

Avian neural crest cells express a melanogenic trait during early migration from the neural tube: observations with the new monoclonal antibody, "MEBL-1"

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Summary

A new monoclonal antibody, MEBL-1, which specifically recognizes melanocytes and their precursors, was used to examine the differentiation of neural crest cells into melanocytes *in situ*. MEBL-1-positive precursors of melanocytes in the trunk were first detected in mesenchyme located above the neural tube at the level of a wing bud at stage 19, when active migration of neural crest cells along the ventral route was almost complete. DiO-labelled neural crest cells, which begin to emigrate from the neural tube at stages 17-18 and then migrate along the dorsolateral route, were found to become MEBL-1-positive. This indicates that MEBL-1 antigens are expressed by neural crest cells immediately following their emigration and neural crest cells become melanogenic. Expression of the MEBL-1 antigens was followed by decrease in, and finally the loss of, HNK-1 antigen expression.

At the cephalic level, MEBL-1-positive precursors of melanocytes were first detected in mesenchyme located above the dorsal side of the mesencephalic neural tube at stage 16. At the vagal level, the precursors were noted first in mesenchyme located above the dorsal side of the rhombencephalic neural tube at stage 18.

MEBL-1 reacted with the cell membrane and pre-melanosome-like granules in addition to the nuclear membrane, endoplasmic reticulum and Golgi apparatus of the migratory precursors. The antigens recognized by MEBL-1 were proteins with relative molecular masses of 135×10^3 and 115×10^3 . We suggest that MEBL-1 may be the earliest marker for melanogenic neural crest cells.

Key words: neural crest cells, melanogenesis, monoclonal antibody, chick embryo.

Introduction

Avian melanocytes in the skin derive from the neural crest (Dorris, 1939; Eastlick, 1939; Rawles, 1945; Le Douarin, 1982; Bagnara, 1987). The migratory route of the precursors of melanocytes following emigration from the neural tube has been clarified by using a chick-quail chimera system (Teillet and Le Douarin, 1970; Teillet, 1971). The precursors move between the ectoderm and dermomyotome, migrate into the epidermis and finally differentiate into melanocytes. The cell lineage of melanocytes has been studied mainly in clonal culture systems of neural crest cells. Although some neural crest cells generate only melanocytes in clonal culture, bi- or multipotential neural crest cells give rise to melanocytes (Cohen and Konigsberg, 1975; Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Dupin et al., 1990). The results of chasing a single

labelled neural crest cell *in situ* also support these findings (Bronner-Fraser and Fraser, 1988, 1989).

The differentiation of melanocytes occurs homogeneously in spherical clusters formed on a cultured neural tube (Loring et al., 1981). The extracellular matrix surrounding the migratory neural crest cells also promotes the differentiation of melanocytes (Loring et al., 1982; Perris and Löfberg, 1986; Tucker and Erickson, 1986; Vogel and Weston, 1988). The avian pigment pattern is determined by local cues that promote the differentiation of melanocytes (Richardson et al., 1989, 1990). The neural tube and extracellular cues thus appear essential for the differentiation of neural crest cells into melanocytes.

A marker that recognizes the early expression of melanogenic traits is needed to determine the mechanisms by which neural crest cells begin to acquire melanogenic traits *in situ*. Recently, the migration and

differentiation of neural crest cells have been the focal point of considerable research using the monoclonal antibodies, NC-1 and HNK-1 (Tucker et al., 1984; Vincent and Thiery, 1984; Rickmann et al., 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987; Lallier and Bronner-Fraser, 1988). However, the differentiation of melanocytes in vitro has been shown to lead to the elimination of the HNK-1 antigen (Vincent and Thiery, 1984). The lack of early, stable and natural markers for the precursors of melanocytes prevented detailed research on the early phase of migration and differentiation of the precursors. Some monoclonal antibodies specific toward melanoma and melanocytes are presently available (Dippold et al., 1980; Mitchell et al., 1980; Ross et al., 1986; Hayashibe et al. 1986; Yanai and Takeuchi, 1987; Mochii et al., 1988) but have not yet been used to study the normal development of melanocytes.

The present study was thus carried out (1) to obtain a new marker for the precursors of melanocytes, (2) to characterize the antigen partially and (3) to perform in situ analysis of the differentiation and migration of the precursors using the monoclonal antibody.

Materials and methods

Animals

Fertilized eggs of White Leghorn, Rhode Island Red and Silkie Bantam were obtained commercially and incubated at 37.5°C until use.

Production of monoclonal antibodies

Dorsal skin was removed from chick embryos at E8 (E: embryonic day), homogenized in Ca^{2+} -, Mg^{2+} -free phosphate-buffered saline (PBS), emulsified with complete Freund's adjuvant and subcutaneously injected at several sites along the flank of a Balb/c female mouse. Three booster shots (containing the same ingredients in incomplete Freund's adjuvant) were administered intraperitoneally at one month intervals. Four days before fusion, the mouse was administered an intraperitoneal injection of the same material without the adjuvant, killed and the spleen removed. Splenocytes were fused with SP2/0 mouse myeloma (ratio 1:5) in the presence of polyethylene glycol 1500 (Boehringer-Mannheim) according to the methods of Köhler and Milstein (1975). The fused cells were plated on 6 plates of 96-well tissue-culture dishes (Coaster).

Undiluted culture supernatants were tested for secreted antibodies by indirect immunofluorescence on cryosections of the dorsal skin, as described below. The cell line was cloned by the limiting dilution method and the monoclonal antibody secreted from the cloned line was named MEBL-1 (see 'Results'). The immunoglobulin subclass of MEBL-1 was determined using a mouse monoclonal antibody typing kit (Serotec) in dot analysis.

