Identification and Characterization of a Bovine Neurite Growth Inhibitor (bNI-220)*

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The poor axonal regeneration that follows lesions of the central nervous system (CNS) is crucially influenced by the local CNS tissue environment through which neurites have to grow. In addition to an inhibitory role of the glial scar, inhibitory substrate effects of CNS myelin and oligodendrocytes have been demonstrated. Several proteins including NI-35/250, myelin-associated glycoprotein, tenascin-R, and NG-2 have been described to have neurite outgrowth inhibitory or repulsive properties in vitro. Antibodies raised against NI-35/250 (monoclonal antibody IN-1) were shown to partially neutralize the growth inhibitory effect of CNS myelin and oligodendrocytes, and to result in long distance fiber regeneration in the lesioned adult mammalian CNS in vivo. We report here the purification of a myelin protein to apparent homogeneity from bovine spinal cord which exerts a potent neurite outgrowth inhibitory effect on PC12 cells and chick dorsal root ganglion cells, induces collapse of growth cones of chick dorsal root ganglion cells, and also inhibits the spreading of 3T3 fibroblasts. These activities could be neutralized by the monoclonal antibody IN-1. The purification procedure includes detergent solubilization, anion exchange chromatography, gel filtration, and elution from high resolution SDS-polyacrylamide gel electrophoresis. The active protein has a molecular mass of 220 kDa and an isoelectric point between 5.9 and 6.2. Its inhibitory activity is sensitive to protease treatment and resists harsh treatments like 9 M urea or short heating. Glycosylation is, if present at all, not detectable. Microsequencing resulted in six peptides and strongly suggests that this protein is novel.

Neurite growth in the mammalian CNS* ceases at the end of the developmental period. Although CNS neurons maintain some ability to rearrange their axonal and dendritic arbors in the adult brain, regeneration of severed CNS axons over long distances is absent. Transplantations of peripheral nerve explants into various parts of the brain and spinal cord revealed that the lack of regeneration is not primarily due to intrinsic properties of CNS neurons but is instead dependent on the microenvironment encountered by the regenerating fibers (1, 2); CNS axons were able to grow over long distances in the peripheral nerve segments, but ceased to grow as they entered the CNS tissue again (2).

Several lines of evidence suggest that the presence of inhibitory factors rather than the lack of growth promoting molecules is responsible for the non-conductive properties of CNS tissue in adult vertebrates (for review, see Ref. 3). In vitro experiments demonstrated that adult optic nerve explants were not invaded by neurites, although high amounts of neurotrophic factors were provided (4). Similarly, cryostat sections of adult CNS tissue were shown to be non-permissive substrates for neurite outgrowth, especially the densely myelinated areas (5–9). Differentiated oligodendrocytes in culture and CNS myelin exerted a strong inhibitory effect on adhesion and outgrowth of primary neurons, neuroblastoma cells, and also for spreading of 3T3 fibroblasts (10–12). Growth cones of rat dorsal root ganglion (DRG) neurons interacting with differentiated oligodendrocytes were arrested and collapsed (13, 14). In vivo experiments demonstrated that regeneration of lesioned axons over long distances could be observed in myelinfree spinal cord or optic nerve, which has been obtained by killing the dividing oligodendrocyte precursors by repeated x-irradiation of newborn rats (15, 16) or by the suppression of the onset of myelinization by immunocytolysis in chicken (17, 18). Moreover, high levels of GAP-43, a protein related to axonal growth (19–21), and a greatly increased structural plasticity as reflected by collateral sprouting of sensory fibers in response to dorsal root lesions could be observed in these myelin deficient zones (22). All these results are consistent with a strong growth-restricting function of adult CNS myelin.

When CNS myelin was separated by ether/ethanol extraction into a lipid and a protein fraction, the neurite growth inhibitory activity was associated with the protein fraction (23). Proteins eluted from gel slices containing molecules with an apparent molecular mass of 35 and 250 kDa (SDS-PAGE) showed a very potent inhibitory activity (23, 24). A monoclonal antibody (mAb IN-1), which has been raised against rat NI-250 (25), neutralizes the neurite growth inhibitory property of differentiated oligodendrocytes and NI-35 and NI-250 (14, 24–30). Immunoprecipitation of CNS myelin proteins by the mAb IN-1 removed more than 50% of inhibitory substrate properties (25, 31). Immunohistochemistry revealed that mAb IN-1 stains white matter and myelin in the whole brain and spinal cord from adult rats (32). In vivo, the application of mAb IN-1 to

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¶ The abbreviations used are: CNS, central nervous system; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; NGF, nerve growth factor; FCS, fetal calf serum; PBS, phosphate-buffered saline; mAb, monoclonal antibody; E, embryonic day; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MAG, myelin-associated glycoprotein.

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PC12 Neurite Outgrowth Assay—The test was performed as described previously (29). Briefly, NGF-primed PC12 cells were detached mechanically, trypsinized for 5 min at 37 °C with 0.5%/w/v trypsin in Ca2+/Mg2+-free Hank’s solution, and plated at a density of 3000–5000 cells/cm2 in culture medium with 100 ng/ml NGF. Assays were stopped 24 h after plating by addition of 0.5% w/v formaldehyde (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.0 mM NaHPO4, pH 7.4) and quantified in duplicates. In six randomly chosen fields per well, we determined the percentage of PC12 cells with neurites shorter than two diameters of the cell body. Treatment with the mAb IN-1 was performed in the same way as for the PC3 fibroblasts (see above). About 150–200 cells were evaluated per condition tested. EC50 values were determined as described in the assays (see above).

