

Latex Beads as Probes of a Neural Crest Pathway: Effects of Laminin, Collagen, and Surface Charge on Bead Translocation

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ABSTRACT In the trunk region of avian embryos, neural crest cells migrate along two pathways: dorsally just under the ectoderm, and ventrally between the neural tube and the somites. Previous work from this laboratory has shown that uncoated latex beads are able to translocate along the ventral neural crest pathway after injection into young embryos; however, beads coated with fibronectin are restricted from the ventral route (Bronner-Fraser, M. E., 1982, *Dev. Biol.*, 91: 50–63). Here, we extend these observations to determine the effects of other macromolecules on bead distribution. The data show that laminin-coated beads, like fibronectin-coated beads, are restricted from the ventral pathway. In contrast, beads coated with type I collagen translocate ventrally after injection. Because macromolecules have characteristic charge properties, changes in surface charge caused by coating the beads may confound interpretation of the results. Electrostatic effects on bead movement were examined by coating the latex beads with polyamino acids in order to predictably alter the initial surface charge. The surface charge before injection was measured for beads coated with amino acid polymers or with various biologically important macromolecules; the subsequent translocation ability of these beads was then monitored in the embryo. Polylysine-coated beads (positively charged) were restricted from the ventral pathway as were fibronectin and laminin-coated beads, even though fibronectin and laminin beads were both negatively charged. In contrast, polytyrosine-coated beads (neutrally charged) translocated ventrally as did negatively charged collagen-coated or uncoated beads. The results demonstrate that no correlation exists between the charge properties on the latex bead surface and their subsequent ability to translocate along the ventral pathway. Therefore, an adhesion mechanism independent of surface charge effects must explain the restriction or translocation of latex beads on a neural crest pathway.

The neural crest is an embryonic structure that arises during the formation of the nervous system as the neural folds appose to form the neural tube. Crest cells subsequently disperse from their site of origin, undergo extensive migrations, and differentiate into a wide variety of cell types. Because of the migratory ability of neural crest cells and the diversity of crest derivatives, this transient structure provides an important system for studying interactions involved in cell movement and differentiation. A variety of cell-marking techniques in combination with neural tube transplantations have been used to map the neural crest migratory routes and sites of localization (1–5). However, little is known about the mechanisms

responsible for the initiation, guidance, or cessation of neural crest cell migration.

The most studied neural crest migratory routes are those in the “trunk” region of the embryo, between the eighth and 28th somites. Trunk neural crest cells migrate along two major routes (Fig. 1): either dorsally underneath the ectoderm, or ventrally between the neural tube and the somites (1). Those cells following the dorsal route eventually give rise to melanocytes. The cells following the ventral route settle in three locations: adjacent to the neural tube where they form the dorsal root ganglia, above the dorsal aorta where they condense into sympathetic ganglia, and lateral to the dorsal aorta

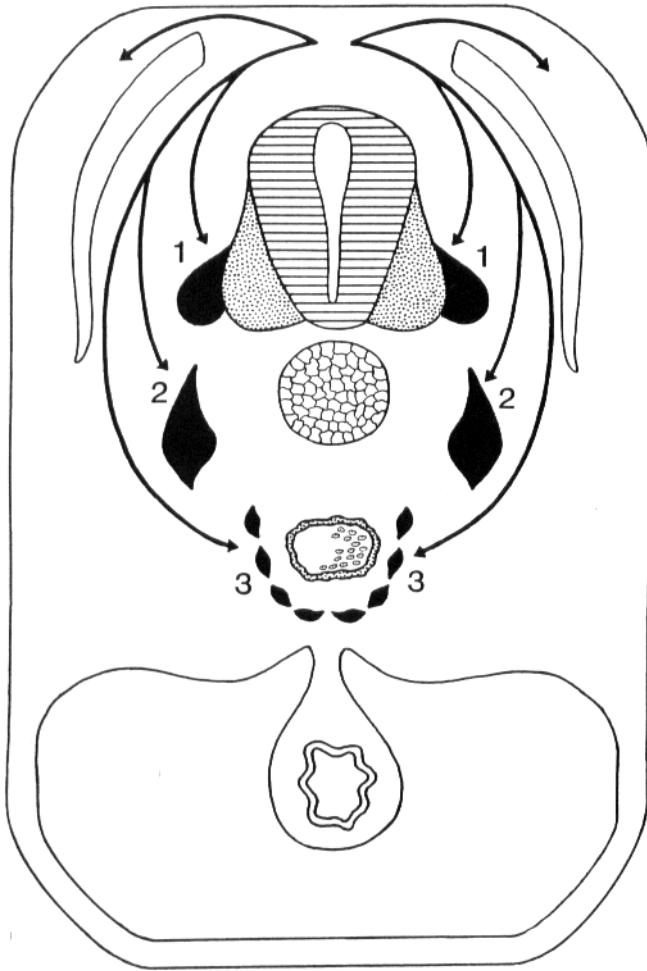


FIGURE 1 Schematic diagram illustrating the pathways of neural crest migration in the trunk region of avian embryos. Some crest cells migrate along the "dorsal" pathway just under the skin. Cells following the dorsal route are precursor to melanocytes. Other neural crest cells migrate along the "ventral" pathway and settle in three sites: (1) lateral to the neural tube where they form the dorsal root ganglia; (2) above the dorsal aorta where they give rise to the sympathetic ganglia; and (3) lateral to and below the dorsal aorta where they form the adrenal medulla and aortic plexuses.

where they form the chromaffin cells of the adrenal medulla.

After emigration from the neural tube in the trunk region, neural crest cells migrate into a narrow, cell-free space which contains extracellular matrix (ECM)¹ molecules. The primary components of this matrix include glycosaminoglycans, fibronectin, and fibrillar material. Of the glycosaminoglycans, hyaluronic acid is present in high concentrations along both the dorsal and ventral pathways at the onset of crest migration (6, 7). Fibronectin, a cell surface and ECM-associated glycoprotein with important functions in cell adhesion and motility, is also present in high concentrations along both the dorsal and ventral migratory routes (8, 9). In addition, the extracellular spaces through which crest cells migrate contain fibrillar ECM components that can be seen by electron microscopy

¹ Abbreviations used in this paper: ECM, extracellular matrix; FN-beads, fibronectin-coated latex beads; LM-beads, laminin-coated latex beads; PL-beads, polylysine-coated latex beads; PT-beads, polytyrosine-coated latex beads.

(10–13). Though not yet fully characterized, this fibrillar material may be predominantly composed of collagen (10, 13).

Previous work from this laboratory has utilized latex beads as probes of neural crest pathways (14). The latex beads were introduced into the trunk somites of avian embryos by means of an injection technique (15); as the sclerotomal cells of the somite dispersed, the beads were exposed to the ventral neural crest pathway during endogenous crest migration. After injection, uncoated latex beads translocated along the ventral pathway and settled adjacent to neural crest sites. By coating the surface of the latex beads with various macromolecules, the inert particles could be used to examine interactions between the applied surface coat and the embryonic milieu. When the bead surface was coated with fibronectin (FN-beads), the FN-beads failed to move ventrally, but remained at the implantation site (14). There thus appears to be a correlation between the presence of fibronectin on the bead or cell surface and restriction of movement along the ventral pathway. Trunk neural crest cells lack cell surface FN (8, 15). On the other hand, somite cells and fibroblasts synthesize FN (16, 17) and both these cell types fail to move along the ventral pathway after injection (18, 19). Therefore, cell surface-associated fibronectin may function to restrict certain embryonic cell types from neural crest sites. Experiments using FN-beads to produce an "artificial cell" with a single surface component offer a simple system to test the correlation between surface fibronectin and inability to translocate. Because FN-beads behave in a manner similar to that of certain cell types with surface fibronectin, the results strengthen this correlation. These studies demonstrate that latex beads can be used as a tool for probing neural crest pathways. By observing the effects of surface macromolecules on bead distribution, one can better characterize the nature of crest pathways and their role in guiding cell movement and localization.

In the present study, latex beads coated with ECM molecules or amino acid polymers were injected onto the ventral pathway; these experiments were designed to probe interactions of the individual molecular species present on the bead surface with the environment along neural crest migratory routes. Coating the beads with macromolecules may alter their surface charge and surface charge, in turn, is well known to affect the binding of cells to artificial substrates. Therefore, it was important to separate the electrostatic interactions due to the surface charge of the latex beads from other adhesive properties. By derivatizing the surface of the beads with a variety of macromolecules that have characteristic adhesive or electrostatic properties and by measuring their surface charge, two questions were addressed: First, is the restriction of FN-beads from the ventral pathway specific for fibronectin, or will other adhesive molecules cause the same effect? Second, does the macromolecular coat alter bead localization by some specific adhesion mechanism, or do less specific electrostatic adhesions explain translocation and restriction of the latex beads? Here, we report that the localization of beads along the ventral pathway was altered by the macromolecular species present on the surface; however, no correlation was found between their translocation ability and surface charge properties before injection. This suggests a possible role for more specific adhesive interactions between the neural crest cell surface and the ECM in the guidance of neural crest movement and localization.

