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Effect of In Ovo Retinoic Acid Exposure on Forebrain Neural Crest: In Vitro Analysis Reveals Up-Regulation of N-CAM and Loss of Mesenchymal Phenotype

KAVITA R. SHANKAR, CHENG-MING CHUONG, TINA JASKOLL, AND MICHAEL MELNICK
Laboratory for Developmental Genetics (K.R.S., T.J., M.M.) and Department of Pathology (C.-M.C.), University of Southern California, Los Angeles, California 90089-0641

ABSTRACT **In a prior study of in ovo exogenous retinoic acid (RA) exposure, we observed a prolonged expression of cell surface N-CAM in cranial neural crest (NC) cells exhibiting migratory failure. In the present studies, we employed an experimental strategy in which embryos were first exposed to exogenous RA in ovo and incubated for 45–60 hr; this was followed by extirpation and in vitro culturing of these same RA-exposed cranial neural tubes. NC cell outgrowth from the explant was assayed, as was the immunohistochemical localization of HNK-1 and N-CAM antigens. In RA-exposed explants, the size of the NC cell outgrowths were comparable to controls. However, almost all NC cells lost their mesenchymal phenotype and were arranged in an "epithelioid" pattern of tightly packed polygonal cells that expressed N-CAM at adjacent cell boundaries. By contrast, control NC cells were flattened and multipolar in shape and expressed HNK-1, rarely co-expressing N-CAM. These observations indicate that RA modulates NC cell N-CAM expression and microanatomical phenotype, a finding consistent with prior in ovo studies of RA-exposure. Several possible explanations are considered.** © 1994 Wiley-Liss, Inc.

Key words: Avian embryo, HNK-1, N-CAM, Neural crest, Retinoic acid

INTRODUCTION

Cephalic neural crest (NC) contributes extensively to the mesenchyme from which most of the craniofacial structures develop (Noden, 1978, 1986; Le Douarin, 1982). Direct observation of progressive neural tube/NC differentiation in live avian embryos using high definition, time-lapse microcinematography has demonstrated that NC cells in the forebrain and anterior midbrain, unlike trunk NC, appear not to migrate in large numbers or as a sheet, but as smaller numbers of individual cells (Jaskoll et al., 1991). This was consistent with the original in situ studies by Johnston (1966). Regardless of numbers, the migratory ability of NC cells depends on down-regulation of N-CAM (Thiery et al., 1982; Bronner-Fraser et al., 1992) and adhesive interactions between themselves and with the extracellular matrix (Boucaut et al., 1984; Thiery et al., 1986; Noden, 1988). Craniofacial abnormalities observed with or without an associated neural tube defect may thus be related to altered NC migration into the presumptive facial region during early embryogenesis (Johnston, 1975; Johnston and Bronsky, 1991; Melnick and Myrianthopoulos, 1987). For example, defective NC cell migration from the forebrain neural folds results in frontonasal mesenchyme deficiencies and precludes normal primary palate formation (Johnston, 1975).

Retinoic acid (RA), a candidate developmental morphogen (Thaller and Eichele, 1987) may, in excess, induce neural tube defects and/or abnormalities of a variety of facial structures, including facial clefting and otomandibular dysplasias (Kochhar, 1961; Morriss and Thorogood, 1978; Wiley et al., 1983; Lammer et al., 1985; Webster et al., 1986). Immunocytochemistry and three-dimensional reconstruction of developing chick embryo neural tubes exposed to exogenous RA in ovo reveal an abnormally prolonged expression of cell surface N-CAM and an apparent inhibition of NC migration (Shankar et al., 1992; Fig. 1). This may be central to understanding the pathogenesis of RA-induced craniofacial anomalies, with or without cranial neural tube defects.

The first objective of the present study was to coculture normal chick embryo cranial and trunk neural tube explants and examine the migratory characteristics of cranial and trunk NC cells. Second, to gain insight into possible mechanisms underlying RA-induced craniofacial malformations, we employed an experimental strategy in which developing embryonic neural tubes, first exposed to exogenous RA in ovo for 45–60 hr, were then explanted, cultured, and analyzed in vitro. To understand the effects of RA on NC cells, we assayed in vitro NC cell outgrowth and the immunohistochemical expression of HNK-1, a marker of migrating NC cells (Abo and Balch, 1981), and N-CAM, the down-regulation of which precedes the migration of NC cells (Thiery et al., 1982; Bronner-Fraser et al., 1992).

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Address reprint requests/correspondence to Dr. Michael Melnick, Laboratory for Developmental Genetics, University of Southern California, DEN 4266, MC-0641, Los Angeles, CA 90089-0641.

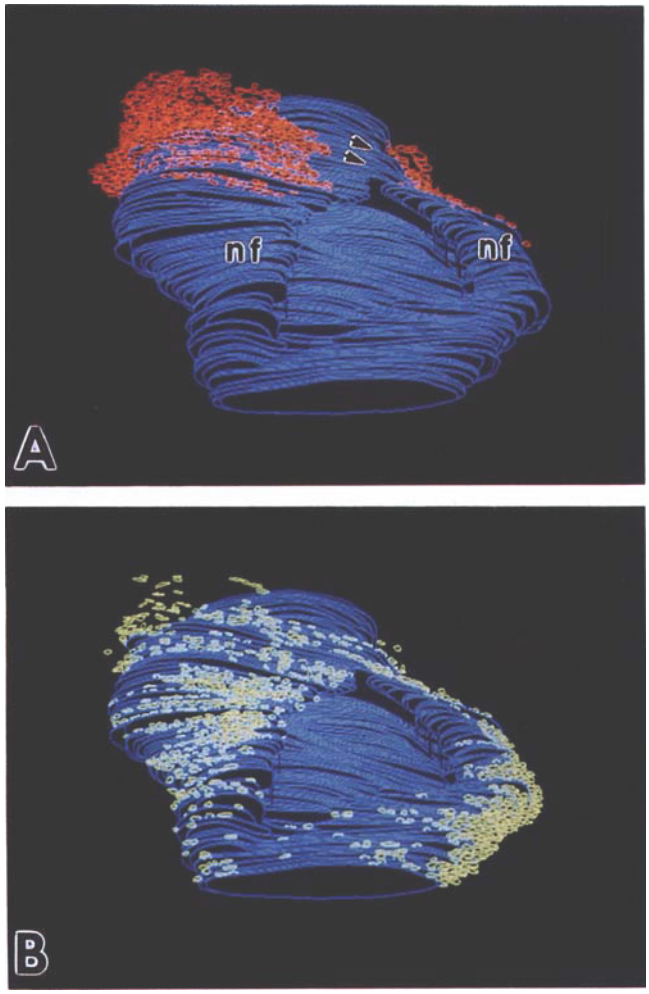


Fig. 1. Three-dimensional reconstruction of Hamburger and Hamilton (1951) stage 10 RA-treated abnormal chick embryo colocalized for HNK-1 (**A**) and N-CAM (**B**) and viewed from the anterior (bottom) to the more posterior (top) mesencephalon. With RA treatment, embryos exhibited dysmorphic neural tubes, i.e., unfused neural folds (nf) with convoluted neuroepithelia, reduced neural structures, and anophthalmia. The neural folds are apposed caudally (arrowheads) with a large population of N-CAM positive cells that are clearly HNK-1 negative.

RESULTS

To study the effect of RA on craniofacial development, we injected 13-cis retinoic acid in ovo (10 μg RA/50 μl DMSO) at zero time of incubation (primitive streak stage 2). DMSO injected and uninjected eggs were used as controls. After 48–50 hr of incubation, embryos treated with RA showed a large opening in the region of the anterior neuropore along with absent or reduced optic lobes, and abnormal shape of the diencephalon (Fig. 2).

Characteristics of NC Cells in Control Cultures

After carefully dissecting away the tissues surrounding the optic lobes and the diencephalon, we explanted the cranial portion of neural tube extending to the midmesencephalic region (Fig. 3A). Initially, NC cells exited only from the dorsal margin of the neural tube. Later, crest cells appeared opposite the cut ends of the neural tube. Based on NC cell morphology, two populations were present. The predominant population close to and distal to the explant exhibited a mesenchymal phenotype consisting of multipolar, spindle-shaped, flattened NC cells (Fig. 4A); at the periphery of the outgrowth, crest cells were confluent with a well delimited migration front except for a few cells that exhibited a multipolar or stellate morphology (Fig. 4A). The outgrowth area of the crest cell population increased gradually with time and can be used as an indication of the degree of cell migration (Newgreen et al., 1979). Time-lapse video demonstrated that close to the explant, the NC cells were a stationary population with a visibly active cytoplasm; near the periphery they were extremely motile and frequently changed their direction of movement. This persistence of movement increased greatly when they formed a quasi-confluent monolayer with frequent cell contacts (data not shown). At times, partial overlapping of crest cell bodies was seen.

