CNTF Protection of Oligodendrocytes Against Natural and Tumor Necrosis Factor–Induced Death

Jean-Claude Louis,* Ella Magal, Seiji Takayama, Silvio Varon

A proportion of developing oligodendrocytes undergo natural cell death by apoptosis, and mature oligodendrocytes die, either by apoptosis or necrosis, in response to injurious signals such as cytotoxic cytokines and complement. Ciliary neurotrophic factor (CNTF), a trophic factor found in astrocytes in the central nervous system (CNS), promoted the survival and maturation of cultured oligodendrocytes. This trophic factor also protected oligodendrocytes from death induced by tumor necrosis factors (apoptosis) but not against complement (necrosis). These results suggest that CNTF functions in the survival of oligodendrocytes during development and may lead to therapeutic approaches for degenerative diseases of the CNS that involve oligodendrocyte destruction.

Natural cell death is important in the cell development and cell turnover of higher vertebrates (*1*). Natural cell death occurs through an active process that requires RNA and protein synthesis (*2*). Cells that undergo natural death experience morphological alterations characteristic of apoptosis—cytoplasmic and nuclear condensation, cytoplasmic vacuolation, membrane blebbing, and DNA fragmentation (*2*)—that are fundamentally different from those observed during degenerative cell death produced by injury—cell swelling and lysis. Cells undergo apoptosis in response to a death-inducing stimulus or to the loss of a stimulus whose function is to suppress the execution of the death program. In the CNS, natural death caused by trophic factor deprivation seems to be the major developmental strategy for controlling the number of neurons that are innervating a specific target (*3*). During postnatal development of the rat optic nerve, newly formed oligodendrocytes also undergo a stringent positive selection that involves successful competition for trophic factors (*4*). Mature oligodendrocytes undergo spontaneous death in vitro unless sufficient amounts of serum or trophic factors are provided to them (*4–6*); trophic support of oligodendrocytes can also be provided by astroglial cells (*5*). Insulin-like growth factors support the survival of both newly formed and mature oligodendrocytes (*4*) and promote their maturation (*7*). Platelet-derived growth factor (PDGF) prevents the death of newly formed but not mature oligodendrocytes (*4*). Oligodendrocytes undergo apoptosis in response to cytotoxic cytokines such as tumor necrosis factors α and β (TNFα and TNFβ) (*8, 9*), which are believed to participate in the immunopathogenesis of multiple sclerosis (*9, 10*). Necrotic death (lysis) of rat oligodendrocytes occurs after exposure to complement (*11*), even in the absence of added cell-specific antibody, because complement reacts directly with myelin to form pores through which calcium ions enter the cell (*12*).

Ciliary neurotrophic factor (CNTF), a trophic factor first isolated from chick eye (*13*), acts on select populations of neurons from the peripheral nervous system and the CNS as a survival-promoting and phenotype-modulating factor (*14*). It also induces the transient expression of glial fibrillary acidic protein in O-2A progenitors from the optic nerve (*15*). We report that CNTF is effective in preventing the natural death of oligodendrocytes in vitro and TNF-induced cell death but does not prevent complement-induced death of oligodendrocytes.

Pure cultures of oligodendrocytes were generated from the CG4 cell line of perinatal O-2A cells (*6*). These cells can be maintained as self-renewing O-2A progenitors in serum-free medium by concurrent treatment with PDGF and basic fibroblast growth factor (bFGF) or by use of medium conditioned by B104 neuroblastoma cells (*16*). Upon interruption of either treatment, CG4 cells differentiate constitutively into oligodendrocytes; addition of 2 to 5% fetal calf serum (FCS) 24 hours after mitogen withdrawal promotes their survival and maturation (*6*). After 3 days in 2% FCS (day zero), oligodendrocyte cultures were washed and transferred to serum-free medium containing the N1 supplement in the absence or presence of human recombinant CNTF (20 ng/ml) (*17*). Cell survival was determined at daily intervals with the MTT assay (*18*), which assesses mitochondrial function in living cells. In the absence of trophic factor, the number of MTT-positive oligodendrocytes rapidly declined; half of the cells died within 36 hours, and few cells survived more than 4 days (Fig. 1A). Addition of CNTF greatly enhanced oligodendrocyte survival; after 6 days 80% of the day-zero oligodendrocytes survived (Fig. 2), and after 12 days 63% were still present. Survival in the presence of CNTF was equal to that in medium containing 2% FCS. Removal of CNTF after 2 days resulted in the death of oligodendrocytes at the same rate as after FCS withdrawal (Fig. 1A). The greater number of cells in CNTF-treated cultures was not due to increased proliferation; fewer than 1% of the cells incorporated bromodeoxyuridine (*6*) during a 24-hour pulse period. High concentrations of insulin can promote the survival (*4*) and development (*7*) of oligodendrocytes. Indeed, exposure of our oligodendrocyte cultures to medium lacking insulin resulted in their enhanced degeneration [16 ± 4% of surviving oligodendrocytes after 2 days in the absence of insulin versus 42 ± 3% in the presence of insulin (5 μg/ml)]. However, CNTF was equally potent in high-insulin (5 μg/ml) or in insulin-free medium (97 ± 3% and 94 ± 4%, respectively, after 2 days).

