Neural Crest Cell Migration: Requirements for Exogenous Fibronectin and High Cell Density

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ABSTRACT Cells of the neural crest participate in a major class of cell migratory events during embryonic development. From indirect evidence, it has been suggested that fibronectin (FN) might be involved in these events. We have directly tested the role of FN in neural crest cell adhesion and migration using several in vitro model systems.

Avian trunk neural crest cells adhered readily to purified plasma FN substrates and to extracellular matrices containing cellular FN. Their adhesion was inhibited by antibodies to a cell-binding fragment of FN. In contrast, these cells did not adhere to glass, type I collagen, or to bovine serum albumin in the absence of FN. Neural crest cell adhesion to laminin (LN) was significantly less than to FN; however, culturing of crest cells under conditions producing an epithelioid phenotype resulted in cells that could bind equally as well to LN as to FN.

The migration of neural crest cells appeared to depend on both the substrate and the extent of cell interactions. Cells migrated substantially more rapidly on FN than on LN or type I collagen substrates; if provided a choice between stripes of FN and glass or LN, cells migrated preferentially on the FN. Migration was inhibited by antibodies against the cell-binding region of FN, and the inhibition could be reversed by a subsequent addition of exogenous FN. However, the migration on FN was random and displayed little persistence of direction unless cells were at high densities that permitted frequent contacts. The in vitro rate of migration of cells on FN-containing matrices was 50 μm/h, similar to their migration rates along the narrow regions of FN-containing extracellular matrix in migratory pathways in vivo.

These results indicate that FN is important for neural crest cell adhesion and migration and that the high cell densities of neural crest cells in the transient, narrow migratory pathways found in the embryo are necessary for effective directional migration.

The mechanisms involved in the directional migration of embryonic cell types is still poorly understood. The properties of migratory cells and their interactions with the environment through which they migrate can be studied particularly effectively with neural crest cells (22, 26, 28, 35, 50).

The neural crest is a transient structure that forms during embryonic development at the dorsal border of the closing neural tube. After extensive migration along a series of defined pathways, neural crest cells accumulate at specific sites (14, 44, and references therein). Thereafter, they differentiate into a variety of different types of tissues such as the peripheral nervous system, numerous cranio-facial structures, and melanocytes (reviewed by Le Douarin, 27).

During most of their migration, crest cells progress through an intricate network of fibrillar structures of differing diameters (5, 29, 48, 49). In avian embryos, it is thought that the 3-nm fibers contain large amounts of glycosaminoglycans especially hyaluronic acid, whereas fibronectin (FN) is present in the 10-nm fibers (31). There are also collagens type I and III possibly associated with the FN fibers (20; Duband et al., Manuscript in preparation). Immunohistological studies with anti-fibronectin antibodies reveal that crest cells migrate in narrow spaces which form transiently between FN-rich basement membranes of adjacent tissues. In all cases, available cell-free spaces are rapidly occupied by the crest cells, whereas compact epithelial tissues, surrounded by a laminin-rich basal lamina, are not

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invaded (14, 44, and Duband et al., Manuscript in preparation).

Grafting of neural crest cells into the middle of the presumptive trunk migratory pathway showed that migration could occur both forwards and backwards (17). Migration was much more restricted when the transplantation was performed in territories where host crest cells had completed their migration (51).

Taken together, all these data suggest that crest cells use transient, narrow, acellular pathways without encountering cues for directionality such as haptotaxis or chemotaxis.

Numerous studies have documented the function of FN as an attachment protein for fibroblastic cells. FN has also been shown to stimulate the migration of fibroblastic cells (3, 53). Mesenchymal cells emigrating from chick heart explants require both exogenous plasma- and cell-derived FN. However, cellular FN was found to be present only as small plaques at the cell surface, whereas cessation of migration correlated with the appearance of a dense fibrillar FN-rich matrix. Stationary anchorage was also promoted by the addition of cellular FN (11).

A general conclusion from these studies of the influence of FN on fibroblastic cells is that it can stimulate adhesion and/or motility, depending on its type and local concentration.

An unusual property of most neural crest cells is that they lack FN, and it has been suggested that they might be particularly sensitive to regulation by exogenous FN (33). Previous in vitro studies indicated that FN could mediate crest cell attachment to collagen and could act as an attractant in Boyden chamber assays (18). Migration on FN-containing substrates was favored (13) but it could be affected by the presence of undersulfated chondroitin sulfate (34).

In this work, we have studied the role of the adhesive glycoproteins FN and LN in the adhesion and motility of neural crest cells in vitro using various extracellular matrix (ECM) components and antibodies to the cell-binding fragment of FN. We found that FN was required for neural crest cell adhesion and motility. In contrast to fibroblasts, crest cell migration was enhanced in FN-rich ECM. An additional requirement for reproducing an in vivo rate of directional migration in these cell culture model systems was to maintain a high cell density similar to that found in vivo.

MATERIALS AND METHODS

Extracellular Matrix Components

COLLAGEN TYPE I: Rat tail tendon collagen was prepared according to Bornstein (6) using an acetic acid extraction procedure.

LAMININ: Laminin was extracted from an EHS sarcoma homogenate and further purified as described previously (47). Laminin was stored at 4°C, at a concentration of 0.2–0.4 mg/ml in 0.05 M Tris-HCl, 0.4 M NaCl, pH 7.4. Fragments of laminin were obtained by digestion with trypsin or with elastase (36).

FIBRONECTIN: Plasma fibronectin was purified from human and chick plasma on a gelatin-sepharose column (16). Fibronectin was eluted from the column with 8 M urea, 0.1 M citrate buffer pH 4.7, 2 mM phenyl methyl sulfonyl fluoride (PMSF) (25), dialyzed against 1 mM NH_4OH to avoid precipitation, lyophilized, and stored at −70°C.

Cellular fibronectin was obtained from confluent chick embryo fibroblast cultures extracted with 1 M urea and 2 mM PMSF (52). The 160-kilodalton (kd) cell-binding fragment was prepared by limited proteolysis with chymotrypsin and was purified by sequential gelatin affinity and Sephacryl S-300 (1.5 x 100 cm) molecular sieve chromatography. Its cell binding activity was determined according to Hahn and Yamada (19). The preparations were over 97% pure according to SDS PAGE.

CHICK EMBRYO FIBROBLAST MATRICES: The procedure of Chen et al. (9) was followed with slight modifications. Secondary fibroblast cultures were grown on square 24 x 24 mm or round 32-mm diameter glass coverslips, or on 100-mm plastic tissue culture dishes (Falcon Labware, Oxnard, CA) to confluence in the presence of Dulbecco's Modified Eagle's Medium (DME) supplemented with 3% fetal calf serum (FCS) (Gibco, Europe Ltd., Scotland) and 50 μg/ml ascorbic acid (Serva, Heidelberg, RFA).

Cultures were washed with phosphate-buffered saline (PBS), pH 7.4, then with 20 mM glycine, 2 mM $MgCl_2$, 2 mM EDTA and 1 mM PMSF, pH 9.6. The cells were lysed in 1% Nonidet P-40 (BDH, Poole, England) in 20 mM glycine, 1 mM PMSF pH 9.6 for 15 min at room temperature. The ECM was washed with 10 mM Tris-HCl, 1 mM PMSF pH 7.5 and PBS.

The ECM deposited by chick fibroblasts appeared as a fibrillar meshwork of FN, and procollagen types I and III (our unpublished observations). It is likely to contain substantial amounts of glycosaminoglycans such as hyaluronic acid, heparan sulfate and chondroitin sulfate (21).

Preparation of Substrates

UNIFORM SUBSTRATES: Purified ECM components were deposited either on glass coverslips (square 24 x 24 mm or round 32-mm diameter), or on bacteriological 60-mm dishes (Falcon Labware). For cell attachment experiments, different substrates were deposited in the same Petri dish within 10-mm circles, and incubated for 30 min at 37°C on the different supports.

Collagen type I in 0.01 M acetic acid was used at a concentration of 150 μg/ml. After neutralization with an equal volume of PBS, the collagen gel formed during the incubation was extensively washed with DME before use. Lyophilized plasma FN was resuspended in DME to a final concentration of 10 μg/ml. FN plus collagen (1:1) solutions were prepared by mixing 150 μg/ml solution of both proteins and allowing polymerization which occurs as rapidly as pure collagen. LN and its fragments were used at 20 μg/ml in DME. PBS, DME, or bovine serum albumin (BSA) (200 μg/ml) in DME were also incubated as controls.

STRIPES: Stripes of FN or LN were obtained by firmly scratching lines in the uniformly deposited substrates with the tip of a sealed Pasteur pipette under a dissecting microscope. All these substrates were washed with DME before use. FN stripes were also labeled with 10 μg/ml tetramethyl-rhodamine isothiocyanate (Research Organics Inc, Cleveland, Ohio) in 0.3 M NaCl 0.05 M borate buffer, pH 9.2, for 30 min at room temperature, and then washed extensively with PBS and DME.