Since previously published work largely characterizes trunk crest cell behavior, it was important to compare the difference between co-cultured cephalic and trunk crest cells. After an initial 16 hr in vitro, significantly fewer NC cells had migrated from the cranial neural tube than from the trunk neural tube (Fig. 3B, C). Moreover, trunk NC cells had migrated greater distances, and our time-lapse video recording revealed them to be far more active and motile than cranial NC cells. By 20 hr in vitro, a larger number of NC cells had migrated from the cranial neural tube (Fig. 4A).

As previously noted, proximal cranial NC cells were flattened, spindle-shaped, and non-randomly oriented, and those at the periphery of the outgrowth were multipolar and flattened. Caudal to the optic lobes, HNK-1 positive cells exhibited strong cell membrane fluorescence (Fig. 4B); positive staining was also seen cranial, caudal, and proximal to the explant. Distal to the explant, HNK-1 staining was sparse and of variable intensity (Fig. 4B); some cells had a punctate staining pattern. HNK-1 negative cells may also include non-NC derived mesenchyme; as reported by others (Vincent and Thiery, 1984), this cannot be entirely avoided in this type of culture. N-CAM was mostly expressed by cells close to the explant; away from the explant, crest cells were more rarely N-CAM positive (Fig. 4C).

Effect of In Ovo RA Exposure on Cephalic NC Cells: Abnormal Morphology and Expression of HNK-1 and N-CAM

The NC cell outgrowth derived from RA-treated cranial neural tube explants substantially differed from the control explants. Crest cells had emigrated from the neural tube by 18–20 hr in vitro and their out-

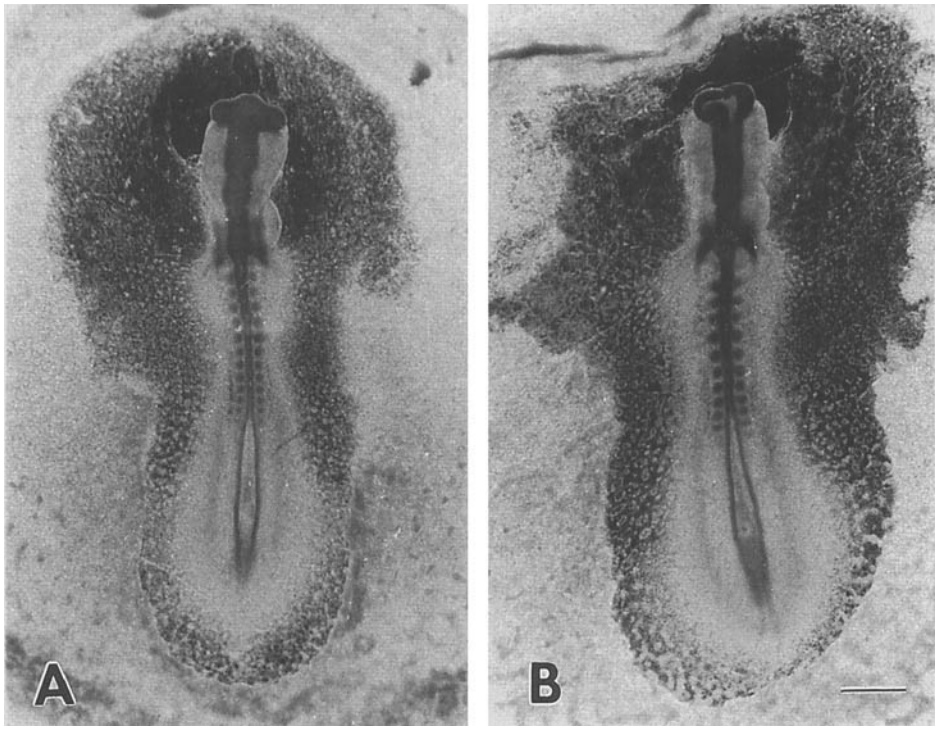


Fig. 2. Examples of fixed control (**A**) and RA-treated (**B**) stage 10+ chick embryos. With treatment, unfused cranial neural folds and abnormal optic lobes are observed. Bar = 600 μm.

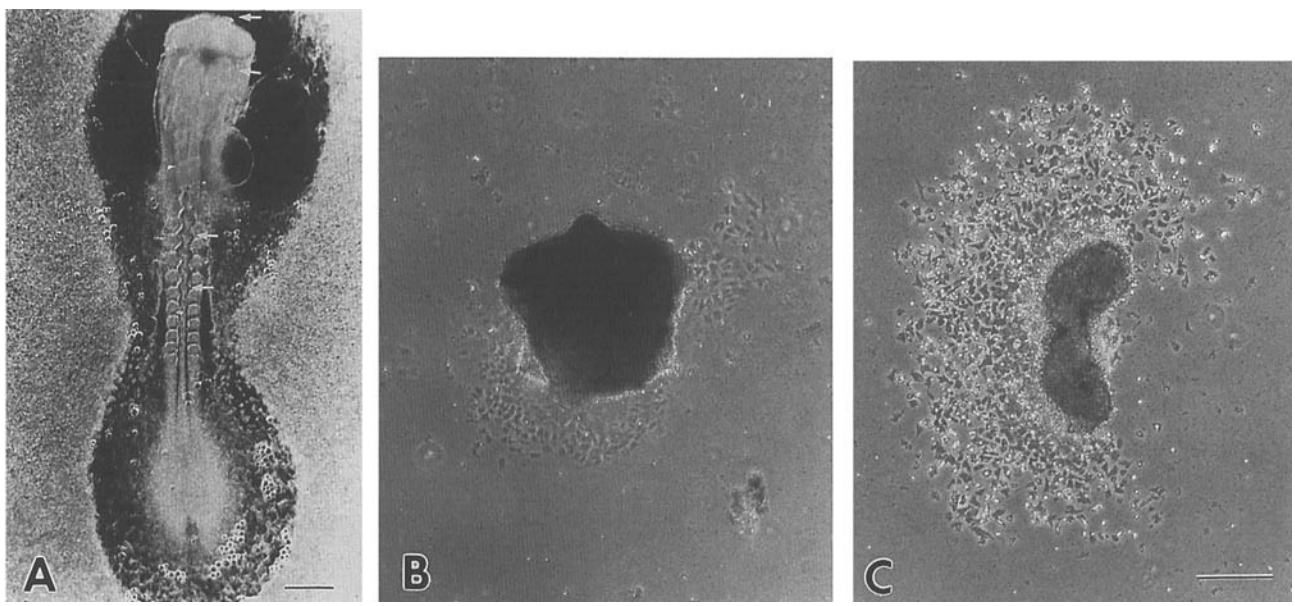
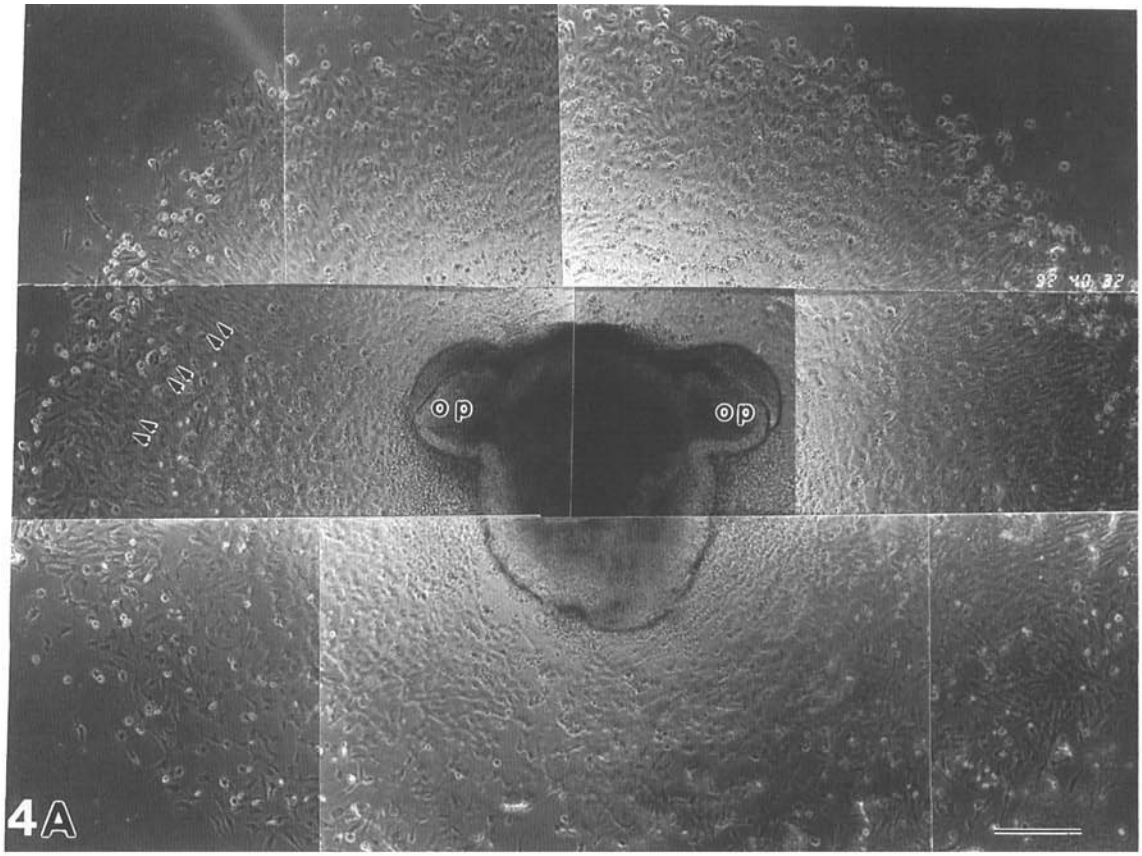