Light microscopic immunohistochemistry

Embryos at different developmental stages were fixed in 2% paraformaldehyde in 0.2 M cacodylate buffer (pH 7.2) for 30 to 60 minutes at 0°C, impregnated with 10% and then 20% sucrose in PBS, embedded in Tissue Tek II compound (Miles Scientific), frozen and then cut into 10 μm thick sections with a cryostat (Frigocut 2800E, Reichert-Jung). Each section was

mounted on a poly-L-lysine-coated slide and treated with MEBL-1 overnight at 4°C, followed by washing in five changes of PBS. The secondary antibody consisting of biotinylated anti-mouse Ig antibodies (diluted 1:25 in PBS containing 1% bovine serum albumin, Amersham) was allowed to react with MEBL-1 for 1 hour at room temperature. The sections were rinsed as above, incubated with FITC-streptavidin (diluted 1:100 in PBS containing 1% bovine serum albumin, Amersham) for 1 hour at room temperature, rinsed again and then mounted using 50% glycerol (Merk) in 0.1 M sodium carbonate, pH 9. They were viewed with a Zeiss standard microscope equipped with a IV FI epifluorescence condenser (Carl Zeiss). Photographs were taken using Kodak Tri-X film of ASA 800.

Double immunolabelling experiments

To follow the process of the differentiation of neural crest cells into precursors of melanocytes, two monoclonal antibodies were used simultaneously for immunohistochemistry. A mixture of HNK-1 (monoclonal antibody specific for migratory neural crest cells; IgM subclass; diluted 1:50 in PBS containing 1% bovine serum albumin; Becton-Dickinson) and MEBL-1 was allowed to react on the sections overnight at 4°C. The sections were then washed five times with PBS and exposed to the second antibodies for 30 minutes at 37°C. The second antibodies consisted of the Texas Red-anti-mouse IgG1 antibody (diluted 1:10 in PBS containing 1% bovine serum albumin; Southern Biotechnology Associates Inc.) and the FITC-anti-mouse IgM antibody (diluted 1:20 in PBS containing 1% bovine serum albumin; Cappel). The slides were washed and mounted as above.

Electron microscopic immunocytochemistry

The trunks were fixed with 0.05% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 hour and then with 4% paraformaldehyde in the same buffer overnight at 4°C. This was followed by impregnation with 10%, 15% and then 20% sucrose in PBS, one day in each case, at 4°C, embedding in Tissue Tek II compound (Miles Scientific), freezing and finally cutting the trunks into 6 μm thick sections with a cryostat. The sections were treated with fresh culture medium for 30 minutes at room temperature, then with MEBL-1 overnight at 4°C and washed in five changes, 10 minutes each time, of PBS containing 10% sucrose. The secondary antibody consisting of horseradish peroxidase (HRP)-labelled anti-mouse Ig F(ab')₂ fragments (diluted 1:25 in PBS containing 1% bovine serum albumin, Amersham) was made to react overnight at 4°C, and then washed in five changes of PBS, 5 minutes each time. The sections were refixed with 0.5% glutaraldehyde in PBS for 5 minutes on ice, followed by washing in ten changes, 5 minutes each, of PBS containing 10% sucrose. 3,3'-diaminobenzidine (DAB)-H₂O₂ solution was made to react for 10 minutes at room temperature, followed by washing in five changes, 5 minutes each, of cold PBS. The sections were postfixed with 2% OsO₄ in H₂O for 1 hour at room temperature, followed by washing in five changes, 5 minutes each, of cold PBS, dehydration in a gradient series of alcohol and embedding in Epon resin. The control samples were treated with fresh culture medium instead of MEBL-1. The ultrathin sections were observed with a HITACHI H-500 electron microscope.

Injection of tracer dye into the neural tube

All injections were a 0.5% solution (w/v) of 3,3'-dioctadecyloxa-carbocyanine perchlorate (DiO; Molecular Probes) in dimethyl formamide. Prior to injection, the dye solution was centrifuged to remove any crystals that might clog the

micropipet tip. The procedure for injection was essentially according to Serbedzija et al., (1989). Briefly, the micropipet was filled with DiO solution and attached to a microinjector (Narishige). The injection pipet was inserted obliquely into the neural tube either anterior to the 14th or posterior to the 25th somite level using a micromanipulator (Narishige). After incubation, the embryos were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 hours and impregnated with PBS containing sucrose, embedded as described above and serially sectioned by a cryostat at 10 μ m. The sections were mounted on poly-L-lysine-coated slides and viewed without coverslips. After being recorded photographically, the sections were immersed in PBS and stained immunohistochemically with MEBL-1.

Neural crest cell cultures

The embryos of stages 15 to 16 (Hamburger and Hamilton, 1951) were removed and the last 7 somite regions were excised with a scalpel. Trunk fragments were incubated for 30 minutes at 0°C with 1% trypsin in Ca^{2+} -, Mg^{2+} -free Tyrode's solution and transferred to Dulbecco's Minimum Essential Medium (MEM) supplemented with 10% horse serum. Each neural tube segment was freed of ectoderm, somites, endoderm and notochord with sharp needles under a dissecting microscope. The neural tubes were cultured in Dulbecco's MEM supplemented with 20% fetal calf serum and 8% chick embryo extracts in 35 mm Petri dishes. Two days later, they were carefully removed from the dishes with a sharp needle. Neural crest cells that had migrated from the dorsal border of neural tube explants were cultured in the same dishes and on days 2 and 5, were washed twice in PBS and fixed with 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, at 0°C for 1 hour. The fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 minutes and immunostained with MEBL-1.

Immunoblotting

The dorsal skin at E8 was solubilized using the sample buffer of Laemmli (1970). Following heat treatment, the samples were analyzed by SDS-polyacrylamide gel electrophoresis (7.5% acrylamide). The gel was treated according to Towbin et al., (1979) for immunoblotting. The blotted nitrocellulose paper was treated with MEBL-1 or fresh culture medium followed by Vectastain ABC-AP kit (Vector Laboratories).

