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The neural cell adhesion molecule (N-CAM) inhibits proliferation in primary cultures of rat astrocytes

(antisense/glia/regeneration/contact inhibition)

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ABSTRACT **Cell proliferation is a key primary process during neural development and also plays an important role in the regenerative response of neural tissue to injury. It has been reported that glial cell proliferation is, at least in part, controlled by a neuronal signal, possibly involving cell surface molecules. We report here that the addition of purified rat neural cell adhesion molecule (N-CAM) to primary cultures of rat forebrain astrocytes inhibits their proliferation. This inhibitory effect can be elicited in cultures grown in chemically defined serum-free medium or in medium that had been supplemented with growth factors. Polyclonal antibodies to N-CAM or their Fab' fragments elicited a similar inhibitory effect. The magnitude of the inhibitory effect of N-CAM was dependent on cell density: it was maximal at low cell densities and weakened progressively as cells approached confluency. Synthetic peptides with sequences identical to a putative homophilic binding region of N-CAM mimicked the effect of purified N-CAM, while peptides of the same length and amino acid composition but with a randomized sequence did not. The addition of N-CAM antisense oligonucleotides to primary astrocyte cultures for 48 h resulted in reduced levels of N-CAM expression. After N-CAM levels on astrocytes were diminished by this treatment, the antiproliferative effect of N-CAM added to the medium was significantly reduced. The combined results suggest that N-CAM homophilic binding may be involved in the control of glial cell proliferation.**

During embryogenesis, the proliferation of glioblasts and their differentiation into several glial cell types appear to be closely coordinated with the development of neurons. During early postnatal development of the rodent brain, glial proliferation continues at significant levels after neuronal proliferation has mostly ceased. In general, glial proliferation in the brain remains strongly inhibited throughout adult life, except in tumorigenesis and after injury. After physical injury to the central nervous system (CNS), astrocytes respond by changes in cell morphology, increased expression of glial fibrillary acidic protein, hypertrophy, and rapid proliferation (1–3). Such reactive astrocytes form glial scars in a process of gliosis that appears to interfere with neuronal regeneration in the adult CNS (4, 5).

Glial proliferation can be influenced by a variety of factors. Stimulatory effects are exerted by various growth factors (6), interleukins 6 (7) and 1β (8), tumor necrosis factor (9), and γ interferon (10). The mitogenic effects of several of these factors can be inhibited by transforming growth factor β (11), which is produced by neurons (12).

Further evidence points to a direct involvement of neurons in the control of glial proliferation. The addition of purified cerebellar granule neurons to cerebellar astrocytes (13) or of hippocampal neurons to glia (14) resulted in a decrease in glial proliferation. This inhibition depended on a proper ratio of neurons to glia of about 4:1, ensuring that astrocytes made surface contact with the added neurons. A cell membrane preparation of cerebellar granule neurons also inhibited proliferation, whereas medium conditioned by such neurons did not, indicating that membrane-associated molecules might be involved in the neuronal control of glial proliferation.

Astrocytes express a variety of adhesion molecules, among them the cell adhesion molecules N-CAM (neural cell adhesion molecule), and L1, and the extracellular matrix protein laminin. N-CAM (15, 16) has an important regulatory role in the developing nervous system and is present in adult nervous tissue. It has been found in astrocytes *in vivo* (17) and in primary astrocyte cultures (18), although normal levels of N-CAM in glia are low in comparison to those in neurons (19). The amount of N-CAM expressed in the whole nervous system (20, 21) and on the surface of astrocytes (22) decreases significantly as the CNS matures compared to the amount seen during prenatal and early postnatal development. After neurotoxin-induced brain damage, however, glial cells reexpress high levels of the highly sialylated embryonic form of N-CAM (23). Peripheral nerve injury leads to the expression of high levels of sialic acid-rich N-CAM in both neural and glial tissue (24). The increased expression of N-CAM after injury and the presence of N-CAM on reactive astrocytes are in accord with the hypothesis that cell adhesion molecules may play a functional role in regeneration.