Immunological Procedures

PREPARATION OF ANTIBODIES AGAINST THE CELL-BINDING FRAGMENT OF FIBRONECTIN: Rabbits were immunized intraperitoneally and subcutaneously with 90 μg of 160-kd cell-binding fragment in complete Freund's adjuvant followed by booster injections of 40 μg at 3-wk intervals in incomplete Freund's adjuvant. The sera were systematically tested 1 wk after each injection by an indirect immunofluorescence technique (see below). After the third injection the sera could be diluted to 1/1,000 or more for visualization of FN in the fibroblast ECM.

The IgG fractions of the sera were isolated by precipitation with 37% saturated ammonium sulfate and ion exchange chromatography on DEAE-trisacryl-M (Industrie Biologique Française, Paris). Most of the IgG was not retained when columns were equilibrated with 25 mM Tris-HCl, 35 mM NaCl pH 8.8 buffer. The IgG fractions were dialyzed against water and lyophilized.

To obtain Fab' fragments of anti-160-kd antibodies, 10 mg/ml IgG in 0.1 M sodium acetate buffer pH 4.5 was incubated with 0.5 mg/ml of pepsin (twice crystallized, Sigma Chemical Co., St. Louis, MO) for 18 h at 37°C, then centrifuged and dialyzed against β-mercaptoethanol and iodoacetamide according to Brackenbury et al. (7). The Fab' monovalent fragments were extensively dialysed for several days against PBS and then against water, lyophilized, and stored at −70°C.

Comparable titers as determined by immunofluorescence were found for the IgG and their corresponding Fab' fragments.

The specificity of these antibodies was assessed by the absence of staining of the ECM when the immunoglobulins were preincubated with chick plasma FN and by immunoblotting.

BLOTTING: Chicken cellular fibronectin was cleaved by 2 or 10 μg/ml chymotrypsin for 60 min at 23°C to generate 160-kd cell-binding and 40-kd collagen-binding fragments; 10 μg of digest was loaded per lane of 4% to 10% SDS polyacrylamide gels and electrophoresed exactly as described previously (19). The polypeptides were electrophoretically transferred to nitrocellulose filters (0.45 μm; Schleiber and Schuell, Keene, NH) in a Bio-Rad (Richmond, CA) Transblot apparatus in 5 mM sodium borate at 8 V/cm for 2 h at 4°C. Antibody binding was assessed by the "Western blotting" procedure described in detail by Burnette (8) with 1:100 to 1:500 dilutions of the 160-kd antibody and 5×10^5 dpm of ^{125}I-protein A (New England Nuclear, Boston, MA). Strong binding to the 160-kd fragment was detected, but none was found to the 40-kd fragment; a control using an anti-40-kd antiserum showed binding only to the 40-kd fragment in autoradiograms prepared from adjacent lanes of the same protein transfers.

Analysis of the binding of the 160-kd antibody to the ECM matrix from chick fibroblasts revealed binding only to the cellular fibronectin band.

ANTI-LN ANTIBODIES: Anti-LN antibodies were obtained by affinity chromatography on a LN-coupled Sepharose column (39).

IMMUNOFLUORESCENCE: Fibroblast cultures were fixed with 3.7% formaldehyde in PBS for 30 min at room temperature and washed with PBS. Anti-160-kd antibodies (3–30 μg/ml of IgG or Fab' fragments depending on the serum batch used) were incubated with cultures in PBS containing 5 mg/ml of BSA for 30 min at room temperature. After washing in PBS, sheep anti-rabbit IgG coupled to fluorescein (dilution 1/150) (Institut Pasteur, Paris, France) was added and incubated for 30 min. After washing, the preparations were mounted with 90% glycerol in PBS containing *p*-phenylenediamine (1 mg/ml) to prevent bleaching (23). LN substrates were examined with the same protocol except that the formaldehyde fixation was omitted. The anti-LN antibody was used at 20 μg/ml. Preparations were examined by epifluorescence with oil immersion objectives on a Leitz orthoplan microscope (Weitzlar, W. Germany). Photographs were taken on Tri-X Kodak film with a Leitz Orthomat camera.

Crest Cell Cultures

PREPARATION OF NEURAL TUBE SEGMENTS: Japanese quail (*Coturnix coturnix japonica*) eggs were incubated at 38°C for approximately 60 h to reach stage 10 to 13 of Zacchei (54), equivalent to 15 to 25 pairs of somites. Since trunk neural crest cannot be excised directly, whole neural tube segments from the most caudal region were cultivated in vitro to allow crest cells to migrate outward onto the substratum (10). Briefly, the embryos were removed and the caudal 5–7 somite regions were excised with a scalpel (Fig. 1*a*). The trunk fragments were incubated for 15 min at room temperature with 200 μg/ml Dispase (Godo Shusei Co. Ltd., Tokyo, Japan) in DME in 35-mm Petri dishes (Falcon Labware). The neural tube segments were freed of the ectoderm, somites, endoderm and, finally, of the notochord with pairs of sharp tungsten needles under a dissecting microscope (Fig. 1*b*). Under these conditions of dissociation, the neural tubes were devoid of any contaminating mesenchymal cells. The neural tubes were incubated in DME supplemented with 10% FCS to inhibit the enzyme and cultured at 37°C in a humidified 7% CO_2-93% air incubator.

PREPARATION OF CREST CELLS FOR SECONDARY CULTURES: Neural tubes were placed in Terasaki plates (one segment per well) and were cultured in 15–20 μl of DME, 10% FCS for 8 and 24 h. Aggregated crest cells were obtained after 48 h of culture in the presence of 10% heat-inactivated FCS (60 min at 56°C).

The cultures were examined individually with an inverted microscope (Invertoscope D, Zeiss, Oberkochen, RFA), and those with a low crest cell density were discarded. The neural tubes were carefully removed with a tungsten needle before the neural crest cells were dissociated with 0.05% crude trypsin (1/250, Gibco Europe Ltd., Scotland) for 10 min at room temperature. Routinely, crest cells from 20–40 explants were pooled, washed, and incubated with DME and 10% FN-free FCS. FN was removed from FCS by chromatography of the serum on gelatin-Sepharose columns (16, 33). A viability >90% was determined by trypan blue exclusion. The cell suspension was adjusted to a density of 2×10^4/ml in DME with 10% FN-free FCS.

ATTACHMENT ASSAYS: Spots of different ECM components on bacteriological dishes were incubated with 100 μl of crest cell suspension (2×10^3 cells) for specified times up to 4 h at 37°C in an air-CO_2 incubator. At the end of the incubation, the cultures were washed extensively with PBS to remove the non-adherent cells and fixed with 3.7% formaldehyde in PBS for 30 min at room temperature.

The FN-containing substrates were treated with IgG or Fab' fragments from preimmune or anti-160-kd sera for 30 min at 37°C and washed with DME before the cell attachment experiments or primary culture (see below). Depending on the titers of the sera, 5 to 10 mg/ml of IgG or 2.5 to 5 mg/ml of Fab' fragments were used. In some experiments the antibodies were also added to the cell suspension before incubation.

All the attached cells were counted by phase microscopy, and each experiment was performed in triplicate. The Fisher *t* test was applied to all the results.

MIGRATION ASSAYS: Crest cells were incubated for 60 min at 37°C on 32-mm circular glass coverslips coated with the appropriate substratum and subsequently mounted in a Sykes-Moore chamber (Bellco Glass Inc., Vineland, NJ). Inhibition of motility was studied with FN substrates pretreated with Fab' preimmune antibodies or with anti-160-kd Fab' antibodies. Time-lapse video microcinematography was performed with a Leitz Orthoplan microscope equipped with a video camera (× 3,400, 0.5 Lux, Sanyo Electronic Co., Ltd., Osaka, Japan) connected to a TV monitor (WV 5400, National Matsushita Co., Osaka, Japan), a time generator, and a time-lapse recorder (NV8030, National Matsushita Co.).

Migration pathways were plotted on the TV monitor screen and then copied onto a transparent paper. The total distance of migration of each cell was measured with a map measurer. 20 to 40 cells were scored for each experiment. The speed of locomotion was calculated, taking into account the magnification on the TV monitor (900-fold for × 20 objective), and the real time recorded simultaneously. The degree of persistence was defined as the ratio between the linear distance and the total distance covered by the cells.

BEHAVIOR OF CREST CELLS IN PRIMARY CULTURE ON DIFFERENT SUBSTRATA: Neural tubes were explanted on glass coverslips (24 x 24 mm) coated with collagen, FN, LN, or fibroblast matrices. If explanted on FN and LN stripes, the neural tube segments were placed perpendicular to the stripes.

Cultures were also established on ECM pretreated with preimmune antibodies or anti-160-kd antibodies. After 17 h in FN-free medium, some of these cultures were washed with DME, and incubated for an additional 7 h in the presence of FN-free medium plus 30 μg/ml plasma FN. In the case of FN stripes, 4 mg/ml of Fab' anti-160-kd antibodies were added 6 h after explanting for 9 h of incubation, then the cultures were washed with DME and cultivated in presence of 30 μg/ml plasma FN.