Results

Production and characterization of a monoclonal antibody, "MEBL-1"

Of the 576 wells initially plated, 483 contained one or more hybrid colonies. Of these, 135 showed immunoreactivity with epidermis, dermis or both. In one well, culture supernatant was found to react with restricted cells in epidermis and dermis at E6 (Fig. 1) and the hybridomas in the well were subsequently cloned for further examination. The secreted monoclonal antibody from the cloned hybridoma was named MEBL-1. To determine whether MEBL-1 would specifically recognize melanocytes/precursors of melanocytes, it was allowed to react with retinal pigment epithelium. Weak staining by MEBL-1 was noted in the retinal pigment epithelium at stage 16 prior to pigmentation (Fig. 2A). The staining became more intense with eye development. The retinal pigment epithelium of stage

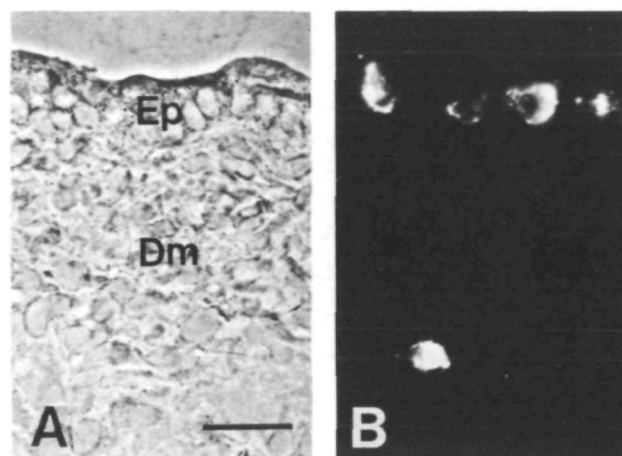


Fig. 1. Phase-contrast (A) and immunofluorescence (B) micrographs of section of dorsal skin at E6, stained by indirect immunofluorescence with MEBL-1. The restricted cells in the epidermis (Ep) and dermis (Dm) are stained with MEBL-1. Bar=25 μ m.

20 with faint pigmentation stained specifically with MEBL-1 (Fig. 2B). Retinal pigment epithelium at E5 also showed strong staining (Fig. 2C). No neural retina, lens, ectoderm or mesenchyme stained with MEBL-1 (Fig. 2A-C). Thus, MEBL-1 specifically recognizes melanocyte and their precursors in retinal pigment epithelium. MEBL-1 was subclassed as IgG1 based on the results of mouse immunoglobulin typing analysis.

Reactivity of MEBL-1 with neural crest-derived melanocytes

After neural tubes at stage 15 had been cultured for 2 days, the neural crest cells had migrated from them (Fig. 3A) and some cells stained weakly with MEBL-1 (Fig. 3B). Following culture for 5 days, most of the cells became heavily loaded with pigment granules (Fig. 3C) on which the reactivity of MEBL-1 was quite evident (Fig. 3D).

The reactivity of melanocytes in a feather filament with MEBL-1 was examined in an embryo of Silkie Bantum. The melanocytes in this filament and skin were MEBL-1-positive (Fig. 3E, F), thus showing MEBL-1 to react with melanocytes and their precursors which differentiate from neural crest cells. The MEBL-1-positive cells in Fig. 1 are thus the precursors of melanocytes derived from neural crest.

Appearance and migration of MEBL-1-positive precursors of melanocytes from trunk neural crest at the level of a wing bud in situ

The differentiation and migration of melanogenic neural crest cells were examined using MEBL-1. MEBL-1-positive precursors of melanocytes in a trunk were first noted in the upper region of the neural tube at wing bud level at stage 19 (Fig. 4A, B). By HNK-1 immunostaining, the active migration of neural crest cells along a ventral route was shown to be virtually

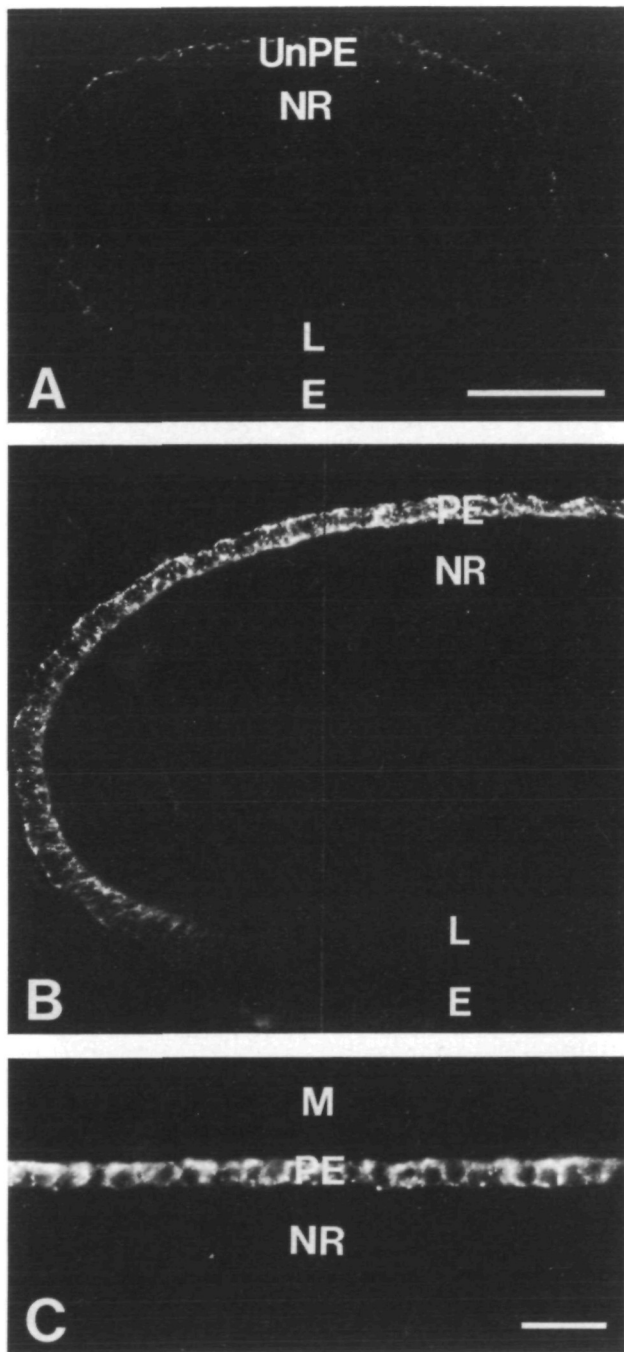


Fig. 2. Immunofluorescence micrographs of sections of embryonic eye, stained by indirect immunofluorescence with MEBL-1. (A) An eye at stage 16. The retinal pigment epithelium (UnPE) not yet showing pigmentation is weakly stained with MEBL-1. (B) An eye at stage 20. The retinal pigment epithelium (PE) showing faint pigmentation is specifically stained with MEBL-1. (C) Retinal pigment epithelium at E5. Only retinal pigment epithelium is strongly stained with MEBL-1. No neural retina (NR), lens (L), ectoderm (E) and mesenchyme (M) at any stage is stained with MEBL-1. Bar(A and B)=100 µm; bar(C)=25 µm.