In this study, we provide evidence to support this hypothesis and show that the addition of soluble N-CAM to primary cultures of astrocytes inhibits their proliferation. We describe experiments suggesting that this inhibitory effect is mediated by homophilic N-CAM binding. A similar inhibitory effect can be elicited by addition of short synthetic peptides with sequences corresponding to parts of the third immunoglobulin-like domain of N-CAM. The inhibitory effect of N-CAM on glial proliferation supports a functional role for N-CAM in the developing and regenerating nervous system.

MATERIALS AND METHODS

Astrocyte Cultures. Primary cultures of astrocytes were prepared from the forebrains of 4- to 5-day-old neonatal rats. After decapitation and removal of the forebrains from the skull, the meninges were carefully peeled off, and forebrain tissue was triturated in sterile phosphate-buffered saline (PBS) by using fire-polished pipettes. The resulting suspension of tissue fragments was incubated in Hanks' balanced salt solution (GIBCO/BRL) with trypsin (2.5 mg/ml), 1 mM EDTA, and DNase (Boehringer Mannheim; 0.1 mg/ml) at 37°C for 15 min. Undissociated tissue was removed by centrifugation and the remaining cell suspension was centrifuged through a cushion of 3.5% (wt/vol) bovine serum albumin to remove cell debris. The

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Abbreviations: N-CAM, neural cell adhesion molecule; FBS, fetal bovine serum; CNS, central nervous system; bFGF, basic fibroblast growth factor.

pellet was resuspended in serum-free medium (SFM) consisting of Dulbecco's modified Eagle's medium, 2 mM L-glutamine, penicillin G sodium (50 units/ml), and streptomycin sulfate (50 μg/ml) (all from GIBCO/BRL) supplemented by 10% (vol/vol) fetal bovine serum (FBS). The cells were transferred to a standard 100-mm tissue culture dish (Falcon) and incubated at 37°C for 1–2 h. After mild shaking, the supernatant containing unattached cells was transferred to a 100-mm collagen-coated dish. Cultures were maintained in SFM supplemented with 10% FBS in 5% CO_2/95% air for ≈7 days before the start of an experiment. Cell samples were checked regularly for the expression of glial fibrillary acid protein, a marker for astrocytes. Only those cultures containing >95% astrocytes were used.

Purification of N-CAM. N-CAM was purified from early postnatal rat brains by affinity chromatography using a cross-reactive anti-human N-CAM monoclonal antibody (a generous gift of John Hemperly, Becton Dickinson Research Center, Research Triangle Park, NC). Purity of the preparations was tested by SDS/gel electrophoresis using standard Coomassie blue staining and Western blot analysis techniques. Protein concentrations were estimated using the Bio-Rad micro Bradford assay. Fab' fragments were prepared from anti-mouse N-CAM polyclonal antibodies as described (25). In some experiments, purified N-CAM was treated with neuraminidase (1 unit/ml; from *Vibrio cholerae*; Calbiochem) in 100 mM sodium acetate/2 mM $CaCl_2$/2 mM EDTA for 24 h at 35°C and pH 5.5 (26) before being added to cell cultures.

Peptide Synthesis. Peptides were synthesized on a Gilson AMS-422 automated peptide synthesizer. The lyophilized peptides were resuspended in water, desalted, and relyophilized at least once before use. Purity and concentrations of synthetic peptides were confirmed using mass spectrometry, analytical HPLC, and amino acid analysis.