Fig. 3. **A:** In this representative control stage 11 chick embryo, the regions used in all cephalic and trunk explant cultures are indicated between the anterior or posterior arrow pairs. **B,C:** Comparison of control cephalic and trunk NC cells after 16 hr of culture. Fewer NC cells migrated from the cranial (B) neural tube during this initial period than from the trunk (C). In the neural tube explants shown, cranial is to the top; the trunk (C) explant is lying on its lateral surface, with dorsal being to the left. (A) Bar = 400 μm; (B, C) Bar = 250 μm.



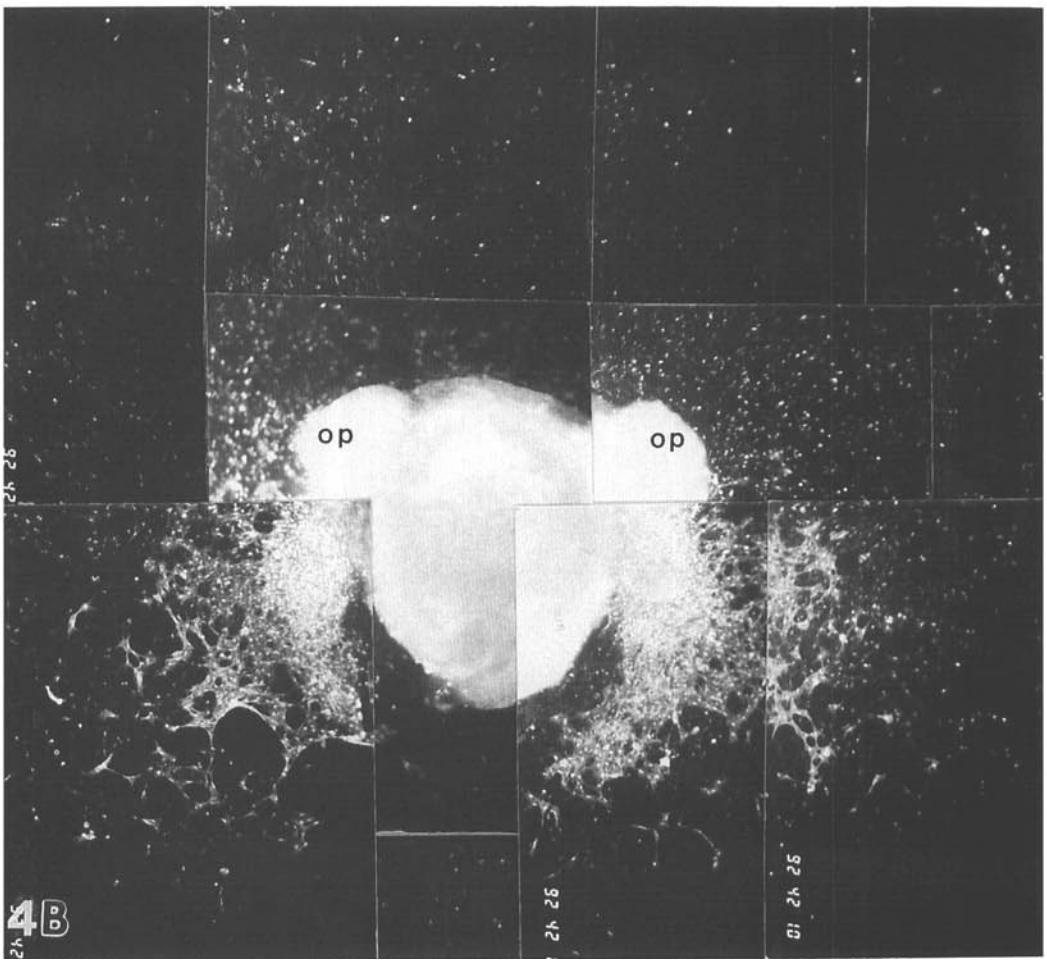


Fig. 4A–B.