Neurite Outgrowth of Chick DRG Explants—22-mm glass coverslips were coated with 0.1 mg/ml collagen (from rat tail, Ref. 42), 1 µg/ml laminin (Life Technologies, Inc.) for 6 h at 37 °C and air-dried. 10 µl of one of the following substrates was coated: 10 µg/ml gel-eluted bni-220, 10 µg/ml semiIII/collapsin 1 (COS supernatant), or an equivalent amount of gel-eluted control protein (120 KDa, bc120) for 2 h at 37 °C. Coverslips were then blocked with F-12/DMEM culture medium (Dulbecco’s MEM NUT MIX F-12 (Ham), Life Technologies, Inc.), containing 10% FCS, 100 units/ml penicillin, and 0.5 mg/ml streptomycin or incubated with hybridoma supernatant (mAb O1 or mAb IN-1) containing 6% FCS for 1 h at 37 °C. Four embryonic day 11–13 chick DRG explants were grown on these coverslips with 200 µl of F-12/DMEM culture medium containing 50 nM NGF. Results are from three independent experiments for SemiIII, laminin, and bc120, and five independent experiments for bni-220.

Collapse Assay—Serial dilutions of purified protein were assayed for growth cone collapse on explanted chick embryonic day 11–13 (E11–13) DRG similar as described previously (43). Briefly, explants were dissected from chick embryos and incubated at 37 °C overnight on a plastic dish coated with 1 µg of laminin (area 1 cm2) in 60 µl of L15 medium (L15 medium = 60 mg/liter imidazole, 15 mg/liter aspartic acid, 15 mg/liter glutamic acid, 15 mg/liter cystine, 5 mg/liter p-aminobenzoic acid, 25 mg/liter fumaric acid, 0.4 mg/liter coenzyme A; pH 7.35) containing 50 ng/ml NGF and 0.5% methocel. The following day, 20 µl of liposomes containing different amounts of gel-eluted protein (24) were applied to the explanted culture. Following 1 h of incubation at 37 °C, the explants were fixed in 4% paraformaldehyde-sucrose/PBS solution. Growth cones were scored as being either spread or collapsed. The percentage of collapsed growth cones was then plotted against the concentration of the purified proteins added to the cultured explants. At least 50 growth cones were counted per explant, and for one condition, experiments were performed in duplicate. Data are the mean of three independent experiments.

Purification of bni-220—All purification steps were carried out at 4 °C. Inhibitory substrate activity of the obtained fractions was routinely determined by the ST3 spreading or by the PC12 neurite outgrowth assay. Bovine spinal cord tissue (30 min post mortem frozen to −80 °C) was disrupted by homogenization and subsequently cut into small pieces. Myelin was prepared by the method of Colman et al. (44). The obtained myelin was then extracted in extraction buffer (60 mM CHAPS, 100 mM Tris-Cl, pH 8.0, 10 mM EDTA buffer, pH 8.0, 2.5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluorid, 0.1 mM aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). To obtain spinal cord extract, the tissue was homogenized directly in CHAPS extraction buffer in a ratio of (1:1; w/v). The homogenate was centrifuged twice at 100,000 × g (Kontron type: K50.13, fixed angle) for 1 h at 4 °C. The clear supernatant (extract) was immediately applied to a Q-Sepharose column (2.6 × 11.5 cm), equilibrated in buffer A (20 mM Tris-Cl, pH 8.0, 8.0% (v/v) CHAPS). Bound proteins were eluted with a five-bed volume linear gradient from 0 to 1 M NaCl in buffer A (100-mM gradient in 50 min). Active fractions containing bni-220 eluted around 0.4 M NaCl and were pooled (p-1 pool) for subsequent application on a Superdex 200 (2.6 × 60 cm) column, equilibrated in buffer B (150 mM NaCl, 20 mM Tris-Cl, pH 8.0, 0.5% (v/v) CHAPS). Active fractions after gel filtration (p-2 pool) were separated by 6% SDS-PAGE (10 × 24 × 0.1 cm gel) under reducing conditions and low constant power (2 watts/gel) to a total of 2500 Vh. Bands and gel regions were identified after Coomassie Blue staining of 0.1% w/v R25 (a 1000-fold dilution) and methanol-acetic acid (95:5) cut out, and extracted in 800 µl of gel elution buffer (0.5% (w/v) CHAPS, 20 mM Tris-Cl, pH 8.0, 10 mM EDTA, pH 8.0, 2.5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluorid, 0.1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A) for at least 48 h at 4 °C (30).

Microsequencing of bni-220—The IN-1 neutralizable active gel-eluted material of several gels was re-run on a 10% SDS-polyacrylamide gel electrophoresis. The gel was incubated in 6 M urea, 0.5% CHAPS, 0.5% SDS, 5% glycerol, 0.05% bromophenol blue at 37 °C for 2 h. The gel was cut into strips, eluted with 50% acetonitrile and 0.1% trifluoroacetic acid, and lyophilized. The strips were re-suspended in 50% acetonitrile and 0.1% trifluoroacetic acid and lyophilized again. The lyophilized material was then microsequenced by the tandem of Dr. C. Haubold and Dr. D. Fendel of the Institute of Chemistry, University of Heidelberg. The following amino acids were determined by Edman degradation: ser, thr, glu, pro, tyr, leu, glu, val, ala, ilu, lys, met, pro, thr, ser, ser, ileu, leu.
gel under reducing conditions, and stained with 0.1% (w/v) Coomassie Blue R250 in 50% methanol and 10% acetic acid. The 220-kDa band was cut out, and endoproteinase Lys-C digestion (1:50 molar ratio) was performed directly in the gel. The sample was acidified and applied to a reverse phase high performance liquid chromatography column, peptide fragments were separated with a linear gradient (0–100%) of 0.04% trifluoroacetic acid and 80% acetonitrile, and fractions containing single peptide species were subjected to automated Edman degradation.