Proliferation Assay. Astrocytes were labeled by addition of 10 μl of 8.72 μM L-[^{35}S]methionine (New England Nuclear; ≈1200 Ci/mmol; 1 Ci = 37 GBq) to 10 ml of culture medium and transferred into 96-well plates (Falcon) usually at a density of 2×10^5 cells per ml (equivalent to 7×10^4 cells per cm^2 or 2×10^4 cells per well). After cell attachment, the culture medium was exchanged for SFM. In antisense experiments, oligonucleotides were added at the same time ($t = 0$). Growth factors, purified N-CAM, or synthetic peptides were added after the cells had remained in SFM for 48 h ($t = 48$ h); at this time most of the cells are synchronized with respect to their position in the cell cycle at the G_1/G_0 boundary (6). After another 12 h (at $t = 60$ h), [^{3}H]thymidine (New England Nuclear; 20 Ci/mmol) was added (0.38 μM, 10 μCi/ml). After an additional 12 h ($t = 72$ h), the wells were drained, radioactivity was precipitated with ice-cold 10% (wt/vol) trichloroacetic acid, and the plates were left to dry. Radioactivity was solubilized overnight by adding 100 μl of 1 M KOH to each well, neutralized with 100 μl of 1 M HCl, transferred to liquid scintillation vials, and measured in 3 ml of Beckman Ready Safe liquid scintillation solution on a Packard 1600 TR counter. To determine levels of [^{3}H]thymidine incorporation relative to the number of cells, a dual dpm counting protocol was used (27) that minimizes the overlap between the ^{3}H and ^{35}S energy spectra and takes into account detection efficiencies for both isotopes. In this paper, proliferation is expressed as [^{3}H]thymidine incorporation normalized to [^{35}S]methionine incorporation (^{3}H dpm/^{35}S dpm).

Blot Analysis. To quantify the amounts of N-CAM present in astrocytes, cultures were grown as described and cellular material was lysed in PBS (pH 7.4) containing 0.5% Nonidet P-40, phenylmethylsulfonyl fluoride (350 μg/ml), aprotinin (2 μg/ml), leupeptin (0.5 μg/ml), and pepstatin A (0.7 μg/ml) at 4°C. Standard Western blot analysis techniques were employed using cell lysates directly or after N-CAM immunoprecipitation. ^{125}I-immunolabeled N-CAM was estimated by densitometry on a Molecular Dynamics PhosphorImager using IMAGEQUANT software.

Oligonucleotide Synthesis. Phosphorothioate oligoribonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer by using phosphoramidite chemistry. Two 24-nt sequences were used: 5'-AGAUCCUUAGUUCGCAGCAUUGUA-3', which was complementary to positions −4 to +20 of rat N-CAM in which the ATG initiation codon is designated as positions 1–3 (28) ("N-CAM antisense"), and 5'-GUCCAUGUACAUCUAGUCAGUUGA-3', which consisted of the same nucleotides arranged in random order ("random antisense"). After deprotection, the oligonucleotides were purified by HPLC, ethanol-precipitated, solubilized in distilled water, and added to the culture medium at the indicated concentrations.

RESULTS

Purified N-CAM and Anti-N-CAM Inhibit Astrocyte Proliferation. Addition of N-CAM purified from rat forebrain to primary cultures of astrocytes (at a density of 7×10^4 cells per cm^2) in chemically defined medium (SFM) inhibited their proliferation in a concentration-dependent manner (Fig. 1*A*). SDS/gel electrophoresis revealed that the purified N-CAM consisted mainly of the polysialylated embryonic form of N-CAM (200–250 kDa) with other molecular forms including a ≈65-kDa hydrolyzed N-CAM fragment present in lower amounts. As shown by testing three preparations of rat N-CAM, maximal (>90%) suppression of proliferation could be effected at concentrations of 10 μg/ml. N-CAM between 2 and 0.2 μg/ml was sufficient for half-maximal (50%) suppression.