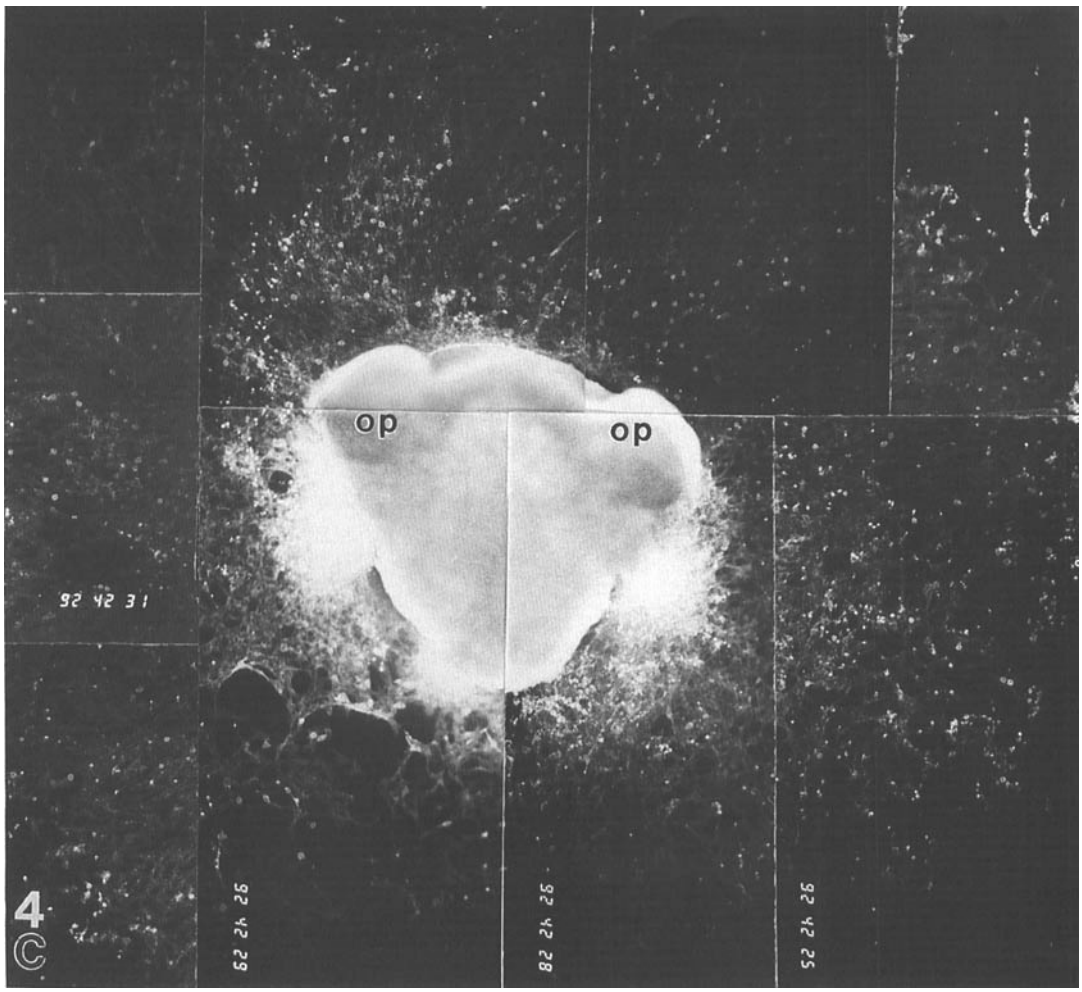


Fig. 4. Composite phase (A) and immunofluorescence (B, C) photomicrographs of a representative control stage 11+ cranial neural tube explant culture. In all explants shown in Figures 3–5, cranial is to the top and the cephalic neural tube is viewed from the ventral surface. **A:** After 20 hr, hundreds of NC cells have emigrated from the explant. The region of flattened mesenchymal-appearing cells is seen a distance from the explant (double arrowheads). **B:** HNK-1 staining. Caudal to the optic lobes, HNK-1 positive cells near the explant exhibited strong cell membrane fluorescence and those crest cells further away from the explant showed a more punctate fluorescent staining pattern. **C:** N-CAM staining. A small population of NC cells close to the explant expressed N-CAM while crest cells away from the neural tube were predominantly negative for N-CAM. op = optic lobes. Bar = 200 μm.

growth size was equivalent to that of the controls. However, unlike the control NC cell population which was composed of predominantly flattened, spindle-shaped, or multipolar cells, in the RA-treated explant, four morphologically distinct cell populations were apparent. First, and most striking, the largest population of NC cells in the outgrowth consisted of a confluent mass of polygonal cells in a "cobblestone" pattern (Figs. 5A, 6B, 8A, 8B) that has previously been defined as "epithelioid" (Mege et al., 1988); these cells differed in their morphology from that seen in the control population (compare Fig. 6A,B and Figs. 7 and 8). These polygonal cells were present both proximal and distal to the explant. Second, on the near periphery of the outgrowth, there were a few spindle-shaped cells mingled with the polygonal population (Figs. 5A, 8C). Third, the extreme periphery was bordered by a loose arrangement of "fibroblast-like" cells with a multipolar, mesenchymal morphology (Figs. 5A, 8C), intermixed with small spherical refractile cells.

With RA-treatment, there was an overall decrease of HNK-1 positive NC cells throughout the NC cell outgrowth as compared to controls (compare Fig. 4B with Figs. 5B, 8D,E,F). Immediately adjacent to the neural tube explant, N-CAM positive cells were not always seen; beyond this narrow region, all cells of the "epithelioid" population showed a relatively uniform expression of N-CAM mainly at adjacent cell boundaries (Figs. 5C, 8G,H); these "epithelioid" cells were negative for HNK-1 (Figs. 5B, 8D–F). However, there were sporadic regions within the "epithelioid" population that were neither N-CAM nor HNK-1 positive (Fig.

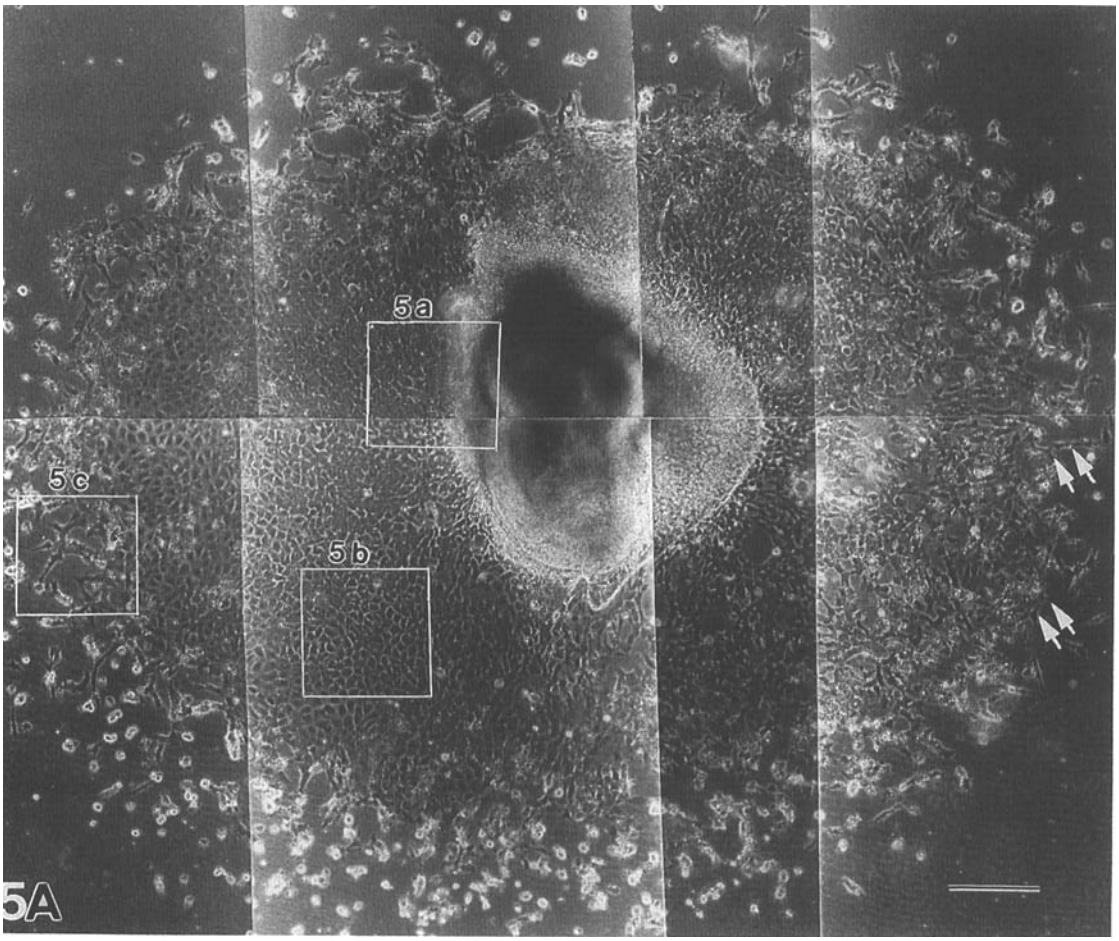


Fig. 5. Composite phase (A) and immunofluorescence (B, C) photomicrographs of a representative abnormal stage 11 + cranial explant first exposed to RA in ovo and then cultured for 20 hr. **A:** Crest cells, close to and a short distance from the explant (boxes 5a and 5b), were closely packed and polygonal in appearance, thereby presenting a "cobblestone" morphology. At the periphery of the NC cell outgrowth (box 5c and more lateral), scattered, flattened fibroblastic cells were observed (arrows). **B:** HNK-1 staining. **C:** N-CAM staining. There was an overall decrease in the HNK-1 positive NC cells as compared to the controls. However, there were small populations of NC cells that co-expressed HNK-1 and N-CAM (arrowheads) close to the explant. The arrows indicate HNK-1 positive cells that are N-CAM negative. Cells of the "cobblestone" population showed a relatively uniform expression of N-CAM on their cell surfaces. Bar = 200 μm.

