Specific Alteration of NCAM-mediated Cell Adhesion by an Endoneuraminidase

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ABSTRACT A phage endoneuraminidase that specifically cleaves alpha-2, 8-linked polysialic acid has been found to be a useful probe for examining the biological role of this sugar moiety on the neural cell adhesion molecule (NCAM). The enzyme caused a 3.3-fold increase in the rate of NCAM-dependent aggregation of membrane vesicles from chicken embryonic brain, without the nonspecific effects previously encountered with the use of exoneuraminidases. The enhancement of aggregation was closely correlated with removal of sialic acid as assessed by electrophoretic mobility. Extension of this analysis to cultures of spinal ganglia indicated that removal of sialic acid by the endoneuraminidase results in an increase in the thickness of neurite bundles. This enhancement of fasciculation was reversed by addition of anti-NCAM Fab, suggesting that the enzyme treatment was not toxic and did not produce nonspecific effects on adhesion. Injection of the enzyme into the eyes of 3.5-d chicken embryos consistently produced a striking array of abnormalities in those parts of the neural retina that contained the highest concentrations of NCAM at the time of injection. These perturbations included a dramatic thickening of the neural epithelium in the posterior eye, a failure of cells in this region to elongate radially, formation of an ectopic optic fiber layer, and an incomplete association of the presumptive pigmented epithelium with the neural retina. These results provide the first direct evidence that the polysialic acid on NCAM has a regulatory effect on adhesion between living cells, and that the amount of this carbohydrate is critical for the normal morphogenesis of nerve tissue.

The neural cell adhesion molecule (NCAM)[1] is a cell-surface glycoprotein that serves as a ligand in the formation of cell–cell bonds (see references 9, 26, and 27 for review). The binding appears to involve the direct interaction between NCAMs on each cell, and therefore represents an example of homophilic adhesion (31). NCAM is expressed on a variety of cell types in the vertebrate embryo, including primitive neuroepithelia (36), neurons (29, 32), glial cells (14, 23), and muscle cells (15). Adhesion of neurons to muscle, glia, and other neurons has been found to be mediated at least in part by NCAM, and appears to be an essential event in the formation of nerve–muscle contacts that lead to synapses (30), guidance of axon growth cones along marginal pathways of the central nervous system (36), and the formation of neurite fascicles (29). In addition, antibodies to NCAM have been shown to alter the histogenesis of the retina in vitro (3).

The complex glycan chains associated with NCAM have a very unusual structure including one or more relatively long unbranched homopolymers of alpha-2, 8-linked sialic acid residues (6, 7, 12, 43). Removal of the sialic acid by commercial exoneuraminidases results in an increase in the apparent binding affinity of the molecule (7, 18). The relationship between binding affinity and sialic acid content is of particular interest, in that the sialic acid content varies widely in vivo both as a function of tissue source (4, 19, 33) and age (8, 24, 25, 33). Therefore, the possibility exists that differences in the carbohydrate moiety of NCAM produce differential affinities

[1] *Abbreviations used in this paper:* endo-N, the soluble endoneuraminidase from K1F bacteriophage; NCAM, neural cell adhesion molecule.

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among cells. As originally proposed by Steinberg (38), such hierarchies of adhesion are potentially an important force in the formation and histogenesis of embryonic tissues.

Until now, this intriguing hypothesis has not been tested, largely because specific reagents have not been available to alter the carbohydrate of NCAM on cells or in tissues. What is known about the role of sialic acid in NCAM function has been interpreted largely from the use of exoneuraminidases, which remove terminal nonreducing sialic acid residues from a variety of proteins, glycolipids, and gangliosides. In addition to this lack of specificity for NCAM, these enzymes are used at acidic pH, a condition which by itself causes a substantial alteration of cell adhesiveness (18).

Recently Vimr et al. (43) described a soluble form of endoneuraminidase (endo-N), which is produced by bacteriophage K1F to cleave the polysialic acid capsule of K1 antigen-positive serotypes of *E. coli.* This enzyme, which has a pH optimum of 7.2 and requires no cofactors, rapidly and specifically degrades linear homopolymers of sialic acid with alpha-2, 8-ketosidic linkages, requires a minimum chain length of 7–9, and primarily releases oligomers of 3–4 sialic acid residues.

Polysialic acid glycans with chain lengths in excess of ten sialosyl residues have so far been described only in bacteria (see reference 42 for review) and in the embryonic and neonatal nervous system (10), where they appear to be associated primarily if not entirely with one or more complex N-asparaginyl-linked carbohydrate chains on NCAM (6, 7, 11, 12, 22, 43). Thus, a polyclonal antiserum made against *Neisseria meningitidis* Group B bacteria, which express polysialic acid identical to the *E. coli* K1 capsule, also reacts specifically with a membrane glycoprotein with the same unusual SDS gel electrophoretic properties as NCAM (43), and immunoprecipitation of NCAM from extracts of brain membranes removes almost all of this carbohydrate antigen. The apparent specificity of endo-N for NCAM led Vimr et al. (43) to suggest that this enzyme could be used as a probe for the detection and selective modification of the NCAM carbohydrate. Finne and Makela (11) have reported a similar endoneuraminidase activity in a virion-bound enzyme, and also conclude that this type of enzyme can be useful in studying the structure and function of sialic acid on NCAM. In this report, we have tested some of these applications of endo-N both in vitro and in vivo, provided strong evidence in support of the suggestion that polysialic acid can regulate NCAM-mediated adhesion, and determined that this carbohydrate is an important determinant in the normal function of NCAM during nerve tissue histogenesis.