MATERIALS AND METHODS

Injection Technique: In most of the experiments described, the latex beads were microinjected into one of the most posterior somites in the trunk region of 2.5-d-old host embryos (20). The latex beads were placed into a micropipette (with an opening of $\sim 30 \mu\text{m}$) which was mounted on a micro-manipulator and connected to a pressure injection apparatus. After puncturing the ectoderm and underlying somite, the particles were expelled into the somitic cavity with a pulse of pressure and the injection needle was withdrawn. The beads were originally contained in the cavity of somite. As the sclerotomal cells of the somite dispersed during the normal course of development, the beads were deposited onto the ventral neural crest pathway adjacent to the ventromedial aspect of the neural tube. In this way, they were reproducibly introduced into a region of the embryo where endogenous neural crest migration was just beginning. Because this method produces no incision adjacent to the neural tube, the ECM material along the ventral pathway remains intact and undisturbed. For all the bead types reported, the distribution of the beads was monitored from 1 to 3 d after injection.

Using the injection technique, it is possible to deposit a small number of beads within the somitic lumen and subsequently onto the ventral neural crest pathway. Approximately 100 pl of fluid are placed into each somite, containing an average of 30 beads. The exact number implanted into any one embryo varies because: (a) beads may sediment in the micropipette; (b) some beads probably leak out into the extraembryonic fluid during the injection; and (c) the resistance of the injection site may vary from embryo to embryo. In fixed and stained sections, single beads are easily identifiable. All sections in the trunk region of the injected embryos are scanned. Therefore, we feel that all beads in the sectioned embryos can be identified, and that the number found represents the number injected.

In one experimental series, latex beads were injected adjacent to the neural tube to control for the somitic injection site. The injections were performed as described above, except that the micropipette was placed just lateral to the neural tube, instead of inside the somite. Though this method sacrifices some of the advantages of the somitic injection site (the beads are not localized in a cavity and an incision is made along the crest migratory pathway), it is possible to deposit a small number of beads in this location by careful pressure injection.

Preparation of the Host: Stage 14–17 White Leghorn chicken embryos (according to the criteria of Hamburger and Hamilton [21]) were used as the hosts for all experiments. The eggs were candled to mark the position of the embryo, then washed with 70% ethanol. A window was made in the shell, the embryo was lightly stained with neutral red to aid visualization, and the vitelline membrane was removed over the injection site. After injection of beads into the embryo, the window was closed with adhesive tape, and the eggs were returned to the incubator until the time of fixation.

Histological Procedures: The embryos were fixed for 4–24 h in Zenker's fixative. After fixation, mercuric chloride crystals were removed by rinsing the embryos overnight in running water. The embryos were dehydrated, embedded in paraplast, serially sectioned at $10 \mu\text{m}$, and stained with hematoxylin and eosin (22).

Preparation of Coated Latex Beads: Monodispersed latex polystyrene beads (2.5% solid) were obtained from Polysciences, Inc. (Warrington, PA). For all experiments, blue-dyed beads were used to aid in visualization of the beads during injection. The mean diameter of the beads was $6.46 \pm 1.6 \mu\text{m}$. This size was chosen to approximate the dimensions of neural crest cells during active migration. Previous experiments demonstrated that beads spanning a 10-fold range of diameters behaved similarly (14).

The latex beads were coated with one of the following substances: collagen type I, laminin, poly-D-lysine, or poly-D-tyrosine. Poly-D-lysine and Poly-D-tyrosine were purchased from Sigma Chemical Co. (St. Louis, MO). At physiological pH, these two polyamino acids are positively and neutrally charged, respectively. Type I collagen from humans (Calbiochem-Behring Corp., San Diego, CA) or rat tails (prepared according to the method of Bornstein [23]) were used for all experiments. The data for both human and rat tail collagen were identical and, therefore, are presented cumulatively. Laminin derived from a rat L-2 tumor was prepared as described elsewhere (24) and was kindly provided by Dr. Erkki Ruoslahti (La Jolla Cancer Research Foundation). The procedure for coating the bead surface with laminin or poly-D-tyrosine was similar to that used in a previous study (14). Briefly, 0.15 mg of the macromolecule was dissolved in $150 \mu\text{l}$ of phosphate saline (0.15 M NaCl, 0.001 M monobasic sodium phosphate, adjusted to pH 7.2). $5 \mu\text{l}$ of latex polystyrene beads was added to the solution and incubated at 22°C for 10 min. The coated beads were centrifuged and resuspended in phosphate saline. To produce collagen-coated beads, $5 \mu\text{l}$ of bead solution was incubated with $150 \mu\text{l}$ of collagen in 0.017 M acetic acid and $30 \mu\text{l}$ of 6% aqueous NaCl at room temperature overnight. Poly-D-lysine was adsorbed to the bead surface according to the procedure of Peng et al. (25). $10 \mu\text{l}$ of latex bead solution was

incubated with $150 \mu\text{l}$ of aqueous poly-D-lysine (1 mg/ml) at 4°C overnight. The beads were washed as described above. After brief sonication or vigorous pipetting with a $50\text{-}\mu\text{l}$ syringe (Hamilton Co., Reno, NV), the coated beads were used immediately for injection.

Indirect Immunofluorescence on the Bead Surface: Antibodies against laminin or collagen type I (sheep IgG) were kindly provided by Dr. David Woodley (University of North Carolina). The anti-laminin, anti-collagen I, anti-chick FN (N. L. Cappel Laboratories, Inc., Cochranville, PA) and fluorescein-conjugated second antibodies (Cappel) were reconstituted in sterile distilled water. Subsequent dilutions were made in phosphate-buffered saline, pH 7.2, containing 0.1% BSA. To reduce nonspecific antibody binding, antiserum to porcine IgG (Cappel) was added to the reaction mixture.

1 or 2 d after injection of uncoated beads or beads coated with various macromolecules, the region containing the beads was dissected from the animal using fine forceps and iridectomy scissors. Excess fluid was removed and the tissue was triturated with a Hamilton syringe. Beads prepared simultaneously but not injected into embryos were used as controls. $100 \mu\text{l}$ of anti-laminin (diluted 1:100) and $100 \mu\text{l}$ of anti-porcine IgG (diluted 1:10) were added to $20 \mu\text{l}$ of the tissue containing the injected beads and the reaction mixture was incubated at 37°C for 15 min. The beads were then centrifuged and the supernatant was removed. $100 \mu\text{l}$ of fluorescent goat-anti-sheep antibody (diluted 1:100) and $100 \mu\text{l}$ of anti-porcine IgG (diluted 1:10) were added to the bead pellet. The mixture was incubated for 15 min at 37°C . After centrifuging the preparation and washing the pellet in phosphate-buffered saline, the bead mixture ($10 \mu\text{l}$) was placed onto a glass slide and coverslipped. Immunofluorescent staining with antibodies to collagen type I (diluted 1:10) or to chick fibronectin (diluted 1:100) was conducted analogously. The binding of fluorescent antibodies to the beads was observed using a Zeiss microscope equipped for epifluorescence (Carl Zeiss Inc., New York). Slides were scanned in their entirety; the number of fluorescent beads in each sample was expressed as a percentage of the total number of beads observed.

Assay for Protein on the Bead Surface: A fluorescamine binding method was used to assay for the presence of proteins and amino acid polymers of the bead surface after coating with laminin, collagen, polylysine or polytyrosine. $20 \mu\text{l}$ of sample containing the beads was placed into $1.5 \mu\text{l}$ of borate buffer (0.2 M sodium borate, pH = 9.5). Noninjected latex beads served as a control. While mixing on a vortex, $500 \mu\text{l}$ of fluorescamine (20 mg/100 ml acetone) was added in five $100\text{-}\mu\text{l}$ aliquots. The beads were then placed on a glass microscope slide, coverslipped, and observed using a Zeiss microscope equipped for epifluorescence. The excitation/emission was 400/475 nm, respectively.

Scanning Electron Microscopy: Collagen-coated or uncoated beads were placed on polylysine-coated coverslips and allowed to adhere to the surface. The beads were prepared for scanning electron microscopy by two methods which yielded indistinguishable results. Some of the polylysine plates containing beads were fixed in 4% glutaraldehyde in phosphate-buffered saline (pH 7.3) for 2 h. The fixed beads were dehydrated, first in ethanol and then in an ethanol-freon series. After sitting in 100% freon overnight, the fixed beads were critical point-dried. Other collagen-coated or uncoated beads were air-dried on the polylysine plates. The fixed or air-dried bead preparations were mounted on aluminum stubs with silver conducting paint. For observation, the beads were sputter coated and viewed on a Hitachi S-500 scanning electron microscope (Hitachi America, Ltd., NY) at 5 kV.

Quantitative Analysis of Bead Distribution: The overall distribution of beads was determined for embryos fixed 2 and 3 days after injection in each series of experiments. By 2 d postinjection, the endogenous neural crest cells have finished their migratory phase and have coalesced into recognizable ganglia. The location of every bead in an embryo was plotted by camera lucida on a two-dimensional grid of an idealized cross-section through an embryo (see Fig. 2). The diagrammatic embryo was divided into 90 sectors. Every bead was counted and superimposed on the grid, thus collapsing the cranio-caudal axis onto the two-dimensional grid. The number of beads in each sector was determined and expressed as a percentage of total beads for that embryo. The data for each bead type was averaged with every embryo given equal weight. The percentage of beads per sector (rounded to the nearest integer) was illustrated on the schematic representation; values below 1% were not shown.