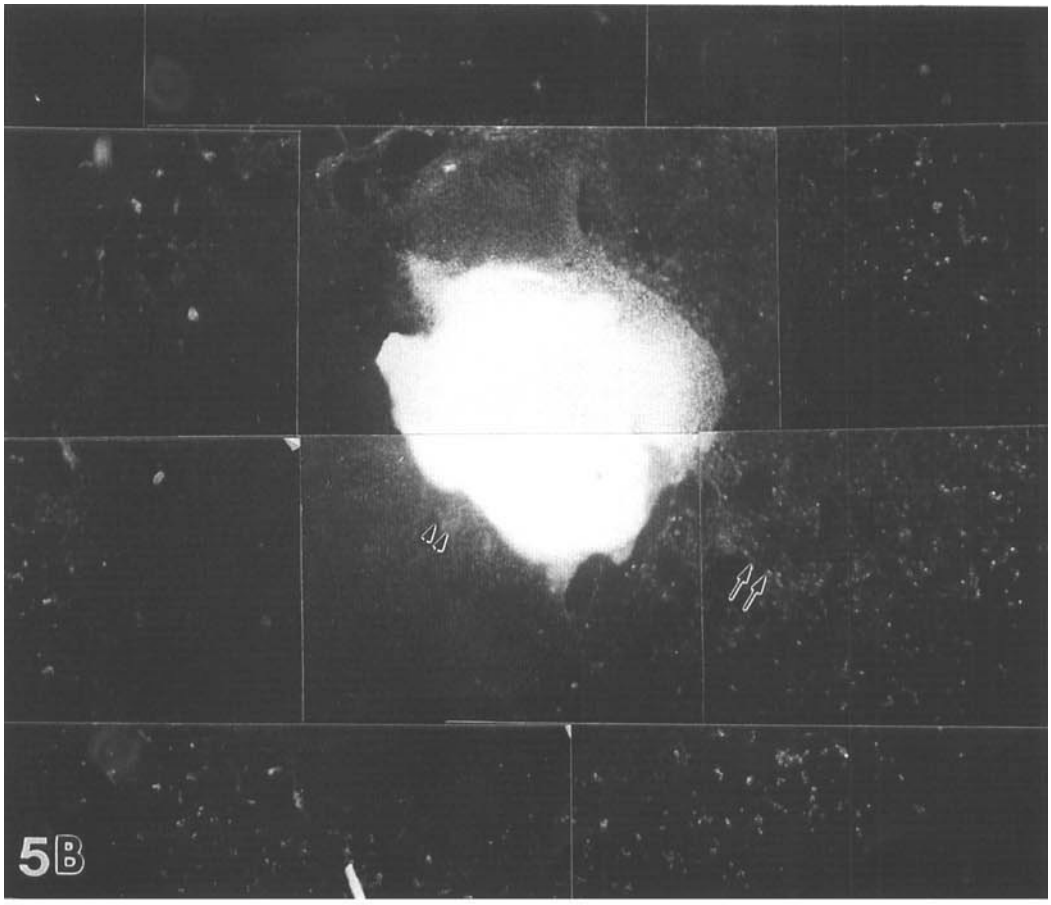
5C). In a few other areas there were N-CAM positive cells interspersed with N-CAM negative cells (Fig. 5C). A few spindle-shaped cells at the periphery of the outgrowth showed a uniform staining of N-CAM over their entire surface with no concentration of stain in particular locations (Fig. 8I). There were occasionally very small scattered subpopulations coexpressing HNK-1 and N-CAM (Fig. 5B,C).

The relative pattern of N-CAM expression in control and RA-treated embryos is shown in Figure 9. After normalization of the proportional data by arcsine transformation, a multiway analysis of variance was performed (Sokal and Rohlf, 1981). The intensity of N-CAM fluorescence in any given area was highly dependent ($P < 0.025$) on the experimental group (control vs. RA-treated). In the controls, the majority of the NC cells were negative for N-CAM; N-CAM was distributed in a small population of NC cells close to the neural tube, with a few cells distal to the neural tube exhibiting weak fluorescence. However, in the RA-treated embryos, more N-CAM expressing cells were observed close to the neural tube. In addition, those cells localized a short distance from the neural tube (regions II and III) also had an elevated, though less intense, level of N-CAM fluorescence.

DISCUSSION

Cephalic NC Cell Outgrowth in Explant Cultures

In ovo, most cranial NC cells migrate ventrally between the surface ectoderm and underlying mesodermal mesenchyme. Many anterior mesencephalic and all prosencephalic crest cells initially remain dorsal to the neural tube and shift rostrally over the roof of the forebrain; these crest cells subsequently disperse ros-



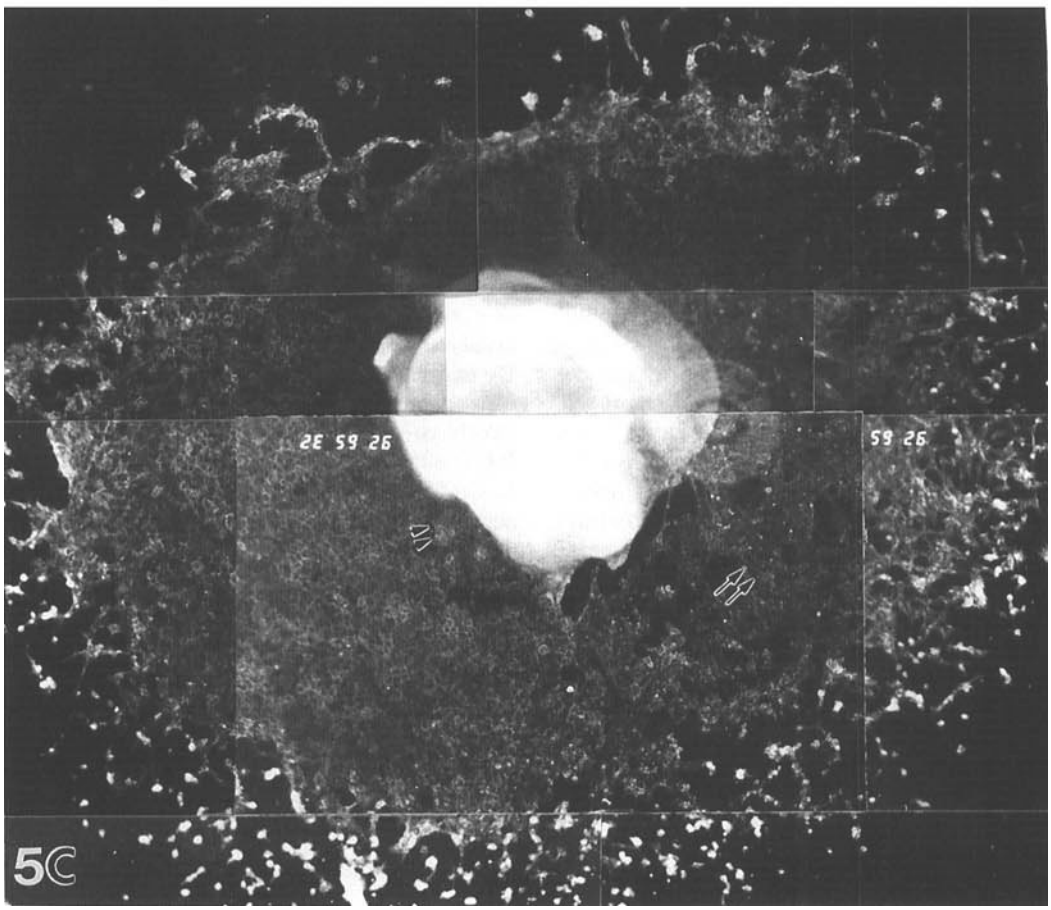


Fig. 5B–C.

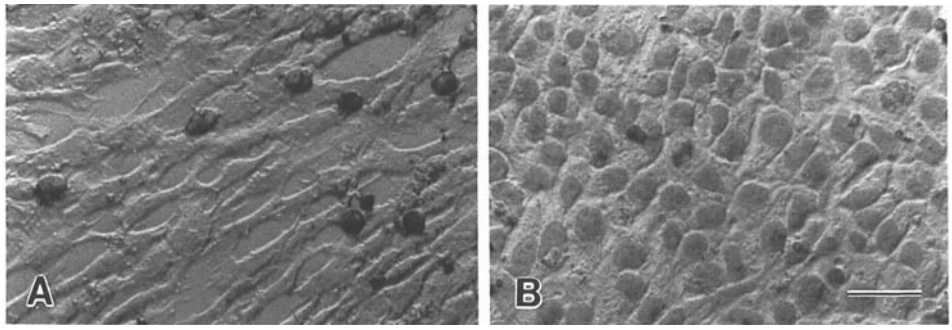


Fig. 6. Higher magnification of toluidine blue stained control (**A**) and RA-treated (**B**) representative NC cells in culture from a region equivalent to that shown in Figure 5A, box 5a. With RA treatment, note the loss of the mesenchymal, spindle-shaped appearance normally observed in control crest populations. The RA-treated cells appear as a confluent mass of polygonal cells in a "cobblestone" pattern. Bar = 25 μm.

trally and laterally between the prosencephalic neuroepithelium and surface ectoderm to form all of the frontonasal and much of the periocular mesenchyme (Johnston, 1975). While there is NC migration of short distances, much of the crest cell movement is secondary to both midbrain flexure and disproportionate growth of the forebrain and eye (Johnston, 1966).