We next assessed the effect of CNTF on the survival of oligodendrocytes generated from rat cerebral cortex cultures. Primary oligodendrocytes died within 6 days in serum-free medium, and CNTF prevented their spontaneous death (Fig. 1B). Thus, oligodendrocytes generated from primary O-2A progenitor cells and from secondary CG4 progenitor cells respond identically to CNTF. Oligodendrocyte survival was dependent on the concentration of CNTF (Fig. 1C) with maximal effect at 10 ng/ml and ED_{50} (median effective dose) at 1 ng/ml (similar to the doses required for the survival of chick ciliary ganglion neurons) (*18*). In addition to promoting the survival of oligodendrocytes, CNTF also enhanced their maturation, as assessed by myelin basic protein (MBP) expression (*19*). Although CNTF did not significantly increase the number of MBP-positive cells (85 to 90% MBP-positive cells in either CNTF or FCS-containing medium from day 2), it increased MBP amounts by 70% per MBP-positive cell after 10 days in culture, compared to only 30% in cultures maintained in 2% FCS (Fig. 1D).

We next investigated the ability of CNTF to prevent toxic death of mature oligodendrocytes. After being cultured for 6 hours with either TNFα or TNFβ, mature oligodendrocytes displayed morphological changes including intense cytoplasmic vacuolation and disruption of the processes, consistent with the occurrence of apoptotic death (*9*). Toxicity due to TNF was progressive, as assessed by the MTT assay, resulting in 60% oligodendrocyte death after 72 hours (Fig. 3A). This toxicity was dose-dependent (maximal at 100 ng/ml; ED_{50} at 25 ng/ml). In contrast, exposure to rat complement (maximal effect at 1:8 dilution of Sigma rat complement; ED_{50} at 1:20 dilution) in the absence of oligoden-

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drocyte-specific antibodies caused an acute swelling of oligodendrocytes, followed by cell lysis; after 24 hours nearly all the somata and membrane processes had disintegrated (Fig. 3A). Treatment of oligodendrocytes with CNTF (20 ng/ml) 24 hours before the start of the toxic treatments fully prevented the morphological alterations caused by exposure to TNFα or TNFβ over a 72-hour period (Fig. 3B). Maximal protection against TNF was achieved at a CNTF concentration of 10 ng/ml, with an ED_{50} at 1 ng/ml (Fig. 3C), the same concentrations as those required for protection of oligodendrocytes against natural death (Fig. 1C). In contrast, CNTF had no effect on the toxicity caused by complement (Fig. 3, B and C). The period beyond which CNTF presentation could no longer protect oligodendrocytes from TNF toxicity was short. The effect of TNF toxicity (tested at 48 hours) was fully abolished when CNTF was added at the time of the onset of TNF treatment or up to 8 hours after it, but not when supplemented after 16 hours or later (Fig. 3D).