MATERIALS AND METHODS

Endoneuraminidase and Antibodies: Rabbit antibodies and Fab fragments were produced against affinity-purified chicken NCAM by methods described previously (2, 19). Endo-N was prepared as the soluble, virion-free form from bacteriophage K1F as described by Vimr et al. (43). The enzyme used in these studies is 200–300-fold purified from K1F-induced lysates of *E. coli*, and has no detectable proteolytic or exoneuraminidase activity. One unit of the enzyme is defined as the amount that degrades 1% of ^{14}C-labeled colominic acid (10–12 sialyl residue chain length) to smaller sialyl oligomers in 1 min at 37°C (43).

Membrane Vesicle Aggregation: The adhesivenses of brain membrane vesicles was analyzed by determining the rate of decrease in particle number using a Coulter ZB1 particle counter (Coulter Electronics Inc., Hialeah, FL) with a 100-μm aperture tube. Membrane vesicles were prepared on the day of each experiment as described previously (19). 25 μl of a 10% vol/vol suspension of vesicles in phosphate-buffered saline (PBS) pH 7.2 containing 20 μg/ml each of DNase and aprotinin was pretreated for 15 min at 4°C with 0–100 U of endo-N in 0.6 ml PBS. Aggregation was carried out at 37°C with rotation at 90 rpm in a 20-ml glass scintillation vial. 50-μl aliquots were diluted in 15 ml 1% glutaraldehyde in PBS before particle counting.

Electrophoretic Characterization of NCAM: The removal of sialic acid from NCAM was monitored by determination of electrophoretic mobility (19) in 7% polyacrylamide gels containing SDS (SDS PAGE, reference 21). Membrane vesicles from the aggregation assay were pelleted and solubilized in 10 vol of sample buffer. Dorsal root ganglia cultures were solubilized by sonication in 10 vol of Nonidet P-40 buffer (0.5% Nonidet P-40 in PBS, pH 7.4), and the NCAM concentrated by immunoadsorption to Bio-Rad Affigel 10 agarose beads coupled with a monoclonal antibody against the NCAM polypeptide. After the proteins were separated by SDS PAGE, they were transferred to nitrocellulose by electrophoresis (41), and the NCAM was detected by incubation with either polyvalent or monoclonal anti-NCAM antibodies and the appropriate second antibody (20). For analysis of the membrane vesicle samples, the second antibody was goat anti-rabbit IgG or rabbit anti-mouse IgG conjugated to peroxidase (Cappel Laboratories, Cochranville, PA), and bound peroxidase was detected using the chromogenic substrate 4-chloro-1-napthol (17). For the dorsal root ganglia samples, ^{125}I-labeled goat anti-rabbit IgG (New England Nuclear, Boston, MA) was used as the second antibody, and bound radioactivity detected using Kodak X-Omat AR X-ray film with an enhancing screen (39).

Analysis of Neurite Fasciculation in Cultures of Spinal Ganglia: Thoracic and lumbar spinal ganglia were obtained from 9-d and 7-d chicken embryos, respectively, dissected from attached connective tissue to minimize fibroblast contamination, and cultured on a collagen gel substrate in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 50 ng/ml 7S nerve growth factor (29). After 19–24 h of culture, in the presence or absence of 66 U/ml of endo-N and/or 0.5 mg/ml anti-NCAM Fab, the extent and degree of fasciculation of outgrowth was evaluated by phase contrast light microscopy. Extent of outgrowth was expressed in terms of distance of radial outgrowth from the ganglion. Fasciculation was estimated as described previously (29), in terms of the relative amounts of outgrowth contained in thick (15–4 μm in diameter), medium (4–1.2 μm), and thin (1.2–0.4 μm) processes. After this analysis, the NCAM contained in the cultures was extracted with 0.5% Nonidet P-40 and analyzed by SDS PAGE as described above.

Effects of Endo-N on Development of the Eye: 72-h chicken embryos were placed in shell-free culture (1). The development of embryos under these conditions is normal up to 18 d. After 12 h, the right eye of each embryo was injected through the dorsal hemisphere by hand with 1 μl of PBS or PBS containing 0.2 U of endo-N. The injections themselves did not change the size or shape of the developing eye relative to the uninjected left eye. After an additional 24 h of incubation, the eyes were removed, fixed in 0.5% glutaraldehyde/2% formaldehyde, processed through alcohol, and embedded in Spurr's plastic as described by Silver et al. (37). 1-μm sections were taken serially through the transverse plane of the eye (sagittal to the head), stained with toluidine blue, and observed by bright field light microscopy.

Histological Localization of NCAM in the Eye: Frozen sections of paraformaldehyde-fixed and sucrose-impregnated tissue were prepared, incubated with monoclonal antibody against the polypeptide chain of chicken NCAM, and stained for bound antibody using peroxidase-labeled goat anti-mouse IgG and diaminobenzidine, as described previously (36). The development of the stain was adjusted to approximately half-maximal levels, in order not to obscure quantitative differences in the amounts of NCAM.