Electrophoretic Mobility of Beads: The electrophoretic mobility of the latex beads was measured using an electrophoresis apparatus (Rank Brothers, Cambridge, England). The beads were suspended in a solution of 0.1 M KCl (neutral pH) and were placed into the electrophoresis chamber. A voltage of 80 V/cm was passed across the chamber. The beads were viewed through an eyepiece graticule of a microscope which was focused on the stationary layer of the fluid within the cylindrical chamber; in this way, the movement of the beads can be observed while the field is on. By measuring the time it takes the bead to travel a fixed distance (one graticule division), one can

directly calculate the electrophoretic mobility. For each bead type, 10 time measurements were taken and averaged. The electrophoretic mobility is related to the velocity of the bead and the applied voltage by the equation

$$\mu = \frac{(L)(D)}{(T_{av})(V)} \quad (\text{Eq. 1})$$

where μ = electrophoretic mobility; L = effective electrical length; T_{av} = average time to pass a graticule division; D = length of one graticule division; V = applied voltage.

The T_{av} term is the only variable and is measured experimentally in the electrophoresis apparatus. After calculating the electrophoretic mobility, the surface potential can in turn be calculated by the expression

$$\xi = \frac{(\mu)(\eta)}{(\epsilon_r)(\epsilon_0)} \quad (\text{Eq. 2})$$

where ξ = surface potential; μ = electrophoretic mobility; η = viscosity; ϵ_r = permittivity of free space; ϵ_0 = the dielectric constant.

The surface charge is related to the surface potential by the expression

$$\sigma = (I)^{1/2} \sinh \left(\frac{F\xi}{2RT} \right) \frac{1}{136} \quad (\text{Eq. 3})$$

where σ = surface charge; I = ionic strength; F = Faraday's constant; ξ = surface potential; R = Gas constant; T = temperature.

The surface charge is expressed in number of charges per unit area.

RESULTS

To examine the possible role of specific surface macromolecules in the localization process of neural crest cells, latex beads were coated with laminin, type I collagen, polytyrosine, and polylysine. After injection into the posterior somites at the onset of endogenous neural crest migration, the subsequent translocation of the coated beads was monitored. A summary of the pattern of localization for each bead type is given in Table I. To further probe the nature of these interactions, the surface charge properties of the beads were determined before injection and compared with their ability to translocate. In addition, the stability of the coat and possible modifications to the bead surface after intraembryonic injection were considered.

Distribution of Latex Beads after Injection Lateral to the Neural Tube

Latex beads are typically injected into the embryonic somites. The distribution of uncoated latex beads injected into the somitic cavity was reported in a previous study (14); a summary of this data is represented in schematic form in Fig. 2*a* for those embryos fixed 2 and 3 d after intrasomitic injection. 97% of the uncoated beads localized in the vicinity of the sympathetic ganglia, ventral nerve cord, or around the dorsal aorta where the adrenal chromaffin cells coalesce. Embryos fixed 1 d after injection were not included in the schematic representation because translocation is not completed at this time; by 2 d after injection, endogenous neural crest cells have finished their migration and have coalesced into ganglionic structures.

To control for any effects that the somitic injection site may have on the results, latex beads were also injected "lat-

TABLE I
Summary of Injection Data

Injected bead type	Injection site	Number	Localization	
			Ventral	Dorsal
			%	
Uncoated beads	Lateral to neural tube	14	97	3
LM-beads	Somite	11	3	97
Collagen beads	Somite	15	90	10
PT-beads	Somite	13	95	5
PL-beads	Somite	9	12	88

Beads coated with laminin, collagen type I, polytyrosine, or polylysine were injected into the somites of 2½-d-old chick embryos. Uncoated latex beads were also injected lateral to the neural tube. The translocation of the various bead types was monitored in embryos fixed 1–3 d after injection. Summarized above are the bead types injected, injection site, the number of embryos, and the percentage of embryos localized in dorsal vs. ventral locations (for embryos fixed two or more days postinjection).

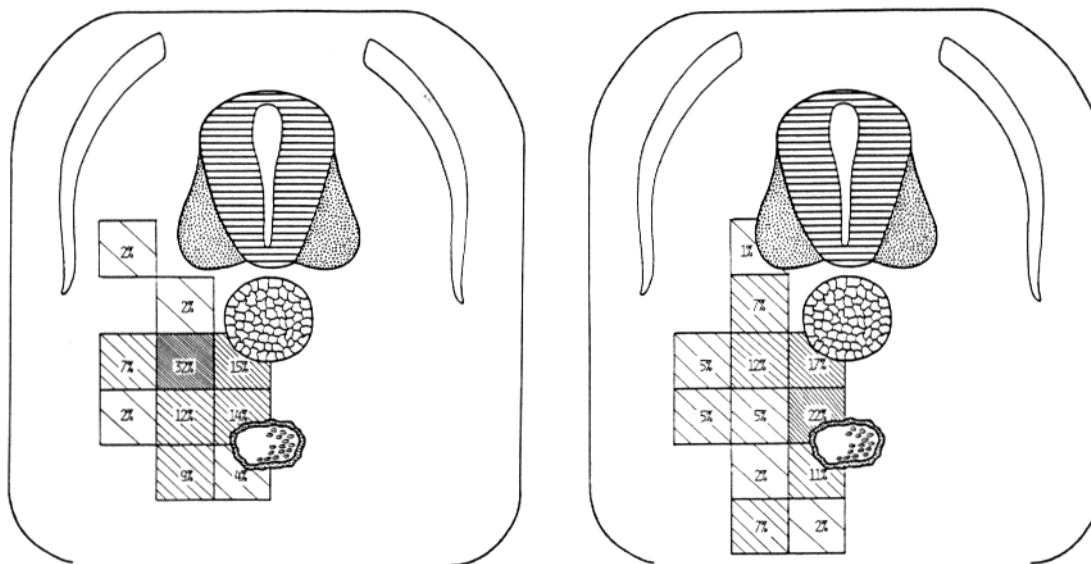


FIGURE 2 Schematic representation of the distribution of uncoated beads in embryos fixed 2 and 3 d after injection. The localization of every bead in all embryos was plotted by camera lucida on a two-dimensional grid of an idealized embryo in cross-section. In each embryo, the percentage of beads in a given sector was determined and then averaged for all embryos. The data are expressed as percentage of beads in a sector, rounded off to the nearest integer; values of <1% are not shown. The density of stippling in each sector is proportional to the percentage of beads localized in that region. (left) Uncoated beads after injection into the somites; $n = 9$. (right) Uncoated beads after lateral injection; $n = 13$.

eral” to the neural tube (between the neural tube and somites) at the onset of host neural crest migration. This injection location places the beads directly onto the ventral pathway, but is more difficult because no natural cavity exists for initially containing the beads. 14 embryos were monitored for up to 3 d after the injection of uncoated beads between the neural tube and somite. The distribution of beads after lateral injection is summarized schematically in Fig. 2*b*. No differences in the pattern of bead localization were found for the lateral injection site vs. the somitic injection site. For those embryos fixed 2 and 3 d after lateral injection, 97% of the beads were found in ventral sites near the sympathetic ganglia, ventral nerve cord, or adrenal chromaffin cells. The beads, therefore, appear to localize in the same regions independent of the implantation site.

Laminin-coated Beads Injected into the Somites

To explore surface adhesion effects which may be involved in restriction from the ventral pathway, latex beads coated with laminin were injected into embryos. The efficacy of coating the beads with laminin was tested with immunofluorescence (see the later subsection *Modifications to the Bead Surface: Immunofluorescent Detection of Laminin*) and with a fluorescamine assay for detecting protein; both methods demonstrated the effectiveness of the coating procedure. The movement of the laminin-coated beads (LM-beads) was traced in 11 embryos fixed 1, 2, and 3 d after injection.

LM-beads did not translocate along the ventral pathway but remained at the implantation site. Fig. 3 is a schematic representation of the distribution of LM-beads in all embryos fixed 2 and 3 d after injection. 97% of the LM-beads were found in dorsal sites—associated with the dermamyotome, ectoderm, and occasionally, lateral to the neural tube. Of the 11 embryos injected with LM-beads, 10 contained LM-beads exclusively in dorsal sites. The one remaining embryo had a few beads (19% of beads in this embryo) in more ventral sites, such as around the notochord; the remainder of the LM-beads in this embryo, however, remained at the implantation site. Fig. 4 shows sections through individual embryos fixed 2 and 3 d postinjection, which reflect the typical distribution of LM-beads. 2 d after injection, LM-beads were predominantly associated with the somitic remnant, with beads occasionally found adjacent to the ectoderm (Fig. 4, *a* and *b*). The results for those embryos fixed 3 d after injection were similar to those fixed after 2 d; the LM-beads were found associated with the dermamyotome or adjacent to the ectoderm (Fig. 4, *c* and *d*). One embryo had LM-beads dorsally adjacent to the neural tube. Thus, LM-beads appear to be restricted from the ventral neural crest pathway in a way that is similar to the case of FN-beads in our previous study (14).

