1 μg/ml antigen concentration using ELISA techniques. Each bar represents 2 determinations and standard deviations are indicated.

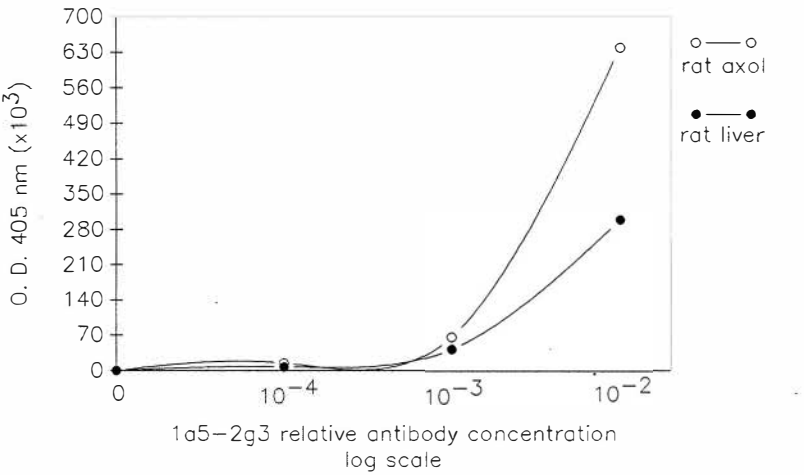

Figure 21. 1A5-2G3 in concentrated defined media preferentially reacts with axolemma compared with liver microsomes. 1A5-2G3 hybridoma supernatant grown in defined media and concentrated 9-fold by filtration as described in Materials and Methods was reacted at the indicated antibody dilutions with rat axolemma (open circles) or liver microsomes (closed circles) at antigen concentration of 1 μg/ml using ELISA techniques. Each point represents 2 determinations; standard deviation is less than the range occupied by the symbols.

by ELISA techniques) depends upon both antibody and antigen concentration. We found that at very high antigen concentrations specificity of binding was lost.

Figures 22 A-C show that 1A5-2G3 grown in defined media retains the capability to inhibit the mitogenic effect of axolemma on Schwann cells. The antibody alone shows a slight inhibitory effect on Schwann cell proliferation; however, even where no inhibition of proliferation by the antibody alone is seen, 1A5-2G3 still significantly inhibits the mitogenic effect of axolemma on Schwann cells. Figure 22 A shows that at an axolemmal protein concentration of 168 μg/ml, undiluted (straight) 1A5-2G3 hybridoma supernatant almost completely inhibits the mitogenic effect of axolemma on Schwann cells; approximately 88% of the mitogenic effect is inhibited. Decreasing the axolemmal protein concentration to 84 μg/ml (Figure 22 B) allows undiluted 1A5-2G3 to completely inhibit the mitogenic effect of axolemma on Schwann cells. At 42 μg of axolemmal protein/ml (Figure 22 C), 1A5-2G3 diluted 1:2 with HF can completely inhibit the mitogenic effect of axolemma to Schwann cells. The inhibition of the mitogenic effect of axolemma on Schwann cells by 1A5-2G3 is thus dependent upon both mitogen and antibody concentration. For a given dose of axolemma, increased amounts of antibody inhibit more mitogenicity; similarly, a given amount of antibody (for example, undiluted) inhibits

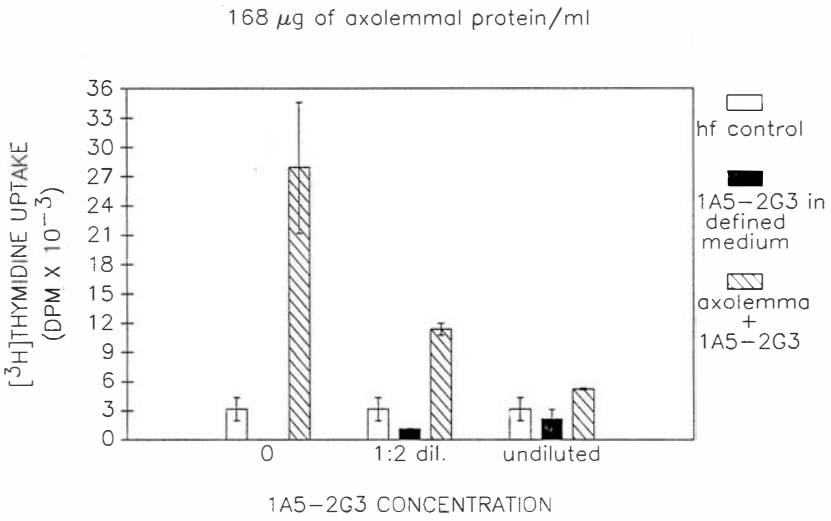

Figure 22a. 1A5-2G3 in defined media inhibits the mitogenicity of axolemma (168 μg/ml). 1A5-2G3 in defined media was incubated at the indicated dilutions with 168 μg/ml of axolemma. Open bars indicate the mitogenic effect of hf alone on Schwann cells; closed bars represent the effect of antibody alone at the indicated dilution; hatched bars represent the mitogenic effect of 168 μg/ml of axolemma treated with the dilution of antibody indicated. Each bar represents five or more determinations; standard deviations are indicated. Where no error bars appear, standard deviation is less than the width of the top of the bar.

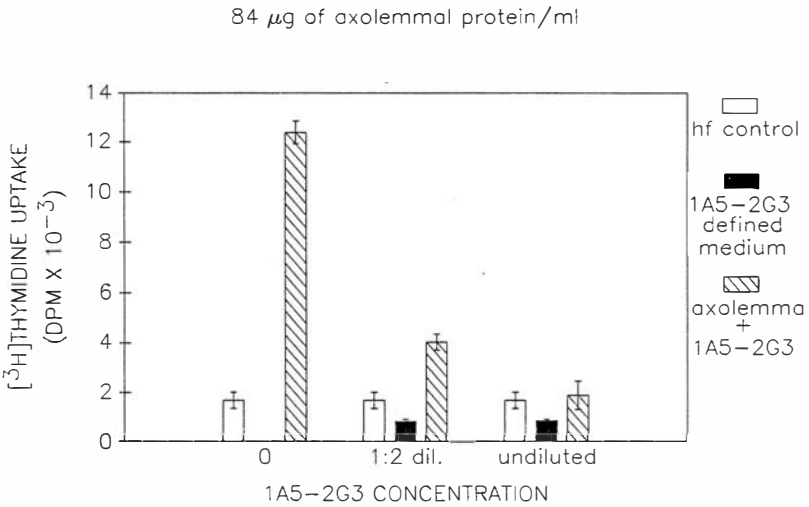

Figure 22b. 1A5-2G3 in defined media inhibits the mitogenicity of axolemma (84 μg/ml). 1A5-2G3 grown in defined media was incubated at the indicated dilutions with axolemma (84 μg/ml). Open bars indicate mitogenic effect of hf only; closed bars represent the effect of antibody at the indicated dilutions; hatched bars represent the mitogenicity of axolemma (84 μg/ml) treated with the indicated dilution of antibody. Each bar represents 5 or more determinations, and standard deviations are indicated.

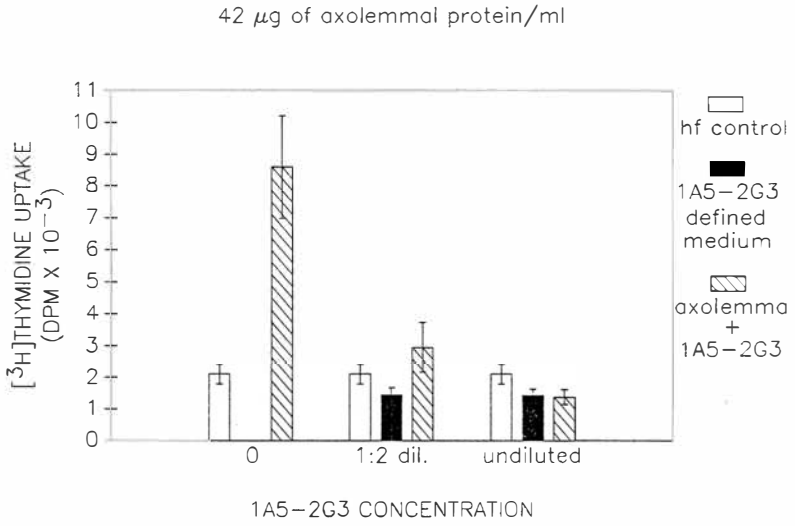

Figure 22c. 1A5-2G3 in defined media inhibits the mitogenicity of axolemma (42 μg/ml). 1A5-2G3 grown in defined media at the indicated dilution was incubated with axolemma at 42 μg/ml. Open bars indicate the mitogenic effect of hf only; closed bars represent the effect of antibody alone at the indicated dilutions; hatched bars represent the mitogenic effect of axolemma (42 μg/ml) treated with antibody at the indicated dilutions. Each bar represents 5 or more determinations and standard deviations are indicated.

smaller doses of axolemma to a greater extent than larger doses.

Figures 23 A-C show that 1A5-2G3 grown in defined media and then concentrated by Amicon filters (as described in methods) retains the ability to inhibit the mitogenicity of axolemma to Schwann cells. We found that the inhibition of concentrated 1A5-2G3 grown in defined media was variable and dependent upon treatment conditions. For example, the inhibition seen in figures 23 A-C using concentrated 1A5-2G3 is not as extensive as that seen in figures 22 A-C using unconcentrated antibody. The reasons for this variability in inhibition of mitogenic activity were not always clear.

Despite the complex behavior of the concentrated antibody, the results shown in figure 23 indicated that molecular weight filtration could be used as a method for concentrating and purifying to a certain extent the IgM 1A5-2G3. Since the antibody was now being produced in hybridoma cultures grown in defined medium, this medium could be concentrated by the Amicon XM300 filter, allowing most of the small molecular weight proteins (<300 kDa) to be removed and the 1A5-2G3 IgM proteins (>900 kDa) to be retained and thus concentrated. These processes would be important if the antibody were to be used for affinity columns, iodination, and other coupling procedures requiring homogeneous molecular species.

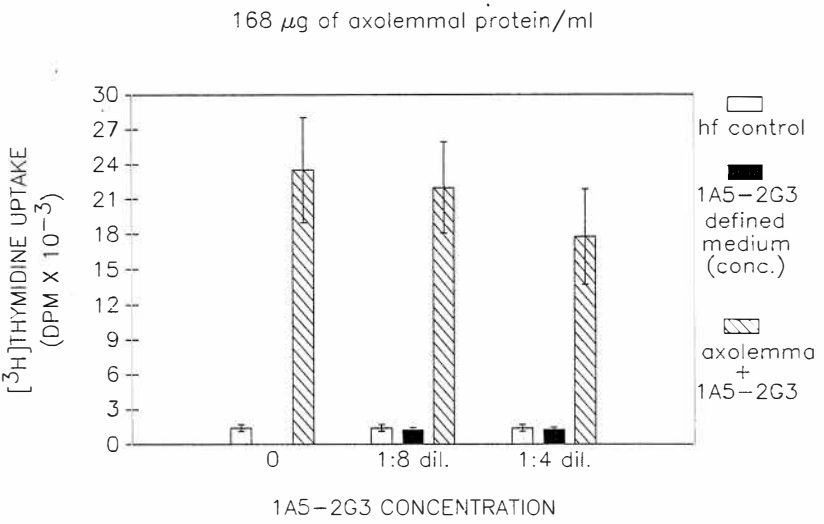

Figure 23a. 1A5-2G3 in concentrated defined media inhibits the mitogenicity of axolemma (168 μg/ml). 1A5-2G3 grown in defined media and concentrated 9-fold by filtration as described in Materials and Methods was incubated at the indicated dilutions with axolemma (168 μg/ml). Open bars indicate the mitogenic effect of hf only; closed bars represent the effect of antibody alone at the indicated dilutions; hatched bars represent the mitogenic effect of axolemma (168 μg/ml) treated with antibody at the indicated dilutions. Each point represents 5 or more determinations and standard deviations are indicated.