complete by this stage (data not shown). MEBL-1-positive precursors increased in number at stages 20-21 and were present in the vicinity of a dermomyotome (Fig. 4C, D). In stage 22 embryos, many of these precursors were found to move along a dorsolateral route tightly bounded on either side by ectoderm and a dermomyotome (Fig. 4E, F). MEBL-1 did not recognize other neural crest-derived tissues, such as dorsal root and sympathetic ganglia.

The timing and locality of appearance of precursors of melanocytes in colored chick embryos of Rhode Island Red and Silkie Bantam were the same as those in embryos of White Leghorn (data not shown).

Timing of the initial emigration of neural crest cells expressing MEBL-1 antigens from the neural tube at the level of a wing bud

The timing of the initial emigration from the neural tube of neural crest cells that express MEBL-1 antigens was determined by injecting a vital dye DiO into the lumen of the neural tube. In embryos injected at stages 12-13 and incubated up to stage 17, many DiO-labelled cells external to the neural tube were found to be present in truncal neural crest derivatives located in ventral, but not dorsolateral routes (Fig. 5A). MEBL-1-positive cells could never be detected in these embryos (Fig. 5B). When incubated up to stage 18, DiO-labelled neural crest cells were found above the neural tube as well as in the ventral route (data not shown). In embryos injected at stages 12-13 and incubated up to stage 20, DiO-labelled cells were found in all truncal neural crest derivatives located in ventral and dorsolateral routes (Fig. 5C), and many DiO-labelled cells in the dorsolateral route were MEBL-1-positive (Fig. 5D). For more detailed examination of the initial timing of MEBL-1-positive cell emigration, embryos were injected with DiO at stage 17 and incubated to stage 20. DiO-labelled cells could be found primarily in the dorsolateral route (Fig. 5E) and were MEBL-1-positive (Fig. 5F). In embryos injected at stage 18 and incubated up to stage 20, DiO-negative and MEBL-1-positive cells were noted in front of DiO- and MEBL-1-positive cells (data not shown). It thus follows that neural crest cells that subsequently differentiate into melanocytes begin to emigrate from the neural tube at stages 17-18 and express MEBL-1-antigens at stage 19.

Replacement of HNK-1 antigen with MEBL-1 antigens in the melanogenic neural crest cell lineage

Double staining with MEBL-1 and HNK-1 on cryosections of stages 20 and 22 embryos was carried out. Identical cells situated above the neural tube stained with both HNK-1 and MEBL-1 (Fig. 6). The number and staining intensity of HNK-1-positive cells at stage 20 were larger and stronger, respectively, than those of MEBL-1-positive precursors (Fig. 6A, B), while the situation was the reverse at stage 22 (Fig. 6C, D). Thus, the MEBL-1 antigen expression in the melanogenic neural crest cell lineage replaces HNK-1 antigen