Collagen-coated Beads Injected into the Somites

To probe the possible cell surface function of the collagen molecule, latex beads were coated with type I collagen and subsequently injected into the trunk somites. The efficacy of the coating procedure was tested by immunofluorescence (see the later subsection *Modification of the Bead Surface: Immunofluorescent Detection of Collagen*), by scanning electron microscopy (see below), and by a fluorescamine assay; all three methods indicated that the beads were effectively coated with collagen. 15 embryos were monitored after the intrasomatic injection of collagen-coated beads. Beads coated with

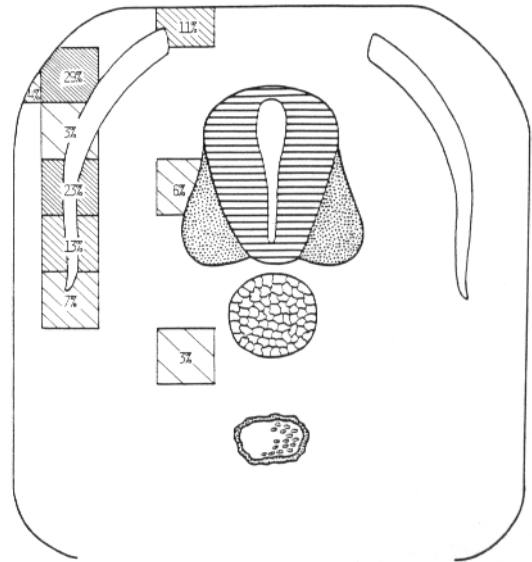


FIGURE 3 Schematic representation of the distribution of LM-beads in embryos fixed 2 and 3 d after somitic injection. Data was analyzed as described in Fig. 2 and Materials and Methods; $n = 6$.

type I collagen translocated ventrally along the pathway taken by endogenous crest cells. The distribution of collagen-coated beads is represented schematically in Fig. 5 for embryos fixed 2 and 3 d after injection. 90% of the beads were found in ventral sites. The collagen beads settled adjacent to and often within sympathetic ganglia or adrenomedullary sites. By 2 d after injection, the beads were generally found above the dorsal aorta or around the notochord, in the vicinity of the primary sympathetic ganglion. Fig. 6 is one example of an embryo fixed 2 days postinjection where the beads have settled within the primary sympathetic ganglion. In ~30% of the embryos fixed at 2 d, the collagen-coated beads were found localized in the primary sympathetic ganglia. In one embryo fixed at 2 d, the beads did not translocate ventrally but remained associated with the dermamyotomal remnant of the somite. In all embryos fixed 3 d after injection, the collagen beads were found ventrally along the pathway—predominantly adjacent to the dorsal aorta where the chromaffin cells coalesce, or next to the sympathetic ganglia. The distribution of the collagen-coated beads was similar to that of uncoated latex beads reported previously (14; see Fig. 2*a*). Unlike the uncoated beads, the collagen-coated beads showed a marked tendency to cluster.

Scanning Electron Microscopy of the Collagen Beads

To examine the arrangement of collagen on the bead surface, collagen-coated beads were viewed with the scanning electron microscopy. The most striking feature of the collagen beads was the fibrils present on the surface. Because of the limited resolution of the scanning electron microscopy the fibrils were most readily observed connecting beads together or radiating from the bead surface (Fig. 7, *a* and *b*). In contrast, no fibrils were seen on or between uncoated latex beads.

Polytyrosine-coated Beads Injected into the Somites

At physiological pH, tyrosine is a neutrally charged amino acid. In order to produce a bead with neutral surface charge,

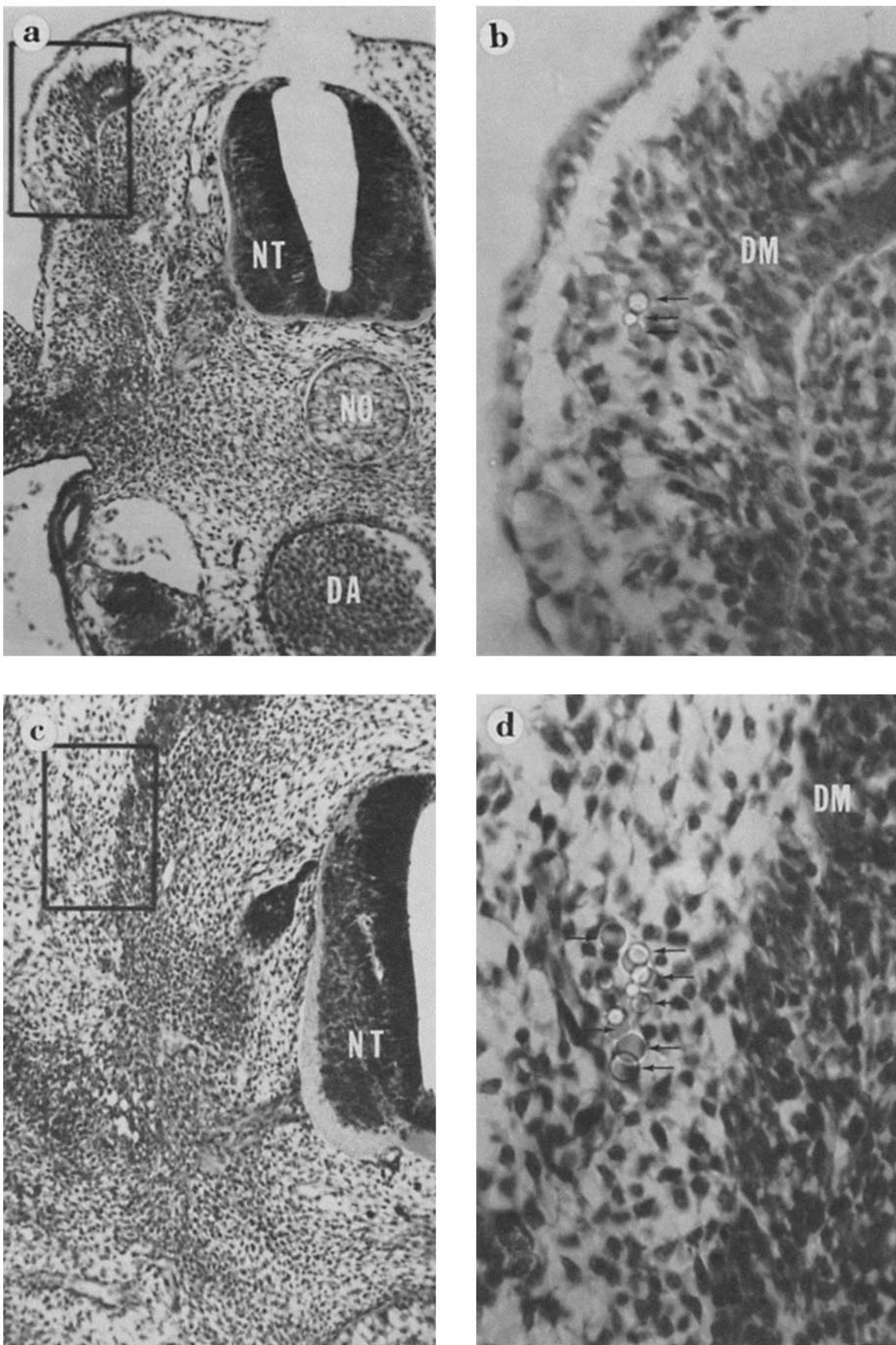


FIGURE 4 Light photomicrographs of transverse sections through embryos after injection of LM-beads into the somites. (a) An embryo 2 d after injection shown at low magnification ($\times 144$); (b) higher magnification of inset in a demonstrates that the LM-beads remain associated with the dermamyotomal remnant of the somite ($\times 570$); (c) another embryo fixed 3 d postinjection ($\times 144$); (d) higher magnification of inset in c shows that the LM-beads associated with the dermamyotome ($\times 570$). *NT*, neural tube; *NO*, notochord; *DA*, dorsal aorta; *DM*, dermamyotome. Arrows indicate the position of the beads.

the latex beads were coated with polytyrosine. The D-form of the polyamino acid was chosen to avoid excessive digestion of the polytyrosine coat. A fluorescamine assay was used to determine the effectiveness of the coating procedure. When observed under the fluorescence microscope, polytyrosine-

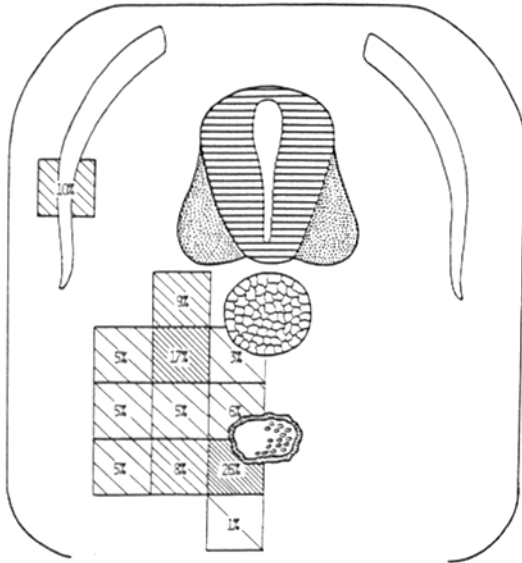


FIGURE 5 Schematic representation of the distribution of collagen-coated beads in embryos fixed 2 and 3 d after injection into the somites. Data was analyzed as described in Fig. 2 and Materials and Methods; $n = 11$.

coated beads were fluorescent whereas uncoated latex beads lacked fluorescence.