SDS-PAGE and Immunoblotting—High resolution SDS-PAGE was carried out using 6% (w/v) SDS-polyacrylamide gels (10 × 24 × 0.11 cm) under reducing conditions (100 mM dithiorthreitol) according to the method of Laemmli (45). Transfer onto Immobilon-P membranes (Millipore) was performed in 20 mM Tris base, 192 mM NaCl, 0.2 g of KH₂PO₄·2.8 g of Na₂HPO₄·12H₂O, and 0.2 g of KCl (dissolved in 1 liter of water) and the washing solution contained 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.4% Tween (3 × 10 min at room temperature). Incubation time for the first antibody (for dilution with 1% gelatin in PBS, see “Antibodies”) was usually overnight at 4 °C. Horseradish peroxidase-conjugated antimouse IgGs secondary antibody (1:2000) was incubated for 1 h at room temperature. Finally, the ECL chemiluminescence system was used for detection (Amersham Pharmacia Biotech).

Two-dimensional Gel Electrophoresis—Gel-eluted proteins from high resolution SDS-PAGE were precipitated by addition of a 9-fold excess of acetone (1 day at −20 °C), dried in a stream of nitrogen and solubilized in 10 μl of buffer that contained 2% (w/v) CHAPS, 0.1% (w/v) Triton X-100, 9 μl urea, and 2% (v/v) amphotolines (one-third volume pH 3–10, two-thirds volume pH 4–8; Millipore).(46). Undissolved material was pelleted for 5 min at 13000 rpm in a microcentrifuge. The supernatant was applied to the isolectric focusing gels (3.5% acrylamide, 9M urea, 2% w/v Triton X-100, 0.3% (w/v) CHAPS, and 6% (v/v) amphotolines (1/3 vol. pH 3–10, 2/3 vol. pH 4–8; Millipore) in 5 cm 0.1 mm capillaries, (46). The isoelectrically focused proteins were separated in the second dimension by 6% (w/v) SDS-PAGE (6 × 8 × 0.0075 cm) under reducing conditions (47).

Detergent Treatment—Gel-eluted fractions after high resolution SDS-PAGE as described above were precipitated by addition of a 9-fold excess of acetone (1 day at −20 °C), dried in a stream of nitrogen, and solubilized in 100 μl of buffer, which contained various concentrations of different detergents. Undissolved material was pelleted for 5 min at 13000 rpm in a microcentrifuge. The supernatant was then tested in the PC12 neurite outgrowth assay.

Urea Treatment—Urea was added to the gel eluted fractions to the desired final concentration. After incubation for 2 h at 37 °C, the solution was tested in the PC12 neurite outgrowth assay.

Trypsin Treatment—Trypsin was added to gel-eluted fractions resulting in a final concentration of 0.1% (w/v), incubated for 1 min at 37 °C and then put on ice. As a control, either heat-inactivated trypsin (5 min, 100 °C) or trypsin inhibitor 0.2% (w/v) together with trypsin 0.1% (w/v) were used and processed in the same way. After centrifugation for 5 min at 13000 rpm in a microcentrifuge, supernatants were analyzed in the PC12 neurite outgrowth assay and by SDS-PAGE.

Heat Treatment—Gel-eluted fractions were incubated for 0, 5, or 30 min at 100 °C and then put on ice. After centrifugation for 5 min at 13000 rpm in a microcentrifuge, supernatants were analyzed in the PC12 neurite outgrowth assay.

To verify the presence of sugars in a possible glycoconjugate, the digoxigenin glycan detection kit from Boehringer Mannheim was used. The principle is as follows. Hydroxyl groups of sugars of glycoconjugates are oxidized to aldehyde groups by mild periodate treatment. Digoxigenin is detected in an enzyme immunoassay using a digoxigenin glycan detection kit from Boehringer Mannheim was used. The principle is as follows. Hydroxyl groups of sugars of glycoconjugates are oxidized to aldehyde groups by mild periodate treatment. Digoxigenin is detected in an enzyme immunoassay using a digoxigenin glycan detection kit from Boehringer. Hydrolysis of O-Linked Sugars—Samples were incubated for 1 h at 37 °C in 0.1 M NaOH.

Chondroitinase ABC Digestion—50 μl of gel-eluted fractions (~0.4 μg of protein) were incubated with 20 milliliters of affinity-purified chondroitinase ABC (Sigma) in 40 mM Tris-Cl, pH 8.0, for 3 h at 37 °C (48) and then put on ice. After centrifugation for 5 min at 13000 rpm in a microcentrifuge, supernatants were analyzed either by SDS-PAGE or in the PC12 neurite outgrowth assay.

Results—Inhibitory Activities Neutralized by mAb IN-1

For the purification of the IN-1 antigen present in bovine CNS myelin, two culture assays in combination with the neutralizing mAb IN-1 were used.

The first bioassay tests the effect of substrates on the spreading behavior of 3T3 fibroblasts, which has been shown previously to be strongly impaired by oligodendrocytes and CNS myelin (10). The second bioassay analyzes substrate effects on the neurite outgrowth response of PC12 cells (29). In order to distinguish between the different inhibitory activities which might occur in CNS myelin, the neutralizing effect of the mAb IN-1 was included as a selection criterion (25).