All these cultures were performed in Rose chambers (40) under a dialysis membrane according to Newgreen et al. (32) and Newgreen and Thiery (33). These conditions favor the immediate attachment of the neural tube to its substrate and greatly facilitate the emigration of crest cells. The Rose chambers were placed in a 37°C humidified chamber mounted on the microscope stage.

These cultures were photographed with an automatic camera (Olympus Optical Co., Ltd., Tokyo, Japan) on Ilford Pan-F 35-mm film with a Zeiss inverted microscope (equipped with phase contrast) and filmed with time-lapse video equipment.

SCANNING ELECTRON MICROSCOPY: Cultures of crest cells on different substrates were fixed with 2.8% glutaraldehyde (Merck, Darmstadt, Germany) in PBS for 48 h. After washing in PBS, cultures were postfixed in 1% OsO_4 in PBS for 60 min, followed by washing in PBS. Samples were dehydrated with a graded ethanol series, absolute ethanol, and acetone, critical point dried (Balzers Union; Switzerland), and coated with gold-palladium (E5000, Polaron Ltd.; England). The specimens were examined with a Jeol JSM-35 scanning electron microscope at 25 kV, and photographed with Ilford FP4, 125 ASA film.

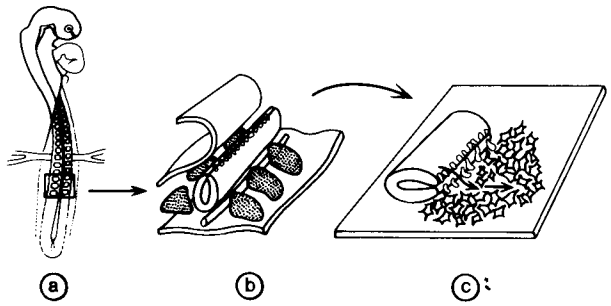

FIGURE 1 Primary neural tube culture technique. (*a*) The most caudal somite-containing region of stage 10 to 13 quail embryos were excised. (*b*) Trunk fragments were incubated with 200 μg/ml dispase in DME for 15 min at room temperature. The neural tube segments were isolated by micromanipulation under a dissecting microscope, then incubated in DME with 10% FCS to inactivate the enzyme. (*c*) Crest cells emigrate from the dorsal border of neural tube explants onto different substrates.

RESULTS

Culture of Crest Cells

After 1 to 2 hours of culture in Rose chambers in normal culture medium containing 10% FCS, crest cells could be identified along the dorsal side of the neural tube. These cells were rounded and blebbed intensively as seen by time-lapse microcinematography, but they rapidly acquired a stellate morphology after reaching the substratum. After 8 h of culture (Fig. 2*a*), several hundred crest cells had appeared; they remained close to the neural tube and only a few cells migrated ahead of the halo of cells. By 24 h (Fig. 2*b*), crest cells had migrated outward and accumulated to form a very dense monolayer of cells. Most crest cells maintained their characteristic stellate morphology with several long, active filopodia. In contrast to typical fibroblast migration, cells remained densely confluent; the front of migration was well delimited except for a few pioneer cells. Frequent, partial overlapping of cell bodies

occurred within this dense cell mass. The size of the halo of cells reached a maximum by 48 h, containing approximately 1,000 cells for a neural tube segment corresponding to 1 pair of somites.

When 10% heat-inactivated FCS was used, crest cells remained more rounded and, within 24 h, formed two-dimensional aggregates rather than uniform sheet of cells. By 48 h, most crest cells formed epithelioid zones of cells separated from each other and from the neural tube (Fig. 2*c*). Many three-dimensional aggregates were also found under these conditions. In FN-free medium, crest cells remained at the edge of the neural tube without any significant emigration (data not shown).

Crest Cell Adhesion

TIME COURSE OF ATTACHMENT: Crest cells cultured for 24 h were dissociated and resuspended at 2×10^4 cells/ml in FN-free medium. Attachment to plasma FN and to the fibroblast ECM substrates was initiated within 5 min after addition of cell suspensions. Crest cells spread very rapidly on the substrate and developed numerous fine filopodia (Fig. 3*a*). In contrast, cells adhered more slowly to LN and LN fragments and very poorly to collagen, to the control substrate coated with BSA, or to bare glass. Most of these crest cells remained rounded with numerous blebs (Fig. 3*b* and *c*).

After 2 h of incubation, the number of attached cells reached a plateau (Fig. 4). Almost 80% of the crest cells adhered to plasma FN and to FN-containing matrices with similar kinetics. LN was substantially less effective than FN, and intact LN was more adhesive than its fragments. More than 35% of the total cell input adhered firmly to LN substrates, whereas <10% bound to a gel of native type I collagen.

AGE AND CULTURE CONDITIONS: Fig. 5 shows that crest cells cultured for only 8 h had properties similar to those cultured for 24 h as described above, although more cell bound to FN within the 2-h assay period (90% for 8-h cells and 80% for 24-h cells). A further decrease in adhesion to FN substrates was observed for cells cultured for 48 h (Fig. 5). Interestingly, the binding to LN was now found to be equal or slightly higher than to FN. In all cases the binding to native collagen, gels, glass, or BSA remained very low.

INHIBITION OF ATTACHMENT BY ANTI-160-KD ANTIBODIES: The time course of attachment of cells to plasma FN substrates in the absence of antibodies or in the presence of preimmune Fab' fragments was identical at all times (Fig. 6*a*).

Preincubation of the substrate with anti-160-kd Fab' fragments resulted in a marked reduction in adhesion (Fig. 6*a*). Complete inhibition was observed when the antibodies were also present in the culture medium (not shown). The few cells that did bind to anti-160-kd-treated fibronectin substrates remained rounded with many blebs at their surface (Fig. 6*b*).

A similar extent of binding of cells was found with fibroblast

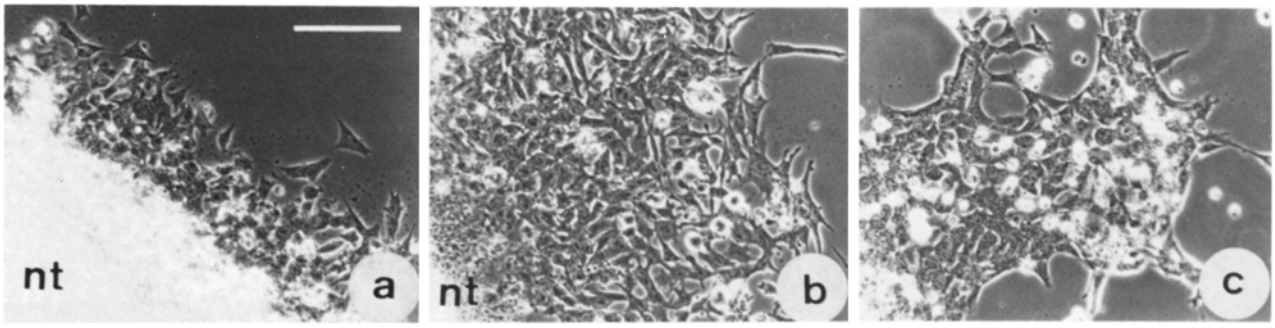

FIGURE 2 Primary culture of neural crest cells. (*a*) After 8 h of culture in DME plus 10% FCS, several hundred crest cells formed a narrow halo in close apposition to the explanted neural tube. (*b*) By 24 h of culture, the outgrowth of crest cells had become a dense monolayer around the neural tube. (*c*) In contrast, after 48 h of culture in DME with 10% heat-inactivated FCS, the crest cells formed two- and three-dimensional aggregates of epithelioid structures. *nt*: neural tube. Bar, 100 μm. × 137.

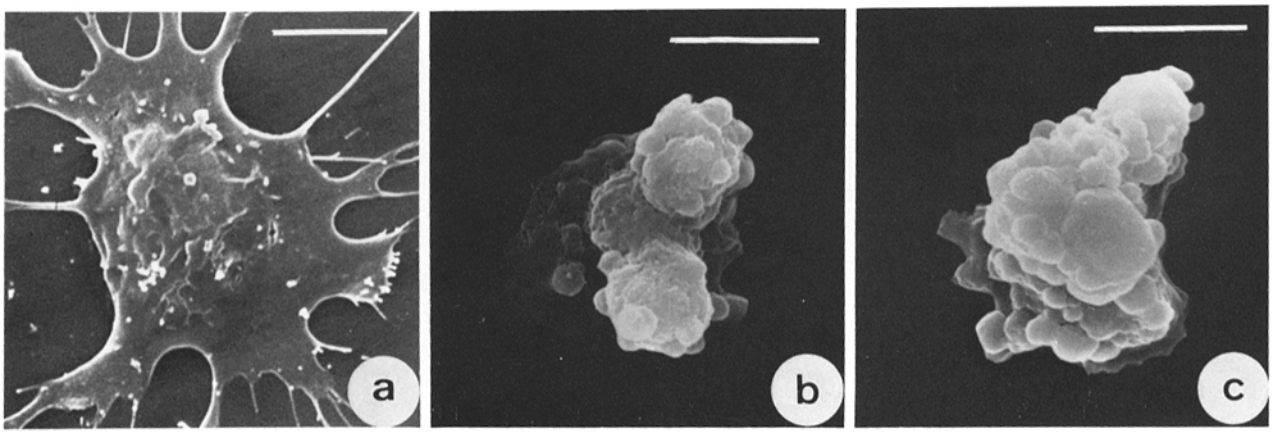

FIGURE 3 Scanning electron micrographs of neural crest cell on different substrates. Cells were obtained from 24-h primary cultures and examined after 2-h subculturing. (*a*) Crest cells attached and spread very rapidly on plasma FN, developing numerous filopodia. On other substrates, the few cells that did not adhere remained rounded and produced numerous blebs on LN (*b*) and on collagen (*c*) substrates. Bars, 5 μm; (*a*) × 2,800; (*b* and *c*) × 3,800.