Freshly prepared polytyrosine-coated beads (PT-beads) were injected into the somites of 2.5-d-old chicken embryos at a time when endogenous neural crest migration was just beginning. The distribution of the PT-beads was monitored in 13 embryos fixed 1, 2, and 3 d after injection. The beads translocated along the ventral neural crest pathway with a time course and distribution pattern similar to that of uncoated latex beads and to endogenous neural crest cells. Fig. 8 is a schematic representation of the distribution of PT-beads in embryos fixed 2 and 3 d postinjection. 95% of the beads localized in ventral sites, typically above the dorsal aorta, adjacent to the sympathetic ganglion, or around the notochord. Fig. 9 shows sections from an embryo fixed 3 d after injection with beads located above the dorsal aorta. In 11 embryos, all beads localized in ventral sites. 2 of 13 embryos had a small number (~20% in each case) of the PT-beads in the dermamyotomal remnant of the somite, although the remainder of beads in these embryos were found in the ventral sites. Thus, the poly-tyrosine coated beads distributed along the ventral pathway analogously to uncoated latex particles.

Polylysine-coated Beads Injected into the Somites

Lysine is a basic amino acid at physiological pH. Latex beads were coated with poly-D-lysine in order to produce beads with a positive surface charge. The D form of the

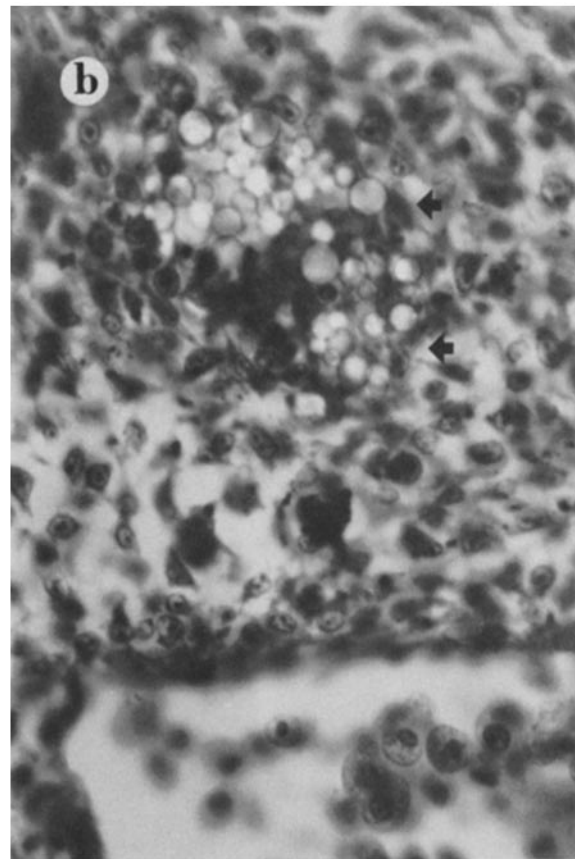
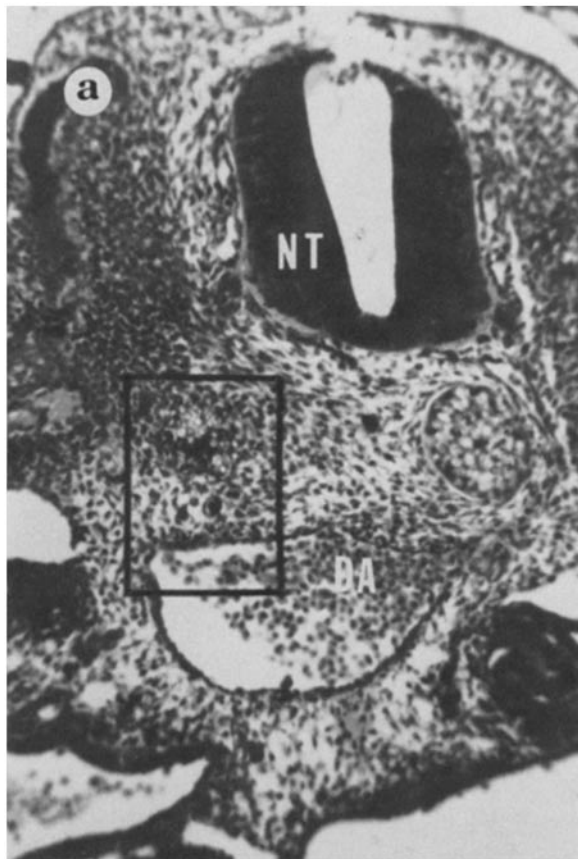


FIGURE 6 Light photomicrographs of an embryo 2 d after injection of collagen-coated beads into the somite. (a) Low magnification ($\times 144$); (b) higher magnification of inset in a shows the collagen beads surrounding and within the primary sympathetic ganglion, just above the dorsal aorta ($\times 570$). NT, neural tube; DA, dorsal aorta. Arrows mark the location of the beads.

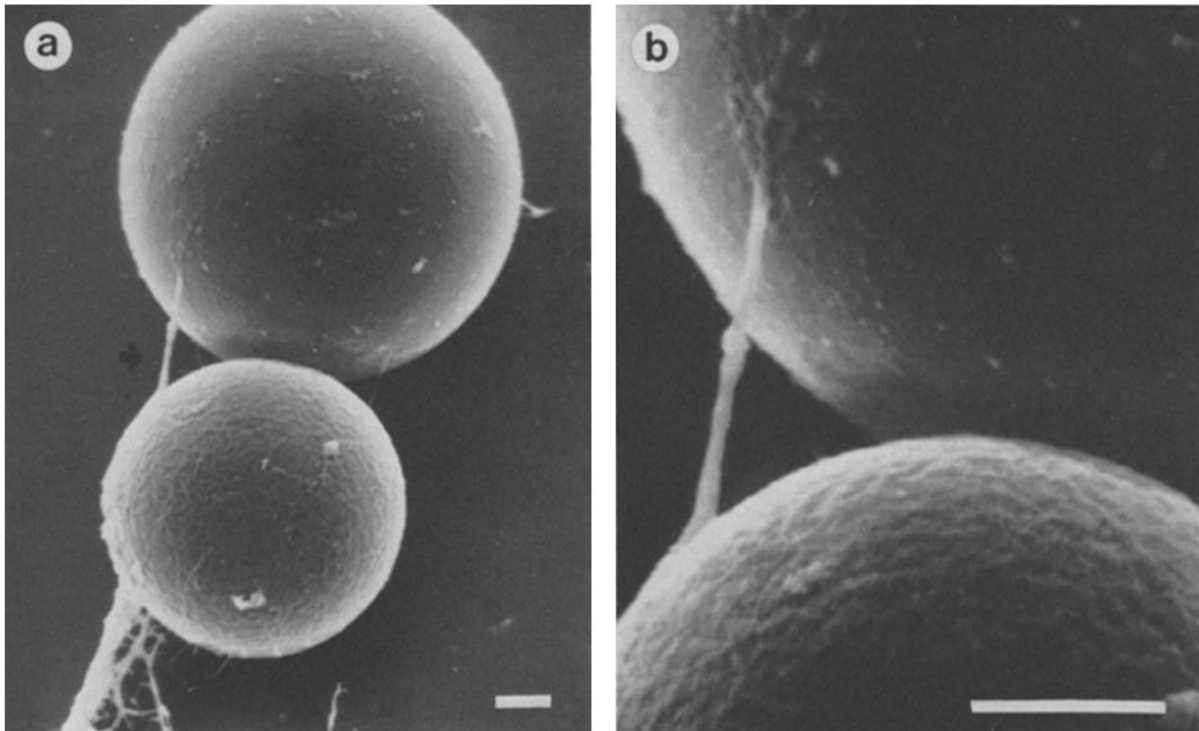


FIGURE 7 Scanning electron micrograph of collagen-coated beads. The collagen-bead surface generally had an irregular texture. Fibrils can be observed coming off the surface of the beads as well as connecting adjacent beads. (a) $\times 8,000$; the arrow indicates the collagen fibril shown at higher magnification in *b*. (b) $\times 23,000$. Bars, $1 \mu\text{m}$.

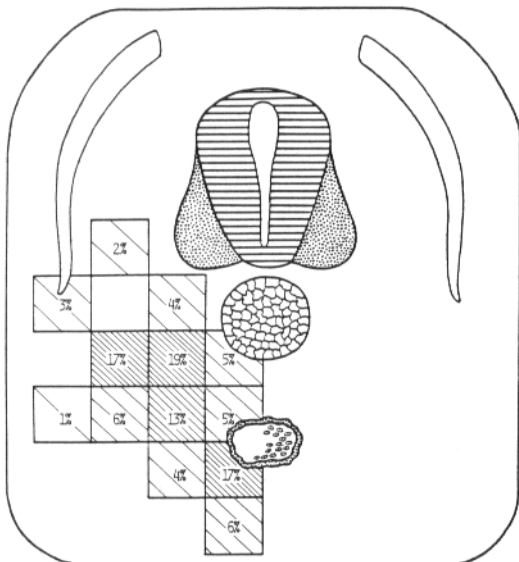


FIGURE 8 Schematic representation of the distribution of PL-beads in embryos fixed 2 and 3 d following somitic injection. Data was analyzed as described in Fig. 2 and Materials and Methods; $n = 9$.

polyamino acid was chosen to avoid excessive digestion of the surface coat. To assay the efficacy of the coating procedure, a fluorescamine assay was used to stain the beads. Like polytyrosine beads described above, the polylysine beads were fluorescent, indicating they were effectively coated.