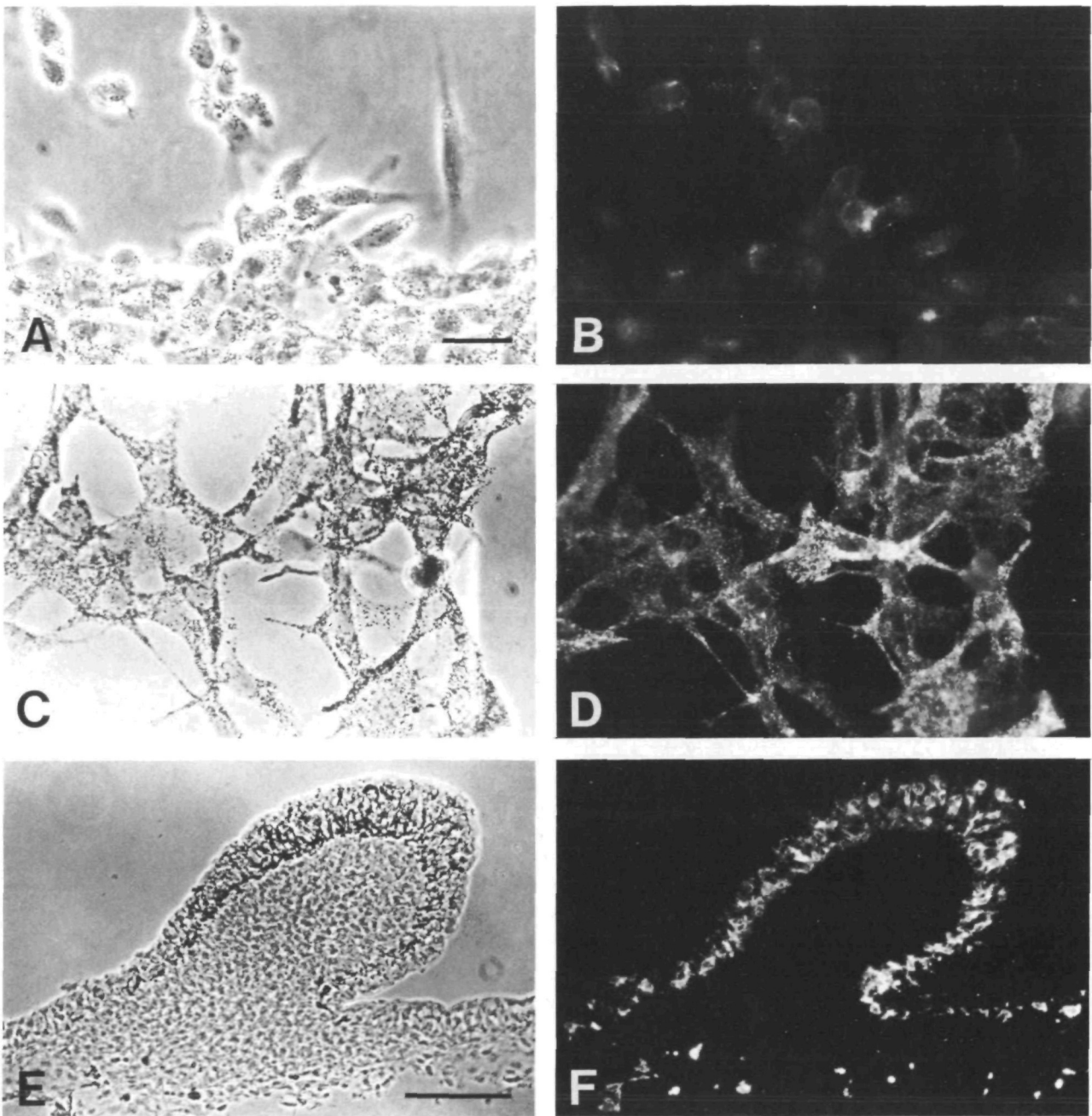


Fig. 3. Phase-contrast (A, C, E) and immunofluorescence (B, D, F) micrographs of cultured neural crest cells (A-D) and section of a feather filament at E10 of Silkie Bantum (E, F), stained by indirect immunofluorescence with MEBL-1. (A, B) Neural crest cells that have migrated from the cultured neural tube for 2 days. (B) Some are weakly stained by MEBL-1. (C, D) Neural crest cells cultured for 5 days. (C) They can be seen to be heavily loaded with pigment granules. (D) They are stained with MEBL-1, particularly so in the pigment granules. Bar(A to D)=25 μ m. (E, F) Many MEBL-1-positive melanocytes can be seen in the epidermis of a feather filament. Bar(E and F)=100 μ m.

expression, which is characteristic of migratory neural crest cells.

The timing of appearance of MEBL-1-positive precursors of melanocytes in cephalic and vagal regions

By stage 14, HNK-1-positive cephalic neural crest cells vacated the top of the neural tube and dorsal side of the

embryo (Fig. 7A-C). In the vagal region (level of somites 1 to 7 in the posterior rhombencephalon), numerous HNK-1-positive neural crest cells had already reached the lateral border of the pharynx. However, in no case could MEBL-1-positive precursors of melanocytes be seen at this stage (Fig. 7D).

In the cephalic region, MEBL-1-positive precursors were first found at stage 16 in the mesenchyme between