These results demonstrate that CNTF can act as a survival factor for oligodendrocytes. The survival effect of CNTF likely is restricted to the apoptotic mode of oligodendrocyte death, either during development or after exposure to environmental signals such as cytotoxic cytokines; CNTF protein and mRNA are found throughout the brain, mostly associated with astrocytes (*20*). The appearance of CNTF during the early postnatal period in the rat brain (*20*) coincides with the time at which oligodendrocytes are generated from O-2A progenitors (*21*), consistent with a role for CNTF as a trophic factor for maturing oligodendrocytes. The inhibition of TNF toxicity by CNTF is consistent with a role for CNTF as a protective factor for oligodendrocytes in inflammatory demyelinating diseases, such as multiple sclerosis in humans (*22*) or experimental allergic encephalitis in rodents, in which oligodendrocytes and the myelin sheaths they produce are damaged (*23*). Indeed, TNFs are associated with active multiple sclerosis lesions (*10*) and abnormally high amounts of TNF and other oligodendrocyte-damaging cytokines, such as interleukin-1 or interleukin-2, are present in the serum and cerebrospinal fluid in

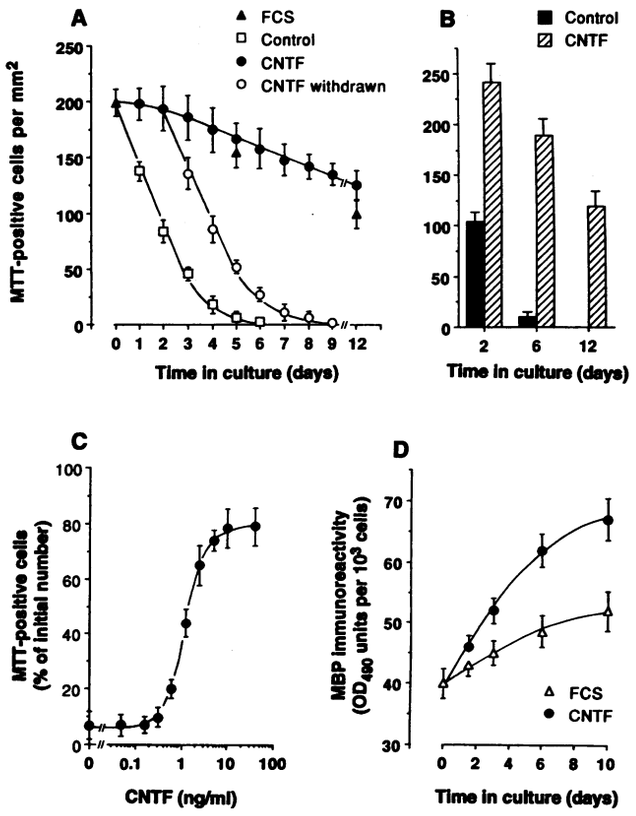

Fig. 1. CNTF promotion of oligodendrocyte survival and maturation. (**A**) Time course with oligodendrocytes generated from CG4 progenitor cells. At day zero, the serum-containing medium was replaced with DNB (open squares) or DNB containing human recombinant CNTF (20 ng/ml) (closed circles). In one set of cultures, the CNTF treatment was stopped after 2 days, and the cells were incubated further in DNB (open circles). In another group of cultures, oligodendrocytes were maintained in DNB plus 2% FCS (closed triangles). Each point is the mean ± SD of 15 to 24 determinations from three separate experiments. (**B**) Time course with primary cultures of oligodendrocytes: DNB (filled columns), CNTF-treated cultures (hatched columns). Each point is the mean ± SD of six to eight determinations. (**C**) CNTF dose-response curve. Oligodendrocytes generated from CG4 cells treated on day zero with different concentrations of CNTF were assessed 6 days later. Each point is the mean ± SD of four to six determinations. (**D**) CNTF-induced increase in MBP. The MBP immunoreactivity was determined in the absence (open triangles) or in the presence (filled circles) of CNTF; OD, optical density. Each point is the mean ± SD of four to six determinations.