Fractions exerting inhibition of cell spreading (3T3 assay) and neurite outgrowth (PC12 assay) that could be neutralized in both assays by the mAb IN-1 were regarded as IN-1 antigen-containing inhibitory fractions. Fig. 1 shows the effect of purified bNI-220 on 3T3 cells (Fig. 1B) and PC 12 cells (Fig. 1E) compared with a control protein (gel-eluted spectrin) on 3T3 cells (Fig. 1A) and PC12 cells (Fig. 1D). In both assays, this inhibitory effect of bNI-220 could be completely neutralized by the addition of the mAb IN-1, but not by a mAb against galactocerebroside (mAb O1) (Fig. 1, C and F).
Anion Exchange Chromatography—The CHAPS extract was chromatographed on Q-Sepharose, a strong anion exchange column. Most of the inhibitory activity bound to the column chromatographed on Q-Sepharose, a strong anion exchange column. Protein concentrations were determined by the Bradford assay for each 5-ml fraction (mg/ml). The relative inhibitory activity (bars) is expressed for each fraction as the percentage of cells that were inhibited (% cells not spread) when 5 μg/cm² protein were coated as a substrate. Fractions eluting at 0.35–0.5 M NaCl showed strong inhibitory activity and were pooled resulting in q-pool-1. The activity of q-pool-1 was neutralized to almost 70% by the mAb IN-1 (Fig. 3A). A second peak of inhibitory proteins, whose activity could not be neutralized by the mAb IN-1, eluted under high salt conditions (q-pool 2, Fig. 3B). Approximately half of the spinal cord extract proteins did not bind to the anion exchanger and were inactive in the fibroblast- and PC12 assay (not shown). B, elution profile of the size exclusion column when pooled active fractions from the Q-Sepharose column (q-pool 1) were loaded onto a Superdex 200 column. A major peak of activity was found at the beginning of the elution, corresponding to standard proteins of 400–800 kDa. These active fractions were pooled resulting in the s-pool 1. A second peak of activity contained proteins of molecular mass between 20 and 100 kDa (s-pool 2). The activity of s-pool 1 and s-pool 2 could be neutralized to about 70% by the mAb IN-1 (Fig. 3).

Size Exclusion Chromatography—Fractions of the first activity peak were pooled (q-pool 1) and subsequently processed on a Superdex 200 column. A major activity peak was found in the high molecular mass region between 400 and 800 kDa. SDS-PAGE of these fractions showed that also lower molecular mass proteins (e.g. 40 kDa) were present indicating the existence of macromolecular complexes (Figs. 2B and 4). Inhibitory activity of these pooled fractions (s-pool 1) was about 25 times enriched in the two assays as compared with the spinal cord extract (EC₅₀ in 3T3 assay: 0.4 ± 0.3 μg/cm²; n = 5; and in PC12 assay: 0.8 ± 0.4 μg/cm²; n = 5) and neutralized by the mAb IN-1 by 70% (Fig. 3A).

A second peak of activity (s-pool 2) contained proteins of molecular mass between 20 and 100 kDa (Figs. 2B and 3B). Its peaks could be eluted with a linear salt gradient (Fig. 2A). The first peak of active fractions eluted at approximately 0.4 M NaCl with an EC₅₀ of 3.0 ± 2.9 μg/cm² (fibroblast assay; n = 5) and 5.3 ± 3.8 μg/cm² (PC12 assay; n = 5). This activity could be neutralized by the mAb IN-1 by about 70% (Fig. 3A), whereas the control antibody O1 showed no effect. A second peak of inhibitory substrate proteins eluted at high salt concentrations (Fig. 2A) but could not be neutralized by the mAb IN-1 (Fig. 3B). A preliminary analysis revealed that proteoglycans may be responsible for this second inhibitory substrate activity.²

² C. E. Bandtlow, unpublished data.
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To identify the proteins responsible for neurite outgrowth inhibition, the eluted fractions from the size-exclusion column were grouped into two pools: one containing the high molecular mass fractions with significant IN-1 neutralizable activity (s-pool 1) and one containing fractions without activity. The protein compositions of these pools were compared on a high resolution 5 or 6% SDS-polyacrylamide gel, along with fractions of the CHAPS-solubilized membrane extracts and of the active q-pool 1 (Fig. 4). Analytical evaluation of the protein pattern in the high molecular mass range (150–500 kDa), revealed a consistent pattern of eight bands. Of these eight bands, only two (bands 2 and 7) were present in the active pools but absent in the inactive pool (Fig. 4). In particular, a band migrating at an apparent molecular mass of 220 kDa became increasingly prevalent as the inhibitory activity was enriched. To analyze if inhibitory activity could be correlated with any of these bands, the eight bands were cut out individually, proteins were eluted from the obtained gel pieces and tested in the two bioassays (Fig. 5). Only the eluted proteins from bands 2 and 7 exerted inhibitory activity. The most potent inhibitory activity was correlated with the 220-kDa band (EC50 in 3T3 assay: 0.13 ± 0.07 μg/cm2, n = 5; EC50 in PC12 assay: 0.23 ± 0.13 μg/cm2, n = 5) and, most importantly, could be completely neutralized by the mAb IN-1 (Figs. 3 and 7). We therefore called this activity bovine neurite growth inhibitor 220, or bNI-220. The estimated elution efficiency of bNI-220 from the preparative gels was around 10%, as determined by estimation of the amount of bNI-220 in the s-pool 1 and the eluate, whereas the elution efficiency of inhibitory activity was below 1% (Table I), suggesting a major loss of activity by this step. The activity correlating with band 7 at molecular mass 400 kDa was minor (EC50 in 3T3 assay: 1.1 ± 0.7 μg/cm2, n = 3) and could not be neutralized by the mAb IN-1 (Fig. 3B).