RESULTS

Enhancement of Membrane Vesicle Adhesion by Endo-N

Measurement of the initial rate of decrease in particle number as cells or membrane vesicles aggregate provides a reproducible measure of their relative adhesiveness (2, 18). The rate of decrease is nearly constant down to ~50% of the original count, and then decreases with a plateau particle count of ~20% of the original value. With membrane vesicles from 11-d chicken embryo brain, a 50% decrease occurred after ~7.5 min (Fig. 1). When the vesicles were treated with endo-N, the apparent rate increased ~3.3-fold over controls, with a 50% decrease in particle number at ~2.5 min. After 30 min the aggregation of both the control and enzyme-

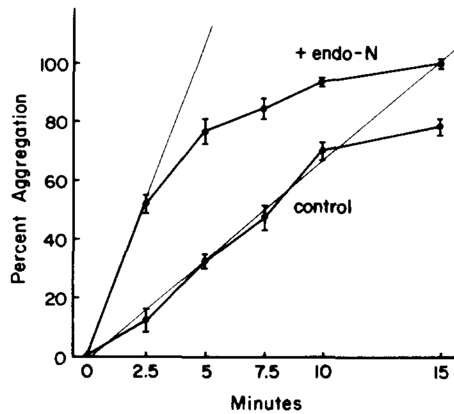

FIGURE 1 Effect of endo-N on the rate of brain membrane vesicle aggregation, expressed as the percentage of the total decrease in particle number after 60 min. Equivalent experiments are shown for untreated vesicles (lower curve) and membranes pretreated with 30 U of endo-N (upper curve). In both experiments the total decrease in particle number averaged 80%. The apparent rate of aggregation was nearly linear for up to a 50% decrease in particle number, and the slope of this line was 3.3 times greater for the enzyme-treated membranes than for control vesicles. The standard errors are of the average of two determinations in each of four independent experiments.

treated vesicles had reached a plateau at ~20% of the original particle count. Vesicles treated with the enzyme buffer alone had aggregation rates indistinguishable from those of untreated membranes.

The correlation of sialic acid removal from NCAM by endo-N with an increase in adhesiveness is illustrated in Figs. 2 and 3. Removal of sialic acid by endo-N under the conditions of the aggregation assay was observed with more than 1 U of enzyme per assay and reached maximal values with 10 U, as judged by the increased gel electrophoretic mobility of NCAM and the appearance of multiple bands on the gel (Fig. 2). This dose relationship was very similar to that observed for the increase in rate of vesicle aggregation (Fig. 3).

Exposure of the membrane vesicles to high levels of endo-N (100 U) for up to 3 h did not change the electrophoretic mobility of NCAM polypeptides beyond the alterations illustrated in Fig. 2, which suggests that lytic activities other than removal of sialic acid, such as a slow rate of proteolysis, were not present in the endo-N preparations.

Increase in Fasciculation of Spinal Ganglion Neurites Cultured in the Presence of Endo-N

Inhibition of NCAM function by anti-NCAM Fab has been shown to decrease the average thickness of neurite bundles that grow out from spinal ganglia in culture, and to slightly augment their extent of outgrowth along the substrate (28, 29). The effect of the Fab on fascicle diameter is believed to reflect directly a reduction in NCAM-mediated adhesion between neurites. On the other hand, the increase in the average length of the fibers has been interpreted as an indirect consequence of reduced tension along individual neurites relative to large bundles (28). The effect of endo-N in cultures of 7-d chicken embryo lumbar ganglia is shown in Figs. 4 and 5. Under the conditions of these experiments, the amount of sialic acid associated with NCAM was greatly reduced, leaving material with an apparent molecular weight of ~150,000 (Fig. 5). In terms of neurite outgrowth, the result was exactly the