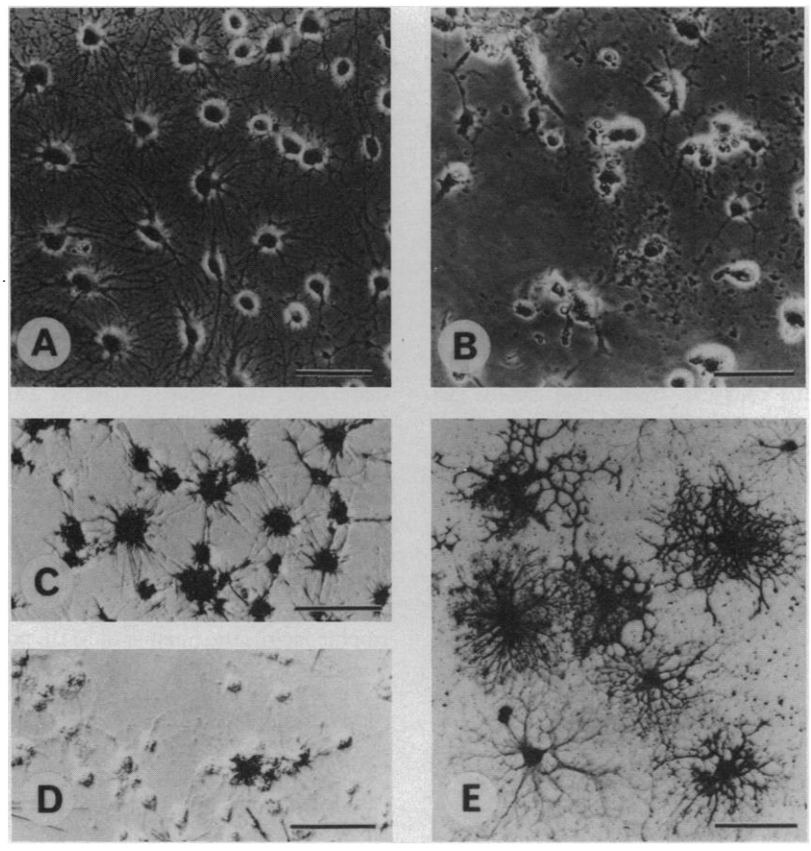

Fig. 2. Morphology of oligodendrocytes generated from CG4 cells and cultured for 6 days in DNB with (**A** and **C**) or without (**B** and **D**) human recombinant CNTF (20 ng/ml). (A and B) Phase-contrast microscopy; (C and D) MTT assay; viable oligodendrocytes transform the MTT into dark blue insoluble crystals. (**E**) Myelin basic protein immunostaining of oligodendrocytes after 6 days in culture with CNTF. Bars, 50 μm.