Further analysis by Western blotting revealed that band 2, bNI-220, could be specifically detected by the mAb IN-1, as shown in Fig. 6, whereas band 6 could be identified as tenasin, band 5 as α spectrin, and band 4 as β spectrin (data not shown). All proteins from these bands were not active in our inhibition assays (Fig. 5B). Proteins from the band 1, 3, and 8 could not be further identified but showed no activity.

bNI-220 was also enriched in the s-pool 1 when the purification protocol started with a myelin fraction instead of a spinal cord tissue homogenate. After preparative gel separation of the s-pool 1 and subsequent gel elution of the 220-kDa band, the eluted protein was highly inhibitory in the two assays and could be fully neutralized by the mAb IN-1 (data not shown). These results confirm that bNI-220 is a CNS myelin protein.

Table 1 summarizes the purification procedure. Starting with 50 g of adult bovine spinal cord tissue, 3 μg of a protein with an apparent molecular mass of 220 kDa (Fig. 4) were obtained. The overall enrichment of activity was around 80-fold as estimated from the fibroblast spreading and PC12 neurite outgrowth assays. The yield of total activity was about 0.2%. 0.5 pmol of bNI-220 was sufficient to inhibit 3T3 fibroblast spreading to 50% (100 ng of a 220-kDa protein is equal to 0.5 pmol) (Fig. 7).
bNI-220 binds to an anion exchange column at pH 8.0 (Fig. 2) and to a cation exchange column at pH 5.0 (data not shown) is also in agreement with a pI of 5.9–6.3.

**Chick DRG Outgrowth Assay**—Since we have observed that bNI-220 is able to inhibit fibroblast spreading and PC12 neurite outgrowth, we decided to also test the substrate effect of bNI-220 on the neurite outgrowth of primary neurons. We took E11–E13 chick DRG explants, an age at which these growth cones were shown previously to avoid oligodendrocytes in culture (52). As shown in Fig. 9, neurite outgrowth on bNI-220 (approximately 100 ng was coated on an area of 0.2 cm² = 2 pmol/cm²) was abolished compared with 100 ng of laminin (same area) or control protein (100 ng of gel-eluted bovine control protein of molecular mass 120 kDa, bC120) (Fig. 9). Preincubation with mAb IN-1 led to a restoration of neurite outgrowth on bNI-220 substrate comparable to neurite outgrowth on the control protein bC120 (Fig. 9) or on polylysine (data not shown), whereas preincubation with the control antibody O1 showed no effect (Fig. 9). No effect of IN-1 or O1 was seen on bC120, indicating that the application of mAb IN-1 was not by itself inducing neurite outgrowth (data not shown). Importantly, the inhibitory activity of semaphorin III/collapsin 1 on neurite outgrowth of chick DRG explants could not be influenced by the mAb IN-1, showing that semaphorin III/collapsin 1 is not an IN-1 antigen (data not shown).

**Growth Cone Collapse Assay**—The effect of bNI-220 on growth cone motility was studied. Different amounts of liposomes containing bNI-220 or bovine tenascin-C (gel-purified like bNI-220) were applied uniformly to chick DRG growth cones in culture. Fig. 10 (A and B) shows a typical growth cone before and after application of liposomes containing bNI-220. The filopodia and lamellipodia of the growth cone retracted and left the growth cone collapsed and immobile (Fig. 10B). Quantification of the collapse response revealed that 78% of the observed growth cones collapsed when exposed to 5 μl of liposomes containing bNI-220 (approximately 50 ng in a volume of 100 μl). In contrast, only 24% of the growth cones collapsed when bovine tenascin-C containing liposomes (same amount) were applied (three experiments, total of 300 growth cones).

**Partial Amino Acid Sequences of bNI-220**—In order to identify this protein and to compare its sequence with that of other proteins, internal sequencing was performed. To this end, gel-eluted bNI-220 from several preparative gels was tested for its biologic activity, pooled, and subsequently re-applied for concentration on a 10% polyacrylamide gel, and stained with Coomassie Blue. Peptides were derived as described under “Experimental Procedures.” The amino acid sequences of these peptides are shown in Table II. A search of the SwissProt and GenEMBL data bases revealed that amino acids 3–11 of peptide 1 are unique to Protease Treatment—To solubilize the IN-1 antigen, detergent was required. If detergent was removed by dialysis or by acetone, proteins precipitated out and no activity could be detected in the supernatant. Reconstitution of 1 μg bNI-220 in various detergents was investigated (Fig. 1LA). Among the three detergents tested, CHAPS was the most favorable one for reconstitution, as shown in the PC12 neurite outgrowth inhib-
independent experiments completely. The results are the means of four growth inhibitory effect of bNI-220 antibody O1, could neutralize neurite outgrowth assay. This is in agreement with the previous finding that CHAPS was the most efficient detergent to solubilize IN-1 neutralizable activity from myelin or spinal cord.

The addition of 0.5% but not 0.1% (w/v) CHAPS was sufficient to dissolve the pellet of acetone precipitated bNI-220 and to restore biological activity. Increasing CHAPS concentration (1% w/v) did not alter inhibition of neurite outgrowth. Although all chosen SDS concentrations (0.1; 0.5; 1% w/v) could solubilize the pellet easily, some inactivation of inhibitory activity by SDS occurred (Fig. 11A). Interestingly, Triton X-100 was able to dissolve the pellet but no activity could be detected (Fig. 11A).