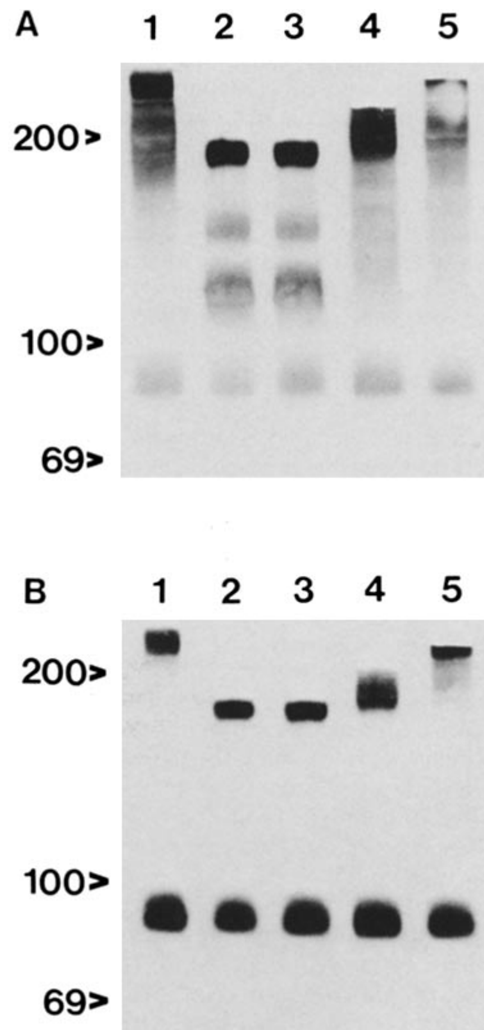

FIGURE 2 Removal of sialic acid from membrane vesicle NCAM by endo-N. Chicken embryo brain vesicle preparations were treated with different amounts of endo-N under the same conditions used to evaluate the effect of the enzyme on vesicle aggregation (Fig. 1). The effect of endo-N on the sialic acid content of NCAM was monitored by SDS PAGE using a polyclonal antibody (*A*) or a monoclonal antibody (*B*) to detect the NCAM. This monoclonal antibody, which reacts with a cytoplasmic region of the molecule (our unpublished results), recognizes only two forms of NCAM in the vesicles, the heavily sialylated M_r 180,000 polypeptide chain, and a spontaneously-generated M_r 90,000 fragment. The latter is unaffected by endo-N and resembles the polysialic acid-free fragment of mouse NCAM described by Gennarini et al. (13). The use of this monoclonal antibody therefore provides a less complex picture of changes associated with polysialic acid. The amounts of endo-N were 0 U (lane *1*), 30 U (lane *2*), 10 U (lane *3*), 3 U (lane *4*), and 1 U (lane *5*). Treatment with 0.3 and 100 U in separate experiments that are not shown resulted in immunoblot profiles similar to that obtained after treatment with 0 and 30 U, respectively. Note that the removal of sialic acid, which increases the mobility of NCAM, is first evident with 1 U of endo-N and reaches a maximum with 10 or more units of enzyme. This is the same dose-response relationship observed in the vesicle aggregation studies (Fig. 3). The migration of the following ^{14}C-radiolabeled proteins is indicated with arrowheads: myosin (M_r 200,000), phosphorylase B (M_r 100,000), and bovine serum albumin (M_r 69,000).

opposite of that obtained with anti-NCAM Fab: the fascicles increased in their average diameter and decreased in both number and radial extent of outgrowth. These results are also

summarized quantitatively in Table I. In contrast to the results obtained with 7-d lumbar ganglia, cultures of thoracic ganglia from 9-d embryos, which contain NCAM with considerably less sialic acid than do the older lumbar ganglia used (4), were not noticeably affected by the enzyme (Table I). The formation of larger bundles with the addition of enzyme was most readily observed on substrates of moderate adhesivity such as

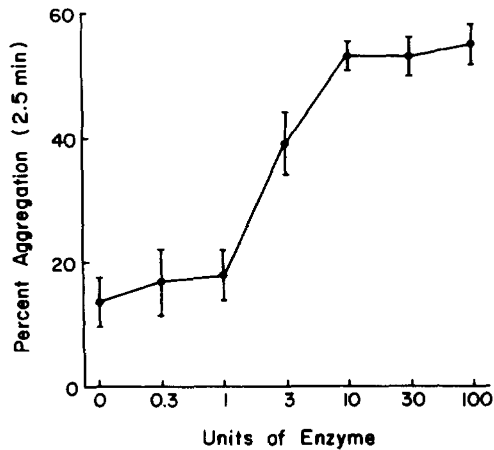

FIGURE 3 Relationship between the amount of endo-N used to treat brain membranes and their initial rate (at 2.5 min) of aggregation (see Fig. 1 and compare with Fig. 2). The error bars indicate standard error of the average of two determinations in each of four independent experiments.

collagen. With a more adhesive substrate, for example a fibroblast monolayer, very little fasciculation occurred with or without enzyme, suggesting that growth cone–substrate interaction was too strong to be overcome by a moderately increased adhesion between nerve fibers. With agar, a relatively poor substrate, the bundles were already large in controls and only slightly thickened by enzyme treatment (data not shown).

If the effects of endo-N specifically reflect an enhancement of NCAM-mediated adhesion, they they should be masked by the addition of anti-NCAM Fab, which strongly inhibits NCAM binding function. As illustrated in Fig. 6, anti-NCAM Fab both decreased neurite fasciculation and increased neurite length, and addition of enzyme did not alter this pattern of outgrowth. These results are also represented quantitatively in Table II.

Effects of Endo-N on Early Development of the Eye

These experiments were carried out by injecting the enzyme into the vitreal body of eyes of 3.5–4-d embryos, and then examining the histology of the eye in serial plastic sections after an additional day of development. During this stage, the eye is growing rapidly in size and is beginning to send optic fibers into the fissure toward the optic chiasm, but has not as yet formed the clearly defined cell and plexiform layers evident in the adult retina. The injected enzyme probably remains largely in the eye, as has been found for antibody Fab