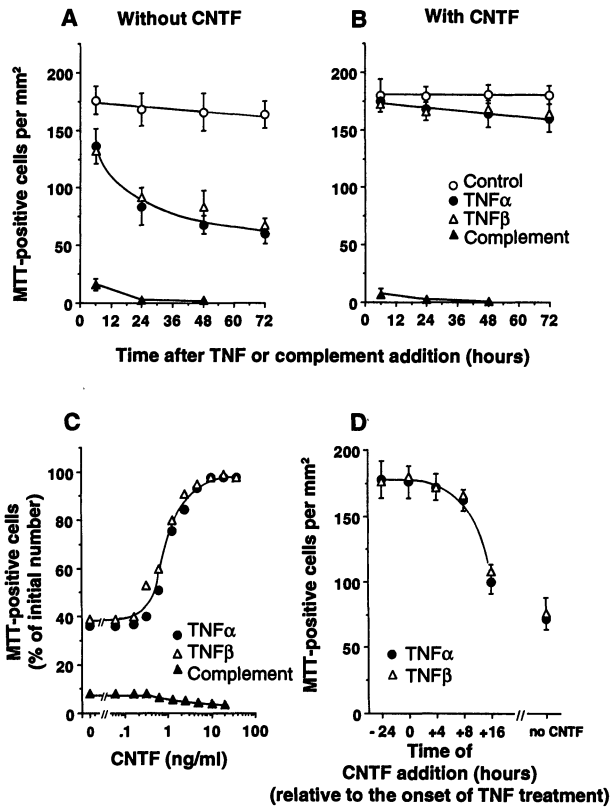

Fig. 3. Effect of CNTF on TNF- and complement-mediated oligodendrocyte toxicity. (**A** and **B**) Time course. Control oligodendrocytes generated from CG4 cells (A) or oligodendrocytes treated with CNTF 24 hours before toxin addition (B) were exposed to TNFα (100 ng/ml) (filled circles), TNFβ (open triangles), or rat complement (1:8 dilution; filled triangles). Cultures that did not receive toxic treatments were used as control (open circles). All treatments were performed in DNB with 2% FCS. Each point is the mean ± SD of 12 to 15 determinations from three separate experiments. In cultures lacking CNTF, the values for cultures treated with TNFs and complement are significantly lower than the values for controls at corresponding times (*t* test; $P < 0.001$). In cultures treated with CNTF, the values for TNFs are not different from control values, whereas the values for complement are significantly lower ($P < 0.001$). (**C**) Dose response. Oligodendrocytes were presented with increasing concentrations of CNTF 24 hours before exposure to TNFα, TNFβ, or complement. Survival was assessed after 48 hours of toxic treatment. Each point is the mean of five to six determinations with SD < 9%. (**D**) Effect of time of CNTF addition. Oligodendrocytes were exposed to TNFα or TNFβ for 48 hours. CNTF was added 24 hours before TNFs, at the time of the onset of TNF treatment, or 4, 8, or 16 hours after TNFs. We assessed survival after 48 hours by counting MTT-positive cells. Toxicity induced by the 48-hour exposure to TNFs (no CNTF) was measured. Each point represents the mean ± SD of six to eight determinations from two separate experiments. The values for CNTF-treated cultures are significantly greater than control values when CNTF was added up to 8 hours after TNF ($P < 0.001$), but not when added 16 hours after the onset of TNF treatments.

human CNS pathological conditions, including multiple sclerosis (*24*), AIDS (acquired immunodeficiency syndrome)-associated neurological diseases (*25*), and head trauma (*26*).

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16. CG4 cells were propagated in Dulbecco's modified Eagle's medium (DME) containing N1 supplement, biotin, and 30% (v/v) conditioned medium from B104 neuroblastoma cells (*6*). Before experiments, CG4 cells were switched to a defined medium consisting of DME-N1-biotin (DNB) plus human recombinant PDGF-BB (2.5 ng/ml) (Intergen) and human recombinant bFGF (10 ng/ml) (gift of Scios, Inc.). One week later, cells were harvested with 0.1% trypsin and 0.2% EDTA, washed twice in DME and seeded in DNB plus PDGF and bFGF at a density of 5000 cells per 6-mm well in 96-well microplates precoated with polyornithine (*6*). The cells were induced to differentiate to oligodendrocytes by removal of the growth factors for 24 hours. The medium was then replaced with fresh DNB containing 2% heat-inactivated FCS; addition of FCS enhances the survival of oligodendrocytes without interfering with differentiation (*6*). The oligodendrocytes generated from CG4 cells were used for experiments 3 days after the addition of FCS. At this time (day zero), the cultures consisted of in excess of 95% galactocerebroside-positive oligodendrocytes, 75% of which expressed myelin basic protein. We generated primary oligodendrocytes cultures from O-2A cultures using the same methods as for CG4 cells (*6*). The effect of CNTF on natural oligodendrocyte death was studied in DNB without FCS; before the addition of CNTF, the day-zero cultures were washed three times with 200 μl of DME to remove residual serum. Studies of TNF and complement toxicity were performed in the presence of 2% FCS to ensure proper survival of control cultures over the 3-day experiments. Media and treatments were replaced after 2 days and every other day thereafter.
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19. We determined MBP immunoreactivity using a direct microculture enzyme-linked immunosorbent assay (ELISA). Cultures were fixed with 4% paraformaldehyde; MBP immunostaining was performed as described (*6*), with rabbit anti-MBP (Chemicon, Temecula, CA) and biotinylated goat anti-rabbit immunoglobulin G, followed by avidin-biotin-peroxidase complex (ABC kit; Vector, Burlingame, CA). We determined peroxidase activity in the incubation medium by using the soluble

chromogen O-phenylenediamine (0.05%) and 0.02% hydrogen peroxide. The absorbance of the reaction product was measured at 490 nm. The wells were then washed extensively with PBS, and MBP-positive cells were visualized by addition of the insoluble chromogen diaminobenzidine (0.05%) and 0.02% hydrogen peroxide. The number of MBP-positive cells per well was counted; the results are expressed as the amount of MBP immunoreactivity (optical density at 490 nm) developed by 1000 MBP-positive cells.
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Inhibition of Neural Crest Cell Attachment by Integrin Antisense Oligonucleotides

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Neural crest cell interactions with extracellular matrix molecules were analyzed with the use of antisense oligonucleotides to block synthesis of integrin subunits. When added to the culture medium of quail neural crest cells, selected antisense phosphorothiol oligonucleotides reduced the amounts of cell surface α_1 or β_1 integrin subunits by up to 95 percent and inhibited neural crest cell attachment to laminin or fibronectin substrata. Differential effects on specific α integrins were noted after treatment with α-specific oligonucleotides. Cells recovered the ability to bind to substrata 8 to 16 hours after treatment with inhibitory oligonucleotides. The operation of at least three distinct α integrin subunits is indicated by substratum-selective inhibition of cell attachment.