The structural stability of bNI-220 was further studied by subjecting the protein to urea and heat denaturation (Fig. 11, B and C). A 2-h treatment with various concentrations of urea showed only a moderate decrease of the neurite outgrowth inhibitory activity: 1 M urea to 92.0 ± 4.2% of inhibitory activity in the PC 12 assay, 4.5 M urea to 72.7 ± 1.8%, and 9 M urea to 63.3 ± 2.2% compared with 94.7 ± 3.5% without urea. This stability of bNI-220 coincided closely with its heat resistance (Fig. 11C), boiling for 5 min had no significant effect on the activity. Only heat treatment for 30 min could completely reduce the activity to control levels.

In contrast to heat and urea, brief treatment of bNI-220 with trypsin (0.1% w/v) abolished neurite outgrowth inhibition (Fig. 11D). As seen in SDS-PAGE, the 220-kDa band was completely degraded by this short trypsin treatment (data not shown). Simultaneous addition of trypsin inhibitor (0.2% w/v) or prior inactivation of trypsin (5 min boiling) did not lead to a decrease of inhibitory activity. Similar results were obtained with proteinase K (data not shown) and were in agreement with previous findings for the inhibitory activity of whole CNS myelin extract (23).

In summary, these findings showed that bNI-220 is a protein whose activity is resistant to denaturing conditions but very sensitive to protease.

Glycosylation of bNI-220—In order to determine the extent of glycosylation of bNI-220, the method of mild periodate oxidation of the hydroxyl groups of sugars to aldehydes was used. Digoxigenin, which was then detected by an enzyme immunoassay, was covalently attached to these aldehydes via a hydrazide group (54). Several proteins including bNI-220 were tested. Results are shown in Fig. 12. Although transferrin and gel-eluted bovine spinal cord tenascin-C (identified by Western blot) could be clearly detected, no positive glycan staining could be observed for gel-eluted bNI-220 (Fig. 12A). Dot-blot experiments showed the same results, suggesting that insufficient transfer of proteins during blotting could be excluded (data not shown). In addition, N-glycosidase F treatment and alkaline hydrolysis (β-elimination) to deglycosylate N- or O-linked sugar resulted neither in a band-shift (Fig. 12B) nor in a
antibody mAb O1 (B) and bNI-220 (A) growth of chick DRG explants on control protein (bC120) (C). Prominent neurite outgrowth, of a E12 DRG explant occurred on the control gel-eluted substrate to control levels (C). Bar, 100 μm.

Fig. 9. Phase contrast photomicrographs of the neurite outgrowth of chick DRG explants on control protein (bC120) (A) and bNI-220 (B and C) preincubated either with the control antibody mAb O1 (B) or the mAb IN-1 (C). Prominent neurite outgrowth, of a E12 DRG explant occurred on the control gel-eluted protein bC120 (A) although less than in the case of laminin (data not shown). Outgrowth on bNI-220 (preincubated with the control antibody O1) (B) or hybridoma culture medium alone (data not shown) was clearly almost absent. mAb IN-1 restored the outgrowth on the bNI-220 substrate to control levels (C). Bar, 100 μm.

Fig. 10. bNI-220 induces collapse of chick DRG growth cones. A representative example of the behavior of a DRG growth cone before (A) and after (B) application of liposomes containing bNI-220. A shows a growth cone with its normal, flat and large lamellipodium and several filopodia. In B, the same growth cone 1 h after application of the bNI-220-containing liposomes showed a completely collapsed morphology. Bar, 10 μm.

decrease of the specific activity of bNI-220 (data not shown). Thus, if bNI-220 is glycosylated at all, its carbohydrate moieties are probably small and short.

bNI-220 Is Not a Chondroitin Sulfate Proteoglycan—Chondroitinase ABC treatment, which was able to digest brevican into a 145-kDa full-length and an 80-kDa truncated protein (data not shown) as described previously (55), led neither to a band shift in the SDS-PAGE (Fig. 12B) nor to a decrease of the inhibitory activity of bNI-220 (data not shown).

DISCUSSION

Several lines of evidence indicate that we have identified a novel myelin-associated neurite growth inhibitory protein (bNI-220). 1) Inhibitory activity was enriched during purification, as monitored in the PC12 neurite outgrowth and 3T3 fibroblast spreading assay in parallel, with the enrichment of the gel-eluted 220 kDa protein, which was absent in inactive fractions. 2) Gel-eluted 220 kDa protein (bNI-220) exerted a potent neurite growth inhibitory activity in the pmol/cm² range. 3) The activity of bNI-220 was specifically neutralized by the mAb IN-1. 4) bNI-220 was specifically detected by the mAb IN-1 on Western blots. 5) bNI-220 is present in spinal cord myelin, in line with the previous finding that mAb IN-1 immunostains CNS myelin (32). 6) Immunoprecipitation of bovine CNS myelin by mAb IN-1 or an IN-1 Fab depleted about two thirds of the inhibitory activity and precipitated a high molecular mass complex comprising several bands including a prominent band at 220 kDa (25, 31). 7) Analytical analysis of the active gel-eluted 220 kDa band by two-dimensional PAGE revealed one major spot, indicating that bNI-220 was purified to homogeneity. 8) Five of six peptides obtained show novel amino acid sequences. Although one of the six bNI-220 peptides shows significant sequence similarities to the NSP/s-rex gene (54, 55), Northern blot signals as well as the recently cloned NI-220 cDNA show that bNI-220 is clearly distinct from the NSP/s-rex protein.3

The EC₅₀ value for highly purified bNI-220 was 5 pmol/cm² for fibroblast spreading, 10 pmol/cm² for PC12, and 2 pmol/cm² for chick DRG neurite outgrowth inhibition. This is in the range of the EC₅₀ for other neurite outgrowth inhibitors (56, 57), or of several positive guidance or outgrowth promoting factors (58). Most probably, bNI-220 acts through a ligand-receptor interaction. The relative high concentration of bNI-220 that was required in the collapse assay might in part reflect technical problems related to the obligatory use of liposomes (loss of material, accessibility, and membrane incorporation).