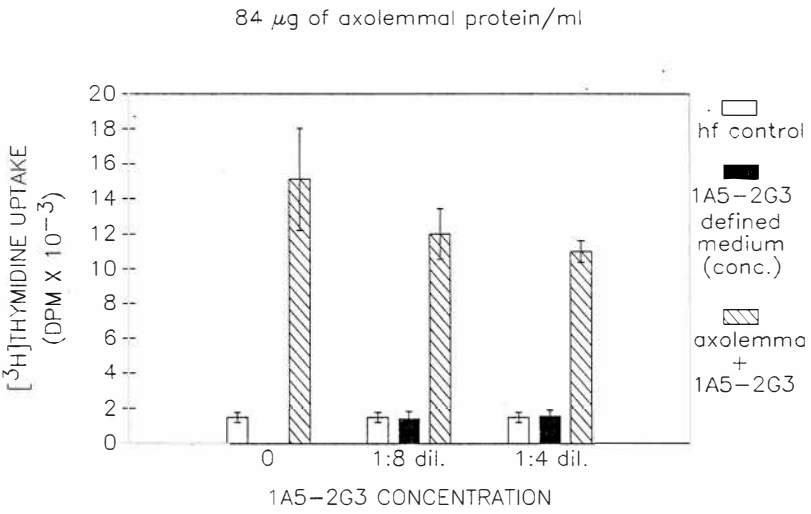

Figure 23b. 1A5-2G3 in concentrated defined media inhibits the mitogenicity of axolemma (84 μg/ml). 1A5-2G3 in defined media and concentrated 9-fold by filtration was incubated at the indicated dilutions with axolemma (84 μg/ml). Open bars indicate the mitogenic effect of hf only; filled bars represent the effect of antibody alone at the indicated dilutions; hatched bars represent the mitogenic effect of axolemma (84 μg/ml) treated with antibody at the indicated dilutions. Each bar represents 5 or more determinations; standard deviations are indicated.

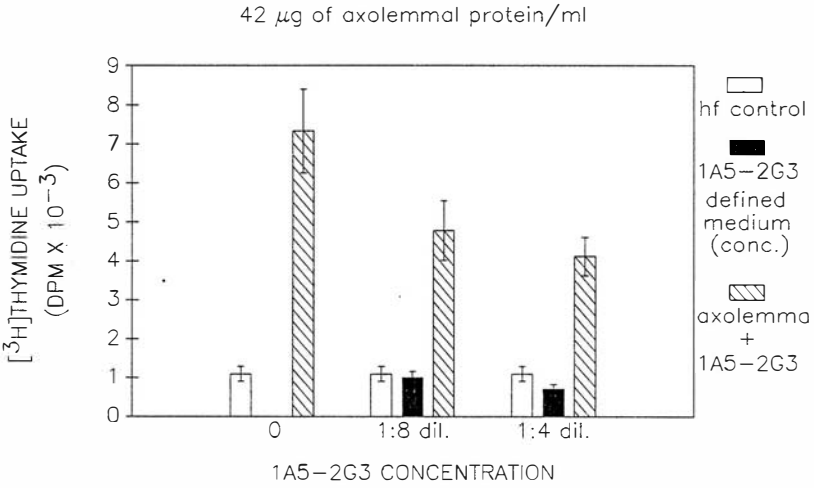

Figure 23c. 1A5-2G3 in concentrated defined media inhibits the mitogenicity of axolemma (42 μg/ml). 1A5-2G3 grown in defined media and concentrated 9-fold by filtration was incubated at the indicated dilutions with axolemma (42 μg/ml). Open bars indicate the mitogenic effect of hf only; filled bars represent the effect of antibody alone at the indicated dilutions; hatched bars represent the mitogenic effect of axolemma (42 μg/ml) treated with antibody at the indicated dilutions. Each bar represents 5 or more determinations and standard deviations are indicated.

To determine whether 1A5-2G3 was inhibiting the mitogenic effect of axolemma on Schwann cells at the site of the cell or at the site of the membrane, we treated Schwann cell cultures with 1A5-2G3 antibody for 24 hours. Separate wells on the same culture plate of Schwann cells received HF only. After removing antibody (or HF), Schwann cell cultures were treated with axolemma. As shown in figure 24, pretreatment of Schwann cell cultures with 1A5-2G3 at concentrations used in previous experiments, does not prevent proliferative response to axolemma. In the experiments described earlier, axolemma and 1A5-2G3 remained on the Schwann cell cultures for the duration of the treatment. It could be argued that the results obtained were due to 1A5-2G3 somehow inhibiting at the cell surface rather than binding to the axolemma; pretreatment of the Schwann cell cultures with 1A5-2G3 seems to exclude this possibility. This result, along with those from ELISA indicate that 1A5-2G3 is binding to axolemma, and inhibiting the mitogenic effect of axolemma to Schwann cells at the site of the membrane, possibly by binding to the mitogen itself.

--1A5-2G3 Immunoaffinity Isolation of the Axonal Mitogen for Schwann Cells:

The above results suggested that 1A5-2G3 might be used as a tool for isolating the axonal mitogen for Schwann

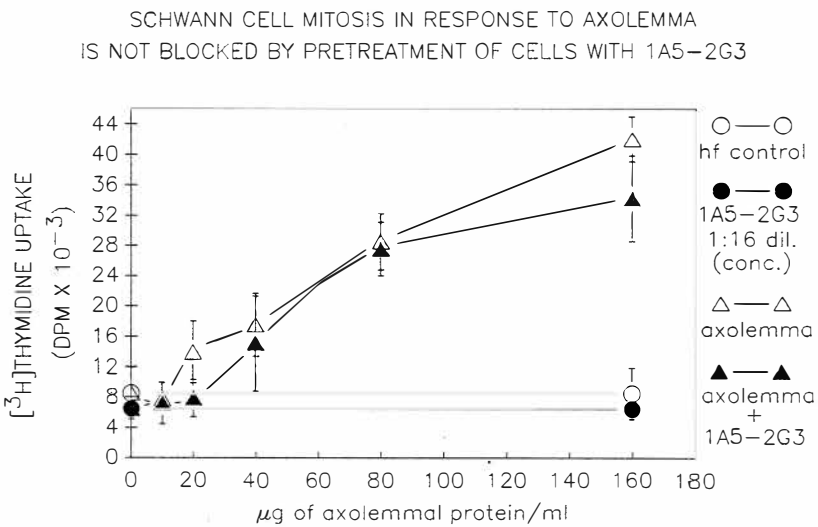

Figure 24. Schwann cell mitosis in response to axolemma is not inhibited by pretreatment of cells with 1A5-2G3. Schwann cell cultures were pretreated with hf or 1A5-2G3 in defined media concentrated 9-fold by filtration at a dilution of 1:16. This dilution of 1A5-2G3 was previously shown to inhibit the mitogenicity of axolemma when axolemma was treated with antibody. Open triangles indicate the mitogenicity of axolemma on untreated cultures at the protein concentrations shown; closed triangles indicate the effect of axolemma on Schwann cell cultures pretreated with a 1:16 dilution of 1A5-2G3. Open circles indicate proliferation of Schwann cell cultures pretreated with hf; filled circles indicate proliferation of cultures pretreated with 1A5-2G3. Each point represents 5 or more determinations; standard deviations are indicated.

cells: 1A5-2G3 bound to axolemma as determined by ELISA techniques; it inhibited the mitogenicity of axolemma and the soluble heparin extract to Schwann cells; and it did not prevent Schwann cells pretreated with antibody from proliferating in response to axolemma. 1A5-2G3 grown in defined medium and filtered as described in methods provided us a source of concentrated antibody which was also purified to the extent that small molecular weight proteins were removed. We coupled concentrated 1A5-2G3 to activated CH-Sepharose 4B as described in methods. This procedure provided us with an affinity column which we used to attempt isolation of the mitogenic activity of the soluble heparin extract.

Mitogenic soluble heparin extract was loaded onto the affinity column as described in methods. The column was centrifuged, eluted fractions collected, and washed with buffer or elution reagents as described in methods. Figure 25A shows that when mitogenic soluble heparin extract was loaded onto the 1A5-2G3 affinity column, no mitogenic activity was recovered from the column. Gel electrophoresis of the eluted fractions using silver staining indicated that the only detectable proteins eluted were in wash-through fractions 4-6. Treatment of the column with glycine/HCl buffer (0.1 M pH 2.5) failed to elute any detectable proteins. This result indicated that the mitogenic activity of the soluble heparin extract had bound to the affinity column. Because of the

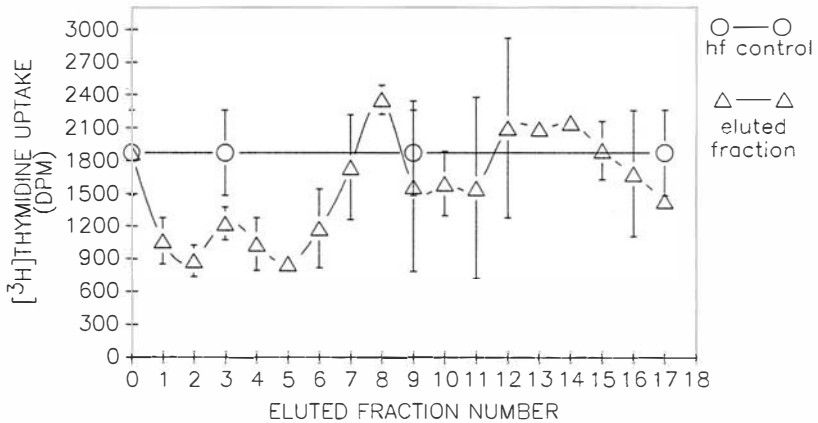

Figure 25a. Mitogenicity of fractions eluted from 1A5-2G3 affinity column. Mitogenic heparin extract was loaded onto a 1A5-2G3 affinity column in a volume of 250 µl as described in Materials and Methods. Eluted volumes collected were 250 µl each. Heparin extract loaded onto the column (8.75 µg/ml) gave a mitogenic response of 3699 ± 510 dpm; a 2-fold dilution (4.375 µg/ml) gave a mitogenic response of 3181 ± 405 dpm. Open circles indicate the mitogenic effect of hf alone (1873 ± 387 dpm); open triangles indicate the mitogenic effect of fractions eluted from the affinity column. Each point represents 2 or more determinations; standard deviations are indicated. Where no error bars appear, the standard deviation is less than the range occupied by the symbol.

centrifugation steps, eluted fractions were of the same volume as those loaded; thus the loss of mitogenic activity could not be due to dilution. Furthermore, all eluted fractions which contained detectable protein by silver stain were in the wash buffer: the glycine/HCl buffer eluted no detectable proteins, so the loss of mitogenic activity was most likely not due to inactivation by acid. It is always possible, however, that a mitogenic factor could remain undetected by protein staining if in small enough quantities.