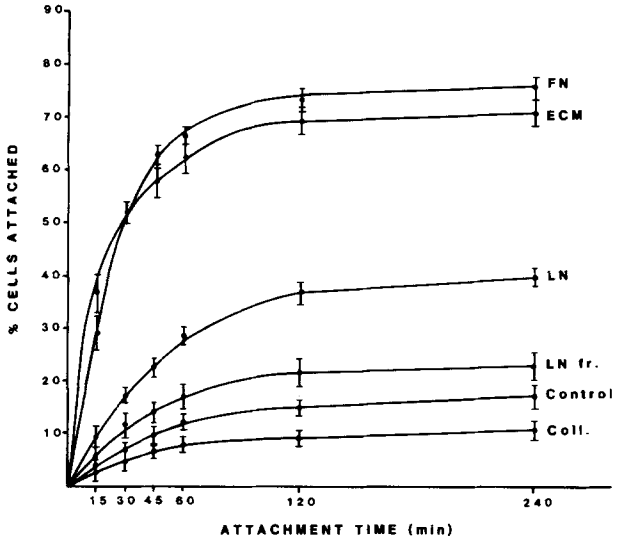

FIGURE 4 Time course of attachment of crest cells to different substrates. Dissociated crest cells obtained from 24-h primary cultures were incubated on different substrates in the presence of DME, 10% FN-free FCS. At each time tested, the cultures were rinsed, fixed, and the attached cells were counted. The results were expressed as percent attached cells of the total number of cells incubated. Each point was the average of triplicates ±SEM. About 70% of the cells had attached to the FN-containing substrates within 2 h of incubation, but in contrast, collagen retained only 8% of the cells. LN was significantly more adhesive than its fragments. Glass or plastic coated with BSA, PBS, or DME served as controls. *FN*: fibronectin; *ECM*: fibroblast extracellular matrix; *LN*: laminin; *LN fr.*: laminin fragments produced by trypsin or elastase; *Coll*: collagen.

ECM as compared to pure plasma FN. When the ECM was pretreated with anti-160-kd Fab' antibodies, a comparable level of inhibition was observed (Fig. 6*a*).

Crest Cell Migration

PREFERENCE FOR FIBRONECTIN-CONTAINING SUBSTRATES: As shown in Fig. 7*a*, very few crest cells could emigrate from a neural tube explanted on a three-dimensional type I collagen substrate. The cells remained rounded, blebbed

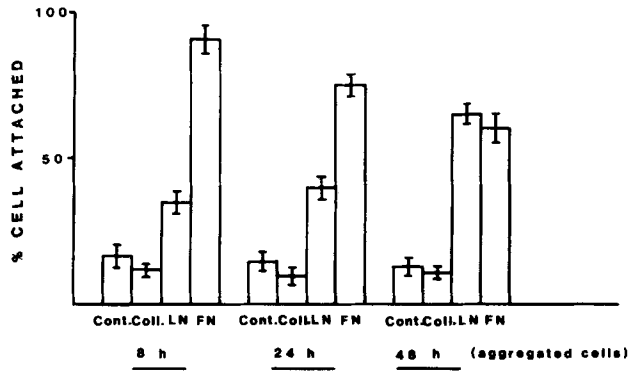

FIGURE 5 Adhesive properties of crest cells as a function of cell culture conditions. Cells were obtained from 8- and 24-h primary cultures in the presence of 10% FCS and from 48-h cultures in heat-inactivated FCS. Trypsin-dissociated cells were incubated for 2 h on the different substrates in FN-free medium. A change in crest cell adhesion to FN and LN was found with increasing time of culture. Crest cells from the 48-h epithelioid aggregates adhered as much to LN as to FN. Data are expressed as in Fig. 4. *Cont*: control.

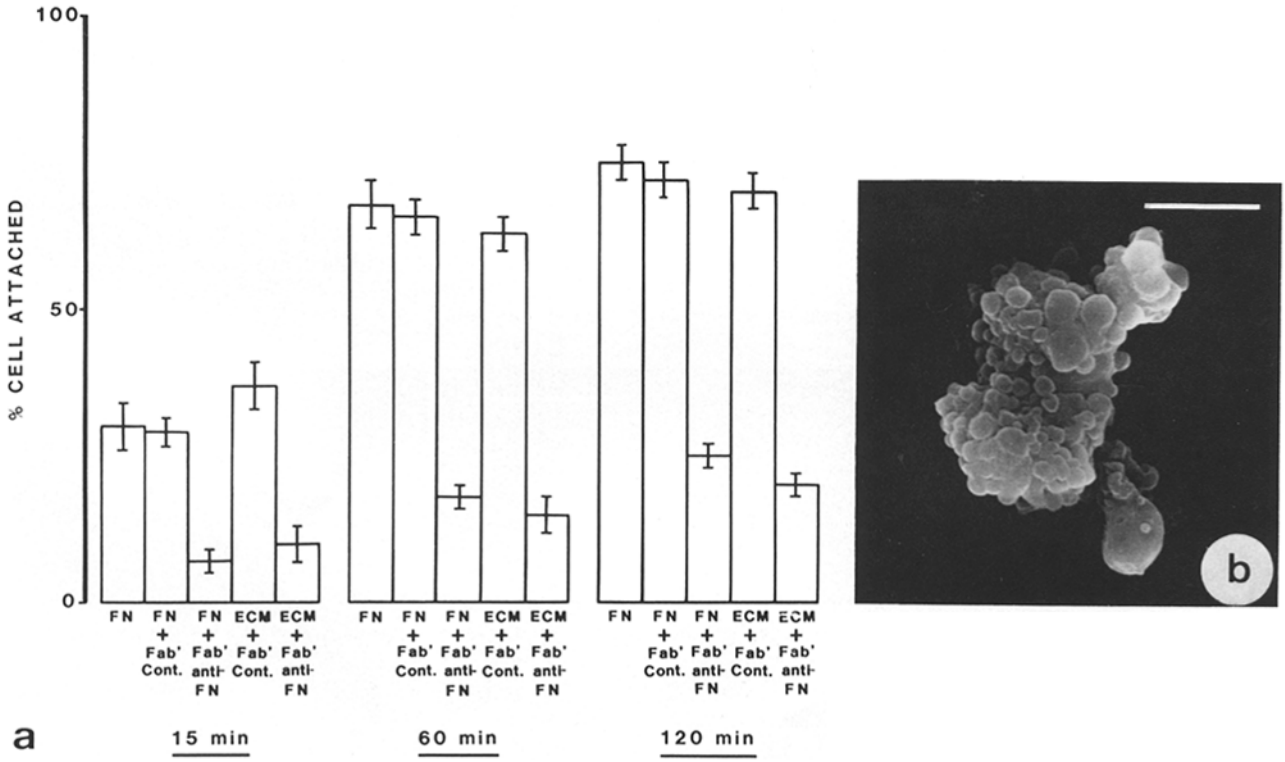

FIGURE 6 Time course of attachment of crest cells in the presence of anti-FN antibodies. (*a*) FN-containing substrates were pretreated with Fab' fragments from preimmune sera (*Fab' Cont.*) or with Fab' anti-160-kdalton (kd) antibodies (*Fab' anti-FN*). Strong inhibition of cell attachment was observed with anti-160-kd antibodies as compared to the controls. Legends as in Fig. 4. (*b*) Scanning electron micrograph of a blebbing crest cell attached to a plasma FN substrate preincubated with Fab' anti-160-kd antibody. Bar, 5 μm. × 3,000.