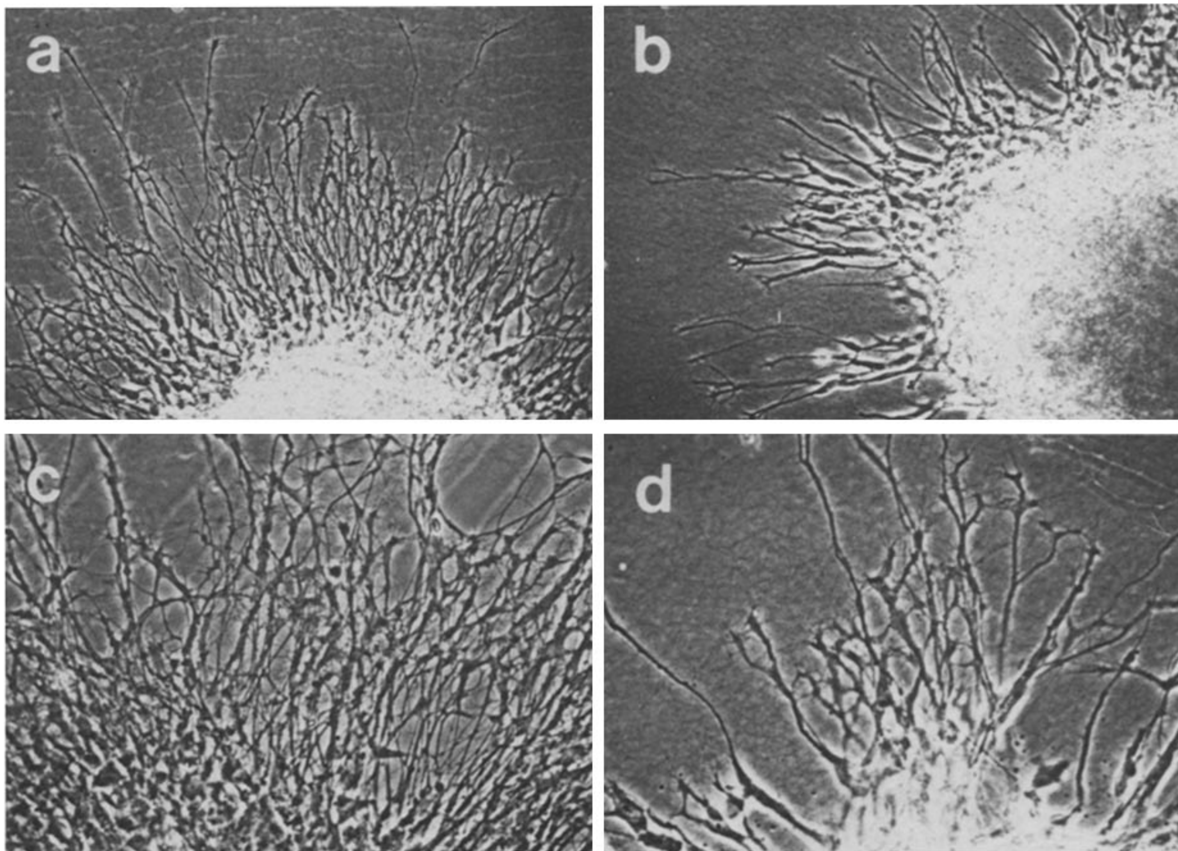

FIGURE 4 Effect of endo-N on the pattern of neurite outgrowth from cultured dorsal root ganglia. With lumbar ganglia from 7-d chicken embryos, the presence of 100 U of endo-N caused an increase in the thickness of neurite fascicles and a slight decrease in the radial extent of the outgrowth. (*a* and *c*) Control cultures; (*b* and *d*) cultures with endo-N. See Table I, lines *1–4* for the same results presented in tabular form. (*a* and *b*) ×100; (*c* and *d*) ×160.

(40), and its estimated initial concentration of ~20 U/ml in the vitreal body is similar to the concentrations used in the aggregation and culture experiments described above.

The injected enzyme was found to have a major effect on the morphogenesis of the retina during this period (Fig. 7). The most noticeable changes were (*a*) a substantial thickening of the neural retina in the dorsal–posterior region, (*b*) failure of the retinal epithelial cells to elongate radially, (*c*) a buckling of the entire epithelium, (*d*) apparent formation of clear zones that may represent a marginal zone with optic fibers, and (*e*) an incomplete association of the pigmented epithelium with the neural retina. Of the seven enzyme-injected and serially sectioned eyes obtained in two independent experiments, five exhibited these types of malformations and two were unaffected. This proportion of unaffected animals is consistent with our previous experience with antibody injections, in which leakage of the injected reagent from the eye prevented a significant perturbation. All of the eight control animals (buffer injection only) had normal eye histology (Fig. 7).

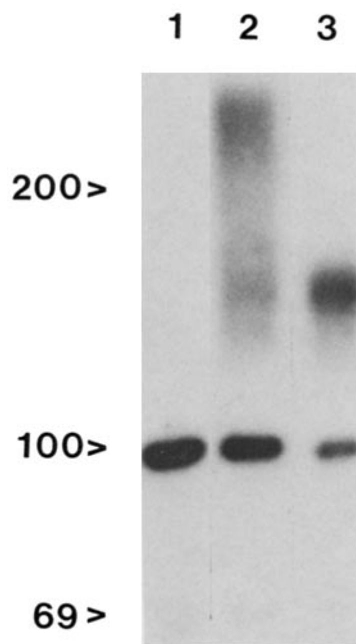

FIGURE 5 Removal of sialic acid from NCAM by endo-N in cultures of dorsal root ganglia. NCAM from untreated cultures had the low and diffuse electrophoretic mobility in SDS PAGE that is characteristic of the heavily sialylated molecule (lane *2*). With cultures containing 100 U of enzyme, the NCAM ran as a sharper band with an apparent M_r of 140,000 (lane *3*), which represents a relatively unsialylated form of the molecule. NCAM from these cultures was initially concentrated on antibody-coated agarose beads. These beads release a 100,000 M_r component that represents eluted antibody and is present in each lane including the control in lane *1*, to which no NCAM was added.

If the enzyme is specific for NCAM polysialic acid, then its effects should be most evident in regions of the eye that express the molecule during the course of the experiment. We have observed that in the neural retinas of both 3.5-d and 5-d embryos there are marked gradients of NCAM expression along both the anterior–posterior and ventral–dorsal axes (Fig. 8), with the highest concentrations being in the dorsal–posterior quadrant. As shown in Fig. 7, this NCAM-rich region is also the part of the eye most clearly affected by endo-N. In fact, the ventral periphery of the eye, including both epithelia as well as the optic fiber layer, appears under the light microscope to be nearly normal, with only a relatively minor overall thickening.

DISCUSSION

This study demonstrates that endo-N, with its neutral pH optimum and specificity for alpha-2, 8 linkages in long linear homopolymers of sialic acid, is a powerful tool for the study of the role of sialic acid in NCAM-medited adhesion. Its use here in vitro has provided new and less ambiguous support for the original suggestion (7) that the carbohydrate moiety of NCAM can modulate the molecule's binding function. More importantly, the present studies with explant cultures and those in vivo provide the first direct evidence that changes in the amount of polysialic acid on NCAM actually affects a physiologically significant adhesion event such as neurite fasciculation, and is a major determinant in the histogenesis of complex nerve tissues.