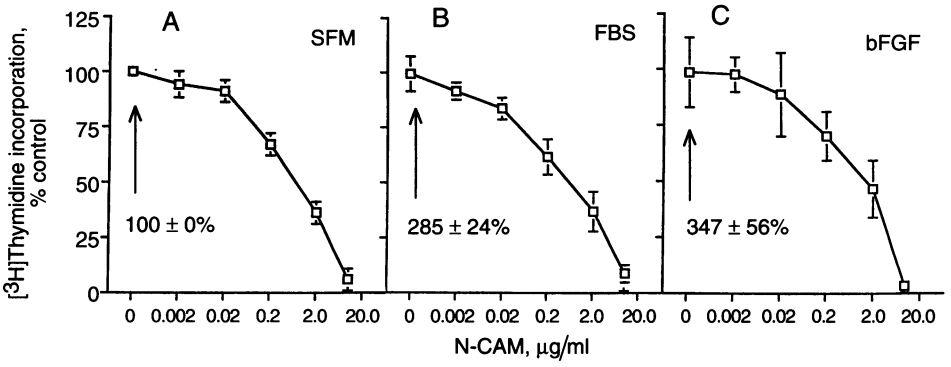

FIG. 1. Inhibition of proliferation as a function of the amount of N-CAM present in the following media: (*A*) SFM ($n = 10$). (*B*) SFM/1% FBS ($n = 5$). (*C*) SFM/basic fibroblast growth factor (bFGF; 20 ng/ml) ($n = 5$). [^{3}H]Thymidine incorporation as a percent of the SFM control with no N-CAM added is shown in each panel. Throughout the paper, [^{3}H]thymidine incorporation is normalized relative to [^{35}S]methionine cell labeling, calculated as the ratio ^{3}H dpm/^{35}S dpm, and expressed as a percentage (mean ± SEM) of the respective control (no N-CAM added) for each of the three conditions. Cell density is 7×10^4 cells per cm^2 throughout.

Addition of N-CAM purified from embryonic chicken brain, which has been shown to bind to rodent N-CAM (26), also inhibited proliferation (data not shown). At an N-CAM concentration of 10 μg/ml, the cells tended to assume a somewhat rounded appearance; at concentrations ≤2 μg/ml (a range that still exhibited substantial inhibitory activity), no gross change in cell morphology was observed.

The addition of 1% FBS (containing various growth factors) and basic fibroblast growth factor (bFGF; Upstate Biotechnology, Lake Placid, NY; 20 ng/ml) to astrocyte cultures led to a marked increase in proliferation compared to cultures in SFM [285 ± 24% for FBS (n = 14) and 347 ± 56% for bFGF (n = 12)]. Addition of N-CAM and either 1% FBS or bFGF (20 ng/ml) also elicited a suppressive effect similar in magnitude to that observed in SFM (Fig. 1 *B* and *C*). Addition of N-CAM (2 μg/ml) treated with neuraminidase to remove sialic acid produced an inhibitory effect comparable to untreated N-CAM {[24 ± 4% [^{3}H]thymidine incorporation vs. SFM control (n = 2)]}. Like purified N-CAM, anti-mouse N-CAM polyclonal antibody (0.1 mg/ml) and Fab′ fragments (0.03 mg/ml) prepared from this antibody exerted an inhibitory effect on astrocyte proliferation [53 ± 15% (n = 2) and 56 ± 13% (n = 4), respectively, vs. SFM].

Contact Inhibition and Dependence of N-CAM Effect on Cell Density. Astrocytes growing in SFM showed different levels of proliferative activity depending on their cell density. Levels of [^{3}H]thymidine incorporation per cell were high at low cell densities, whereas confluent or nearly confluent cultures proliferated more slowly (presumably due to the phenomenon of "contact inhibition," Fig. 2*A*). When N-CAM was added to these cultures, the inhibitory effect of N-CAM on proliferation was critically dependent on cell density and thus on baseline proliferative activity. Inhibition was maximal at lower cell density (1.8 × 10^4 cells per cm^2) when cells are actively proliferating, whereas N-CAM was virtually ineffective at higher cell densities (28 × 10^4 cells per cm^2) when cultures are confluent.