When the polylysine coated beads (PL-beads) were injected into the posterior somites in the trunk region, they behaved differently from either uncoated latex beads or the polytyrosine beads. Instead of translocating along the ventral neural

crest pathway, the PL-beads remained primarily at the implantation site. In all nine of the embryos into which PL-beads were injected, the majority of beads were found associated with the remnants of the somitic mesenchyme or adjacent to the ectoderm. Fig. 10 is a schematic representation of the distribution of PL-beads in embryos fixed 2 and 3 d after injection. In these embryos, 88% of the beads were found in dorsal sites, associated with the dermamyotome, sclerotome, or adjacent to the ectoderm. Examples of cross-sections containing PL-beads are illustrated in Fig. 11. Fig. 11, *a* and *b* shows an embryo 2 d after injection, with PL-beads associated with and actually embedded in the dermamyotome. The presence of beads within the somitic epithelium was only observed in the case of positively charged beads. The mechanism by which the PL-beads cross the basement membrane and become incorporated into the epithelium remains unclear. Another embryo fixed 3 d after injection is depicted in Fig. 11, *c* and *d*. Again, even at this later time, the PL-beads were found in the dermamyotome and not further ventrally along the neural crest pathway. Occasionally, the PL-beads were found contiguous to ectodermal tissue. The data indicates that positively charged polylysine on the bead surface caused restriction of beads from the ventral neural crest pathway. These results are similar to those obtained for beads coated with the adhesive glycoproteins, fibronectin and laminin. The adhesion of PL-beads to the cells at the implantation site is not surprising, since this molecule is well known to adhere to embryonic cells by means of strong electrostatic interactions.

Electrophoretic Mobility and Surface Charge of Latex Beads with Various Surface Coats

The above results indicate that positively charged PL-beads behave similarly to LM- and FN-beads and remain at the

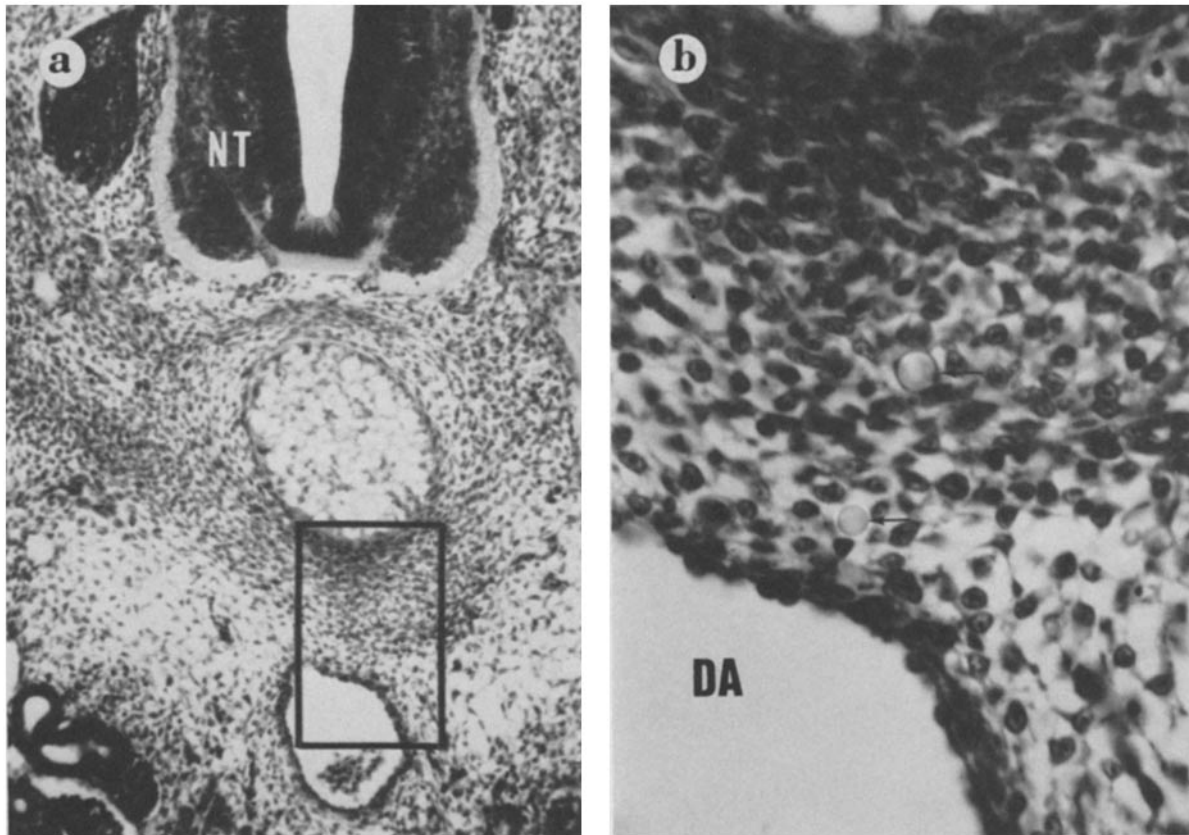


FIGURE 9 Light photomicrograph of a transverse section through a chicken embryo 3 d after injection of poly-D-tyrosine-coated latex beads into the posterior somites. (a) At low magnification, the PT-beads are found on the ventral pathway below the notochord and above the dorsal aorta. $\times 144$. (b) A high magnification view of the inset in a. $\times 570$. NT, neural tube; DA, dorsal aorta; arrows indicate the position of the beads.

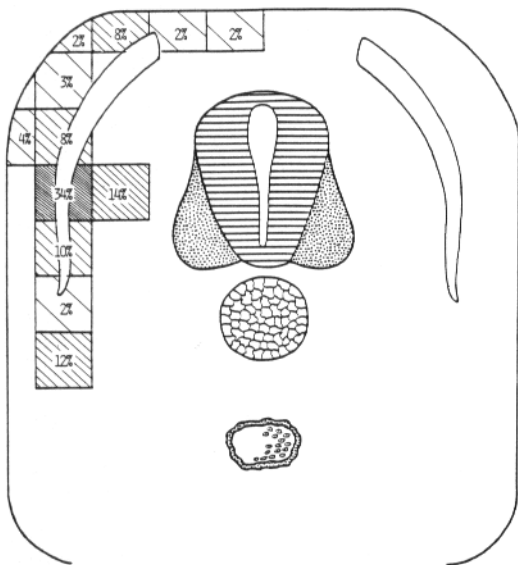


FIGURE 10 Schematic representation of the distribution of PL-beads in embryos fixed 2 and 3 d following injection into the somites. Data was analyzed as described in Fig. 2 and Materials and Methods; $n = 7$.

implantation site after injection onto a neural crest pathway. Two possible explanations may account for this: (a) LM- and FN-beads may adhere to the implantation site by some specific adhesion mechanism; or (b) FN- and LM-beads may

carry a positive charge and thus remain at the implantation site because of nonspecific electrostatic interactions. To distinguish between these possibilities, it was necessary to determine the surface charge of the beads used in this and previous studies. The surface charge can be determined by measuring the electrophoretic mobility of the latex beads.

The electrophoretic mobility of beads coated with a variety of macromolecules was measured in an electrophoresis chamber. When a constant voltage is placed across the chamber, neutrally charged beads do not move, negatively charged beads move toward the anode, and positively charged beads move toward the cathode. The speed at which the beads move can be used to calculate the surface charge of the beads (using Eqs. 1–3 in Materials and Methods). Uncoated latex particles and latex beads coated with the following types of molecules were tested: poly-D-lysine, poly-D-tyrosine, bovine serum albumin, collagen, fibronectin, and laminin. The data are summarized in Table II. The data shows that the PL-beads were positively charged and the PT-beads were neutrally charged as predicted. All of the beads coated with naturally occurring macromolecules were negatively charged. Ranked from least to most negatively charged, the bead types were collagen, bovine serum albumin, uncoated, fibronectin, and laminin.