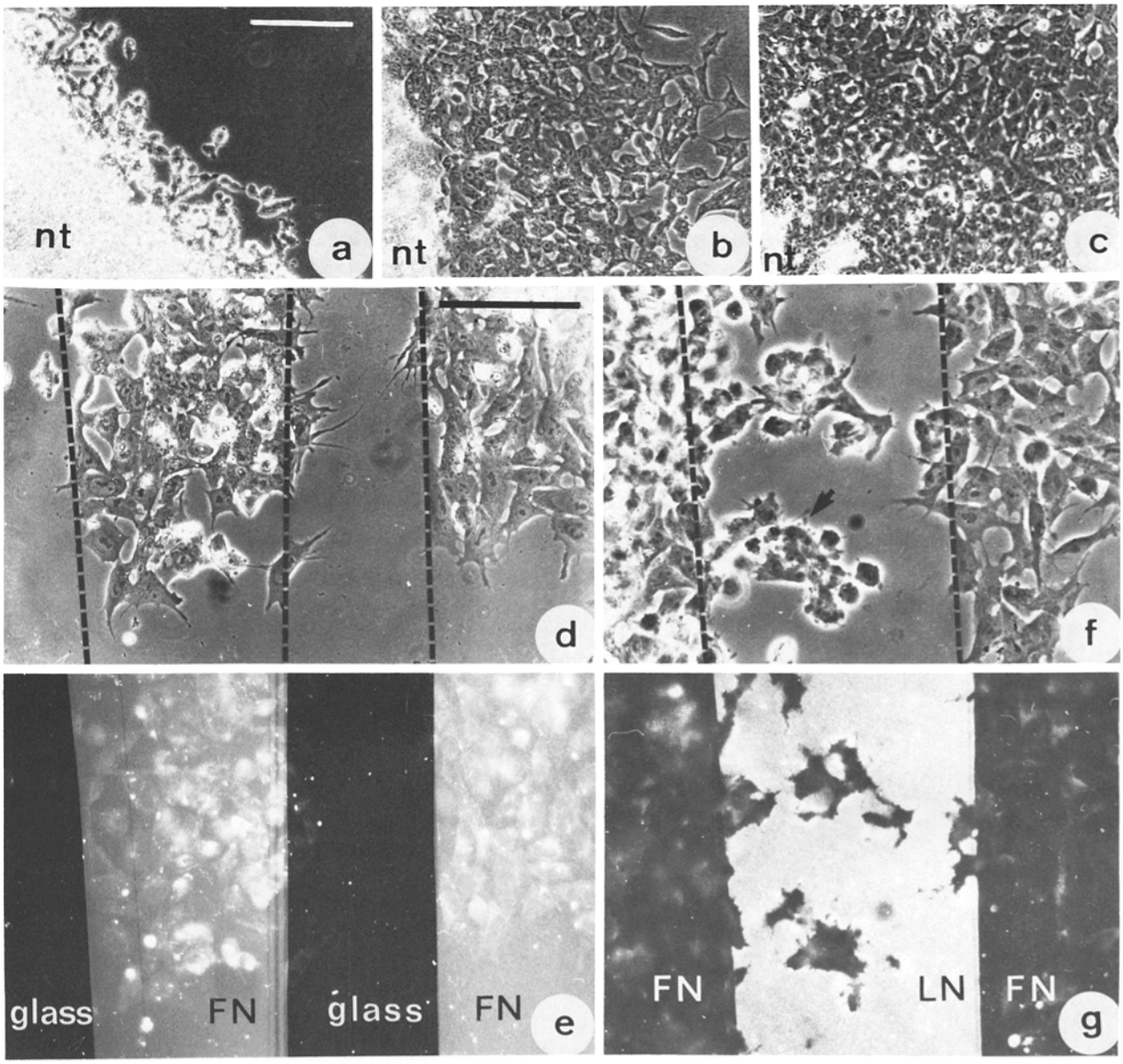

FIGURE 7 Selective migration of crest cells on FN-containing substrates. Crest cells were cultured for 24 h in DME with 10% FN-free FCS (*a–e*). (*a*) Very few rounded and blebbed crest cells emigrated from the neural tube on type I collagen. (*b*) Many more crest cells migrated on a 1:1 collagen-FN mixture. (*c*) Plasma FN was the best substrate for migration; crest cells migrated more rapidly from the neural tube and acquired a flattened shape. Note that the halos of emigrating cells were much wider on FN-containing substrates. (*d*) Crest cells on alternating glass and FN stripes migrated exclusively on the FN substrate. (*e*) Same field, after staining with anti-FN antibodies, examined by immunofluorescence microscopy. (*f*) Crest cells cultured in DME with 10% FCS on LN stripes. Normal FCS was used instead of FN-free FCS since in the absence of FN crest cells could not emigrate from the neural tube. The crest cells migrated preferentially on the regions of glass devoid of a LN substrate where FN was deposited from the normal FCS. Crest cells which were accidentally trapped on LN stripes remained rounded and aggregated rapidly. Note active blebbing of the latter (arrow). (*g*) Same field after immunological localization of LN by incubation with anti-LN antibodies. *nt:* neural tube Bar, 100 μm. (*a, b, c*) × 137; (*d, e, f, g*) × 200.

continuously, and moved very slowly. Crest cells also migrated poorly from neural tubes around which a collagen gel had been polymerized. In contrast, when a 1:1 mixture of FN and collagen was used as substrate (Fig. 7*b*), many more crest cells migrated and formed a dense halo. Crest cells remained congregated into confluent, two-dimensional aggregates except at the front of migration.

A pure fibronectin substrate (Fig. 7*c*) caused crest cells to initiate their migration more rapidly and to assume a flattened shape, suggesting that their adhesiveness was much higher than to a collagen substrate.

When crest cells in FN-depleted medium were confronted with alternating FN and glass substrates, they migrated exclusively on the FN stripes (Fig. 7*d* and *e*). Crest cells migrated very poorly on alternating glass/LN stripes when cultured in the presence of FN-free serum. In normal FCS, crest cells migrated preferentially in regions containing FN derived from the serum, although some cells with a round morphology collected on the LN stripes (Fig. 7*f* and *g*).

A direct side-by-side comparison between substrates coated with equal amounts of plasma FN and LN substrates could not be achieved, since FN or LN stripes bound enough of the

protein added in the second step to obscure the alternating substrates.

MIGRATION AND REORGANIZATION OF THE ECM NETWORK: Cell-free fibroblast matrices allowed the first crest cells to emigrate within 30 min, partly as a consequence of faster attachment of the neural tube. Crest cells migrated as a dense mass, except for the pioneer cells which elongated after anchoring their filipodia to the FN-rich fibrillar matrix. Consequently, a progressive orientation of the three-dimensional fibrillar meshwork (Fig. 8*b* and *c*) occurred ahead of the pioneer cells (Fig. 8*d* and *e*). These fibers no longer appeared to be radially oriented in the proximal region of the neural tube, where the fibrils were in much thicker bundles and arranged in an alveolar pattern on crest cell surfaces (Fig. 8*f* and *g*).

INHIBITION OF CREST CELL MIGRATION WITH ANTI-160KD ANTIBODIES: When the fibroblast ECM was pretreated with anti-160-kd Fab' antibodies, crest cells failed to emigrate effectively, and they accumulated at the edge of the neural tube explant. After 17 h of culture, only a few rounded