Figure 25B shows the pH profile of the eluted fractions from the column. Glycine/HCl elution buffer was applied starting at fraction #5, but the pH of eluted fractions did not change until fraction #9. Thus there is a lag over the length of the column between what is loaded and what is eluted. As mentioned above, protein was detected only in fractions 4-6; these should not have been affected by pH changes.

The same lA5-2G3 affinity column was treated with cycles of wash buffer and elution reagents as described above; elution reagents included: 0.15 M NaCl + ammonia (pH 11), 1 M HCl, and 5 mg/ml heparin. None of these elution reagents lead to release of detectable protein from the affinity column as determined by silver staining. However, when the lA5-2G3 affinity column was treated with 6 M urea (pH 1.8), a single polypeptide species was eluted as detected by silver staining (figure 26). This result

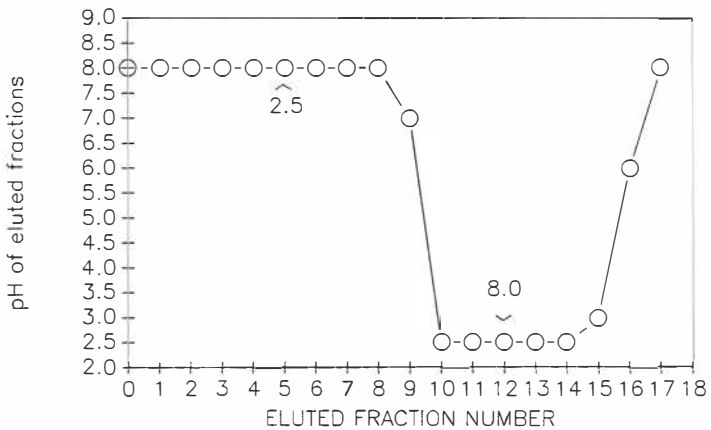

Figure 25b. pH profile of fractions eluted from 1A5-2G3 affinity column. Fractions were eluted from 1A5-2G3 affinity column with glycine/HCl buffer (0.1 M pH 2.5) as indicated in Materials and Methods. pH of eluted fractions was determined by litmus paper. Arrowheads indicate where glycine/HCl buffer was applied (2.5) and where column was equilibrated back to pH 8 with wash buffer (0.1 M Tris + 0.5 M NaCl) (8.0).

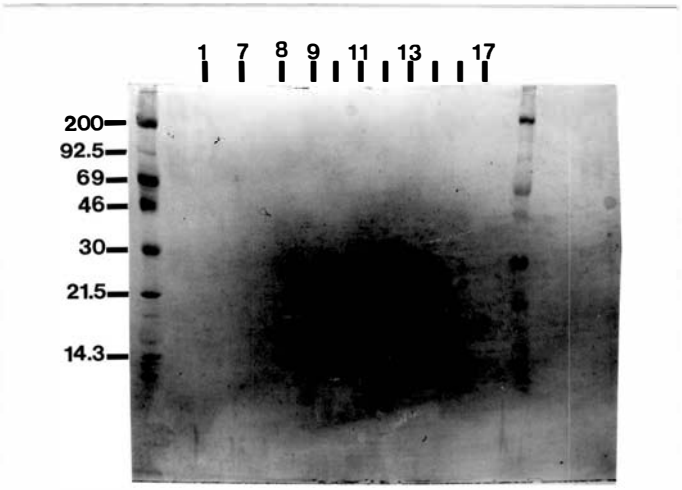

Figure 26. Elution of a single polypeptide species from 1A5-2G3 column using urea. A 1A5-2G3 column which had been previously loaded with heparin extract was treated with wash buffer: 0.1 M Tris + 0.5 M NaCl, pH 8 (fractions 1-4); 6 M urea, pH 1.8 was then applied to the column (fractions 5-12); the column was then equilibrated with wash buffer as above (fractions 13-20). The eluted fractions indicated were separated on SDS-PAGE in a 11-23% acrylamide gradient and stained using silver stain; molecular weights are indicated in kilodaltons. The only detectable protein by silver staining technique occurred in fraction 8; however, no detectable difference existed between SDS-PAGE of mitogenic heparin extract and extract protein not binding to the column. The pH of eluted fractions was 8.0 for fractions 1-8; 1.8 for fractions 9-16; 3.0 for fraction 17; 5.0 for fraction 18; 7.0 for fraction 19; and 8.0 for fraction 20.

indicated the specificity of the 1A5-2G3 column; only one fraction contained detectable protein eluted from the column, and the one fraction contained only one polypeptide species.

Figure 27 shows that after dialysis and sterile filtration, fraction #8 shown in figure 26 is retained. Due to dilution from dialysis and filtration the protein content is decreased. In addition, we see that lane 1 in figure 27, containing the same material as fraction #8 in figure 26, shows minor bands not seen previously. This is due to the longer development time used for the silver staining in figure 27 to allow for detection of the bands in lanes 2 and 3. The minor bands seen in lane 1 of figure 27 could be related to the major band in two ways: 1) the higher molecular weight band could be a dimer of the main band; 2) the lower molecular weight bands could be degradation products of the main band (this could occur due to the different acid and base treatments to which the column had been exposed). A third possibility is that the minor bands seen in lane 1 are not related to the major band, and are proteins which bind to a small degree to the column.

Detection of a single protein band in lane 3 of figure 27 indicated that the major band in lane 1 could survive dialysis and sterile filtration and thus could be tested for mitogenicity to Schwann cells. Not surprisingly, when tested on Schwann cells, eluted fraction

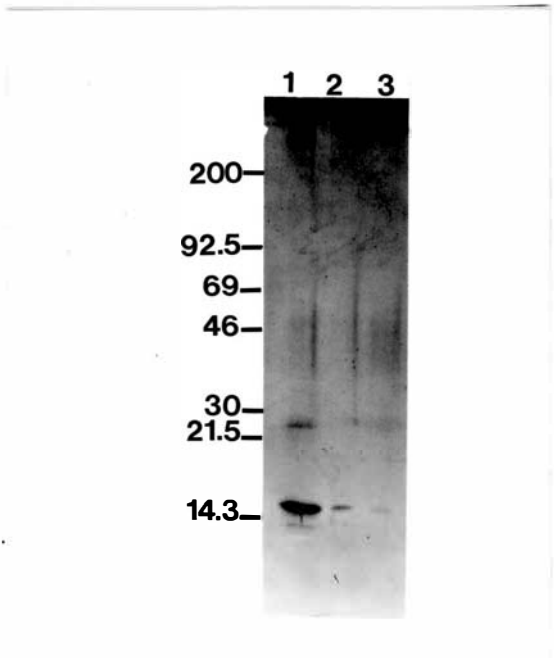

Figure 27. Dialysis and sterile filtration of protein eluted from 1A5-2G3 affinity column. Protein was eluted from 1A5-2G3 affinity column using urea as described; fraction 8, which contained the eluted protein, was separated by SDS-PAGE on an 11-23% acrylamide gradient and stained using silver stain. Lane 1 indicates the eluted fraction in urea; lane 2 dialysed fraction; lane 3 dialysed fraction which had been sterile filtered. Molecular weights are indicated in kilodaltons.

#8 was not mitogenic. If the single protein band seen in fraction #8 were the mitogen, 6 M urea most likely denatured it; denaturation often times irreversibly inactivates proteins. This result left us with the paradox of tight binding to the affinity column: the tight binding might allow for specificity, but it might also necessitate use of harsh denaturants such as urea to uncouple the ligand from the antibody.

The concentrated 1A5-2G3 appears to inhibit the mitogenicity of axolemma to Schwann cells in a manner similar to immunoprecipitation titrations; that is, very high concentrations of antibody actually inhibit the mitogenicity of axolemma less effectively than lower concentrations. This type of behavior is shown in figure 28. Concentrated 1A5-2G3 was incubated with axolemma as described; a 100-fold dilution of the antibody had a greater inhibitory effect than a 50-fold dilution on the mitogenicity of axolemma to Schwann cells. It can be seen that at the lowest concentration of axolemma tested, the 50-fold dilution of 1A5-2G3 had no perceptible inhibitory effect, while the 100-fold dilution of antibody inhibited almost 60% of the mitogenicity of axolemma.

Despite the complicated behavior of the concentrated antibody and the harsh conditions needed to remove proteins from the affinity column, we attempted to demonstrate that the results we obtained were not due to nonspecific IgM binding properties. Purified IgM recognizing an undefined

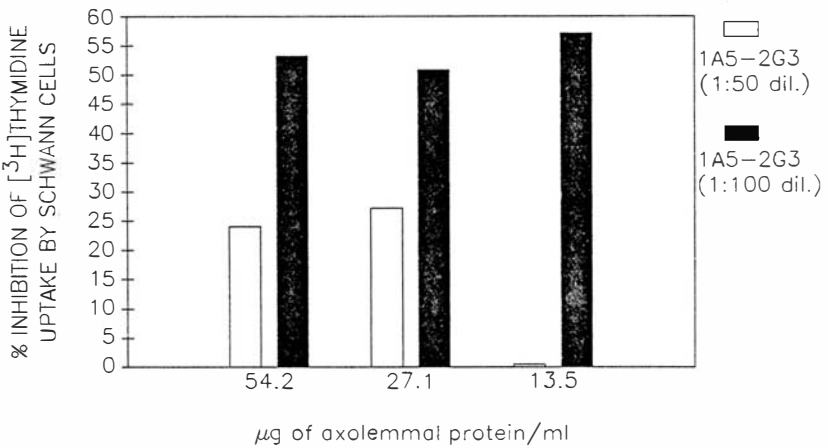

Figure 28. Inhibition of axolemmal mitogenicity by 1A5-2G3 in concentrated defined media. 1A5-2G3 grown in defined media and concentrated 40-fold by filtration was incubated at the indicated dilutions with axolemma. The mitogenic response of axolemma treated with antibody at the indicated dilutions was compared with untreated axolemma at the same protein doses to calculate the % inhibition of proliferation due to antibody treatment. No detectable inhibition of proliferation was observed at 13.5 μg/ml of axolemmal protein using a 1:50 dilution of antibody.

epitope (Sigma TEPC 183) was used as a nonspecific IgM to investigate the specificity of inhibition of lA5-2G3.

Figure 29 shows that lA5-2G3 inhibits the mitogenicity of axolemma to Schwann cells to a greater extent than does Sigma IgM. The same amount of lA5-2G3 and Sigma IgM was used for inhibiting as determined by ELISA. Sigma IgM appears to have a slight inhibitory effect, but it is significantly less than the effect of lA5-2G3.

Further support for the specificity of lA5-2G3 as an affinity tool for isolating the axonal mitogen for Schwann cells is shown in figure 30. As indicated in figure 30, almost 90% of the mitogenic activity associated with the Sigma IgM-coupled resin could be recovered in the supernatant; this indicated that little or no mitogenic activity bound to the Sigma IgM resin. In contrast, just under 50% of the mitogenic activity applied associated with the lA5-2G3-coupled resin could be recovered in the supernatant; thus, mitogenic activity bound to the lA5-2G3 resin.