Co-culture of control forebrain and trunk neural tube explants in a shared environment support the filmed observations in live specimens (Jaskoll et al., 1991). Our present in vitro culture experiments indicate that, during the initial period of culture, forebrain NC cells migrate in significantly smaller numbers than trunk NC cells and over shorter distances per unit time. One proposed explanation is that the molecular characteristics of migrating cranial NC cells is different from that of trunk NC cells (Jaskoll et al., 1991).

Recent studies suggest that differences in migratory behavior could arise from different cell-surface properties of cranial and trunk NC (Lallier et al., 1992; Lallier and Bronner-Fraser, 1991). These include different divalent cation requirements and integrins which differ in their functional properties (Lallier et al., 1992). The HNK-1 bearing glycoprotein and the characteristics of cell attachment to fibronectin, laminin, and collagen appear to be different for cranial and trunk NC cells (Lallier and Bronner-Fraser, 1991). In addition, antibody perturbation that interfered with cranial NC pathways gave rise to neural tube defects and abnormal distribution of cranial NC cells, but no detectable effects on trunk NC migration were observed, suggesting differences in the nature of cues controlling NC migration in different regions (Bronner-Fraser, 1985, 1987).

Cellular and Molecular Characteristics of RA-Pre-Exposed Forebrain NC Cells

In control cultures of forebrain neural tube, proximal NC cells exhibited strong cell membrane HNK-1 fluorescence; distally, HNK-1 staining was punctate. N-CAM was solely expressed by cells close to the explant, rarely colocalizing with HNK-1; away from the explant, crest cells were N-CAM negative. This pattern of staining was similar to our prior in ovo study, where HNK-1 cells were present ventral and dorsal to the optic lobes and only a few N-CAM positive cells were observed close to the neural tube (Shankar et al., 1992). Distal to the explant, the cell population that was either HNK-1 negative or regionally punctate could represent either contaminating non-NC derived mesenchymal cells (Vincent and Thiery, 1984) or, more likely, NC cells which have lost their HNK-1 immunoreactivity upon reaching more distal destinations, as one observes in situ with intact branchial arches (Vincent and Thiery, 1984). The demonstration that the latter phenomenon could also occur in vitro suggests the presence of an NC cell subpopulation with an early determined developmental fate.

The size of the NC cell outgrowth from nearly all forebrain neural tube explants exposed to RA in ovo was similar to that of controls (compare Figs. 4A and 5A). This suggests that in ovo RA exposure was not toxic to the NC population. Further, almost all NC cells were arranged in a cobblestone ("epithelioid") pattern of tightly packed polygonal cells that nearly exclusively expressed N-CAM at adjacent cell boundaries. By contrast, control NC cells were flattened and spindle or multipolar in shape and expressed HNK-1, rarely co-expressing N-CAM. These data indicate that RA is associated with a prolonged NC cell expression of N-CAM, a finding consistent with our prior in ovo observations (Shankar et al., 1992).

Previous studies have demonstrated that vitamin A–induced craniofacial defects often arise from abnormal migration of NC cells (Morriss and Thorogood, 1978; Thorogood et al., 1982; Webster et al., 1986; Pratt et al., 1987; Balbas Moro et al., 1993). A key feature of NC cell migration away from the dorsal neural tube is the down-regulation of N-CAM, as determined by immunochemical methodologies (Thiery et al., 1982; Bronner-Fraser et al., 1992). Cell-cell adhesion inhibits cell migration; thus, small changes in the amount of N-CAM can have significant effects on migration. Indeed, a two-fold increase in the amount of N-CAM on

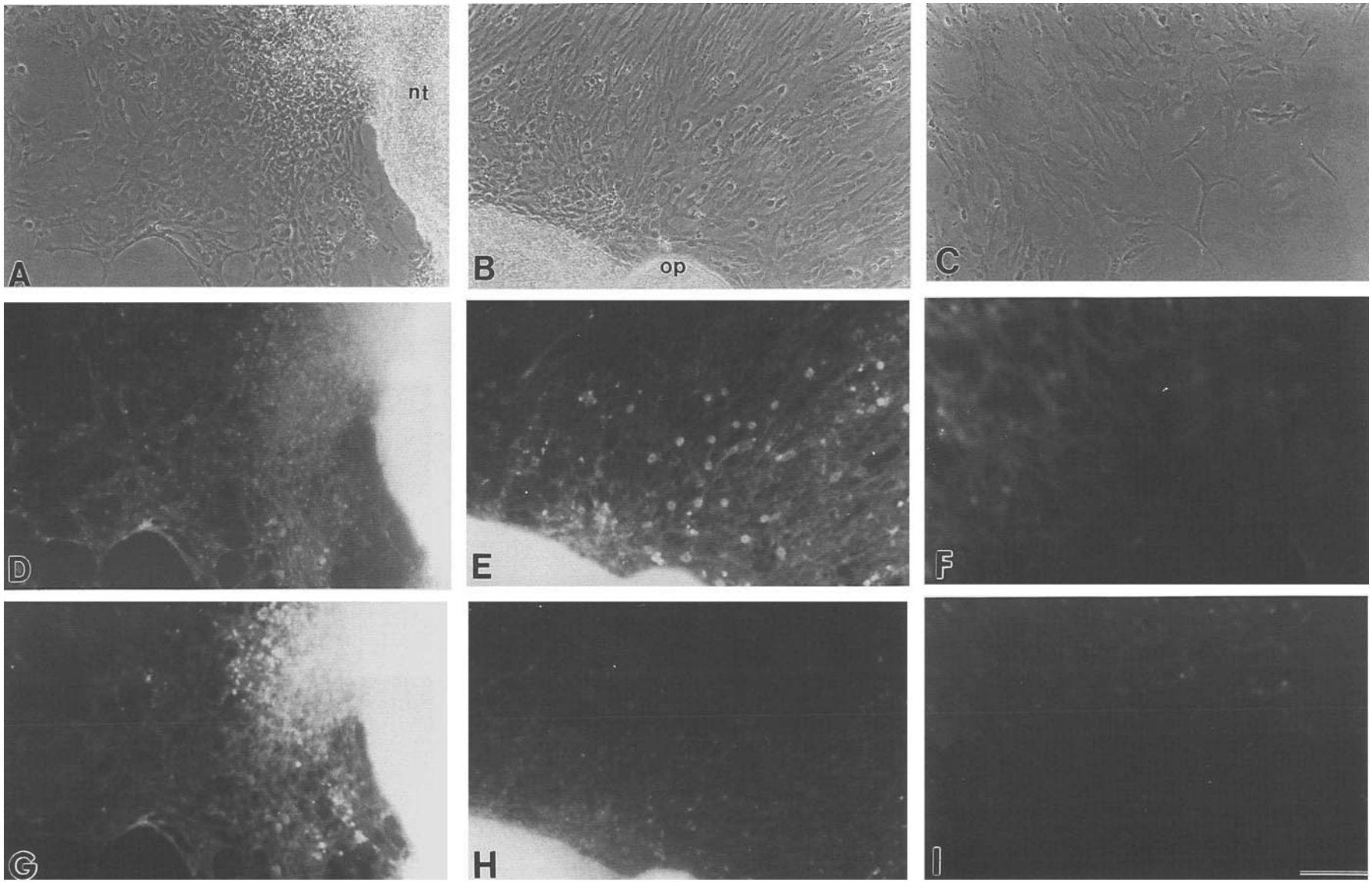


Fig. 7. Higher magnification phase (A–C) and double immunofluorescent (D–I) views of control explant taken at regions approximately equivalent to those outlined in boxes in Figure 5A. **A,D,G:** Higher magnification views of a region caudal to the optic lobes and lateral to the neural tube (nt) explant; **B,E,H:** Higher magnification views of a region cranial to the optic (op) lobe; **C,F,I:** Higher magnification views of a region on the periphery of the explant. D–F: HNK-1 positive cells are clearly visible in D and E. G–I: Clusters of N-CAM positive NC cells caudal to the optic lobes in G. Bar = 100 μm.