A critical point in the evaluation of endo-N preparations as a tool for studying NCAM sialic acid in tissues is the absence of nonspecific effects, either intrinsic to the enzyme or as a result of contamination. As indicated in the introduction, studies from other laboratories suggest that NCAM is the primary if not the sole carrier for the form of polysialic acid that is sensitive to endo-N. The work of Lyles et al. (22) indicates that a small portion of the polysialic acid–bearing material in perinatal mouse brain cannot be removed by antibody against NCAM. The NCAM of perinatal mice has relatively little sialic acid relative to embryos, and in our experience even polyclonal antisera fail to react with all the NCAM in a given sample. It is therefore likely that these results reflect an underestimate of the fraction of total poly-

TABLE I. *Effect of Endo-N on Neurite Outgrowth in Culture from Dorsal Root Ganglia**

Ganglia	Enzyme present‡	Fascicles§ 20–5 μm	Fascicles§ 5–1 μm	Fascicles§ 1–0.3 μm	Outgrowth‖	
	h		*% ± SD*		*h*	*mm ± SD*
E7 Lumbar	—	19 ± 4	37 ± 3	44 ± 5	19	0.5 ± 0.07
	16–19	42 ± 4	33 ± 10	25 ± 3	19	0.3 ± 0.04
	—	11 ± 5	32 ± 3	57 ± 6	24	0.9 ± 0.03
	16–24	36 ± 6	42 ± 4	22 ± 4	24	0.7 ± 0.05
E9 Thoracic	—	29 ± 6	47 ± 4	24 ± 3	24	1.1 ± 0.08
	16–24	24 ± 5	43 ± 5	33 ± 3	24	1.1 ± 0.10

* See Fig. 4 for representative micrographs.
‡ Enzyme was added after 16 h of culture and remained in the culture dish thereafter. Observations were made at 19 and 24 h after culture.
§ The amount of fasciculation was evaluated by scoring the percentage of outgrowth contained in fascicles of the indicated diameters (27).
‖ Expressed as the radial distance of outgrowth from the edge of the ganglion. Standard errors are shown for values obtained in scoring 10–15 ganglia in each of three independent experiments.

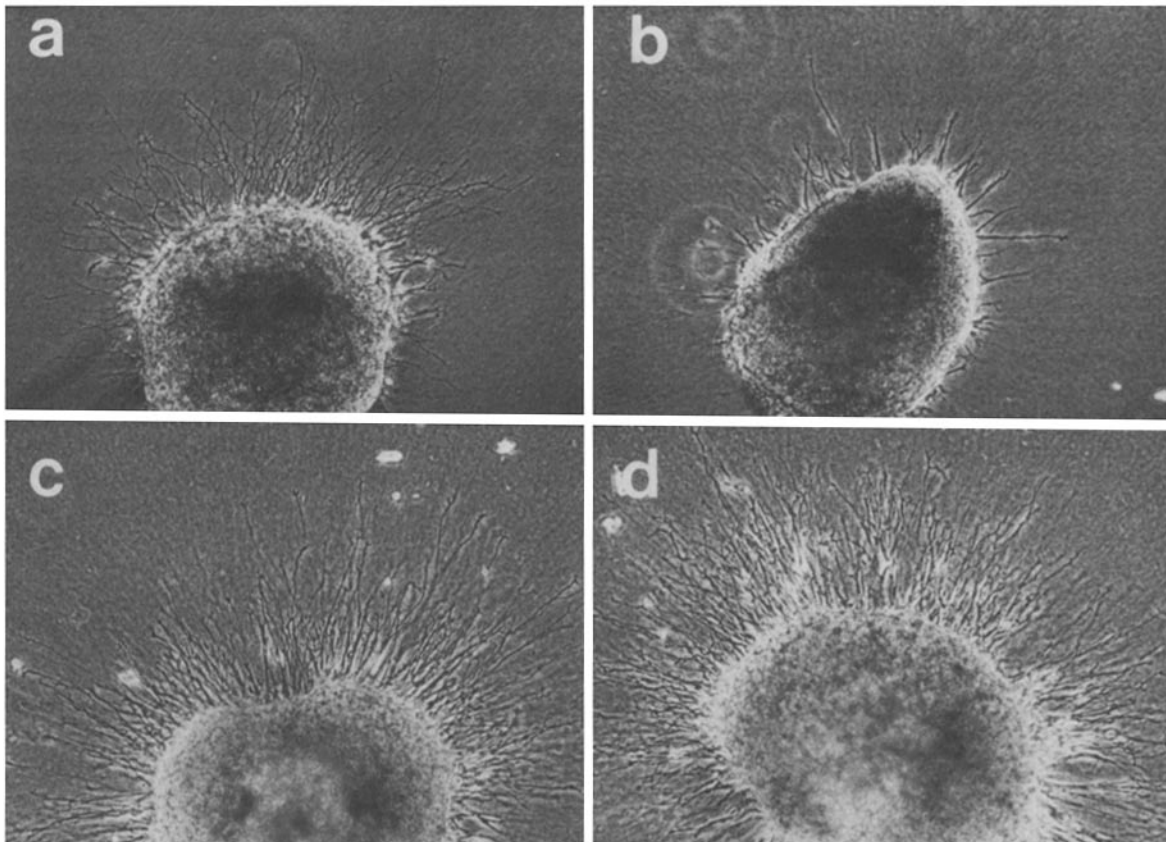

FIGURE 6 Dominance of anti-NCAM Fab over endo-N-induced effects on neurite outgrowth from cultured lumbar dorsal root ganglia. (*a*) Control culture without antibody or enzyme. (*b*) Culture with 100 U enzyme showing thicker and shorter fascicles. (*c*) Culture with longer and thinner fascicles produced by inhibition of adhesion by anti-NCAM Fab. (*d*) Outgrowth identical to that in c which was produced in the presence of both the enzyme and Fab. The quantified results are presented in Table II. ×50.