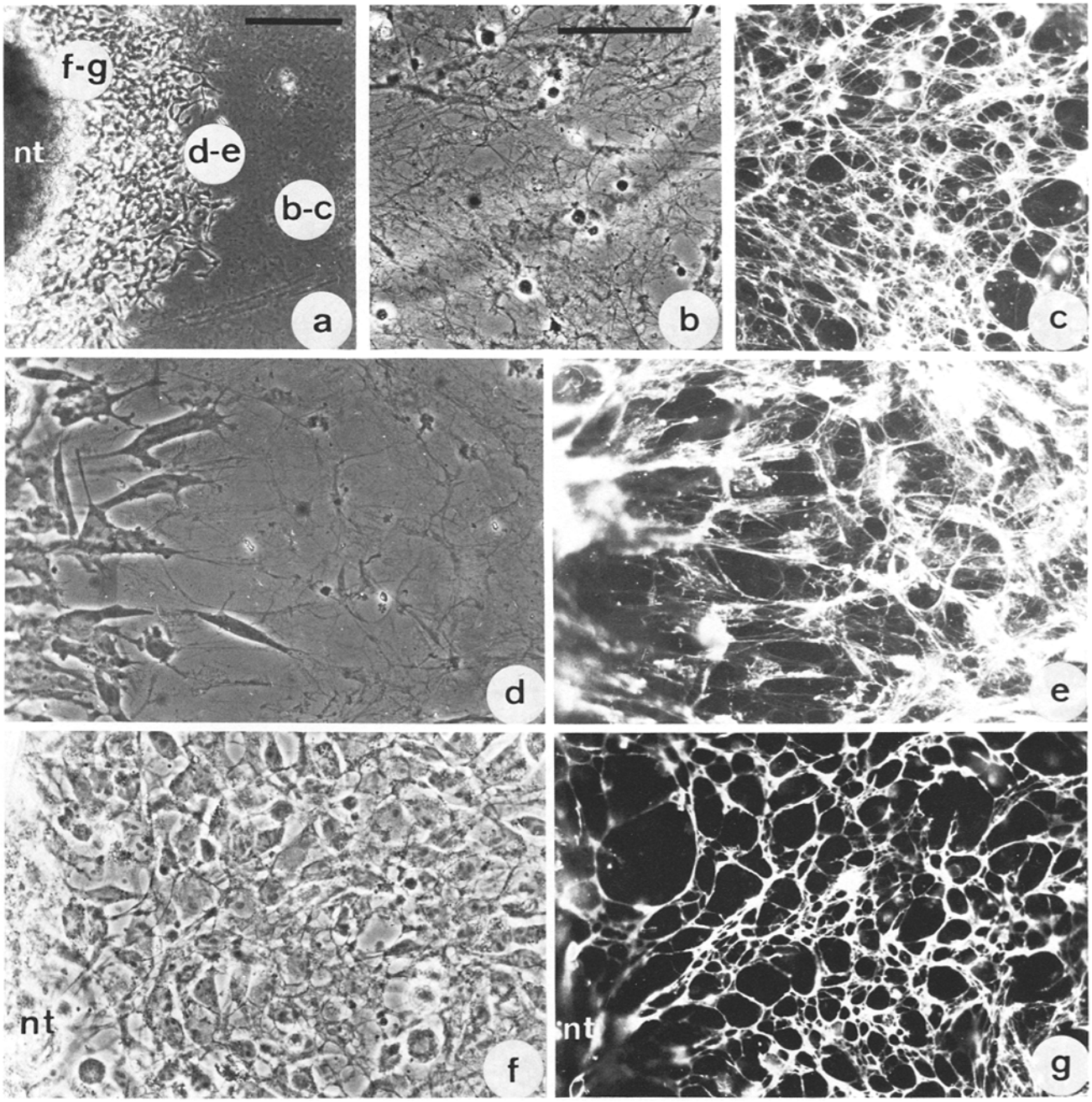

FIGURE 8 Restructuring of the ECM network. (*a*) Overview on the neural crest cell halo after 24 h of culture on ECM with 10% FN-free FCS. *b–g* refer to areas examined at higher magnification by phase and immunofluorescence microscopy. (*b* and *c*) FN localization at a site distant from crest cells. A random orientation of the three-dimensional fibrillar matrix was found in the absence of cells. (*d* and *e*) Field immediately adjacent to the front of migration. Radially oriented bundles of FN-containing filaments formed as a consequence of the migration of pioneer crest cells. (*f* and *g*) Region proximal to the neural tube; thicker bundles of fibers forming an alveolar structure were found mostly above the dense monolayer of crest cells. *nt:* neural tube. Bar, 100 μm. (*a*) × 137; (*b–g*) × 200.

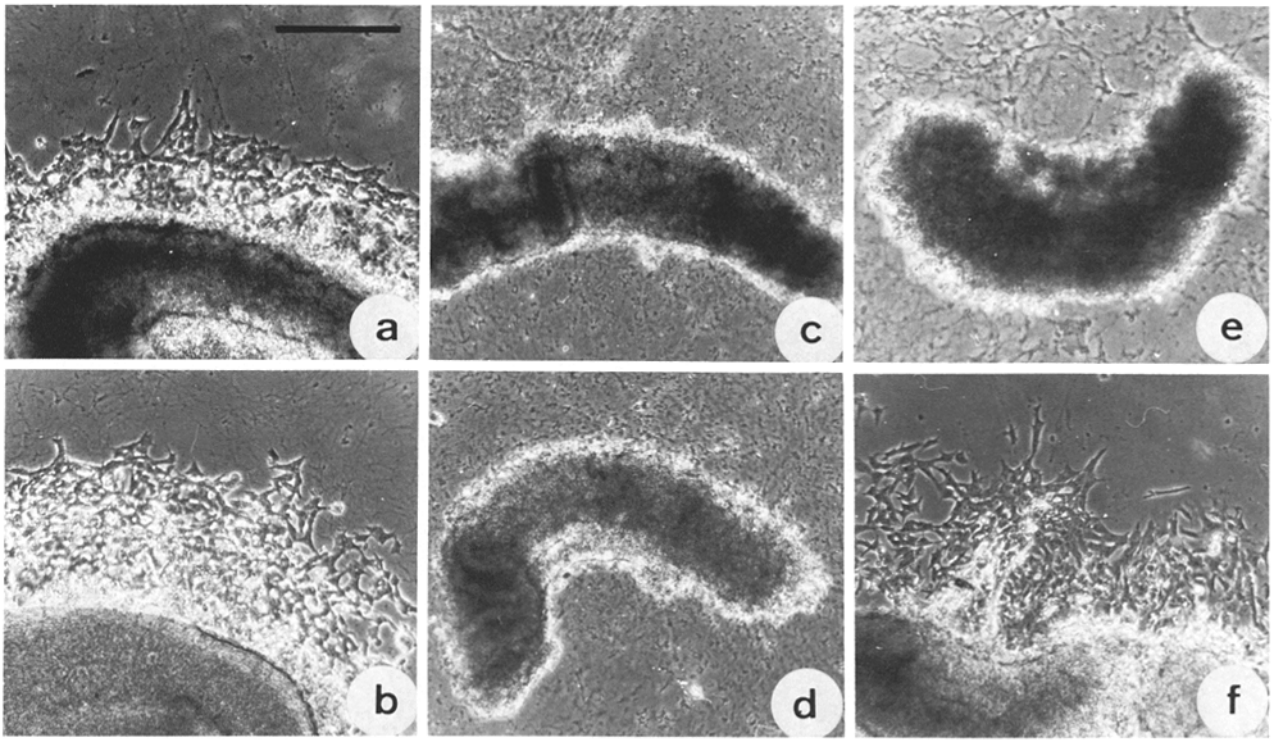

FIGURE 9 Primary crest cell cultures on ECM treated with anti-160-kd antibody and plasma FN. All figures are phase contrast micrographs of cultures with DME and 10% FN-free FCS. (*a*) When cultures were established on ECM substrate coated with preimmune Fab' for 17 h, crest cells migrated outward from the neural tube as in the absence of antibody; (*b*) the number of crest cells increased around the same neural tube explant at 24 h, forming a halo comparable in size to that of untreated cultures (see Fig. 8 *a*). (*c* and *e*) In contrast, at 17 h of culture, crest cells did not emigrate on ECM treated with anti-160-kd antibody. (*d*) When the culture in *c* was incubated under the same conditions for an additional 7 h, no crest cells appeared. (*f*) On the other hand, addition of plasma FN after 17 h to culture the cells in panel *e* for an additional 7 h resulted in migration of crest cells similar to that of the control (*b*). Bar, 200 μm. × 80.

cells were found (Fig. 9*c* and *e*). In contrast, a much wider area was occupied by well-spread crest cells in control explants (Fig. 9*a*).

Rinsing with DME and addition of plasma FN to the medium at a final concentration of 10 μg/ml allowed crest cells to resume their migration and to form a halo of migrating cells comparable to that observed in controls after an additional 7 h of culture (Fig. 9*f*), whereas no crest cells appeared in the cultures maintained in the presence of antibodies (Fig. 9*d*).

Time-lapse Analyses

ISOLATED CELLS: Dissociated crest cells prepared by trypsinization of primary culture outgrowths were plated at low cell density and monitored continuously. On collagen and LN, crest cells remained rounded and blebbed extensively. Their speed of locomotion was also very low (see Table I), although an appreciable persistence of movement was observed especially with laminin (Fig. 10*a* and *b*). In contrast, on FN substrates, individual crest cells migrated more rapidly (20 μm/h) but frequently changed their direction of movement (Fig. 10*c*). In the presence of anti-160-kd Fab' antibodies, the speed of locomotion was progressively reduced with increasing concentrations of the antibodies; at 5 mg/ml antibody, the crest cells were almost paralyzed, although the small amount of residual migration showed a relatively greater persistence of movement (Fig. 10*d*).

DENSE CELL POPULATIONS: Crest cells migrating on FN stripes remained confluent at all times in culture (Fig. 11*a*),

TABLE I

Parameters of Isolated Crest Cell Migration

Substrate	Speed	Effective distance	Degree of persistence
	μm/h	μm/h	
Collagen	6.0 ± 0.4	2.0 ± 0.2	0.34
Laminin	8.4 ± 0.9	3.6 ± 0.6	0.43
Fibronectin	20.5 ± 0.7	3.3 ± 0.4	0.16
FN + Fab' anti-160 kd (2.5 mg/ml)	9.5 ± 0.7	3.2 ± 0.3	0.33
FN + Fab' anti-160 kd (5.0 mg/ml)	3.9 ± 0.5	2.2 ± 0.3	0.55

The absolute speed of locomotion was calculated taking into account the magnification on the TV monitor and the real time simultaneously. The effective distances were defined as the linear distance between initial and terminal points in 17- to 20-h cultures. The degree of persistence was calculated as the ratio between the effective distance and the total distance traversed by the cells. Average ± SEM were obtained for at least 30 cells in each experiment.

except for a few pioneer crest cells at the migratory front, which escaped from the cell mass but remained on the FN stripes. The movement of isolated pioneer cells resembled that of individual, dissociated crest cells plated on an FN substrate (Fig. 10*c*) with frequent changes in the direction of migration (cell *1* in Fig. 11*b* and *c*). These directional changes were observed both when the cells reached the boundary between FN and glass and when the cells were at random positions within the FN stripes. In contrast, cells within the cell mass migrated with a substantially better persistence of movement,

since their attempts to change direction appeared to be prevented by the surrounding cells (e.g. cell *2* in Fig. 11 *b* and *c*).