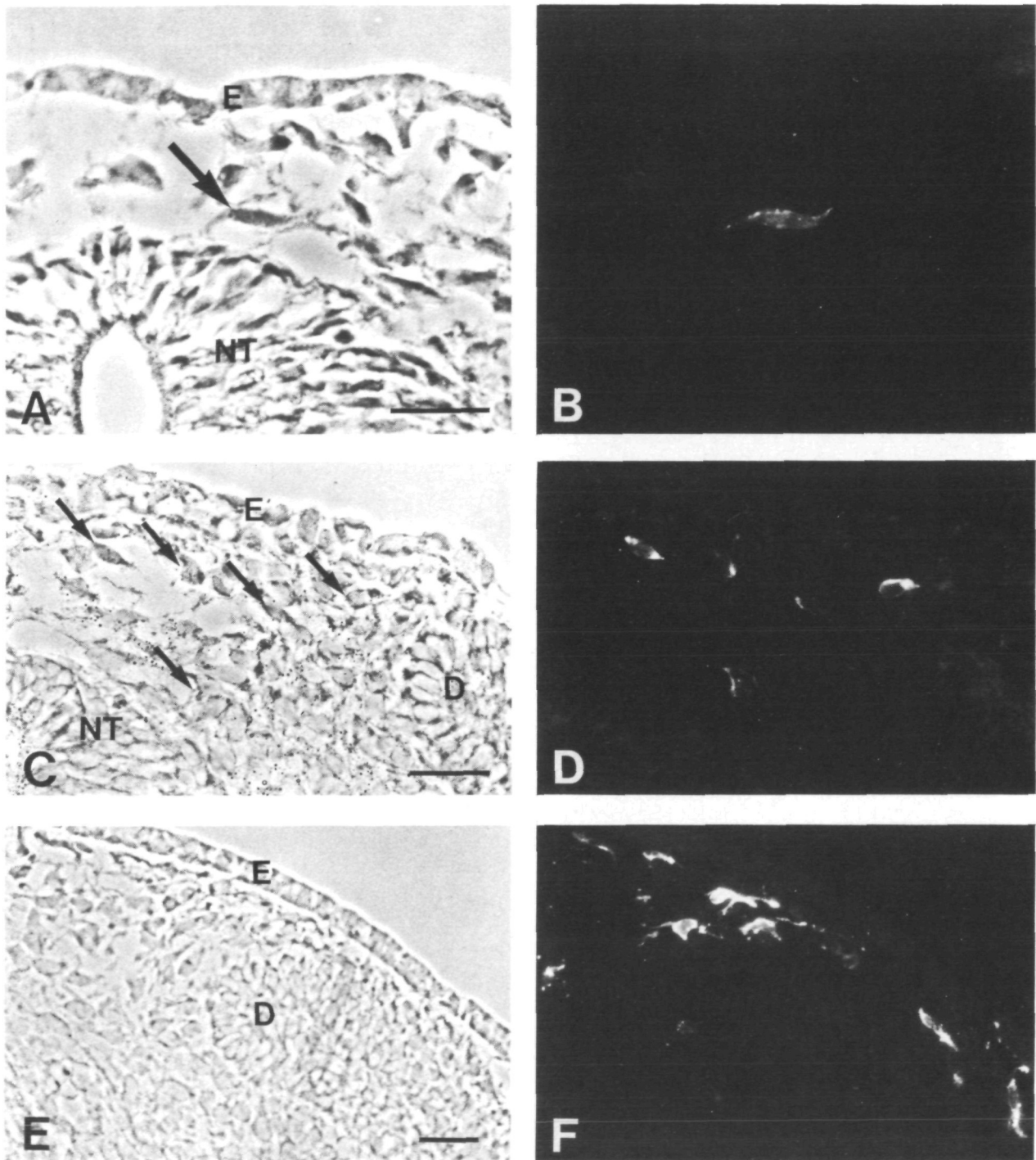


Fig. 4. Phase-contrast (A, C, E) and immunofluorescence (B, D, F) micrographs of sections of trunk, stained by indirect immunofluorescence with MEBL-1. (A, B) Trunk at stage 19. A MEBL-1-positive precursor (an arrow in "A") was first noted in mesenchyme located above neural tube (NT) at wing bud level. Ectoderm; E. (C, D) Trunk at stage 20. MEBL-1-positive precursors (arrows in "C") lie toward the dermomyotome (D). (E, F) Trunk at stage 22. MEBL-1-positive precursors can be seen along the dorsolateral route which is tightly bounded by ectoderm and dermomyotome. Bar=25 μ m.

the dorsal mesencephalon and ectoderm (Fig. 8). In the vagal region, a few MEBL-1-positive precursors were first detected at stage 18 in the mesenchyme between the posterior rhombencephalon and ectoderm (Fig. 9).

The timing of emigration of MEBL-1-positive precursors from the cephalic and vagal neural tube was examined by injecting DiO into the lumen of the neural tube. Although a few cells labelled with DiO after stage

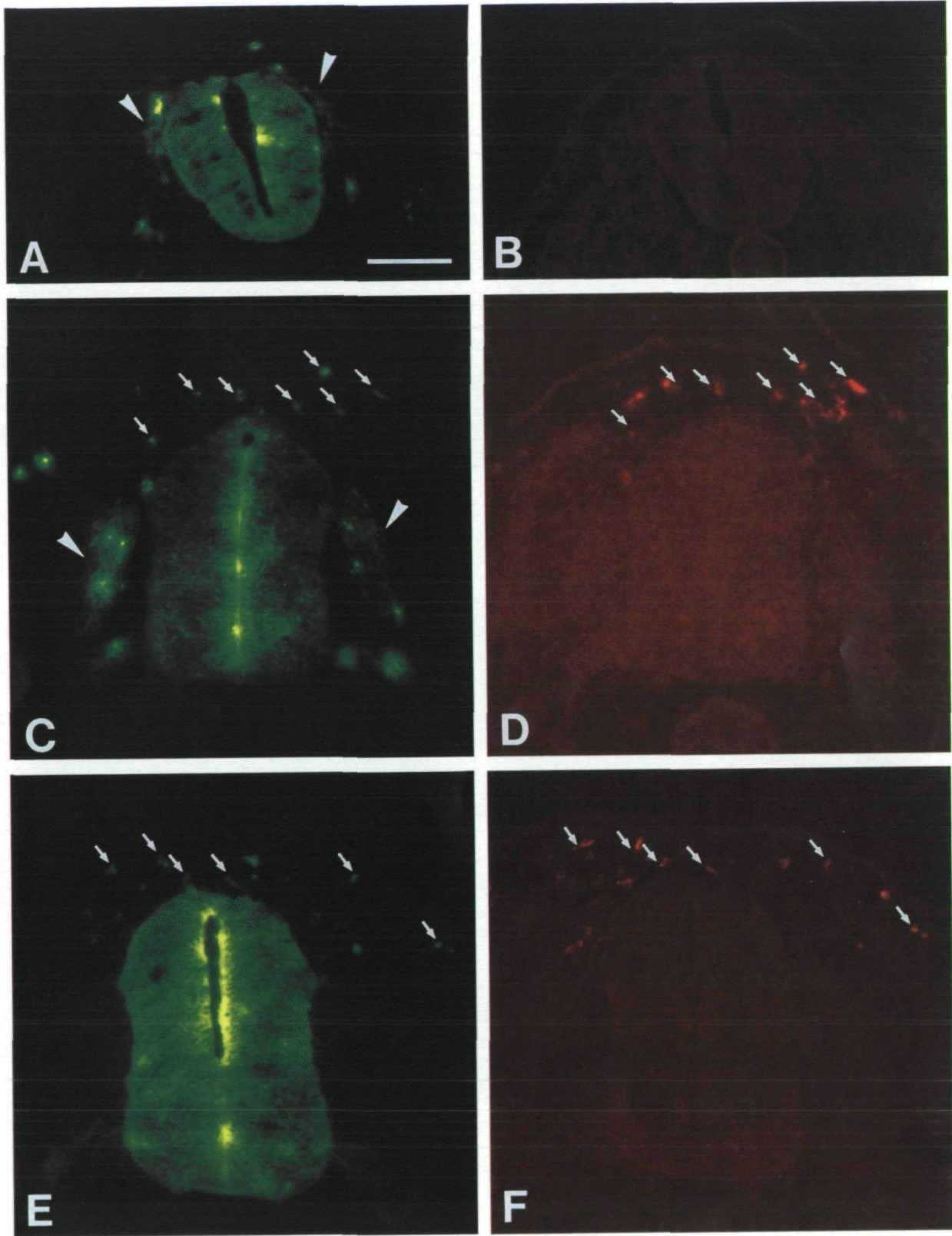


Fig. 5. Fluorescence micrographs of trunk neural crest cells labelled with DiO (A, C, E) and MEBL-1 (B, D, F). (A, B) The same transverse section through an embryo injected with DiO at stage 13⁻, incubated until stage 17. (A) DiO-labelled cells were observed along the ventral (arrowheads), but not dorsolateral route. (B) No MEBL-1-positive precursors could be found at this stage. (C, D) The same transverse section through an embryo injected with DiO at stage 12⁺, incubated until stage 20. (C) DiO-labelled cells were observed in all truncal neural crest cell derivatives located in the ventral (arrowheads) and dorsolateral (arrows) routes. (D) DiO-labelled cells along the dorsolateral route were MEBL-1-positive (arrows). (E, F) The same transverse section through an embryo injected with DiO at stage 17, incubated until stage 20. Many of DiO-labelled cells (arrows in "E") were MEBL-1-positive (arrows in "F"). Bar=100 μ m.