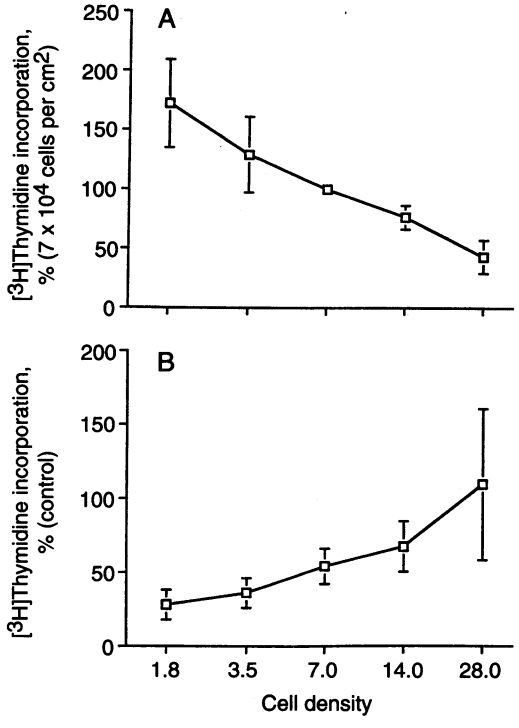

FIG. 2. Dependence of baseline proliferation levels (*A*) and inhibition of proliferation by N-CAM (2 μg/ml) (*B*) on cell density [expressed as number of cells (×10^{-4}) per cm^2]. (*A*) Results are expressed as percent [^{3}H]thymidine incorporation relative to a density of 7 × 10^4 cells per cm^2 (the density used in all other experiments). (*B*) [^{3}H]Thymidine incorporation expressed as percent of the baseline (control) proliferation level (see *A*) for the corresponding cell density (n = 5 throughout).

Antiproliferative Activity of Peptides. Several synthetic peptides, some known to inhibit N-CAM homophilic binding (29), were added to astrocyte cultures. At a concentration of 0.1 mg/ml, the peptide KYSFNYDGSE, identical to an N-CAM homophilic binding region located in the third immunoglobulin-like domain of chicken N-CAM, inhibited proliferation, while a peptide of the same length and amino acid composition but with randomized sequence (GFNSSYEDYK) had no effect (Fig. 3). The direct homologue to the chicken peptide from rat N-CAM KHIFSDDSSE had a similar inhibitory effect, while the corresponding random peptide SFSISDEDHK did not affect proliferation.

Partial Reversal of the Inhibition of Proliferation After Adding Antisense Oligonucleotides. The addition of N-CAM antisense oligonucleotides for 48 h resulted in significantly reduced N-CAM expression (P < 0.02, Table 1). A random antisense oligonucleotide had, on average, no effect on N-CAM expression. Neither oligonucleotide led to changes in the gross morphology of astrocytes or had an influence on the baseline level of proliferation (Fig. 4). Addition of N-CAM (2 μg/ml) to astrocyte cultures that had been preincubated with 200 nM N-CAM antisense oligonucleotides for 48 h revealed a significantly (P < 0.02) weaker inhibitory effect of N-CAM on proliferation. Addition of N-CAM to astrocytes preincubated with the random antisense oligonucleotide revealed no such effect.

DISCUSSION

Control of glial cell proliferation is an important factor in neural development, regeneration, and tumorigenesis. The experiments described in this paper provide evidence for a functional role of N-CAM in the control of glial proliferation. While it is currently unknown whether N-CAM influences glial cell proliferation during development *in vivo*, the effect of N-CAM on astrocyte proliferation *in vitro* opens potential avenues for controlling the formation of glial scars after CNS injury, which may in turn aid neuronal regeneration.

The experimental results indicate that the proliferation of rat forebrain astrocytes in primary culture can be strongly inhibited by addition of purified N-CAM to the culture medium. Whether other cell types or cell lines expressing