It is noteworthy that crest cells did not move with leading lamellipodia analogous to fibroblasts, but instead with thin, firmly anchored filopodia; therefore contact inhibition of movement of the type described for fibroblasts was not observed. Collisions were followed immediately by activation of other filopodia that allowed the cell to modify its trajectory.

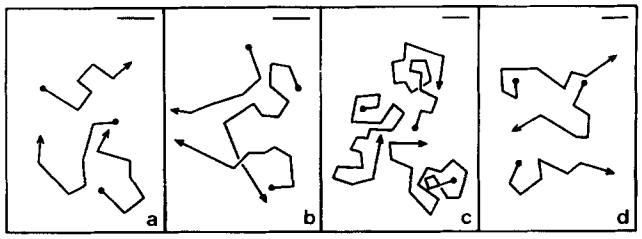

FIGURE 10 Migration of isolated neural crest cells. Cells were cultured in DME with 10% FN-free FCS. Behavior on collagen (*a*), LN (*b*), plasma FN (*c*) and plasma FN previously treated with anti-160-kd Fab' (*d*). Tracks were recorded from time-lapse video films. Three representative tracks corresponding to 19 h of culture are shown for each condition. Note the increased rate of randomly directed motility on the FN substrate in *c*. Bar, 25 μm.

The addition of anti-160-kd antibodies to migrating crest cells provoked a strong decrease in their speed of locomotion and also resulted in more erratic movement (Fig. 12). The front of migration progressed for 83 μm for the first 6 h; thereafter it was reduced to 21 μm for the next 9 h during treatment with antibodies. Migration resumed after addition of FN to the culture, allowing crest cells to traverse 54 μm over the next 8 h. Under these conditions, crest cells left the original stripes of FN since the substrates were now uniformly coated with FN.

Table II summarizes the data obtained with FN-containing substrates. The average speed of locomotion was highest on the fibroblast ECM, and was drastically reduced in the presence of anti-160-kd antibodies. A similar degree of persistence of migration was found for cells within dense cell masses on plasma FN or on fibroblast matrices.

DISCUSSION

This study has analyzed the factors required in vitro to produce the characteristic directional cell migration of neural crest cells

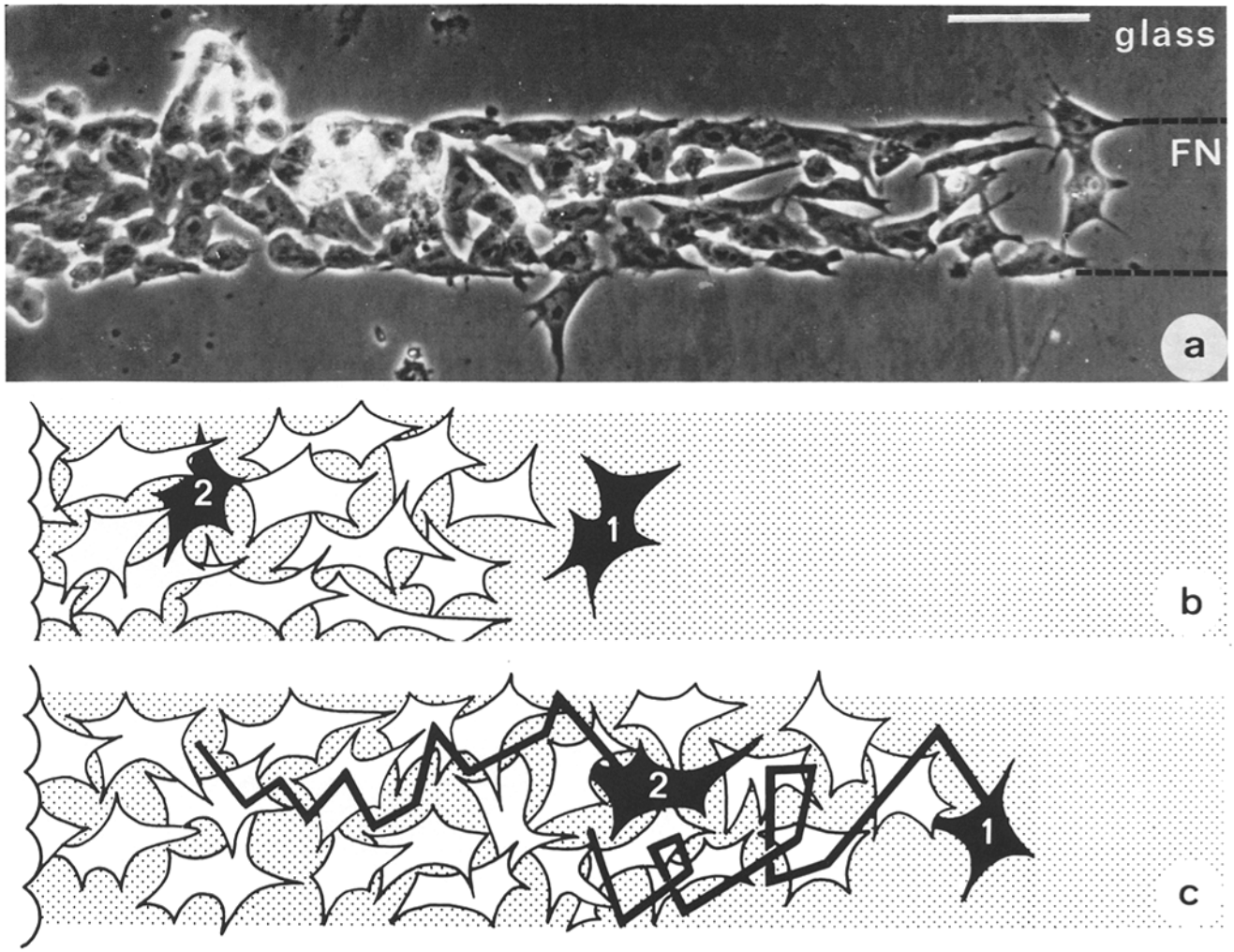

FIGURE 11 Video analysis of crest cell migration on FN stripes. (*a*) Crest cells migrated strictly on FN stripes after 24 h of culture; most crest cells remained in close contact with partial overlapping of cell bodies. (*b* and c) Tracks of a cell surrounded by other cells (*2*); it showed a persistence of movement as compared to a pioneer cell (*1*) which changed directions much more frequently, and even occasionally migrated backwards. Dotted region: plasma FN stripes; undulating line at left corresponds to neural tube edge. Bar, 50 μm; × 380.

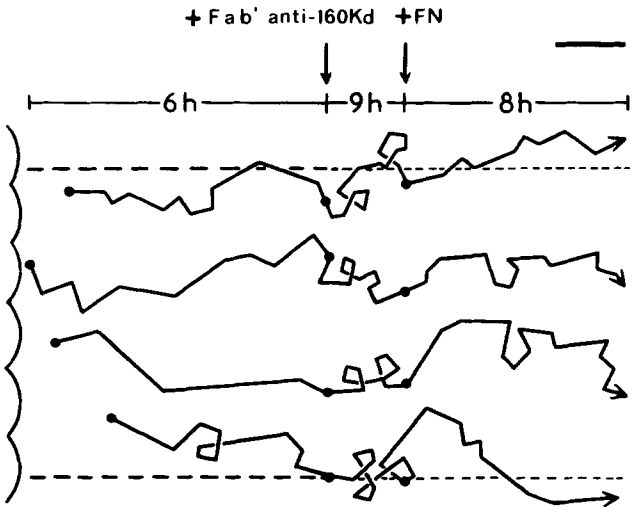

FIGURE 12 Inhibition of crest cell migration on plasma FN stripes treated with anti-160-kd antibody, and recovery after addition of FN. Tracks beginning from the neural tube showed normal migration for the first 6 h. Addition of anti-160-kd Fab' antibodies for the subsequent 9 h resulted in random migration. Oriented migration resumed after addition of plasma FN, although the crest cells could leave the stripes since the substrate was now uniformly coated with FN. Undulating line: edge of neural tube; dashed lines: limits of the FN stripe. Bar, 20 μm.