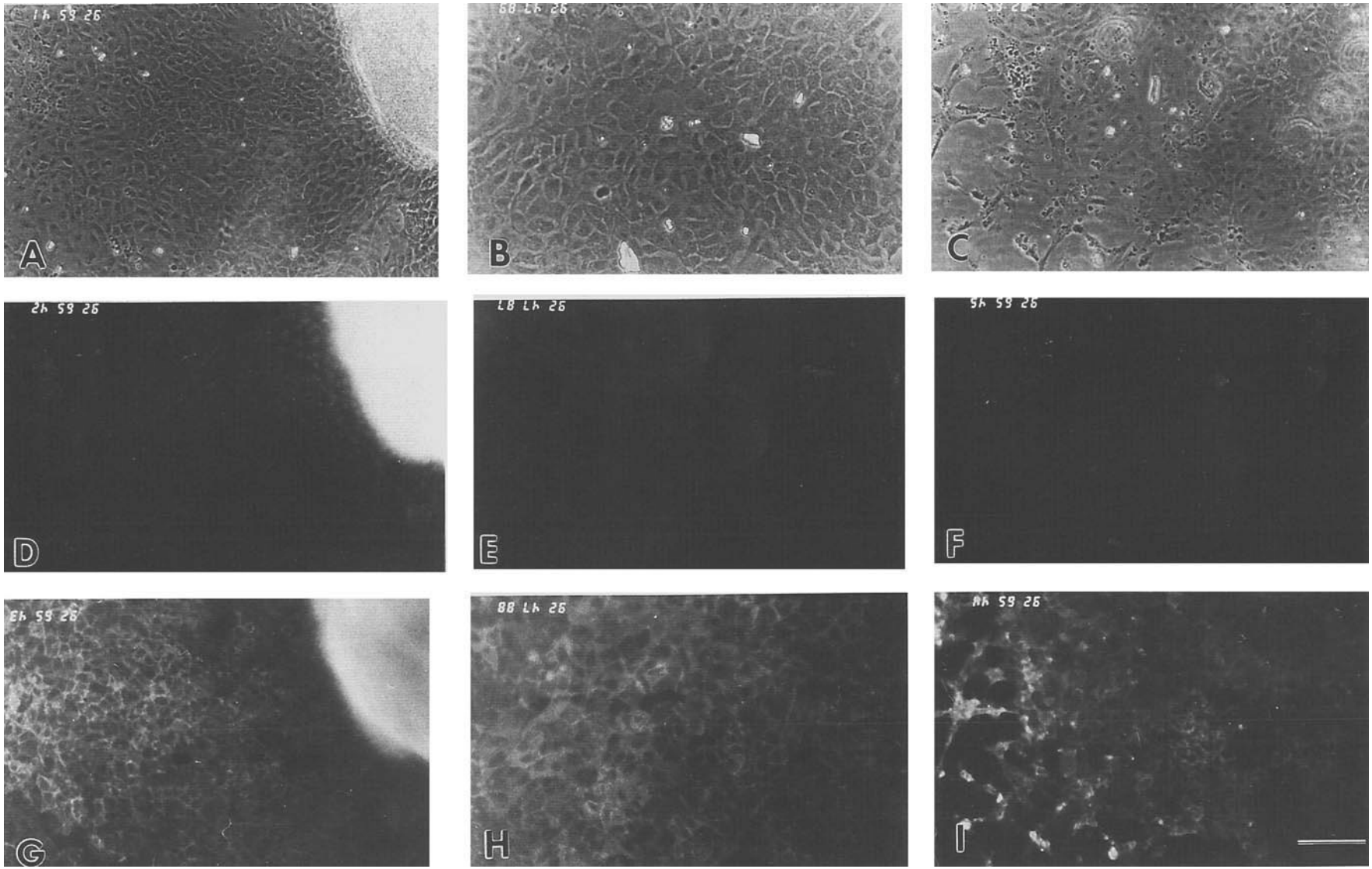


Fig. 8. Higher magnification phase (A–C) and double immunofluorescent (D–I) views of RA-treated explant. **A,D,G:** Higher magnification views of region shown in Figure 5A, box 5a; **B,E,H:** Higher magnification views of region shown in Figure 5A, box 5b; **C,F,I:** Higher magnification views of region shown in Figure 5A, box 5c. D–F: HNK-1 negative NC cells. G–I: Clusters of N-CAM positive NC cells. A subpopulation of confluent polygonal cells that are N-CAM positive, but do not stain for HNK-1 are seen. Bar = 100 μm.

the cell surface produces a 30-fold increase in the rate of cell-cell adhesion (Hoffman and Edelman, 1983; Mayford et al., 1992). Evidence indicates that RA and the homeobox genes might regulate the expression of adhesion molecules either directly or indirectly (Husman et al., 1989; Jones et al., 1992; Chuong et al., 1992). Several possible mechanisms exist through which RA may modulate N-CAM expression: N-CAM may be transcriptionally regulated through RA or homeobox response elements (Jones et al., 1992); RA may inhibit the normal internalization of N-CAM by endocytotic mechanisms (Bailey et al., 1991), prolonging its presence on NC cell surfaces; RA may be an important element in the pathway of N-CAM alternative RNA splicing, a major mechanism of cell surface N-CAM modulation that alters N-CAM affinity, prevalence, mobility, and distribution on the surface (Cunningham et al., 1987; Lyons et al., 1992). These possibilities are, of course, neither exhaustive nor mutually exclusive. They share a reasonably predictable common outcome, the abnormal prolongation of the presence of N-CAM on NC cell surfaces and a consequent diminution of NC cell migration.

Developmental Plasticity and RA

It is heuristic to consider the results of our experiments within the context of Smith-Gill's (1983) models of developmental plasticity. Smith-Gill presents two broad classes: phenotypic modulation and developmental conversion. Phenotypic modulation (variation) occurs as a result of environmental factors affecting the rate or degree of expression of a genetically controlled developmental program, not its alteration. Developmental conversion, by contrast, results when extrinsic factors induce alternative genetically controlled developmental pathways leading to alternative "morphotypes," or what the teratologist might term aplasias or dysplasias. The distinction, of course, is not trivial; it is key to understanding the pathogenesis associated with an environmental perturbant-like RA. Our present experimental strategy appears to be informative in this regard. Indeed, our results would suggest that RA has effected a developmental conversion. The initial NC cell population appears to have lost its mesenchymal phenotype as a result of early extrinsic RA exposure and transdifferentiated into an "epithelioid" morphotype that is consistent with the up-regulation of cell adhesion molecules.

For RA to exert its effects, exposure must occur at a critical period of embryonic sensitivity, such as early embryogenesis. At the time of gastrulation/neurulation, two situations obtain. First, the neural plate is regionalized and cells are clonally committed to form distinct structures (Nieukwoop et al., 1985; Saxen, 1989; Waddington, 1933; Fraser et al., 1990). Second, enzymes that can synthesize RA from precursor compound and endogenous retinoids are present in primitive streak stage embryos (Thaller and Eichele, 1987; Durston, et al., 1989; Wagner et al., 1990; Rossant et