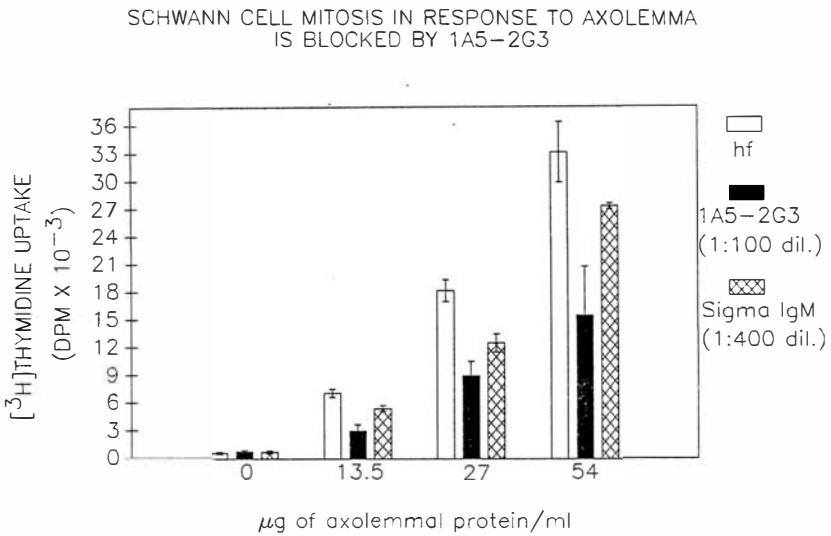

Figure 29. Schwann cell mitosis in response to axolemma is inhibited by 1A5-2G3 to a greater extent than by non-specific IgM. Axolemma was treated with 1A5-2G3 or Sigma IgM TEPC 183 (as a non-specific IgM) at the indicated antibody dilutions. ELISA techniques determined that the indicated dilutions of antibody contained the same amount of IgM. Open bars represent the mitogenic effect of axolemma at the indicated protein concentrations; closed bars indicate the effect of axolemma treated with 1A5-2G3; crossed bars indicate the effect of axolemma treated with Sigma IgM. Each bar represents 5 or more determinations and standard deviations are indicated.

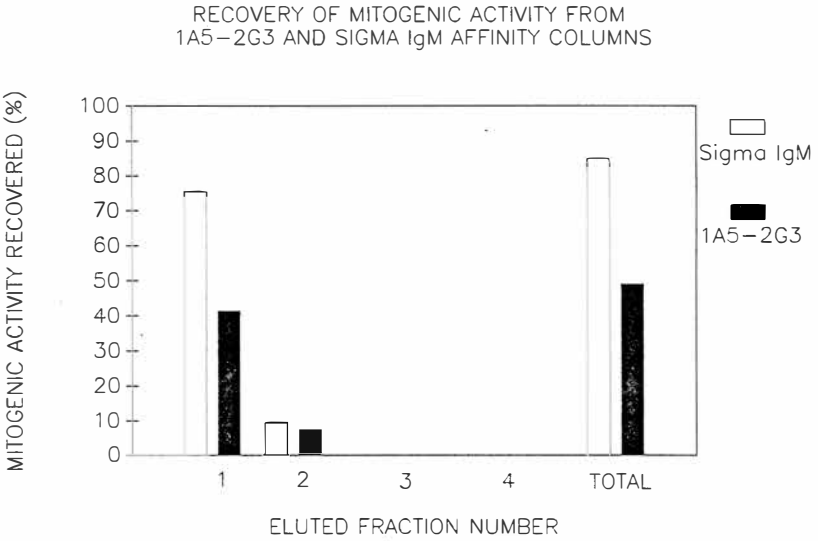

Figure 30. Recovery of mitogenic activity from 1A5-2G3 and Sigma IgM columns. Mitogenic heparin extract was added to 1A5-2G3 and Sigma IgM resins as indicated in Materials and Methods. Resins were centrifuged and the supernatants removed and sterile filtered. Resins were washed as described and supernatants collected and sterile filtered. Supernatants from the recovered resins were tested for mitogenicity and compared with the mitogenicity of the starting heparin extract to determine % of starting mitogenic activity recovered from each resin. No detectable mitogenic activity was recovered from fraction 3 or 4 of 1A5-2G3 resin (filled bars) or from fraction 4 of the Sigma IgM resin (open bars). The total cumulative mitogenic activity recovered from each resin is indicated as "total".

DISCUSSION

In this discussion I will relate our in vitro studies of axonal stimulation of Schwann cell proliferation to in vivo events. Our results from biochemical characterization of the axolemmal mitogen for Schwann cells will be presented and summarized in the form of a model depicting the molecular nature of the mitogen. Finally, the evidence of a monoclonal antibody which inhibits the mitogenicity of axolemma to Schwann cells will be discussed as an important tool for isolating to homogeneity the molecule responsible for axonal promotion of Schwann cell proliferation.

Base extraction of non-mitogenic axolemmal proteins:

The studies of Sobue and Pleasure (1985) had shown that the adhesion of axolemmal fragments to Schwann cells was closely linked to axolemmal induction of Schwann cell mitosis. Using ^{125}I-labelled axolemma, Sobue and Pleasure (1985) showed that adhesion of axolemmal fragments to Schwann cells was specific and time dependent. In an attempt to extend the studies of Sobue and Pleasure (1985), we fluorescently labelled axolemma with FITC and developed a fluorescent binding assay to measure the extent of

binding of axolemma membrane to Schwann cells in culture. Further investigations using the fluorescently labelled axolemma led us to an important control experiment: if fluorescently labelled axolemma were to serve as a valuable probe to how normal axolemma interacted with Schwann cells, then methods used to fluorescently label the axolemma should not drastically alter its properties. Meador-Woodruff et al. (1985) had shown that FITC conjugation did not alter the mitogenicity of axolemma-enriched fractions; thus we had a rationale for correlating results using unlabelled axolemma with those using FITC labelled axolemma.

One difference between the protocol used by Meador-Woodruff et al. (1985) and the method we used to conjugate FITC to axolemma was the use of pH 9 buffer. In the studies of Meador-Woodruff et al. (1985), solid FITC powder was added to axolemma which had been treated with 0.075 M final concentration of sodium carbonate/bicarbonate buffer at pH 9. As indicated above, Meador-Woodruff et al. (1985) found that this treatment did not alter the mitogenicity of axolemma. We wanted to ensure that the FITC-treated axolemma remained sterile, so we dissolved the FITC powder in 0.5 M sodium carbonate/bicarbonate buffer at pH 9, and then sterile filtered the solution. The sterile FITC was then added to axolemma to make the mixture 0.275 M in final sodium carbonate/bicarbonate buffer. All other

aspects of the treatment were similar to those carried out by Meador-Woodruff et al. (1985).

When we treated axolemma with the same buffer used to conjugate FITC to axolemma (0.27 M sodium carbonate/bicarbonate, pH 9) in the absence of fluor, we obtained a surprising result. Treatment of axolemma with 0.27 M sodium carbonate/bicarbonate buffer at pH 9 resulted in a recovered pellet containing 50% of the starting protein and 95% of the starting mitogenic activity. This result indicated that the axolemmal mitogen is ionically unchanged at pH 9; thus, it will remain associated with the membrane while other proteins are removed.

Alkaline extraction has been previously shown to solubilize membrane proteins from red blood cells by the use of NaOH (Steck and Yu, 1973) and from rat liver microsomes using Na_2CO_3 (Fujiki et al., 1982). Philipson et al. (1987) have shown that alkaline extraction of canine cardiac sarcolemmal vesicles using NaOH results in extraction of 33% of the membrane protein. Reconstitution of the alkaline extracted membranes resulted in recovery of 50% of the protein of vesicles reconstituted from control sarcolemma, yet total Na^+-Ca^{2+} exchange activity recovered was unchanged; the authors thus postulated that high pH extracts specific proteins from the membrane, leaving the protein(s) responsible for Na^+-Ca^{2+} exchange fully active and retained in the membrane.

Although the pH 9 supernatant could not directly be tested for mitogenicity on Schwann cells due to the toxicity and molar strength of the buffer, washing with isotonic saline in a 10 kDa molecular weight cutoff Centricon tube allowed the supernatant to be added to cells without toxic effect. The pH 9 extracted supernatant of axolemma contains 50% of the protein in the starting axolemma, yet after washing with isotonic saline, the mitogenicity of this supernatant represents only 8 $\pm$ 2% of the mitogenicity of the starting axolemma. This result further supports the idea that pH 9 extraction of axolemma leaves the vast majority of mitogen undisturbed and membrane-bound while removing non-mitogenic protein from the axolemmal membrane. Retention of binding of the axolemmal mitogen to the membrane after pH 9 extraction indicates that the mitogen may be basic in nature and thus contain regions that are highly positively charged. For example, in proteins the only ionizable groups of amino acids which would have positive charge remaining at pH 9 would be the lysyl amino group of lysine (pK_a: 10.8) and the guanidine group of arginine (pK_a: 12.5) (Palmer, 1985). Retention of the axolemmal mitogen in the membrane after base extraction might therefore indicate that the molecule, if a protein, contains large numbers of lysines and/or arginines.

Enzymatic cleavage of sulfated proteoglycans solubilizes axolemmal mitogenic activity:

The idea that the axolemmal mitogen for Schwann cells had basic characteristics and contained regions that were highly positively charged had added significance in light of experimental result of Ratner et al. (1985). The work of Ratner et al. (1985) demonstrated that interfering with proteoglycan synthesis of neurons or treatment of neuritic membranes with heparitinase significantly depleted the mitogenicity of these membranes to cultured Schwann cells. Since heparitinase cleaves the glycosidic bonds of sulfated glycosaminoglycans, we postulated that heparitinase might deplete the mitogenicity of neurite membranes to Schwann cells (Ratner et al., 1985) by releasing a positively charged mitogen bound to the sulfated heparan sulfate proteoglycans.

When axolemma was treated with heparitinase, a soluble extract was obtained which was mitogenic to Schwann cells. Solubilization of mitogenic activity for Schwann cells was significant in itself; earlier attempts to solubilize the mitogenicity of axolemma to Schwann cells using traditional detergents were not consistently reproducible (Cassel et al., 1982; Dinneen, 1985). Glaser (1978) predicted that "the greatest obstacle in all of these systems will no doubt be the difficulty in fractionating membrane proteins by novel techniques so that they can still interact with

live cells while avoiding the toxic effects of detergents." It seems that the toxicity of detergents to Schwann cells accounts for the variable results of Cassel et al. (1982) and Dinneen (1985) in attempting to solubilize the mitogenicity of axolemma to Schwann cells. Even though the work of Cassel et al. (1982) included steps to remove detergent by dialysis, axolemma extracts solubilized by deoxycholate sometimes appeared toxic to the cells. Another explanation for why detergents might not allow active soluble mitogen to be recovered could lie in the tendency for some detergents to complex with proteins, thereby unfolding and inactivating them by denaturation (Helenius and Simons, 1975).