TABLE II

Behavior of Dense Crest Cell Populations on FN-containing Substrates

Substrate	Speed	Effective distance	Degree of persistence
	μm/h	μm/h	
FN stripes	18.5 ± 1.4	13.8 ± 0.7	0.67
FN + Fab' anti-160 kd	8.2 ± 0.6	2.3 ± 0.4	0.27
FN + Fab' anti-160 kd + FN	14.1 ± 0.9	6.8 ± 0.7	0.48
Fibroblast ECM	48.3 ± 2.1	22.0 ± 1.9	0.46
Fibroblast ECM + Fab' anti-160 kd	9.8 ± 0.7	5.2 ± 0.6	0.53

Description of parameters and expression of results as in Materials and Methods and legend to Table I.

along narrow paths at rates similar to those found along the narrow crest migratory pathways in vivo. Our major findings are: (*a*) avian trunk neural crest cells preferentially utilize FN over LN or collagen for adhesion and motility; (*b*) adhesion and motility are inhibited by anti-FN cell-binding fragment antibodies in both simple and complex matrices; (*c*) in vitro manipulation of crest cells can produce cells capable of utilizing LN for adhesion but not for migration; (*d*) directional migration on FN requires an unusually high cell density; (*e*) rates of directional migration on purified FN were half those found in vivo, and rates equal to those found in vivo require other as yet undefined factors in the extracellular matrix.

Adhesive Properties of "Young" Crest Cells

The first neural crest cells that emigrated in trunk neural tube cultures adhered rapidly to plasma FN and to FN-containing ECM substrates but were poorly adhesive to glass coated with BSA, with collagen type I or with LN. Almost 80% of the crest cells attached within 2 h, a number substantially higher than in an earlier report using collagen and FN in calf serum (18). Part of this difference could be the result of using neural crest cells of differing axial origin. For example, it was shown that none of the crest cells from trunk levels used in the present work could synthesize FN in vitro, whereas more than 20% of the cranial crest cells were able to deposit a FN-rich matrix (33). Plasma FN, deposited as an amorphous substrate, had cell attachment properties very similar to those of cellular FN associated with collagens and proteoglycans in the fibrillar meshwork of fibroblast ECM. In addition, fibroblast ECM treated with specific monovalent antibodies to the FN cell-binding fragment became very poorly adhesive. Taken together, these results strongly suggest that FN is an essential component for the adhesion of crest cells to substrates in vitro. Trunk crest cells in vivo encounter an ECM deposited by the ectoderm, the somite, and the neural tube which has a similar chemical composition to the ECM deposited by fibroblasts (21, 33, 38). It therefore appears likely that in vivo crest cells also bind to FN, which is present in all the migration pathways.

Temporal Changes of Crest Cell Adhesive Properties

With increasing time in culture, crest cells accumulated at the periphery of the neural tube as a result of both active cell proliferation and continuous release from the neural epithelium. These older crest cells became progressively less adhesive to FN-containing substrates, whereas their capacity to bind to LN increased.

These changes in adhesive properties can be manipulated experimentally, since young crest cells grown on poor substrates were found to aggregate and to acquire equal or better adhesiveness to LN than to FN. Such poorly adhesive substrates could be obtained by coating glass with BSA or collagen although very few crest cells could emigrate from the neural tube in the absence of FN. In contrast, when heat-inactivated FCS was used, many crest cells appeared and formed two- and three-dimensional clusters of closely juxtoposed cells. We found that FN heated at 56°C, a temperature very close to its thermal denaturation (2), also produced the same effect (not shown). Therefore, denaturated FN appeared to provide a substrate low enough to adhesion to permit rapid cell-to-cell adhesion while still allowing migration.

At the cessation of migration in vivo, crest cells were shown to accumulate in restricted areas where FN had disappeared. Thereafter, crest cells regrouped into epithelial structures with specialized junctions (37). Furthermore, the cell adhesion molecule (N. CAM) (41, 43) appeared at the surface of aggregating crest cells both in vitro and in vivo (45). Therefore, it is tempting to suggest that our in vitro aggregation culture mimics the microenvironment necessary for development of the ganglia of the peripheral nervous system. It remains to be determined in vivo whether LN is also involved in the formation of ganglia as suggested for kidney tubular epithelium (15). Most importantly, further work should determine whether the loss of FN and the expression of N. CAM are the consequence or the cause of the rapid shift in crest cell adhesive properties.

In Vitro Migration

Of the substrates tested, plasma FN and FN-containing fibroblast ECM were the most effective in stimulating locomotion. However, the speed of locomotion was higher on ECM and reached 50 μm/h, a value which was also estimated in the

embryo (4, 14, 44). In vitro, the fibroblast ECM was found to be reorganized by the migrating crest cells; filopodia from pioneer crest cells induced the formation of thick bundles from thin filaments. These cells had a tendency to follow radially arranged fibers, but the ECM structure was further modified to form an alveolar network above the regions of highest crest cell density.

Although there is some controversy as to whether this mechanism of structural reorganization may apply in vivo in the case of crest cells (29, 48), a similar mechanism has been found in the migration of endocardial cushion tissue (24, 30). Therefore, the speed of locomotion could be increased by the presence of fibers aligned in the direction of movement; other ECM factors besides FN, responsible for this increase, remain to be identified.

The persistence of movement of isolated cells differed, especially on the FN-containing substrates. On plasma FN, crest cells frequently changed their direction of movement, whereas a greater persistence was found on fibrillar matrices. On the latter, we observed that isolated crest cells had a tendency to migrate in association with fibers that became progressively oriented in the direction of migration (see below). On plasma FN, persistence of movement increased greatly when the crest cells formed a quasi-confluent monolayer with frequent cell contacts. Any attempt to change direction appeared to be prevented by the presence of surrounding cells. Crest cells did not exhibit either a contact inhibition of movement as originally defined by Abercrombie and Heaysman (1) or a contact inhibition of overlapping (46). Indeed, we noticed that crest cells within dense cultures overlapped frequently without their motility being affected.

An important finding was that the addition of anti-cell binding fragment antibodies induced a rapid rounding up of cells and arrest of their locomotion; this effect was reversed by addition of plasma FN. Since the antibody was specific, it is likely that FN present in the ECM promoted cell migration and that the blockade of its cell binding site by antibodies inhibited adhesion and migration.

In vivo, crest cell migration was considerably delayed in several FN-rich pathways (13, 33, 42, 44). Therefore, it should be considered that, even though FN may be present in the matrix to provide a suitable adhesive substrate for crest cells, emigration may be prevented by the presence of other components such as chondroitin sulfate (12, 34).

Mechanism of Migration

The in vitro social behavior of crest cells and previous in vivo studies of the structure of the route of migration allow us to propose a model in which crest cells are led to their final destinations by migration as confluent and increasingly crowded populations of cells along narrow, transient, FN-containing pathways. As presented diagrammatically in Fig. 13, FN is required for adhesion and motility. The ECM provides both space and substrate for migration, while unidirectionality of migration results from the unique property of crest cells to display persistence of movement at high cell density.

However, at the present time, we cannot exclude short range contact guidance of the type observed in our in vitro experiments as an additional factor contributing to unidirectional progression. Previous studies on the behavior of crest cells (17) and on the distribution of FN (44) indicated that haptotaxis or chemotaxis is unlikely to operate in vivo. Therefore, further studies might most profitably focus on the roles played by cell proliferation and by the intrinsic ability of crest cells, but not of other cell types such as fibroblasts (11, 17), to migrate in the FN-rich ECM in vivo and in vitro.

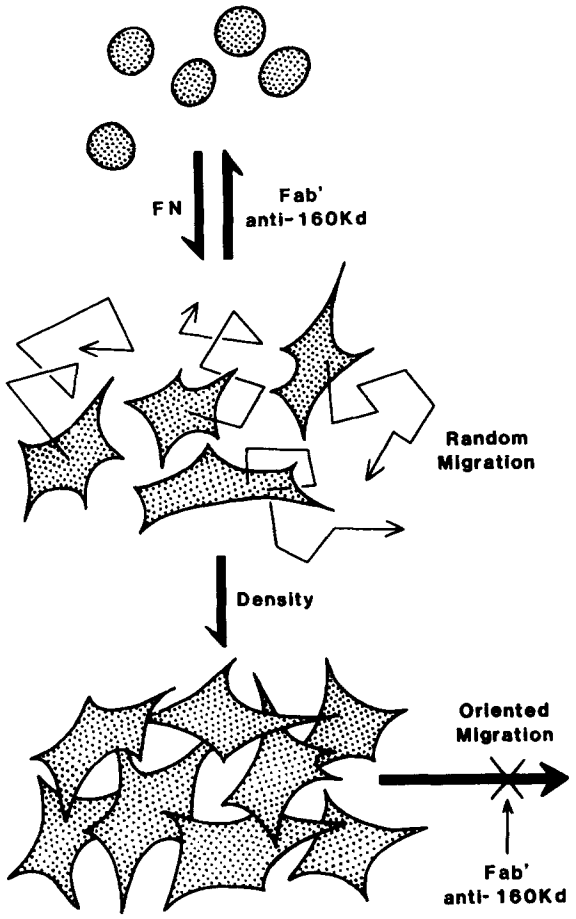

FIGURE 13 Requirements for oriented neural crest cell migration.

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