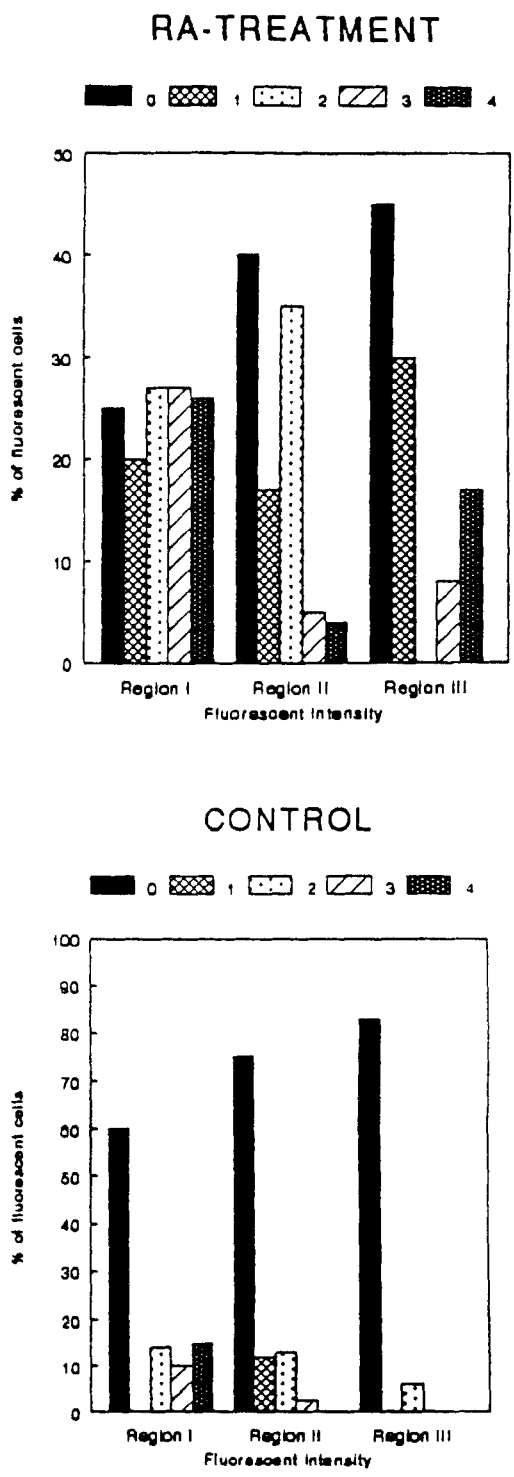


Fig. 9. Up-regulated N-CAM expression in vitro. The percentage of the total number of cells expressing N-CAM in three different regions was plotted against the intensity of immunofluorescence, 0 indicating no fluorescence, and 4 indicating the most fluorescent stain. The three different regions, region I, region II, and region III for the control and RA-treated group, are equivalent to those outlined in Figure 5, boxes 5a, 5b, and 5c, respectively. The data were obtained from the following: Control: region I ≈ 250 cells, region II ≈ 200 cells, region III ≈ 120 cells; RA-treated: region I ≈ 200 cells, region II ≈ 150 cells, and region III ≈ 100 cells.

al., 1991). In our study, we injected a single dose of RA at the primitive streak stage (3 to 5 hr of development; stage 2) and did not expose the embryo to additional RA later, either in ovo or in vitro. The single dose of RA in ovo was sufficient to disrupt neurulation and to induce an alternative morphotype in an in vitro environment normally permissive to mesenchymal structure and function. This environmentally induced developmental conversion of mesenchymal to "epithelioid" morphotype appears to be associated with an untimely up-regulation of cell adhesion molecules.

Epithelial-mesenchymal transformation in either direction is a major developmental process (Edelman, 1988; Hay, 1990; Mattern et al., 1993; Vanderburg and Hay, 1993). A number of elegant studies employing mesenchymal cells transfected with cDNAs for CAMs demonstrate that the up-regulation of cell surface CAMs is a key factor in the transdifferentiation to an epithelium-like phenotype (e.g., Mege et al., 1988; Mattern et al., 1993; Vanderburg and Hay, 1993). Tightly packed sheets of cells assume polygonal boundaries positive for CAMs, what is termed an "epithelioid phenotype" (Mege et al., 1988; Mattern et al., 1993; Vanderburg and Hay, 1993). It is noteworthy in the present study that NC cells exposed in ovo to RA display strikingly similar characteristics. We make no claim that this cell population is a genuine epithelium, only that it has lost its typical mesenchymal phenotype and come to resemble an epithelioid morphotype. The molecular details of this developmental conversion remain to be elucidated. Nevertheless, the fact that it resulted from a single early in ovo RA hit and could not be rescued by a developmentally permissive environment over 20 days in vitro strongly suggests that the altered developmental pathway is irreversible. Recent studies would predict that the ultimate fate of such a cell population in ovo is cell death (Sulik et al., 1988; Alles and Sulik, 1990; Osumi-Yamashita et al., 1992). Frontonasal NC-derived mesenchyme transdifferentiation and apoptosis should be explored as a possible pathogenetic mechanism induced by a wide variety of environmental perturbations. As Poswillo (1976) pointed out long ago, the number of teratologic mechanisms are likely to be far smaller than the number of teratologic agents.

EXPERIMENTAL PROCEDURES

In Ovo RA Application

Fertile White Leghorn eggs (K and R Hatchery, Westminister, CA) obtained at zero time of incubation were placed horizontally and cleaned with 70% alcohol. All injections were done at zero time of incubation which corresponded to early or mid-gastrulation (primitive streak; Hamburger Hamilton stage 2). Using a sterile 26 gauge needle, two holes were made. One, on the blunt end of the egg where the air sac was situated, the other on the surface of the egg facing up. In each case, only the bevel of the needle was inserted. Next, using a 26 gauge needle and a tuberculin syringe fitted to a stepper pipette, 0.05 ml of 10 μg of 13-cis RA dissolved in 50 μl of dimethyl-sulphoxide (DMSO; Sigma Chemical Co., St. Louis, MO) was injected through the second hole onto the blastoderm. This hole was sealed with tape and eggs were placed in the incubator at 37.5°C. The dosage was established in a series of preliminary experiments designed to obtain the maximum number of live abnormal embryos with minimal mortality.

Cephalic NC Cell Cultures

Control (n = 60) and RA-treated (n = 20) chick embryos were incubated as described above. Eggs incubated between 48–60 hr were then cleaned and the egg shells from the top were carefully removed. The blastoderms were cut and the embryos transferred into a 60 mm Corning Petri dish (VWR, Cerritos, CA) containing HBSS (Hanks Balanced Salt Solution; GIBCO, Gaithersburg, MD) (Fig. 2A). Hamburger Hamilton (1951) stages 10 to 12 embryos were recovered and the surrounding membranes were cut with microdissecting scissors under the dissecting microscope. Embryos were incubated in Dispase (Biomedical Products Division, Bedford, MA) for 15 min at 4°C followed by 6 min at 37°C. Using a transfer pipette, embryos were gently transferred into a Petri dish containing DMEM (Dulbecco's Modified Eagle's Medium; Gibco) with 10% fetal calf serum (FCS; Gibco) and 1:1,000 gentamicin and incubated at room temperature for 30 min. During this incubation period, the entire neural tube was carefully isolated from the surrounding ectoderm and somites with tungsten needles. Next, the cranial neural tube was dissected at the caudal mesencephalon and an equivalent length of thoracic neural tube, which served as an internal control, was also dissected (Fig. 2A). The cranial and trunk neural tube explants were placed on fibronectin (100 μg/ml; Gibco) coated Petri dishes, and incubated for 18–27 hr in 500 μl of DMEM containing 3% heat-inactivated FCS and 1:1,000 gentamicin at 37°C in a humidified 5% CO_2 air incubator. After 18–25 hr, the explants were examined with the Olympus TM-2 inverted microscope equipped with phase contrast optics.

Photographic Documentation

Explants were photographed using one of the following procedures: (1) explants were photographed on the Olympus TM-2 using phase contrast optics and TMY 400 or TMAX 400 film; (2) time lapse video recording was performed with an Olympus TM-2 inverted microscope equipped with a video camera (Cohu solid state camera) connected to a TV monitor (Sony, Japan), a time generator, a time lapse recorder (Hitachi), and a NEC Multisync computer equipped with the image analysis program.

Immunochemistry

Selected explants were fixed overnight at 4°C in alcohol: acetic acid (99:1), washed for 30 min in PBS

(phosphate buffered saline, pH 7.1), and incubated in 5% normal goat serum for 30 min. Explants were then incubated overnight in a humidified chamber at room temperature in a mixture of HNK-1 (1:10 in PBS; Becton and Dickinson, Mountain View, CA) and rabbit anti-chick N-CAM (1:50 in PBS). All antibody dilutions are in PBS. Cultures were then washed in three changes of PBS, 10 min each, and incubated in a mixture of Texas Red goat anti-rabbit (1: 100; Biomeda, Foster City, CA) and biotinylated goat anti-mouse IgM (1:50; Vector, Burlingame, CA) for 1 hr at room temperature. The cultures were then washed three times in PBS and incubated in FITC-conjugated streptavidin (1:40; Zymed, South San Francisco, CA) for 1 hr at room temperature. Explants were washed in PBS and mounted in media containing o-phenylenediamine and coverslipped. Fluorescent photography was done on the Olympus TM-2 epifluorescence microscope with TMAX 400 film.

Following immunofluorescent evaluation, the coverslipped explants were removed in PBS and the explants were stained with 0.01% toluidine blue for 5 min, evaluated on the Edge high definition microscope, and photographed with TMax 100 film.

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