Although heparitinase solubilized some of the mitogenic activity of axolemma to Schwann cells, the heparitinase treated pellet was also still mitogenic. Furthermore, heparitinase-solubilized extracts were more mitogenic to Schwann cells as the dose of protein was decreased. This appeared to be due to the toxic effect of heparitinase at high doses; as the dose of soluble extract was increased the amount of heparitinase (all of which presumably remained soluble) was also increased, and had adverse effects on the cells. Nevertheless, solubilization of the mitogenicity of axolemma to Schwann cells using heparitinase helped further characterize the nature of the mitogen. Results using heparitinase indicated 1) that cleavage of the glycosidic bonds of sulfated proteoglycans

released the mitogen from these proteoglycans and/or 2) cleavage of the glycosidic bonds released the sulfated proteoglycan-mitogen complex from the surface of the axolemmal membrane. Figure 31 illustrates a model which we developed to explain the molecular aspects of the axolemmal mitogen and its association with the membrane surface. We can see that the effect of breaking the glycosidic bonds of sulfated proteoglycans (by use of heparitinase) on releasing the mitogen from the membrane surface depends on how the sulfated proteoglycan is associated with the axonal membrane. Kjellen et al. (1980, 1981) showed that proteoglycans which are associated with the membrane ionically or plasma-membrane anchored can both exist on the same tissue surface. By cleaving the glycosidic bonds of sulfated proteoglycans found in axolemma, we can see from figure 31 that the mitogen might be released from the proteoglycan itself, or if the proteoglycan is ionically associated with the axolemmal surface, the mitogen-proteoglycan complex might be released.

Heparin solubilizes the mitogenic activity of axolemma:

Considering the result of heparitinase treatments of axolemma along with that of the pH 9 experiments, (which indicated that the mitogen could be ionically associated with the sulfated proteoglycans) we predicted that the mitogen could be displaced by heparin. Kraemer (1977)

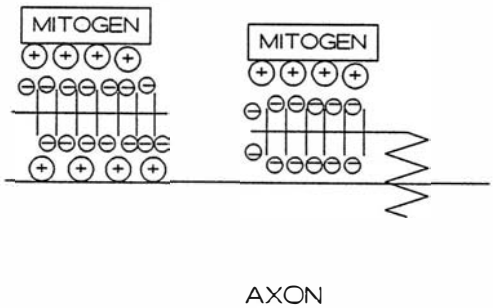

Figure 31. Molecular model of interaction of axolemmal mitogen with surface heparan sulfate proteoglycan. The axolemmal mitogen is represented as a positively charged molecule (rectangle) associated with negatively charged heparan sulfate proteoglycan. Heparan sulfate proteoglycan can be anchored in the lipid bilayer (right) or ionically associated with the membrane surface (left).

showed that heparin can displace heparan sulfate from cell surfaces. In addition, heparin shares structural homologies with heparan sulfate (Alberts et al., 1983), including carbohydrate composition of the repeating disaccharide units, and thus might serve as an effective competitor for binding the mitogen molecule. Since heparin is more highly sulfated than heparan sulfate (Margolis and Margolis, 1979), it should be an effective chelator of any positively charged molecules associated with heparan sulfate proteoglycans.

As shown in figure 6, treatment of axolemma with heparin results in solubilization of mitogenic activity. Although the heparin treated axolemma pellet is still mitogenic to Schwann cells, the mitogenic heparin extract has a higher specific mitogenic activity than the treated or starting axolemma. Thus, heparin treatment of axolemma differentially displaces the mitogen: non-mitogenic proteins are displaced by heparin too, but the mitogenic activity is enriched in the heparin extract. As shown in figure 11, gel electrophoresis of the heparin extract indicates one protein which is most highly enriched in comparison to the heparin treated and untreated axolemma; whether this enriched protein is responsible for the correlated enrichment in mitogenic activity requires further investigation. Recent preliminary findings (DeCoster and DeVries, unpublished observations) indicate that when mitogenic heparin extract is separated into three

molecular weight fractions by Centricon filtration (see Materials and Methods), mitogenic activity is recovered only in the >30 kDa fraction. Further analysis of these separated fractions is required to interpret this result: the mitogen itself could be >30 kDa in molecular weight, or a <30 kDa mitogen might have complexed with heparin to remain above the filter.

In contrast to the possible inhibitory effect of heparitinase, heparin itself has no effect on Schwann cell proliferation at the doses of protein shown in Figure 6 (50 μg of heparin/ml at the highest dose of soluble extract protein). This result differs from that of Ratner et al. (1988), who found that a neuronal cell surface protein obtained from neonatal rat brain was mitogenic to Schwann cells, but that this mitogenic effect was half maximally inhibited by the presence of 0.5-0.7 μg of heparin/ml. One possible explanation for this difference is that the neuronal mitogen for Schwann cells is developmentally altered. Heparin has been shown to potentiate the mitogenicity of acidic fibroblast growth factor more than 100-fold (Gimenez-Gallego et al., 1986) and to protect both acidic and basic fibroblast growth factor from inactivation (Gospodarowicz and Cheng, 1986). Recent results of Chen and DeVries have shown that the heparin extract of axolemma which is mitogenic to oligodendrocytes (Chen and DeVries 1989a) can be protected from heat inactivation by heparin. In addition, as shown in Figure 10 of Results, the

mitogenic activity of heparin extract to Schwann cells does not appear to be completely destroyed be heat treatment of 100° C for 10 minutes. In contrast, the mitogenicity of axolemmal membrane to Schwann cells is completely destroyed by heating at 80° C for 10 minutes (DeVries et al., 1982). Thus, slight developmental alterations in the neuronal mitogen for Schwann cells might drastically change the mitogenic effect in the presence of heparin.

Solubilization of mitogenic activity from axolemma using heparin provides a third method of characterizing the molecular nature of the mitogen. Evidence from pH 9 extraction, heparitinase treatment, and heparin treatment of axolemma all support the molecular model for the axonal mitogen to Schwann cells shown in figure 31. By comparing the effects of the different treatments on the mitogenicity of axolemma to Schwann cells, we can provide the following "clues" about the molecular nature of the mitogenic molecule: 1) Extraction of non-mitogenic protein from axolemma by pH 9 treatment indicated that the axonal mitogen is basic in character; some region of the molecule may be highly positively charged. 2) Treatment of axolemma with heparitinase solubilized mitogenic activity, indicating that cleavage of the glycosidic bonds of sulfated glycosaminoglycans released the mitogen from heparan sulfate proteoglycans and/or the mitogen-proteoglycan complex from the membrane surface. 3) Treatment of axolemma with heparin solubilized mitogenic

activity, indicating that heparin (which is highly sulfated and thus negatively charged), chelated the (positively charged) mitogen from the sulfated heparan sulfate proteoglycans and/or displaced the mitogen-proteoglycan complex from the membrane surface.

Solubilization of the mitogenic activity from axolemma using heparin provides us with additional information about the molecular nature of the mitogen. As shown in Table 4, about 15% of the mitogenic activity of axolemma for Schwann cells was solubilized using heparin; the heparin treated pellet was still mitogenic. We found that heparin treated pellets re-extracted with heparin lost little of their mitogenic activity, and that these additional heparin extracts contained only small amounts of protein and mitogenic activity. It therefore seems that most of the heparin-extractable mitogenicity can be removed from axolemma by one treatment. This provides us with two possibilities to explain the mitogenic activity of axolemma: 1) axolemma contains more than one type of mitogen molecule for Schwann cells, one which is heparin extractable and one which is not or 2) axolemma contains only one type of mitogen molecule for Schwann cells, but some of these molecules are associated with heparin displaceable proteoglycans, and some associated with proteoglycans anchored in the lipid bilayer of the axon plasma membrane. Studies by Kjellen et al. (1980, 1981) have demonstrated that both heparin displaceable and

anchored proteoglycans can exist on the same tissue surface. Proteoglycans of neuronal origin have been identified, and these proteoglycans seem to be predominantly linked to heparan sulfate (Lander et al., 1982; Hampson et al., 1984; Maresh et al., 1984; Morris, 1984). Anchored proteoglycans may be displaced by limited trypsin cleavage at the membrane surface (Kjellen et al., 1981). It would be of interest to determine if this type of treatment could remove heparin resistant mitogenic activity from the axolemmal surface.

We have presented evidence which supports the model of a positively charged mitogen for Schwann cells associated with the negative charge of sulfated proteoglycans at the axolemmal surface. Solubilization of the mitogen has been achieved using heparitinase and heparin. Heparin appears to offer a preferable way of isolating the axolemmal mitogen for Schwann cells, since heparin-treated axolemma results in isolation of a soluble extract which has a higher specific mitogenic activity than the recovered pellet and the untreated axolemma. Preliminary evidence from gel electrophoresis of this soluble mitogenic extract showed one protein band which appeared greatly enriched compared to the recovered pellet.

Isolation of the axonal mitogen for Schwann cells using monoclonal antibodies:

Two properties of the mitogenic heparin extract prompted us to use this extract to raise monoclonal antibodies against the axolemmal mitogen for Schwann cells. 1) The heparin extract was higher in specific mitogenic activity than untreated axolemma; although not homogeneous, figure 12 shows that the gel electrophoresis pattern of heparin extract is much less complicated than untreated axolemma. 2) Solubility of the heparin extract is a great advantage in itself: the extract can be passed over affinity columns to remove and isolate the mitogen molecule.

As described in results, we have isolated a monoclonal antibody (IgM: 1A5-2G3) which inhibits the mitogenic effect of axolemma and heparin extract to Schwann cells. Comparison with a non-specific source of IgM (Sigma TEPC 183) demonstrates that a significant portion of the inhibitory effect of 1A5-2G3 is specific. Using 1A5-2G3 coupled to Sepharose, one major polypeptide species can be removed from the heparin extract; this removal correlates with the loss of mitogenic activity. Nearly all mitogenic activity can be recovered from heparin extract interacted with Sigma IgM-coupled Sepharose; less than 50% is recovered when heparin extract is interacted with 1A5-2G3-coupled Sepharose. The inability to bind all mitogenic activity to 1A5-2G3-coupled Sepharose may indicate a lack of antibody coupled to the resin. Alternatively, heparin extract and 1A5-2G3-coupled Sepharose might need to be

interacted for long periods of time to achieve optimal binding of the mitogen to the antibody. The work of Noronha et al. (1989) for example, indicated that antigen needed to be cycled through the affinity column several times to obtain optimal binding.

The preliminary evidence using monoclonal antibody 1A5-2G3 indicates that this IgM can be used as a tool for isolating to homogeneity the mitogenic activity of the heparin extract and thus elucidating the molecular identity of the axonal mitogen for Schwann cells. The fact that 1A5-2G3 can inhibit 100% of the mitogenic activity of untreated axolemma at certain doses indicates that the axolemmal mitogen for Schwann cells may be of a single molecular type. Solubilization of heparin resistant axolemmal mitogenicity (possibly by use of trypsin as mentioned above) would allow testing this hypothesis: if both heparin displaceable and heparin resistant mitogens could be bound by 1A5-2G3 coupled Sepharose, molecular similarities would be established.

Removal of bound polypeptide from the 1A5-2G3-coupled Sepharose in an active mitogenic form is the most favorable condition for interpreting what has bound to the column. Thus far, we have only been able to achieve removal of affinity-bound protein by the use of urea, the denaturing properties of which often inactivates the bioactivity of molecules. However, by interacting mitogenic heparin extract with 1A5-2G3-coupled Sepharose and analyzing the

proteins not bound to the resin, we should be able to obtain important information about the molecular identity of the axonal mitogen for Schwann cells. Further, removal of the single polypeptide species from affinity-resin, even if denaturants must be used, should still allow analysis by Western blotting and N-terminal sequencing techniques to determine whether the axonal mitogen for Schwann cells we have isolated is of the group of previously known mitogenic factors, or is a novel protein.