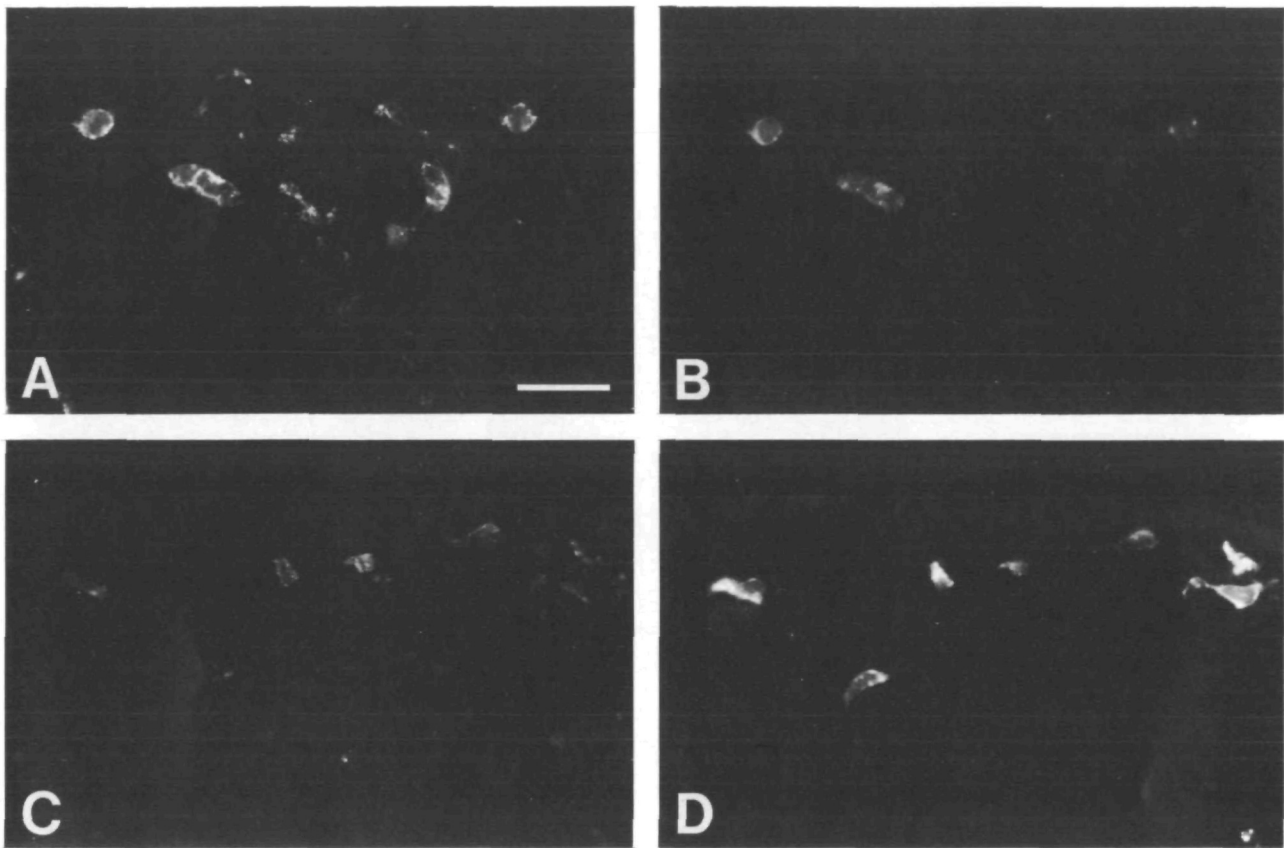


Fig. 6. Immunofluorescence micrographs of sections of trunk, double-stained by indirect immunofluorescence with HNK-1 (A, C) and MEHL-1 (B, D). (A, B) Trunk at stage 20. Identical cells above the neural tube are stained with both HNK-1 and MEHL-1. Relative staining intensity with HNK-1 exceeds that with MEHL-1. (C, D) Trunk at stage 22. Relative staining with MEHL-1 exceeds that with HNK-1. Bar=25 μ m.

14 in cephalic region were stained by MEHL-1 (data not shown), we could not determine the precise emigration timing of the precursors from the neural tube by this method (see "Discussion").

Immunoelectron microscopic localization of the MEHL-1 antigens in migratory MEHL-1-positive precursors of melanocytes

To determine the intracellular localization of the MEHL-1 antigens, MEHL-1-positive precursors were observed. Strong immunoreactivity was evident in cell membranes, in a portion of the endoplasmic reticulum and Golgi apparatus, and in nuclear membranes of the precursors (Fig. 10). Premelanosome-like granules of the precursors were also noted to be immunoreactive (inset in Fig. 10). Pigment granules in melanocytes from cultured neural crest cells were MEHL-1-positive (Fig. 3C, D). Thus, MEHL-1 antigens eventually become localized in the cell membranes and (pre)melanosomes of melanocytes and their precursors.

Identification of the MEHL-1 antigens

Identification of the MEHL-1 antigens in the trunk at stages 22 to 24 was unsuccessful because of deficiency of migratory MEHL-1-positive precursors of melanocytes. Immunoblot analysis of dorsal skin at E8 indicated that

the MEHL-1 antigens comprises a protein of $135 \times 10^3 M_r$ (Fig. 11A). Furthermore, another protein was found to be present at the position of $115 \times 10^3 M_r$, overlapping that of a non-specific band (Fig. 11A, B).

Discussion

To elucidate the mechanisms underlying the restriction of multipotential neural crest cells, a molecular marker is required that definitely recognizes only a specialized cell lineage and can be followed from an early stage of differentiation. MEHL-1 cloned in the present study specifically recognized the melanocyte lineage, allowing the early process of differentiation of melanogenic neural crest cells in situ to be followed.