PL-, FN-, and LM-beads were all restricted from the ventral pathway. Yet the surface charge of PL-beads is positive, whereas that of both fibronectin and laminin is negative. LM-beads were, in fact, the most negatively charged of all the coated beads produced. Because both positively charged beads and some negatively charged beads fail to translocate along

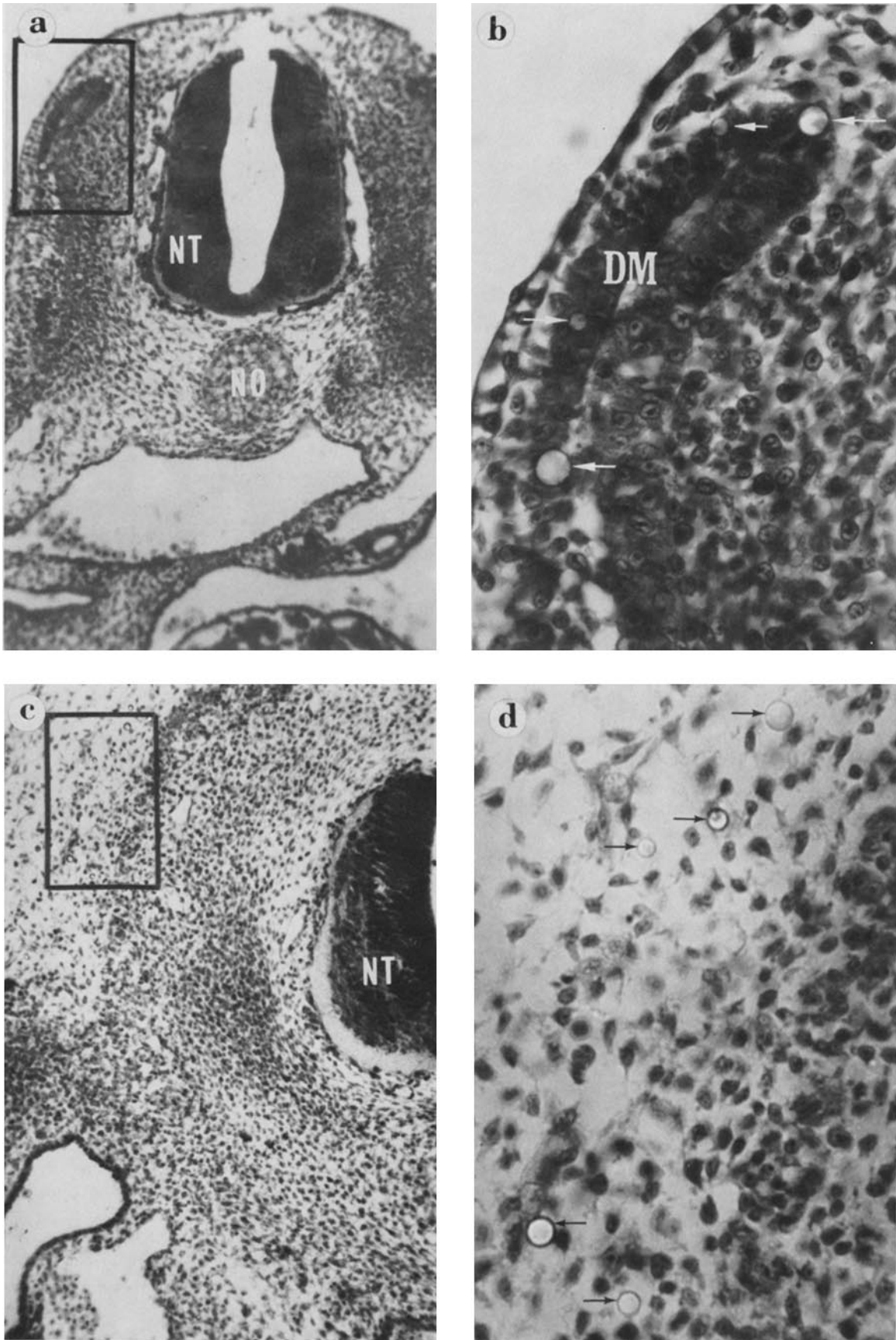


FIGURE 11 Light photomicrographs of transverse sections through embryos after injection of poly-D-lysine-coated beads. (a) Low magnification of an embryo 2 d postinjection. $\times 144$ (b) Higher magnification of inset in a shows that the beads did not move ventrally, but remained associated with the dermamyotomal remnant of the somite $\times 570$. (c) Another embryo fixed 3 d following injection. $\times 144$. (d) Higher magnification of inset in c shows the beads associated with the cells of the dermamyotome $\times 570$. *NT*, neural tube; *NO*, notochord; *DM*, dermamyotome. Arrows mark the position of the beads.

TABLE II
Surface Properties of Coated Latex Beads

Latex bead surface coat	Surface charge charges/Å ² × 10 ⁻⁵	Electrophoretic mobility μm/5 per V/cm	Ventral translocation
Polylysine	+3.1	+1.4	No
Polytyrosine	0.0	0.0	Yes
Collagen	-1.6	-0.8	Yes
BSA	-2.0	-1.0	Yes
Uncoated	-2.3	-1.1	Yes
Fibronectin	-2.5	-1.2	No
Laminin	-5.9	-2.6	No

Summary of the electrophoretic mobility, surface charge, and translocation ability of the bead types used in this and a previous study (14).

the ventral pathway whereas other negatively charged beads do translocate ventrally, there exists no correlation between the sign of the surface charge and subsequent bead localization.

Modifications to the Bead Surface

Because the distribution of latex beads depends upon surface properties, it seems likely that the bead surface interacts with the embryonic milieu. It is, therefore, possible that the surface coat might be modified by interactions within the embryo. The experiments below address (a) if the surface coat remains on the beads after injection; and (b) if additional substances are added to the bead surface within the embryo.

IMMUNOFLUORESCENT DETECTION OF LAMININ ON THE LM-BEAD SURFACE: The presence of laminin on the bead surface was examined before and after injection. Using immunofluorescence, the presence of various proteins and glycoproteins can be detected on the bead surface (14). Freshly prepared LM-beads were incubated with an antibody to laminin, and subsequently with a second fluorescent antibody. Virtually 100% of the beads coated with laminin were brightly fluorescent indicating that at the time of injection, all LM-beads possessed laminin of their surfaces. As a control, uncoated latex beads or beads coated with other macromolecules (collagen, polytyrosine, polylysine) were processed in the same manner; these were nonfluorescent.

By 2 d postinjection, host neural crest migration is completed, gangliogenesis has started, and beads within the embryos have localized. This time point was, therefore, selected for recovery of injected beads. The portion of the embryo containing the LM-beads was excised, macerated, and processed immunohistochemically to detect laminin. After processing, 71 ± 7% of the LM-beads removed from embryos (*n* = 8) retained their antibody staining (Fig. 12, *a* and *b*). For those beads which did not bind fluorescent antibody, it is not clear whether the laminin was cleaved from the bead surface, or if the antigenic site was masked by other molecules that bound to the laminin or to the bead surface. Control LM-beads (not injected but the same batch as those injected into embryos) retained 100% antibody staining.

IMMUNOFLUORESCENT DETECTION OF COLLAGEN ON THE COLLAGEN-BEAD SURFACE: For collagen-coated beads, the presence of collagen before and after injection was determined using an antibody to type I collagen. Using immunofluorescence, freshly prepared collagen beads were stained with the collagen antibody, followed by a second fluorescent antibody. Before injection, virtually 100% of the

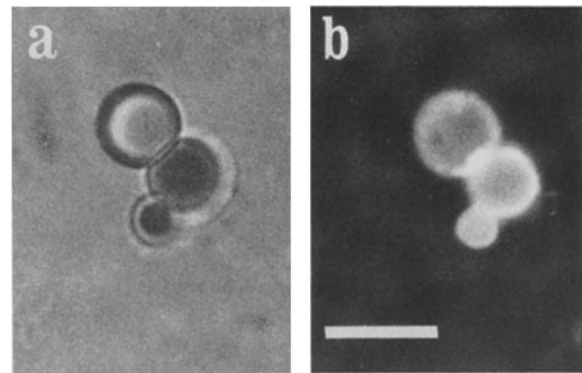


FIGURE 12 Light and fluorescence photomicrographs of LM-coated beads after immunohistofluorescent staining with an antibody to laminin. The LM-beads were injected into embryos, removed 2 d later, and stained with antilaminin. Many of the beads retained antibody staining 2 d following injection. Bar, 10 μm.

collagen-coated beads were antibody positive. Uncoated and laminin-coated beads exhibit no collagen immunofluorescence. The collagen beads were injected into embryos (*n* = 4), excised, and processed 1 or 2 d after injection. 72 ± 6% of the beads retained antibody staining after 2 d *in vivo*. This result is similar to that observed for LM-coated beads described above.

IMMUNOFLUORESCENT DETECTION OF HETEROLOGOUS MOLECULES AFTER INJECTION: In order to determine if the beads accumulated fibronectin after injection *in vivo*, beads (uncoated or coated with laminin, collagen, polytyrosine, and polylysine) were recovered 2 d after implantation and processed with antibodies to chick fibronectin. After removal of collagen beads from embryos (*n* = 6), 15% ± 13% of the recovered collagen-coated beads were fibronectin positive. None of the other bead types exhibited detectable fibronectin immunofluorescence.

As an assay for the accumulation of laminin on beads after intraembryonic injection, uncoated beads and beads coated with collagen, polytyrosine, and polylysine were injected into embryos and recovered 2 d later. After processing with antibodies for laminin, about 4 ± 6% of embryos (*n* = 6) injected with collagen-coated beads stained positively for laminin. Laminin staining was not detectable on beads with other surface coats. It was not possible to assess whether or not other bead types picked up collagen after intraembryonic injection due to lack of antibody cross-reactivity.