TABLE II. *Effect of Endo-N and Anti-NCAM Fab on Neurite Outgrowth from E7 Lumbar Ganglia**

Added reagents		Fascicles			
Endo-N	Anti-NCAM Fab	20-5 μm	5-1 μm	1-0.3 μm	Outgrowth
			% ± SD		mm ± SD
−	−	8 ± 3	35 ± 4	57 ± 7	0.8 ± 0.04
+	−	33 ± 4	46 ± 4	21 ± 3	0.6 ± 0.04
−	+	1 ± 1	20 ± 2	79 ± 6	1.3 ± 0.05
+	+	2 ± 2	18 ± 3	80 ± 5	1.3 ± 0.04

* See Table I for footnotes and Fig. 6 for representative micrographs. The antibody was added at the beginning of the culture, endo-N after 16 h, and the ganglia scored after 24 h.

sialic acid that is associated with the embryonic form of NCAM.

Several observations in the present studies support the interpretation that endo-N-induced effects can be directly attributed to the removal of sialic acid from NCAM. First, prolonged incubation of NCAM or NCAM-containing extracts with endo-N, well beyond the time required to complete the cleavage of sialic acid polymers, did not noticeably alter the gel pattern of NCAM polypeptides. Because NCAM is readily degraded by a variety of proteases, this observation suggests that the enzyme preparation is free of proteolytic activities that might by themselves alter adhesion. The possibility of some type of nonspecific toxicity or unidentified enzymatic activity appears unlikely in that endo-N had no noticeable effect on cultures of spinal ganglia in which NCAM-mediated adhesion was blocked by anti-NCAM Fab. Also, in the same cultures, the ability of endo-N to enhance fasciculation depended on the use of ganglia that produced NCAM with a high sialic acid content. Although a decrease in the extent of fascicle outgrowth can be produced by a variety of toxic conditions, previous studies have demonstrated that the elongation of neurites can also be diminished by an increase in fasciculation (28). Finally, the striking localization of endo-N-induced effects on eye development in regions that express high amounts of NCAM is consistent with NCAM being the unique substrate for this enzyme in a vertebrate embryo.

While the results in aggregation and neurite fasciculation assays reflect an obvious cell–cell adhesion, the possibility remains that some of the effects observed in vivo might be the result of an NCAM activity other than adhesion. In our previous studies on NCAM in vivo (36, 40), the attribution of NCAM function to cell–cell contact was based on detailed anatomical descriptions in which the positions of certain cells and their mutual expression of NCAM suggested a selective affinity between them, and antibodies against NCAM were found to alter these positions. A similar analysis for NCAM sialic acid will require the ability to characterize the sialic acid content of the molecule in tissues. Monoclonal antibodies have been described that react with sialic acid–dependent determinants on NCAM (5, 16). However, these antibodies also cross-react with other cell surface glycoconjugates and

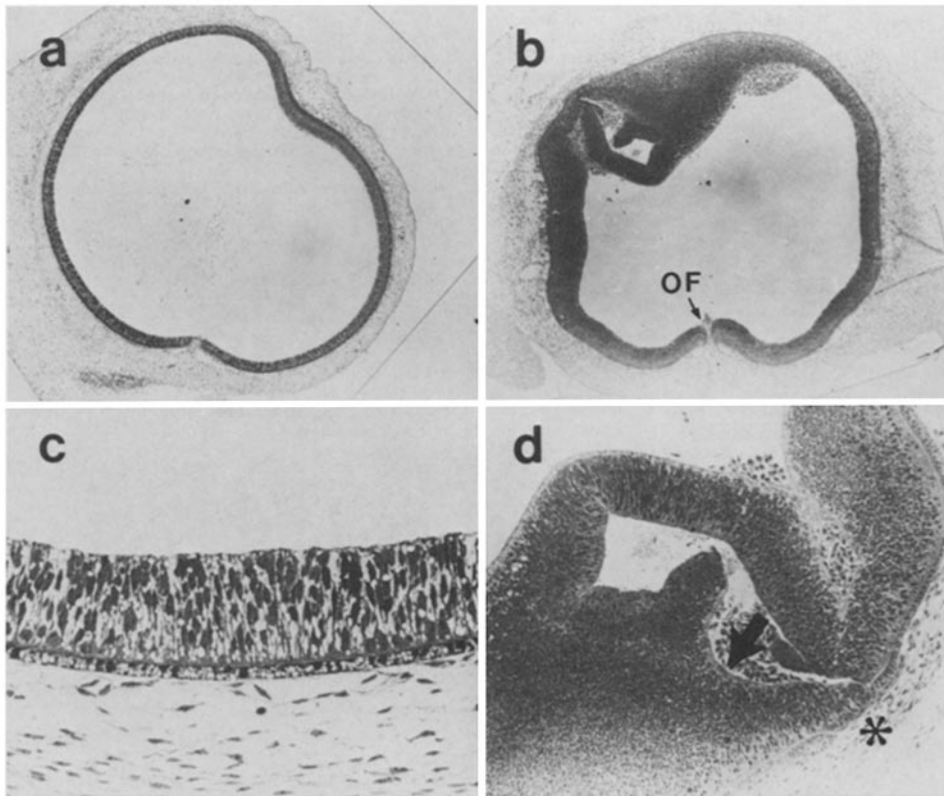

FIGURE 7 The effects of intraocular injection of endo-N into a 3.5-d chicken embryo, as observed in sagittal plastic sections by light microscopy. Control eyes, injected with buffer alone, are shown in *a* at ×37 and *c* at ×375. In Fig. 7, *a* and *b*, the dorsal area of the eye is at the top of the photograph, and the ventral area is at the bottom. The location of the ventral optic fissure is indicated by the initials *OF*. Enzyme-injected eyes are shown in *b* at ×37 and *d* at ×125. Note the thickening of the dorsal posterior pole of the neural retina of the enzyme-injected embryo, the more packed and rounded morphology of the cells in this thickened region, the clear zone which resembles an ectopic marginal zone (arrowhead), and the regions of ventricular neural retina that are not associated with the presumptive pigmented epithelium (asterisk).