The neural crest is a migratory cell population that arises in the dorsal neural tube shortly after tube closure. These cells migrate throughout the embryo along well-defined pathways, then differentiate into a variety of cell types including neurons and glia of the peripheral nervous system, pigment cells, and connective tissue of the face. During migration, neural crest cells contact extracellular matrices containing fibronectin, laminin, collagens, proteoglycans, and tenascin (*1*). Interactions with these molecules are primarily by means of integrins in the β_1 family (*1–3*). Antibodies to the β_1 subunit of chick integrin (*2*, *3*) and several of its ligands in the extracellular matrix (ECM) (*4*, *5*) perturb avian cranial neural crest cell migration both in situ and in vitro. However, relatively little is known about the nature of the integrins on these avian cells.

Integrins are heterodimeric transmembrane glycoproteins composed of noncovalently linked α and β subunits, which mediate interactions between embryonic cells and their surrounding extracellular matrices (*6*). The function of integrins has been studied by antibody inhibition of binding or by transfection of antisense cDNA to block synthesis (*2*, *3*, *7*, *8*). The former method is limited by the paucity of specific blocking antibodies for individual integrin subunits and by possible nonspecific effects. Although transfection with large antisense probes overcomes some of these problems, cDNAs are typically incorporated into only a small percentage of the cells, making the second approach useful for cell lines but not embryonic tissue. An alternative approach is to utilize short antisense oligonucleotides; these are taken up actively by large percentages of cells and decrease the amount of the targeted mRNA or protein (*9*). Phosphorothioate-modified oligonucleotides are particularly useful due to their increased stability: these oligonucleotides have a half-life of greater than 1 hour; the half-life of unmodified oligonucleotides is less than 5 min (*10*).

Here, we have combined molecular, cellular, and biochemical techniques to examine the nature of the integrins involved in quail neural crest cell interactions with ECM molecules. First, we have used antisense phosphorothiol oligonucleotides to perturb integrin synthesis and hence integrin-mediated interactions between quail neural crest cells and the ECM. Second, we have used a functional assay for neural crest cell attachment to specific ECM molecules to test which of several candidate oligonucleotides against integrin α and β subunits inhibit cell attachment in a substratum-specific manner. Finally, we used biochemical analysis to demonstrate decreased amounts of cell surface α and β integrin subunits on neural crest cells after treatment with particular antisense oligonucleotides.

Antisense oligonucleotides (Table 1) were tested for their ability to inhibit the production of the β_1 and various α subunits. The antisense oligonucleotide directed to the β_1 integrin subunit (1B1) encompasses a region (*11*) of high similarity to β_1 subunits from other species but low homology to other β subunits. Because little sequence information is available for avian α subunits, antisense oligonucleotide "guess-mer" sequences were selected from known sequences from mammalian integrins. Oligonucleotides 2aG through 5aG were selected from regions of highest nucleotide sequence identity among integrin α subunits (*12*); for example, 2aG was from the conserved COOH-terminus, a region of sequence identity in mammalian α subunits. Other antisense oligonucleotides (10a1 through 18a5) were selected according to sequence data available for individual mammalian α subunits (α_1, α_4, and α_5). Because these oligonucleotides were determined by sequence information from other species, they do not necessarily correspond to the same α subunits in avian integrins, for which sequence information is currently unavailable. Consequently, we tested the specificity of these antisense oligonucleotides in avian cells with functional and biochemical assays described below.

The ability of isolated neural crest cells to attach to individual ECM components was quantitated by the use of a centrifugal attachment assay (*3*, *13*), which provides reproducible results for small numbers (on the order of hundreds) of embryonic cells. Quail neural crest cells were derived from neural tubes removed from the trunk region

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