DISCUSSION

In the present study, latex beads coated with a variety of ECM molecules and poly amino acids were injected into embryos in order to explore interactions between the cell surface and the embryonic substrata that may be involved in neural crest localization. The results demonstrate that the pattern of bead distribution along the ventral neural crest pathway is altered by the nature of the surface coat. Two distinct patterns of localization were observed: one class of beads (coated with laminin or polylysine) remained primarily associated with the dermamyotomal cells of the implantation site; the second class of beads (coated with collagen and polytyrosine) translocated to ventral sites, usually around the sympathetic ganglia or dorsal aorta. The initial surface charge of beads coated with various macromolecules was measured and compared with

their subsequent ability to translocate along the ventral pathway. The results suggest that initial surface charge properties alone cannot account for the restriction or translocation of beads coated with laminin or fibronectin.

It is intriguing that laminin-coated and polylysine-coated beads, which were injected into the somitic lumen, were later found superficial to the dermamyotome. Thus, their final localization was on the opposite side of the somitic epithelium to their implantation site. The mechanisms responsible for this distribution pattern remain unclear. Possibly, the beads sorted to this superficial site during reorganization of the somitic epithelium into the dermatome and myotome. The lamination of the somite is itself an important and poorly understood phenomenon. Perhaps the use of techniques similar to those utilized here to probe neural crest localization may also offer some insight into the mechanisms responsible for somitic development.

In the majority of experiments, the latex beads were introduced into embryos by injection into the somitic cavity. The beads were initially localized in the lumen of the somite and were subsequently released onto the ventral neural crest pathway as the sclerotomal cells dispersed. Upon release, they presumably intermingled with neural crest cells (2). Endogenous neural crest cells initiate their migration from the dorsal neural tube and may primarily migrate ventrally between the neural tube and somites or between the somites (26). Therefore, it seems important to consider the effects of the "somitic" implantation site on the localization of injected beads. To control for possible effects related to the injection site, latex beads were also injected "laterally", i.e., placed adjacent to the neural tube as crest migration was beginning. The final distribution of beads was indistinguishable for both the lateral and the somitic injection sites. The fact that similar results were obtained for both injection sites adds credibility to the idea that beads localize according to their surface properties and not as a function of their implantation site. The somitic injection site remains preferred because the injected particles can be initially contained within the somitic cavity in a reproducible manner.

The microinjection method for implanting cells or inert substances into embryos makes it possible to explore some aspects of cell surface-ECM interactions during development. By using latex beads, the system can be reduced to simpler components because the beads lack motility and metabolic activity. The beads can be coated with a single surface molecule and, unlike living cells, cannot turnover their surface coat. However, it remains possible that this initially defined coat is altered with time in the embryo. For example, additional macromolecules may be absorbed onto the bead surface or portions of the surface coat may be enzymatically cleaved. Since both the initial surface coat and possible modifications incurred by interactions with embryonic tissues may be important for final localization of latex beads after injection, it was important to examine potential alterations. Immunofluorescence was used to assess the surface properties of laminin- or collagen-coated beads recovered after two days in ovo; ~70% of the beads were found to retain their coat. For those beads which were not antigenic, it remains unclear if the surface coat was enzymatically removed, or masked by the binding of other molecules. A small percentage of collagen-coated beads were found to absorb fibronectin and laminin following intraembryonic injection. For other bead types, however, no accumulation of fibronectin or laminin was

observed at levels detectable by immunofluorescence. Thus, the majority of the beads maintain their original surface coat, supporting the idea that the initial surface properties are largely responsible for the subsequent distribution of the beads. It should be noted, however, that some physical properties, such as surface charge, can only be determined before injection. Subtle changes in surface properties are, therefore, likely to escape detection by our present techniques.

The mechanisms responsible for the initiation of migration, the movement along defined pathways, and final localization of neural crest cells, are not yet understood. Because latex beads can localize in sites adjacent to neural crest cells, it seems possible that the mechanisms of neural crest localization are largely independent of active motility, and primarily involve properties of the cell surface. In other systems as well, inert particles can undergo cell-like displacements. Wiseman (27) placed metal, glass, and plastic spheres into tissue masses, and found that these inert particles were moved to the interior of the tissues. Obviously the particles did not themselves actively move into tissues, but rather were mixed by the movement of cells around them allowing them to respond to their surface properties. It has been suggested that tissues sort out according to simple physical interactions that could be mediated by nonspecific adhesive events (28). In the differential adhesion hypothesis (29, 30), Steinberg suggests that cellular adhesive properties can guide tissue movements because cells which are motile and adhesive will tend to assemble in a manner which maximizes their adhesive interactions. An example of the fact that cells tend to maximize adhesive interactions comes from the work of Carter (31) and others (32, 33), who have found that cultured cells placed on a gradient of adhesivity will move in the direction of increasing adhesiveness. Gradients of adhesiveness may account for cell movement in some developing systems as well; for example, amphibian pronephric duct migration has been interpreted as responding to a traveling gradient of adhesiveness (34). In a situation where inert particles are gently mixed by an external force, latex beads could also respond to an adhesive gradient. Therefore, the mixing produced by the movement of neighboring cells within the embryo together with a gradient of adhesiveness, could account for translocation of some bead types. While no adhesive gradients have as yet been detected along neural crest pathways, this phenomenon could account for orientation in cell migration and bead translocation.

ECM molecules may be important for mediating adhesive cell interactions during many morphogenetic events by providing an appropriate substratum on which cells can move and by mediating adhesions between the cell and the substratum. For example, the migration of cultured fibroblasts, which synthesize fibronectin and adsorb it onto their cell surface (35, 36), appears to be stimulated by addition of exogenous fibronectin (37); this response is amplified in transformed fibroblasts which have lost cell surface fibronectin. Therefore, the balance between fibronectin on the cell surface and in the substratum may determine the migratory behavior of these cells. Other ECM molecules like laminin and collagen may also be involved in cell migration and guidance. Laminin is a glycoprotein which is distinct from fibronectin by many criteria including amino acid composition and absence of immunological cross-reactivity (38). It has been found in a wide variety of basement membranes (39-43) and may mediate the attachment to basement membranes of certain epithelial cell types that cannot utilize fibronectin (44-46). Type I

collagen, which is produced by the epithelial cells of the neural tube that lie adjacent to the ventral neural crest pathway (10), is probably a component of the fibrillar material found in the ECM during crest migration.

During embryogenesis, the composition of the ECM is dynamic. Fibronectin is present in high concentrations around the neural tube and somites as crest cells are undergoing their migration (8, 9). After neural crest migration and during gangliogenesis, the levels of fibronectin in the ECM are reduced (8). The loss of this adhesive glycoprotein from the ECM may help shift the balance of adhesions to favor crest cell aggregation. Temporal changes in cell surface molecules also appear to contribute to changes in adhesiveness along neural crest routes. The neural cell-adhesion molecule can be identified by immunohistochemical staining in the dorsal neural tube before neural crest migration (47). During active crest migration, no neural cell-adhesion molecule staining is observed; however, staining returns in neural crest regions after the cells condense to form ganglia. Thus, the dynamic nature of the cell surface and the ECM along neural crest pathways may cause changes in adhesiveness which contribute to neural crest cell localization by a differential adhesion mechanism (29).

Recent experiments using a vibrating probe (48) have measured small electrical fields around developing embryos both in the chick primitive streak (49) and in the *Xenopus* neurula (50). In *Xenopus*, currents entering near the neural tube and exiting from the ventral side of the embryo have been measured. It has been proposed that such small electrical fields may represent a guiding force for neural crest cells. In tissue culture, both avian (51) and *Xenopus* (52, 53) neural crest cells respond to electric fields by orienting perpendicular to the field and migrating toward the cathode. The charged latex beads used in the present study do not appear to be influenced by these postulated weak electrical fields. Positively charged beads, if responding to this electric field, would be expected to move toward the cathode, i.e., ventrally; negatively charged beads should move dorsally toward the anode; and neutrally charged beads should not move at all. In contrast, positively charged beads remained dorsally after injection onto a neural crest pathway, whereas both negatively and neutrally charged beads moved to more ventral sites. Thus, if electric fields are present in avian embryos, they are not sufficiently strong to overcome electrostatic or other interactions between the beads and their surrounding environment.

The present study examined the distribution along a neural crest pathway of latex beads coated with single species of ECM molecule. In localization along the ventral pathway, it is unlikely that a single macromolecule functions alone but rather that a synergism of numerous surface molecules function together to guide developmental processes. Before one can understand the complex interrelationships, it is first necessary to investigate individual components. The results clearly show that the molecules present on the surface do affect localization of latex beads, since LM-beads remained at the implantation site whereas collagen beads moved to the most ventral sites. The data suggest that movement of the beads coated with ECM molecules does not result from surface charge effects alone; rather, the macromolecular coat appears to confer cell-like properties to the bead surface. By using an injection technique for in vivo implantation of latex beads with defined surface properties, one can explore the role of adhesion in localization along an embryonic pathway.

Although neural crest cells are inherently migratory, it appears that much of their localization in distinct sites could be independent of cell motility; rather, crest cells may localize in response to the adhesive properties of their cell surfaces.

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