Another possible way of identifying separated protein trophic factors after denaturation has been outlined by Carnow et al. (1985) and Rudge et al. (1987). These investigators found that after separating neuronotrophic factors by SDS-PAGE and blotting the proteins onto nitrocellulose, cultured neurons would preferentially associate with certain separated proteins of distinct molecular weight. This method was used to help identify the molecular weight of the trophic factor. In theory, this method might be used to identify the axonal mitogen for Schwann cells: separated axolemmal proteins blotted onto nitrocellulose might cause the association and proliferation of Schwann cells in distinct molecular weight regions, thus helping us to identify the axolemmal mitogen for Schwann cells.

Relevance to CNS glial proliferation:

Since the axolemmal source of mitogen described in this work is CNS-derived, our laboratory was interested in determining whether heparin extract of axolemma is mitogenic to oligodendrocytes. Chen and DeVries (1989) had previously shown that axolemma is mitogenic to oligodendrocytes. When heparin extract mitogenic to Schwann cells was tested on oligodendrocytes, it was found to cause proliferation in these cell types as well (Chen and DeVries, 1989a). This result indicates that the same or similar molecules may be mitogenic to both PNS and CNS glia. However, the results of Mason et al. (1989a) showed that plasma membrane from the CNS granule cells could cause Schwann cells but not oligodendrocytes to proliferate. Thus the PNS and CNS glia might differentially respond to a given membrane as a mitogen. Obviously, purification to homogeneity of the mitogenic molecule(s) by immuno-affinity techniques would clarify this issue.

Relation of autocrine growth factors to the axolemmal mitogen:

Many of the previous experimental results which imply the axon as a direct mitogen for glial cells may need to be reinterpreted now that there is evidence of an autocrine growth factor for Schwann cells (Porter et al., 1987). Bunge (1987) has proposed that the axon may actually serve as a substrate to which a Schwann cell autocrine factor

binds. Once the autocrine factor binds to the axon, the axonal surface provides a mitogenic signal to Schwann cells.

Many results which have been interpreted as describing the characteristics of the axonal mitogen for Schwann cells can also be conceived as describing the characteristics of an axonal "presentation factor". Heat and trypsin lability of the mitogenic factor in axolemma (DeVries et al., 1982) may suggest the protein nature of an axonal mitogen, or alternatively, an axonal protein necessary for presentation of a Schwann cell autocrine factor. Salzer et al. (1980a) originally interpreted the recovery of mitogenic activity of trypsinized neurites as regeneration of the axonal mitogen by neuronal cells. However, in light of recent ideas about a Schwann cell autocrine factor, this result could be as well interpreted as recovery of mitogenicity via regeneration of a presentation factor at the neuronal surface which is required for binding the autocrine factor.

The results of Ratner et al. (1985) could also be interpreted as heparan sulfate proteoglycans are important for the axonal mitogen or for the axonal presentation molecule which binds an autocrine factor. Either function of the sulfated proteoglycans would by supported by the findings of Margolis and Margolis (1979) that much of the heparan sulfate found in the brain is attached to membranes in fractions that appear to be enriched in plasma membranes. This would be consistent with the idea that

heparan sulfate serves as a "presenter" on the surfaces of cells. Heparan sulfate has been found on the surface of many cell types (Kraemer, 1971; Kraemer and Smith, 1974; Buonassisi and Root, 1975; Akasaki et al., 1975; Underhill and Keller, 1977; Oldberg et al., 1977). Thus, in the case of axons, heparan sulfate might present a neuronal mitogen to Schwann cells, or alternatively, present a Schwann cell autocrine factor to the cells in mitogenic form. Finally, our results showing that a monoclonal antibody inhibits the mitogenicity of axolemma to Schwann cells (DeCoster and DeVries, 1989a) could also be explained by an antibody which inhibits the binding of an autocrine factor to a presentation molecule.

It can be seen from the above examples that many descriptions of the axon containing a mitogen for Schwann cells could also be explained by the axon containing a molecule which binds autocrine factors for Schwann cells. It seems that the issue of axon as mitogen for Schwann cells or substrate for autocrine factor cannot be resolved until individual mitogens and their receptors are isolated. In this respect, isolation of nerve growth factor (NGF) and its receptor has provided an interesting model for how binding of a growth factor by one cell can permit another cell type to utilize the bound molecule. Johnson et al. (1988) have provided the model of Schwann cells making NGF and binding this molecule at low affinity NGF receptors. NGF bound by Schwann cells is then given up to the high

affinity NGF receptors found on axons during the process of axonal outgrowth in development and regeneration. The Schwann cell serves as a relevant substrate for NGF: neurons need to be attracted to Schwann cells during development and regeneration for ensheathment and myelination to occur. In a similar fashion the axon may be an appropriate substrate for binding a Schwann cell autocrine factor: Schwann cells need to proliferate in response to axons so that ensheathment and myelination of all axons can be accomplished.

Likely molecular explanation for the axon as a mitogen for Schwann cells:

In light of the above discussion, binding of a Schwann cell autocrine factor to the axonal surface may explain why axons are mitogenic to Schwann cells. However, recent attempts at isolating the axonal molecule(s) involved in Schwann cell proliferation provide evidence that makes the role of an autocrine factor more difficult to envision. Our work at solubilizing the mitogenic activity of axolemma for Schwann cells (DeCoster and DeVries, 1988, 1989) using heparin indicates that molecules in solution, which were once associated with the axonal surface, can still provide a mitogenic signal to Schwann cells. The work of Ratner et al. (1989) has shown that the mitogenic activity of embryonic rat brain to Schwann cells can be extracted and

bound to a heparin sepharose column. The mitogenic activity bound to the heparin column can then be eluted using NaCl, indicating that the mitogen is no longer associated with proteoglycans. Although the molecular identity of this mitogenic activity is not known, it is difficult to envision how a soluble heparin-binding molecule, when added to Schwann cell cultures would help present an autocrine factor to these cells; as the molecules of a soluble extract are separated into their different components, the most likely explanation for their mitogenic activity is that they themselves are mitogens.

The ultimate resolution of axon as mitogen proper or substrate for autocrine factor seems to lie in isolation of axonally-derived molecules of a single species. If a single molecular species can cause Schwann cell proliferation, the most likely mode is via binding to a receptor on the Schwann cell surface. Precedents for such a mechanism of action include platelet-derived growth factor (PDGF) which stimulates DNA synthesis and cell division in Swiss 3T3 cells in the absence of any other synergistic factor (Rozengurt, 1986). We have used a monoclonal antibody raised against the mitogenic heparin extract to begin the preliminary steps of isolating the axonal mitogen for Schwann cells to a single molecular species (DeCoster and DeVries, 1989a). The monoclonal antibody (1A5-2G3), which we are using to isolate the axolemmal mitogen, can remove mitogenic activity from the

soluble heparin extract. However, when coupled to CH-Sepharose resins, 1A5-2G3 binds protein which is only displaced by denaturants such as urea. The denaturing effect of urea most likely irreversibly inactivates any mitogenic activity of the bound protein. However, isolating this protein may allow N-terminal amino acid analysis and comparison with other known growth factors to determine if the axonal mitogen for Schwann cells belongs to a class of previously described mitogens or is novel in character.

Summary:

Using the initial model system of FITC-labelled axolemma to study axon-Schwann cell interactions, we came upon the important finding, through a control experiment, that pH 9 extraction of axolemma dissociates only nonmitogenic protein from the membrane: approximately 95% of the mitogenic activity for Schwann cells remains associated with the membrane pellet. By treating the axolemma with heparitinase, we obtained a soluble extract mitogenic to Schwann cells; this result was of significance in light of the drawbacks of traditional techniques using detergents, which were often toxic to the cells. We were able to obtain a soluble extract mitogenic to Schwann cells by treating axolemma with heparin. The heparin solubilized extract had a higher specific mitogenic activity than the

starting material. The results of the mitogenic behavior of axolemma to Schwann cells after treatments with pH 9 buffer, heparitinase, or heparin all supported a general molecular model of the axonal mitogen: a positively charged mitogen molecule associated with the negative charge of sulfated proteoglycans at the axonal surface.

Using the mitogenic heparin extract of axolemma, we raised and isolated a monoclonal antibody (IgM: 1A5-2G3) which inhibited the mitogenicity of untreated axolemma and the heparin extract to Schwann cells. Coupling of 1A5-2G3 to Sepharose has allowed preliminary isolation of mitogenic activity from the heparin extract. Since heparin extract is also mitogenic to oligodendrocytes, purification of the axonal mitogen using immuno-affinity techniques should clarify the differences or similarities of the proliferative signals which axons communicate to CNS and PNS glia. Determination of the molecular identity of these proliferative signals will help our understanding of the normal developmental course of nervous system function.

Major abnormalities in the nervous may occur because of the effect of proliferative signals on glia. Neurofibromatosis would appear to involve the inappropriate proliferation of Schwann cells with the consequent formation of tumors (Schwannomas). In the case of multiple sclerosis, it might be the lack of oligodendrocyte proliferation after neuronal damage that prevents the type of regeneration we see Schwann cells provide to the

peripheral nervous system. Understanding the control of glial cell proliferation, which is so essential to normal development and regeneration of the nervous system, will most certainly depend on the identification of the molecules causing these cells to divide.

LIST OF REFERENCES

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Abercrombie, M., and Johnson, M. L., Quantitative histology of Wallerian degeneration. I. Nuclear population in rabbit sciatic nerve, J. Anat., 80 (1946) 37-50.

Abercrombie, M., and Santler, J., An analysis of growth in nuclear population during Wallerian degeneration, J. Cell. Comp. Physiol., 50 (1957) 429-450.

Abraham, S. N., Goguen, J. D., Sun D., Klemm, P., and Beachey, E. H., Identification of two ancillary subunits of Escherichia coli type 1 fimbriae by using antibodies against synthetic oligopeptides of fim gene products, J. Bacteriol., 169 (1987) 5530.

Abraham, S. N., Goguen, J. D., and Beachey, E. H., Hyper-adhesive mutant of type 1 fimbriated Escherichia coli associated with formation of fimH organelles (fimbriosomes), Infect. Immun., 56 (1988) 1023.

Aguayo, A. J., Peyronnard, J. M., Terry, L. C., Romine, J. S., and Bray, G. M., Neonatal neuronal loss in rat superior cervical ganglia: retrograde effects on developing preganglionic axons and Schwann cells, J. Neurocytol., 5 (1976) 137-155.

Akasaki, M., Kawasaki, T. and Yamashina, I., The isolation and characterization of glycopeptides and mucopolysaccharides from plasma membranes of normal and regenerating livers of rats, FEBS Lett., 59 (1975) 100.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D., Molecular Biology of the Cell, Garland Publishing Inc., New York (1983).

Asbury, A. K., Schwann cell proliferation in developing mouse sciatic nerve, J. Cell Biol., 34 (1967) 735-743.

Baichwal, R. R., Bigbee, J. W., and DeVries, G. H., Macrophage-mediated myelin-related mitogenic factor for cultured Schwann cells, Proc. Natl. Acad. Sci. USA, 85 (1988) 1701-1705.

Baron-Van Evercooren, A., Kleinman, H. K., Seppa, H. E. J., Rentier, B., and Dubois-Dalcq, M., Fibronectin promotes rat

Schwann cell growth and motility, J. Cell Biol., 93 (1982) 211-216.