We showed that not only fibroblast spreading and PC12 neurite outgrowth but also neurite extension of DRG neurons and collapse of growth cones are affected by bNI-220. The monoclonal antibody IN-1 specifically neutralized the neurite outgrowth inhibitory substrate effect of bNI-220. This result confirms the inhibitory substrate effect of bNI-220 also on neurite outgrowth of primary neurons. Previous data have shown that collapse can be prevented by the mAb IN-1 (36, 59).

Size fractionating of rat myelin proteins by SDS-PAGE revealed two highly nonpermissive protein fractions of molecular mass 35 and 250 kDa (23). Rat NI-250, against which the mAb IN-1 was originally raised, is the putative rat homolog of bNI-220. In the present purification, we observed a second peak of activity that could be neutralized by the mAb IN-1. These fractions contained proteins of molecular mass between 20 and 100 kDa (SDS-PAGE), suggesting a lower molecular mass form of the IN-1 antigen also in bovine spinal cord. Unfortunately,
this activity was very unstable and could not be preserved for more than a few days, making its further analysis difficult.

In addition to the IN-1 neutralizable activity, which accounted for approximately 50% of total inhibitory activity of spinal cord or CNS myelin extract as determined by the fibroblast spreading and PC 12 neurite outgrowth assay, we could detect other inhibitory activities which could not be neutralized by mAb IN-1. MAG, which is present in CNS myelin, has been shown to be inhibitory in vitro for neurite outgrowth of cerebellar neurons, adult (but not perinatal) DRG neurons, embryonic hippocampal neurons, and the neuroblastoma cell line NG 108 (11, 59, 60) and can cause growth cone collapse of postnatal day 1 hippocampal neurons (60). McKerracher and colleagues (11) suggested that MAG may be a major neurite growth inhibitory constituent in CNS myelin. However analysis of the available MAG-deficient mice did not support this hypothesis. Two independent studies showed that there was no significant difference in neurite growth on myelin purified from MAG$^{-/-}$ and MAG$^{+/+}$ mice in vitro (36, 60). In vivo, axonal regeneration of the corticospinal tract after thoracic spinal cord lesion comparing wild type and MAG-deficient mice showed in one study a very small improvement (60) and in the other study no improvement at all (36). In contrast, axonal regrowth increased significantly in spinal cord and optic nerve and to a similar extent in the wild type and MAG-deficient mice, after in vivo application of the mAb IN-1 (36). These results show that in vivo the IN-1 antigen plays an important inhibitory role for axonal regeneration. No in vivo experiments with antibodies against MAG are available so far. In our assays, previous results indicated that neither PC12 neurite outgrowth nor 3T3 fibroblast spreading were affected by immunoaffinity-purified MAG (29). The same MAG preparations were able to induce outgrowth of perinatal DRG neurons, as described previously (61). Thus, the assays used in the present study did not detect the inhibitory activity of MAG and, therefore, the MAG con-

![Fig. 11. bNI-220 is stable to denaturing conditions but sensitive to proteases.](image1)

**A**, effects of detergents on PC12 neurite outgrowth inhibitory activity: After gel elution, bNI-220 proteins were precipitated and redissolved in 20 mM Tris-Cl, pH 8.0, containing various concentration of CHAPS, SDS, or Triton X-100. All chosen detergent concentrations (except 0.1% CHAPS) could dissolve the pellet (1 µg of protein); CHAPS solubilization showed the best activity recovery, whereas Triton X-100 blocked inhibitory activity. **B**, effect of urea on PC12 neurite outgrowth inhibitory activity: The activity of 1 µg/cm² bNI-220 in standard 0.5% CHAPS-buffer was measured as a function of urea concentration. Urea was added directly to the samples, which were incubated for 2 h at 37 °C and then coated on culture dishes for the PC12 assay. **C**, effect of heat on PC12 neurite outgrowth inhibitory activity: The activity of 1 µg of bNI-220 in standard 0.5% CHAPS buffer was measured as a function to heat incubation. Samples were heated to boiling for the indicated time durations, chilled on ice, and coated on culture dishes for the PC12 assay. **D**, effect of trypsin on PC12 neurite outgrowth inhibitory activity. The activity of bNI-220 in standard 0.5% CHAPS buffer was measured when 0.1% trypsin or trypsin + trypsin inhibitor (0.2%) were added. Samples were mixed, incubated for 1 min at 37 °C, chilled on ice and coated at 4 °C on culture dishes for the PC12 assay. Data are the means ± S.E. of three experiments.