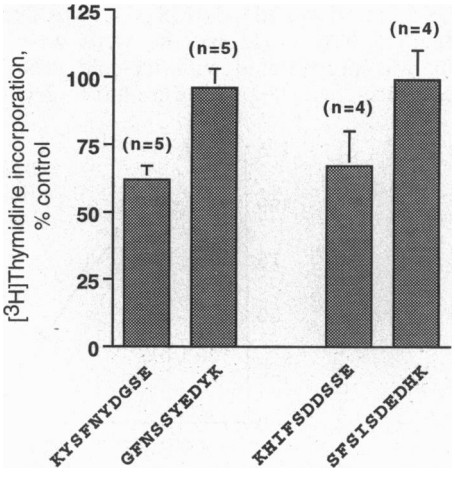

FIG. 3. Inhibitory effect of synthetic peptides with sequences containing a putative N-CAM homophilic binding site. Peptides are added at 0.1 mg/ml to SFM, with astrocytes at a density of 7 × 10^4 cells per cm^2. Bars (from left to right): chicken N-CAM peptide, the corresponding random peptide, rat N-CAM peptide, and the corresponding random peptide.

Table 1. N-CAM expression in antisense experiments

Condition	N-CAM level, % control
Control	100 ± 0 (6)
+ N-CAM antisense	65* ± 9 (5)
+ random antisense	87 ± 18 (6)

Levels of N-CAM expression (sum of all molecular forms, mostly 140-kDa and 120-kDa forms) in antisense oligonucleotide experiments as determined by Western blot analysis. Data are expressed as mean ± SEM (n), where n is the number of experiments. *, $P < 0.02$ vs. control (paired sample Student's t test).

N-CAM are susceptible to N-CAM deserves investigation. Preliminary experiments (unpublished results) indicate that C6 glioma cells, which express N-CAM on their cell surface, also decrease their rate of proliferation in response to externally added N-CAM when grown in SFM at low cell densities. The effect on astrocytes is mimicked by polyclonal N-CAM antibodies and N-CAM Fab' fragments. This suggests that the phenomenon does not result from crosslinking of N-CAM at the cell surface but probably is the result of a signal mediated by homophilic binding to N-CAM at the glial surface. This conclusion is also supported by the finding that N-CAM peptides can have the same effect. The results of the antisense experiments suggest that a threshold amount of N-CAM at the glial surface is required.

It is unlikely that N-CAM added in solution acts by disrupting cell–cell adhesion itself given the observation that the effect is maximal at low cell density. The finding that N-CAM becomes progressively less effective in inhibiting proliferation as cell density increases may be because baseline proliferation levels of cells in confluent culture are already low and, therefore, cannot be suppressed further. The quiescence of confluent cultures could also be due in part to an autocrine effect via secretion of an inhibitory substance into the culture medium (e.g., ref. 30). Alternatively, the effect of N-CAM may be related to changes in N-CAM expression as cells approach confluency (31). There is additional evidence that N-CAM-mediated cell–cell interactions provide a signal to slow proliferation. For example, N-CAM has been implicated in density-dependent inhibition of growth ("contact inhibition;" ref. 32) in a mouse fibroblast cell line (33). In these experiments, addition of an anti-N-CAM monoclonal antibody immobilized on beads to actively proliferating fibroblasts significantly inhibited their [^{3}H]thymidine incorporation. It is thus possible that contact inhibition and the effect of N-CAM on proliferation are mediated by similar cellular mechanisms.