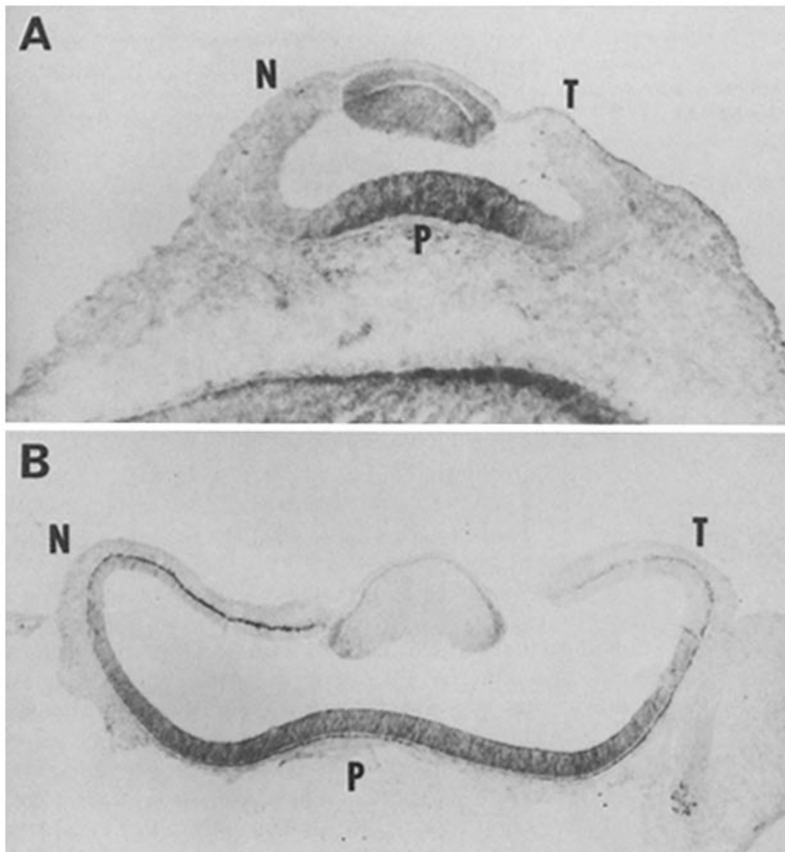

FIGURE 8 Distribution of NCAM in the retina of a 3.5-d (*A*) and a 5-d (*B*) chicken embryo, as visualized by immunoperoxidase staining of coronal frozen tissue sections along the nasal(*N*)–temporal(*T*) axis in the more dorsal part of the eye. There is an increasing gradient of staining toward the posterior (*P*) of the eye at both ages. A similar distribution with increasing NCAM toward the posterior eye is observed in sections perpendicular to those shown here, that is, in the dorsal–ventral plane produced by sagittal sections through the head. This staining pattern of NCAM in the dorsal posterior quadrant coincides with the sites of major abnormalities produced by the presence of endo-N during this period of eye development (Fig. 7). Staining is also seen at the brain margin in (*A*), as reported previously (37). (*A*) ×100; (*B*) ×60.

therefore are not useful for histological analyses. Studies are now in progress to determine if polyclonal antibodies against bacterial silalic acid, which have been reported to require a minimum chain length of 10 residues for recognition of the antigen (11), might prove to be suitable for immunohistological studies. In any case, the presence of the NCAM polypeptide, gross characterizations of carbohydrate content by electrophoretic mobility, and a thorough examination of endo-N

perturbation of tissues during a variety of developmental events are likely to provide sufficient information to define at least some of the effects of polysialic acid on cell–cell adhesion in the embryo.

Assuming that most NCAM-associated phenomenon either directly or indirectly involve cell adhesion, it is interesting to speculate on possible explanations for the perturbations produced by endo-N in the developing eye. The most obvious abnormality appears to be a local increase in the number of undifferentiated cells. Such an effect could have been caused either by increased mitosis, coalescence, or a decrease in the normally high rate of cell death that occurs in the posterior pole of the retina at this stage of eye development (34, 35). The possibility that cell–cell contact could alter mitosis has been proposed in a wide variety of systems, both in terms of inhibition and stimulation. On the other hand, an absence of interactions might lead to selective cell death, and it is possible that artificially increased adhesion might allow survival of a greater number of retinal cells. Similarly, it can be argued that perturbations of cell differentiation and the displacement of fiber layers and pigmented epithelium could directly reflect altered adhesive hierarchies required for orderly histogenesis. It may be that some of these abnormalities are secondary effects caused, for example, by distortions resulting from the apparent increase in cell number. As one approach to distinguishing these variables and examining their causal relationship, we are presently carrying out a detailed description of endo-N–induced effects on eye development, using a variety of stages and durations, different dosages of enzyme, and electron as well as light microscopy.

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