![Fig. 12. Glycosylation of bNI-220.](image2)

**A**, the first three lanes show a silver-stained 6% SDS-polyacrylamide gel of bNI-220 (lane a, 500 ng), transferrin (lane b, 100 ng), and tenascin-C (lane c, 500 ng) run under reducing condition. The next three lanes show a DIG glycan staining of the parallel, blotted gel: lane a$, bNI-220; lane b, transferrin; lane c, tenascin-C. Molecular masses are indicated on the left. **B**, a 6% SDS-polyacrylamide gel of bNI-220 after different treatments is shown: a, no treatment; b, N-glycosidase F (2 units; 14-h incubation at 37 °C); c, alkaline hydrolysis (1 h at 37°C in 0.1 M NaOH); d, chondroitinase ABC (20 milliunits; treatment for 4 h at 37 °C). No band-shifts can be observed in any of these experiments. A band shift for fetuin (positive control) after N-glycosidase treatment was observed (data not shown).
taining fractions obtained in the present study did not correlate with inhibitory active fractions.

Other potential candidates for observed inhibitory activities might be members of the tenasin family (62), proteoglycans (55), or simply not yet identified molecules. Especially tenasin-R, which, in the CNS, is produced by oligodendrocytes and some subtypes of neurons (63, 64), has in vitro, like tenasin-C (62), repulsive effects on growth cones, however without leading to growth cone collapse (65). The identified gel-eluted bovine tenasin-C from our fractions showed inhibitory properties in our assays only at relatively high protein concentration (20 μg/cm²) for immunoaffinity-purified tenasin-C in the fibroblast assay. Tenasin-C knockout mouse show no detectable phenotype and seem to have a normal CNS anatomy (66, 67). Regeneration or recovery in these mice has not been studied.

Chondroitin sulfate proteoglycans, such as neurocan (68), NG2 (69), or phosphacan (70, 71), were shown to inhibit neurite outgrowth of specific primary neurons in vitro; however, no in vivo data are available. GP55, a 55-kDa, GPI-linked membrane glycoprotein of the Ig superfamily, was purified from adult chicken brain. It has a strong growth-inhibitory effect for neurons but does not affect fibroblast spreading (55, 72, 73). Therefore, GP55 can be excluded as a possible candidate exerting our detected inhibitory activities.

When extract or partially purified fractions are tested, our bioassays reflect the sum of growth-promoting and growth-inhibiting substrate effects of different molecules present in the extract. An example is shown by the first step of purification where the total activity of q-pool 1 is bigger than that of the starting material, although a second inhibitory activity (q-pool 2) has been purified away. We, therefore, assume that growth-promoting substrates were present in the starting material and subsequently purified away. The real enrichment factor may thus be considerably higher than the one shown in Table I. In addition, the biological activity may be reduced during several purification steps, gel elution, and dialysis.

The findings that bNI-220 is relatively stable to denaturing conditions, similar to GP55, which is still active after SDS-PAGE and electroelution (72), but very sensitive to proteases is also interesting with regard to the observation that rat and human glioblastoma cells can easily spread and migrate on CNS myelin substrates in vitro and in white matter in vivo (74, 75). Protease activity may be a way in which these highly infiltrating brain tumor cells overcome the inhibitory substrate effect of CNS myelin (74, 75).

Carbohydrate moieties on bNI-220 could be not demonstrated, suggesting that bNI-220 is not glycosylated. The detection limit using periodate for detection of sugar moieties varies for each glycoprotein and lies between 10 and 300 ng (53). Therefore, 0.5 μg of protein, which was used in our experiments, should be well above this limit. However, very minor glycosylation or the absence of adjacent hydroxyl groups in the sugar moieties, which are necessary for oxidation of the hydroxyl group to the aldehyde (first reaction step in the detection), might be reasons why we have found bNI-220 not to be glycosylated. The same is true for the band-shift experiments where small and short carbohydrate moieties would not be detected. The absence of a size reduction of bNI-220 (band shift) after chondroitinase ABC treatment and the finding that bNI-220 is not heavily glycosylated show that bNI-220 does not belong to the class of proteoglycans.

Although there might be several in vitro neurite growth inhibitory activities (unidentified activities from the present purification, MAG, tenasin-C, molecules in the CHAPS-insoluble material; Ref. 76), data from the present and from previous studies indicate that the IN-1 antigen bNI-220 is one of the most potent of them. Perhaps the most striking evidences for this hypothesis is the observation that in vivo application of the mAb IN-1 to lesioned nerve fiber tracts in adult rats resulted in long distance regrowth of fibers in the CNS (spinal cord, optic nerve, septo-hippocampal tract) (16, 33–35). 5–10% of these regenerating fibers, as it was often observed after IN-1 application, were apparently sufficient for large improvements of specific reflex and locomotor functions after spinal cord injury in adult rats (37). The relatively low number of regenerating fibers may point to the presence of other inhibitors at the lesion site and in the adult CNS. The biological function of the IN-1 antigen and other inhibitors may be a general stabilizing, growth-restricting effect in the adult CNS once the extremely complex structure and wiring of the CNS are developed (3, 77, 79).

The purification of bNI-220 has allowed us to obtain six peptide sequences and to clone the corresponding cDNA (80). All six peptides were found on the corresponding cDNA, confirming that they were derived from the same polypeptide. Sequence analysis of the open reading frame shows a novel protein with transmembrane domains, a large extracellular part, and expression in oligodendrocytes (80). Despite the fact that one of the six bNI-220 peptides shares 9 out of 11 amino acids with the human NSP (54) or the chick homolog s-rex (55), molecular analysis of partial cDNAs derived from bNI-220 peptide shows only 64% sequence identity to NSP/s-rex cDNA in a small 0.6-kilobase region. In addition, Northern blot signals using probes derived from the partial bNI-220 cDNA reveal a completely different pattern to that of NSP/s-rex, suggesting that the two proteins are only partly homologous but are distinct proteins.8 At present, however, the biological significance of this similarity, if any, is unclear.

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