Bar-Shavit, Z., Ofek, I., Goldman, R., Mirelman, D., and Sharon, N., Mannose residues on phagocytes as receptors for the attachment of Escherichia coli and Salmonella typhi, Biochem. Biophys. Res. Commun., 78 (1977) 455.

Beuche, W., and Friede, R. L., The role of non-resident cells in Wallerian degeneration, J. Neurocytol., 13 (1984) 767-796.

Bigbee, J. W., Yoshino, J. E., and DeVries, G. H., Morphological and proliferative responses of cultured Schwann cells following rapid phagocytosis of a myelin-enriched fraction, J. Neurocytol., 16 (1987) 487-496.

Bosch, E. P., Assouline, J. G., Miller, J. F., and Lim, R., Glia maturation factor promotes proliferation and morphologic expression of rat Schwann cells, Brain Res., 304 (1984) 311-319.

Bradford, M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72 (1976) 248-254.

Bradley, W. G., and Asbury, A., Duration of synthesis phase in neurolemma cells in mouse sciatic nerve during degeneration, Exp. Neurol., 26 (1970) 275-282.

Brockes, J. P., Fields, K. L., and Raff, M. C., Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve, Brain Res., 165 (1979) 105-118.

Brockes, J. P., Lemke, G. E., and Balzer, D. R., Purification and preliminary characterization of a glial growth factor from bovine pituitary, J. Biol. Chem., 255 (1980) 8374-8377.

Brockes, J. P., Mitogenic Growth Factors and Nerve Dependence of Limb Regeneration, In: Neuroscience, Abelson, P. H., Butz, E., and Snyder, S. H. (eds.), American Association for the Advancement of Science, Washington, DC (1985) 58-73.

Bunge, R. P., Glial cells and the central myelin sheath, Physiological Reviews, 48 (1968) 197-251.

Bunge, R. P., Cellular and Non-Cellular Influences on Myelin-Forming Cells, In: Neuronal-glial Cell Interrelationships, Sears, T. A. (ed.), Springer-Verlag, New York (1982) 115-130.

Bunge, R. P., Tissue culture observations relevant to the study of axon-Schwann cell interactions during peripheral nerve development and repair, J. Exp. Biol., 132 (1987) 21-34.

Buonassisi, V. and Root, M., Enzymatic degradation of heparin-related mucopolysaccharides from the surface of endothelial cell cultures, Biochem. Biophys. Acta 385 (1975) 1.

Carnow, T. B., Manthorpe, M., Davis, G. E., and Varon, S., Localized Survival of Ciliary Ganglionic Neurons Identifies Neuronotrophic Factor Bands on Nitrocellulose Blots, J. Neurosci., 5 (1985) 1965-1971.

Cassel, D., Wood, P. M., Bunge, R. P., and Glaser, L., Mitogenicity of Brain Axolemma Membranes and Soluble Factors for Dorsal Root Ganglion Schwann Cells, J. Cell. Biochem., 18 (1982) 433-445.

Chen, S. J., and DeVries, G. H., Mitogenic Effect of Axolemma-Enriched Fraction on Cultured Oligodendrocytes, J. Neurochem., 52 (1989) 325-327.

Chen, S. J. and DeVries, G. H., Mitogenicity of an axolemma-derived soluble extract on oligodendrocytes, Trans. Amer. Soc. Neurochem., 20 (1989a) 247.

Cline, M. J. and Swett, V. C., The interaction of human monocytes and lymphocytes, J. exp. Med., 128 (1968) 1309.

Cravioto, H., The role of Schwann cells in the development of human peripheral nerves, J. Ultrastruct. Res., 12 (1965) 634-651.

DeCoster, M. A., and DeVries, G. H., Solubilization and Characterization of the Axonal Mitogen for Cultured Schwann Cells, Trans. Amer. Soc. Neurochem., 19 (1988) 247.

DeCoster, M. A., and DeVries, G. H., Evidence that the Axolemmal Mitogen for Cultured Schwann Cells is a Positively Charged, Heparan Sulfate Proteoglycan-Bound, Heparin-Displaceable Molecule, J. Neurosci. Res., 22 (1989) 283-288.

DeCoster, M. A., and DeVries, G. H., A Monoclonal Antibody Blocks the Axonal Mitogen for Schwann Cells, Trans. Amer. Soc. Neurochem., 20 (1989a) 241.

Detskey, P. Z., Bigbee, J. W., and DeVries, G. H., Isolation and Characterization of Axolemma-Enriched Fractions From Discrete Areas of Bovine CNS, Neurochem. Res., 13 (1988) 449-454.

DeVries, G. H., Salzer, J. L., and Bunge, R. P., Axolemma-enriched fractions isolated from PNS and CNS are mitogenic for cultured Schwann cells, Dev. Brain Res., 3 (1982) 295-299.

DeVries, G. H., Minier, L. N., and Lewis, B. L., Further Studies on the Mitogenic Response of Cultured Schwann Cells to Rat CNS Axolemma-Enriched Fractions, Dev. Brain Res., 9 (1983) 87-93.

Dinneen, M. P., Proliferation of Schwann cells induced by axolemmal and myelin membranes, doctoral thesis, Virginia Commonwealth University, 1985.

Eccleston, P. A. and Silberber, D. H., FGF is a mitogen for oligodendrocytes, Brain Res., 353 (1985) 315-320.

Ferrara, N., Ousley, F., and Gospodarowicz, D., Bovine brain astrocytes express basic fibroblast growth factor, a neurotropic and angiogenic mitogen, Brain Res., 462 (1988) 223-232.

Friede, R. L., and Johnstone, M., Response of thymidine labeling of nuclei in gray matter and nerve following sciatic transection, Acta Neuropathol., 7 (1967) 218-231.

Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B., Isolation of Intracellular Membranes by Means of Sodium Carbonate Treatment: Application to Endoplasmic Reticulum, J. Cell Biol., 93 (1982) 97-102.

Gamble, H. J., and Breathnach, A. S., An electron microscope study of human foetal peripheral nerves, J. Anat., 99 (1965) 573.

Glaser, L., Cell-Cell Adhesion Studies with Embryonal and Cultured Cells, Rev. Physiol. Pharmacol., 83 (1978) 89-122.

Gospodarowicz, D., and Cheng, J., Heparin protects basic and acidic FGF from inactivation, J. Cell. Physiol., 128 (1986) 475-484.

Hall, S. M. and Gregson, N. A., The effects of Mitomycin C on the process of regeneration in the mammalian peripheral nervous system, Neuropath. appl. Neurobiol., 3 (1977) 65-78.

Hampson, I. N., Shant, K., and Gallagher, J. T., Heterogeneity of cell-associated and secretory heparan sulfate proteoglycans produced by cultured human neuroblastoma cells, Biochem. Biophys. Acta, 801 (1984) 306-313.

Hanson, M. S. and Brinton, C. C. Jr., Identification and characterization of E. coli type-1 pilus tip adhesion protein, Nature, 332 (1988) 265.

Harrison, R. G., Neuroblast versus sheath cell in the development of peripheral nerves, J. comp. Neurol., 37 (1924) 123-206.

Hedfors, E., Holm, G., and Petterson, D., Activation of human peripheral blood lymphocytes by concanavalin A-dependence of monocytes, Clin. exp. Immunol., 22 (1975) 223.

Helenius, A. and Simons, K., Solubilization of membranes by detergents, Biochem. Biophys. Acta 415 (1975) 29-79.

Johnson, E. M. Jr., Taniuchi, M., and DiStefano, P. S., Expression and possible function of nerve growth factor receptors on Schwann cells, TINS, 11 (1988) 299-304.

Joseph, J., Further studies in changes of nuclear population in degenerating non-myelinating and finely myelinated nerves, Acta Anat., 9 (1950) 279-288.

Kazura, I.W., Negendank, W., Guerry, D., and Schreiber, A. D., Human monocyte-lymphocyte interaction and its enhancement by levamisole, Clin. exp. Immunol. 35 (1979) 258-268.

Kjellen, L., Oldberg, A., and Hook, M., Cell-surface Heparan Sulfate. Mechanisms of proteoglycan-cell association, J. Bio. Chem., 255 (1980) 10407-10413.

Kjellen, L., Pettersson, I., and Hook, M., Cell-surface heparan sulfate: An intercalated membrane proteoglycan, Proc. Natl. Acad. Sci. USA, 78 (1981) 5371-5375.

Klemm, P. and Christen, G., Three film genes required for the regulation of length and mediation of adhesion of Escherichia coli type 1 fimbriae, Mol. Gen. Genet., 208 (1987) 439.

Kraemer, P. M., Heparan sulfates of cultured cells. I. Membrane-associated and cell-sap species in Chinese hamster cells, Biochemistry, 10 (1971) 1437.

Kraemer, P. M. and Smith, D. A., High molecular weight heparan sulfate from the cell surface, Biochem. Biophys. Res. Commun., 56 (1974) 423.

Kraemer, P. M., Heparin releases heparan sulfate from the cell surface, Biochem. Biophys. Res. Commun., 78 (1977) 1334-1340.

Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T_4, Nature 227 (1970) 680-685.

Lander, A. D., Fujii, D. K., Gospodarowicz, D., and Reichardt, L. F., Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan, J. Cell Biol., 94 (1982) 574-585.

LeDouarin, N., The Neural Crest, (1982) Cambridge University Press.

Lemke, G. E., and Brockes, J. P., Identification and purification of glial growth factor, J. Neurosci., 4 (1984) 75-83.

Lim, R., Miller, J. F., Hicklin, C. J., and Andresen, A. A., Purification of bovine glia maturation factor and characterization with monoclonal antibody, Biochemistry, 24 (1985) 8070-8074.

Lohrmann, H. P., Novikovs, L., and Graw, R. G. Jr., Cellular interactions in the proliferative response of human T and B lymphocytes to phytomitogens and allogeneic lymphocytes, J. exp. Med., 139 (1974) 1553.

Lown, J. W., The molecular mechanisms of the antitumor action of the mitomycins, in: Mitomycin C: Current Status and New Developments, Carter, S. K. and Crooke, S. T. (eds.), Academic Press, New York (1979) 1-18.

Maresh, G. A., Chernoff, E. A. G., and Culp, L. A., Heparan sulfate proteoglycans of human neuroblastoma cells: affinity fractionation on columns of platelet factor-4, Arch. Biochem. Biophys., 233 (1984) 428-437.

Margolis, R. K. and Margolis, R. U., Structure and Distribution of Glycoproteins and Glycosaminoglycans, in: Complex Carbohydrates of Nervous Tissue, Margolis, R. U. and Margolis, R. K. (eds.), Plenum Press, New York (1979).

Martin, J. R., and Webster, H. deF., Mitotic Schwann cells in developing nerve: Their changes in shape, fine structure, and axon relationships, Dev. Biol., 32 (1973) 417.

Mason, P. W., Bigbee, J. W., and DeVries, G. H., Cerebellar Granule Cells Contain A Membrane Mitogen For Cultured Schwann Cells, J. Cell Biol., 108 (1989) 607-611.

Mason, P. W., Chen, S. J., and DeVries, G. H., Evidence for two distinct proliferative signals for PNS and CNS glia,

Trans. Amer. Soc. Neurosci., 20 (1989a) 248.

Maurer, L., and Orndorff, P. E., A new locus, pilE required for the binding of type 1 piliated Escherichia coli to erythrocytes, FEMS Microbiol. Lett., 30 (1985) 59.