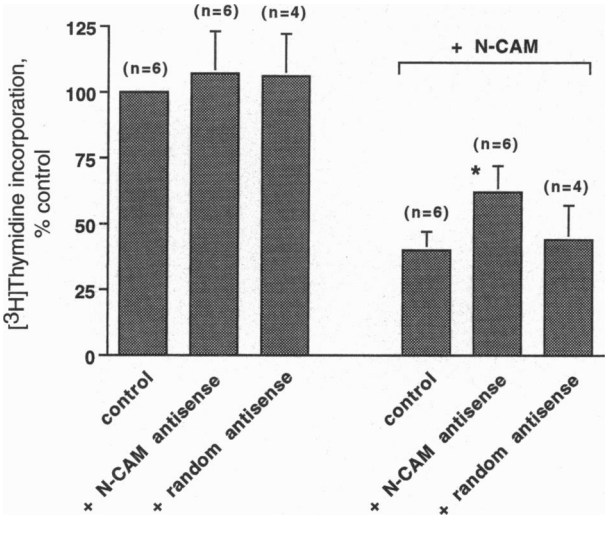

FIG. 4. Effect of N-CAM and random antisense oligonucleotides on the inhibition of glial proliferation by N-CAM. Left side (no N-CAM added): addition of 200 nM N-CAM antisense or random antisense oligonucleotides alone has no effect on proliferation. Right side (N-CAM added): SFM (control) containing N-CAM (2 μg/ml) produces inhibition, which is partially reversed by addition (48 h prior to N-CAM) of 200 nM N-CAM antisense but not by addition of 200 nM random antisense oligonucleotides. *, $P < 0.02$ vs. control + N-CAM (paired sample Student's t test).

The inhibitory effect on proliferation of short synthetic peptides with sequences located in the third immunoglobulin-like domain of N-CAM lends additional support to the hypothesis that N-CAM homophilic binding can lead to decreased proliferation. This result suggests that binding to this region of N-CAM is sufficient to elicit a signal leading to an intracellular response. It has been shown that the decapeptide sequence KYSFNYDGSE is directly involved in chicken N-CAM homophilic binding (29). In solution, this peptide disrupted N-CAM homophilic binding and inhibited N-CAM-dependent neurite outgrowth from retinal cells (34). In our experiments, the peptide mimics the effect of whole purified N-CAM, suggesting that binding of this peptide to N-CAM is sufficient to elicit a signaling event across the cell membrane. Despite some sequence dissimilarity, both chicken and rat N-CAM peptides inhibited proliferation, consistent with highly similar structural properties. It is pertinent that high cross-species binding of chicken and rodent N-CAM has been observed (26). Our experiments do not preclude the possibility that other regions of N-CAM are also involved in N-CAM homophilic binding and may elicit the inhibition of glial proliferation.

When the level of N-CAM expression by astrocytes is diminished by adding specific antisense oligonucleotides, the effect on proliferation elicited by N-CAM added to the medium is reduced. Because of the specificity of antisense probes, this finding suggests that expression of N-CAM on the glial cell surface is required for the inhibitory effect of soluble N-CAM and supports the notion that homophilic N-CAM binding mediates the decrease of astrocyte proliferation. The antisense oligonucleotides were added to the culture medium at relatively low concentrations to reduce possible nonspecific effects. For example, we found that oligonucleotide concentrations in the micromolar range reduced baseline proliferation levels irrespective of their sequence. Previous studies using antisense probes against basic fibroblast growth factor in astrocyte cultures (35) have shown that phosphorothioate oligonucleotides can be effective within a few hours. In the present experiments, however, long incubation periods with N-CAM antisense oligonucleotides were required before significant reductions in N-CAM levels could be observed (see ref. 36); this may be due to slow N-CAM turnover rates (37). Even after 48 h, only a gradual reduction in N-CAM levels was achieved. This finding would account for the significant but only partial reversal of the N-CAM effect on proliferation after N-CAM antisense oligonucleotide treatment.

Renewed glial proliferation and gliosis are important in the response of neural tissue to injury. A reduction in the amount of gliosis after CNS injury may be a significant factor in permitting axonal regeneration across a lesion site. Neuronal regeneration in the adult CNS can occur if regenerating axons are presented with an appropriate environment (38). Injury to the CNS of neonatal rats results in less extensive gliosis compared to the adult (4), possibly facilitating neuronal regeneration. Moreover, gliosis in the adult can be reduced by transplantation of immature astrocytes into the lesion site (39). Our experiments raise the possibility that this might be due to the high levels of N-CAM expressed by immature astrocytes. Recent observations (L. Krushel, O.S., K.L.C., and G.M.E., unpublished) on rat brain lesions indicate that the introduction of N-CAM or N-CAM peptides at the lesion site leads to a reduction in gliosis in the local area. Further elucidation of these responses and definition of the signaling pathways that mediate the inhibition of glial prolifera-

tion should contribute to the design of rational therapies to enhance regeneration by CNS neurons.

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