Maurer, L. and Orndorff, P., Identification and characterization of genes determining receptor binding and pilus length of Escherichia coli type 1 pili, J. Bacteriol., 169 (1987) 642.

McGarvey, M. L., Baron-Van Evercooren, A., Klienman, H. K., and Dubois-Dalcq, M., Synthesis and effects of basement membrane components in cultured rat Schwann cells, Dev. Biol., 105 (1984) 18-28.

Meador-Woodruff, J. H., Yoshino, J. E., Bigbee, J. W., Lewis, B. L., and DeVries, G. H., Differential proliferative responses of cultured Schwann cells to axolemma and myelin-enriched fractions. II. Morphological studies, J. Neurocyt., 14 (1985) 619-635.

Morris, J. E., Isolation of the major chondroitin sulfate/dermatan sulfate and heparan sulfate proteoglycans from embryonic chicken retina, Arch. Biochem. Biophys., 235 (1984) 127-140.

Naot, Y., Tully, J. G., and Ginsburg, H., Lymphocyte activation by various Mycoplasma strains and species, Infect. Immun., 18 (1977) 310-317.

Naot, Y. and Ginsburg, H., Activation of B lymphocytes by mycoplasma mitogen(s), Immunology 34 (1978) 715-720.

Naot, Y., Merchav, S., Ben-David, E., and Ginsburg, H., Mitogenic activity of Mycoplasma pulmonis. I. Stimulation of rat B and T lymphocytes, Immunology, 36 (1979) 399-406.

Noronha, A., Hammer, J., McLenigan, M., Kobiler, D., and Quarles, R., Immuno-affinity purification of the myelin-associated glycoprotein, Trans. Amer. Soc. Neurochem., 20 (1989) 217.

Ofek, I., Mirelman, D., and Sharon, N., Adherence of Escherichia coli to human cells mediated by mannose receptors, Nature, 265 (1977) 623.

Ofek, I., and Beachey, E. H., Mannose binding and epithelial cell adherence of Escherichia coli, Infect. Immun., 22 (1978) 247.

Oldberg, A., Hook, M., Obrink, B., Pertoft, H., and Rubin, K., Structure and metabolism of rat liver heparan sulfate, Biochem. J., 164 (1977) 75.

Palmer, T., Understanding Enzymes, Ellis Horwood Limited, Chichester, England (1985).

Pellegrino, R. G., Politis, M. J. and Spencer, P. S., Axon-associated Schwann cell mitosis during peripheral nerve regeneration in vivo, J. Cell Biol., 91 (1980) 93a.

Pellegrino, R. G., and Spencer, P. S., Schwann Cell Mitosis in Response to Regenerating Peripheral Axons In Vivo, Brain Res., 341 (1985) 16-25.

Peters, A., and Muir, A. R., The relationship between axons and Schwann cells during development of peripheral nerves in the rat, Q. J. Exp. Physiol. Cogn. Med. Sci., 44 (1959) 117-130.

Peters, A., Palay, S. L., and Webster, H. deF., The Fine Structure of the Nervous System: The Neurons and Supporting Cells, Philadelphia: W. B. Saunders (1976) 190-230.

Philipson, K. D., McDonough, A. A., Frank, J. S., and Ward, R., Enrichment of Na^{+}-Ca^{2+} exchange in cardiac sarcolemmal vesicles by alkaline extraction, Biochem. Biophys. Acta, 899 (1987) 59-66.

Ponniah, S., Abraham, S. N., Dockter, M. E., Wall, C. D., and Endres, R. O., Mitogenic stimulation of human B lymphocytes by the mannose-specific adhesin on Escherichia coli type 1 fimbriae, J. Immunol., 142 (1989) 992-998.

Porter, S., Clark, M. B., Glaser, L., and Bunge, R. P., Schwann Cells Stimulated to Proliferate in the Absence of Neurons Retain Full Functional Capability, J. Neurosci., 6 (1986) 3070-3078.

Porter, S., Glaser, L., and Bunge, R. P., Release of autocrine growth factor by primary and immortalized Schwann cells, Proc. Natl. Acad. Sci. USA, 84 (1987) 7768-7772.

Raff, M. C., Abney, E., Brockes, J. P., and Hornby-Smith, A., Schwann Cell Growth Factors, Cell, 15 (1978) 813-822.

Raff, M. C., Hornby-Smith, A., and Brockes, J. P., Cyclic AMP as a mitogenic signal for cultured rat Schwann cells, Nature 273 (1978a) 672-673.

Ratner, N., Glaser, L., and Bunge, R. P., PC 12 cells as a source of the neurite-derived cell surface mitogen which stimulates Schwann cell division, J. Cell Biol., 98 (1984) 1150-1155.

Ratner, N., Bunge, R. P., and Glaser, L., A Neuronal Cell

Surface Heparan Sulfate Proteoglycan Is Required for Dorsal Root Ganglion Neuron Stimulation of Schwann Cell Proliferation, J. Cell Biol., 101 (1985) 744-754.

Ratner, N., Elbein, A., Bunge, M. B., Porter, S., Bunge, R. P., and Glaser, L., Specific asparagine-linked oligosaccharides are not required for certain neuron-neuron and neuron-Schwann cell interactions, J. Cell Biol., 103 (1986) 159-170.

Ratner, N., Wood, P. W., Bunge, R. P., and Glaser, L., in: Glial Neuronal Communication in Development and Regeneration, Althaus, H. H., and Seifert, W. (eds.), Springer, New York (1987).

Ratner, N., Hong, D., Lieberman, M. A., Bunge, R. P., and Glaser, L., The neuronal cell-surface molecule mitogenic for Schwann cells is a heparin-binding protein, Proc. Natl. Acad. Sci. USA, 85 (1988) 6992-6996.

Romine, J. S., Bray, G. M., and Aguayo, A. J., Schwann cell multiplication after crush injury of unmyelinated fibers, Arch. Neurol., 33 (1976) 49-54.

Rosenstreich, D. L., Farrar, J. L., and Dougherty, S., Absolute macrophage dependency of T-lymphocyte activation by mitogens, J. Immunol., 116 (1976) 131.

Rozengurt, E., Early Signals in the Mitogenic Response, Science, 234 (1986) 161-166.

Rudge, J. S., Davis, G. E., Manthorpe, M., and Varon, S., An examination of ciliary neuronotrophic factors from avian and rodent tissue extracts using a blot and culture technique, Dev. Brain Res., 32 (1987) 103-110.

Salit, I. E. and Gotschlich, E. C., Type 1 Escherichia coli pili: characterization of binding to monkey kidney cells, J. Exp. Med., 146 (1977) 1182.

Salzer, J. L., and Bunge, R. P., Studies of Schwann cell proliferation. I. An Analysis in Tissue Culture of Proliferation during Development, Wallerian Degeneration, and Direct Injury, J. Cell Biol., 84 (1980) 739-752.

Salzer, J. L., Bunge, R. P., and Glaser, L., Studies of Schwann cell proliferation. III. Evidence for the Surface Localization of the Neurite Mitogen, J. Cell Biol., 84 (1980a) 767-778.

Salzer, J. L., Williams, A. K., Glaser, L., and Bunge, R. P., Studies of Schwann cell proliferation. II. Characterization of the Stimulation and Specificity of the Response to a Neurite Fraction, J. Cell Biol., 84 (1980b)

753-766.

Schwann, T., Mikroskopische Untersuchungen uber die Uebereinstimmung in der Struktur und dem Wachsthum der Thiere und Pflanzen, Berlin (1839) G. E. Reimer.

Seeger, R. C. and Oppenheim, J. J., Synergistic interaction of macrophages and lymphocytes in antigen-induced transformation of lymphocytes, J. exp. Med., 132 (1970) 44.

Shook, L. B., and Schwartz, P. L., Manual for hybridomas and monoclonal antibodies: Workshop on Hybridomas and Monoclonal Antibodies, Massey Cancer Center, Richmond VA (1986).

Skoff, R. P., Price, D. L., and Stocks, A., Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. I. Cell proliferation, J. Comp. Neurol., 169 (1976) 291-311.

Sobue, G., Kreider, B., Asbury, A., and Pleasure, D., Specific and potent mitogenic effect of axolemmal fraction on Schwann cells from rat sciatic nerves in serum-containing and defined media, Brain Res., 280 (1983) 263-275.

Sobue, G., and Pleasure, D., Adhesion of axolemmal fragments to Schwann cells: a signal- and target-specific process closely linked to axolemmal induction of Schwann cell mitosis, J. Neurosci., 5 (1985) 379-387.

Steck, T. L. and Yu, J., Selective solubilization of proteins from red blood cell membranes by protein perturbants, J. Supramol. Struct., 1 (1973) 220-232.

Taggart, R. T., and Samloff, I. M., Stable Antibody-Producing Murine Hybridomas, Science, 219 (1983) 1228-1230.

Takada, H., Hirachi, Y., Hashizume, H., and Kotani, S., Mitogenic activity of cytoplasmic membranes isolated from L-forms of Staphylococcus aureus, Microbiol. Immunol., 24 (1980) 1079-1090.

Takada, H., Hirachi, Y., Hashizume, H, and Kotani, S., Mitogenic effect of Cytoplasmic Membranes and a Cytoplasmic Fraction of Staphylococcus aureus L-Forms on Human Peripheral Blood Lymphocytes, Microbiol. Immunol., 25 (1981) 317-326.

Tennyson, V. M., Electron microscopic study of the developing neuroblast of the dorsal root ganglion of the rabbit embryo, J. Comp. Neurol., 124 (1965) 267.

Terry, L., Bray, G., and Aguayo, A., Schwann cell

multiplication in developing rat unmyelinated nerves- a radioautographic study, Brain Res., 69 (1974) 144-148.

Thomas, G. A., Quantitative histology of Wallerian degeneration. II. Nuclear populations in two nerves of different fiber spectrum, J. Anat., 82 (1948) 135-145.

Underhill, C. B. and Keller, J. M., Heparan sulfates of mouse cells: Analysis of parent and transformed 3T3 cell lines, J. Cell. Physiol. 90 (1977) 53.

Webster, H. deF., Trapp, B. D., Sternberger, N. H., and Quarles, R. H., Myelin-forming glial cells: morphological and immunocytochemical observations, In: Development of the nervous system, Garrod, D. R., and Feldman, J. D. (eds.), Cambridge University Press (1981) 265-288.

Weston, J. A., The migration and differentiation of neural crest cells. In Abercrombie, H., Bracht, J., and King, T., eds., Advances in Morphogenesis, Vol. 8, New York, Academic Press (1970) 41-114.

Wood, P. M., Separation of functional Schwann cells and neurons from normal peripheral nerve tissue, Brain Res., 115 (1976) 361-375.

Wood, P. M. and Williams, A. K., Oligodendrocyte proliferation and CNS myelination in cultures containing dissociated embryonic neuroglia and dorsal root ganglion neurons, Dev. Brain Res., 12 (1984) 225-241.

Wood, P. M., and Bunge, R. P., Evidence that axons are mitogenic for oligodendrocytes isolated from adult animals, Nature, 320 (1986) 756-758.

Yoshino, J. E., Dinneen, M. P., Lewis, B. L., Meador-Woodruff, J. H., and DeVries, G. H., Differential Proliferative Responses of Cultured Schwann Cells to Axolemma- and Myelin-enriched Fractions. I. Biochemical Studies, J. Cell Biol., 99 (1984) 2309-2313.

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