Commitment, emigration and differentiation of melanogenic neural crest cells

MEHL-1-positive precursors of melanocytes were found in mesenchyme located above the neural tube at all cephalocaudal levels examined (Figs 4, 8, 9). This shows that the neural crest cells express MEHL-1 antigens early in their migration from the neural tube and thus become melanogenic. The present results on the timing of the appearance of MEHL-1-positive

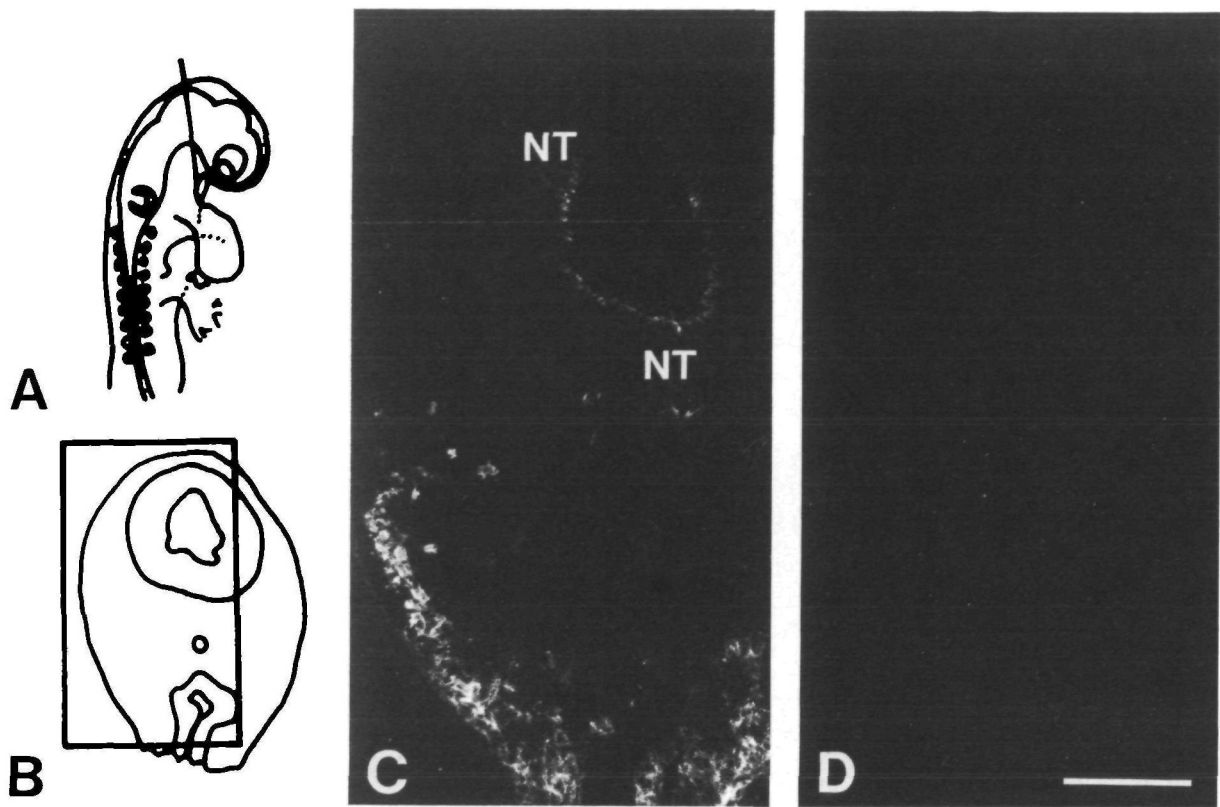


Fig. 7. Immunofluorescence micrographs of sections of the mesencephalic region at stage 14, stained by indirect immunofluorescence with HNK-1 and MEBL-1. (A) The axial level corresponding to B-D can be seen. (B) Schematic diagram of a section; the square indicates an area from photomicrographs C-D. (C) HNK-1 immunostaining. Many HNK-1-positive neural crest cells can be seen to have already left the top of the neural tube (NT). (D) MEBL-1 immunostaining of the neighbouring section. No MEBL-1-positive precursors are present. Bar=150 μ m.

precursors of melanocytes along the cephalocaudal axis are summarized in Fig. 12. MEBL-1-positive precursors were first noted in the mesencephalic region at about stage 16, although mesencephalic neural crest cells appeared first and start migrating by stage 10 (Noden, 1975). Following the appearance of the cephalic MEBL-1-positive precursors, MEBL-1-positive precursors at vagal and wing levels could be seen progressively from about stage 18 and 19, respectively. Thus, melanogenic differentiation proceeds cephalocaudally as shown in the formation of other neural crest-derivatives such as the dorsal root ganglion. MEBL-1 antigens were seen to be expressed much later than the reported timing of emigration of neural crest cells (Noden, 1975; Thiery et al., 1982). This prompted the authors to determine the emigration timing of melanogenic neural crest cells from the neural tube.

Serbedzija et al., (1989) showed by DiI staining that trunk neural crest cells, which migrate along a dorsolateral route, emigrate from the neural tube at approximately stage 20. By labelling trunk neural crest cells with DiO and immunostaining with MEBL-1 in the present study, these cells, which emigrate from neural tube at the wing bud level after stages 17-18 and migrate along dorsolateral route, were confirmed to be MEBL-1-positive precursors of melanocytes (Fig. 5) which no longer express the HNK-1 antigen. In contrast, most

neural crest cells which differentiate into peripheral nerve and chromaffin cells emigrate from neural tube before stages 17 (Fig. 13).

The emigration timing of neural crest cells at the vagal and cephalic that become melanocytes could not be determined precisely with DiO injection, because the lumen of cephalic and vagal neural tube is too large to label the entire neuroepithelium with DiO and distinguishing neural crest cells from blood cells, which are often simultaneously labelled by injected DiO, is difficult. There are two possibilities for the timing of emigration from cephalic and vagal regions of neural crest cells that become melanogenic: (1) all cephalic neural crest cells emigrate from the neural tube up to stage 14, with some remaining in the space between the mesencephalon and ectoderm up to stage 16, which then express melanogenic traits and migrate along a dorsolateral route, and (2) neural crest cells that become melanogenic emigrate from the neural tube after about stage 16 and soon express melanogenic traits. Although the first possibility cannot be excluded, there are no reports indicating neural crest cells that remain above the neural tube. Taking into consideration the emigration timing of trunk neural crest cells mentioned above, the second possibility would thus appear more valid.

It is presently considered that the developmental

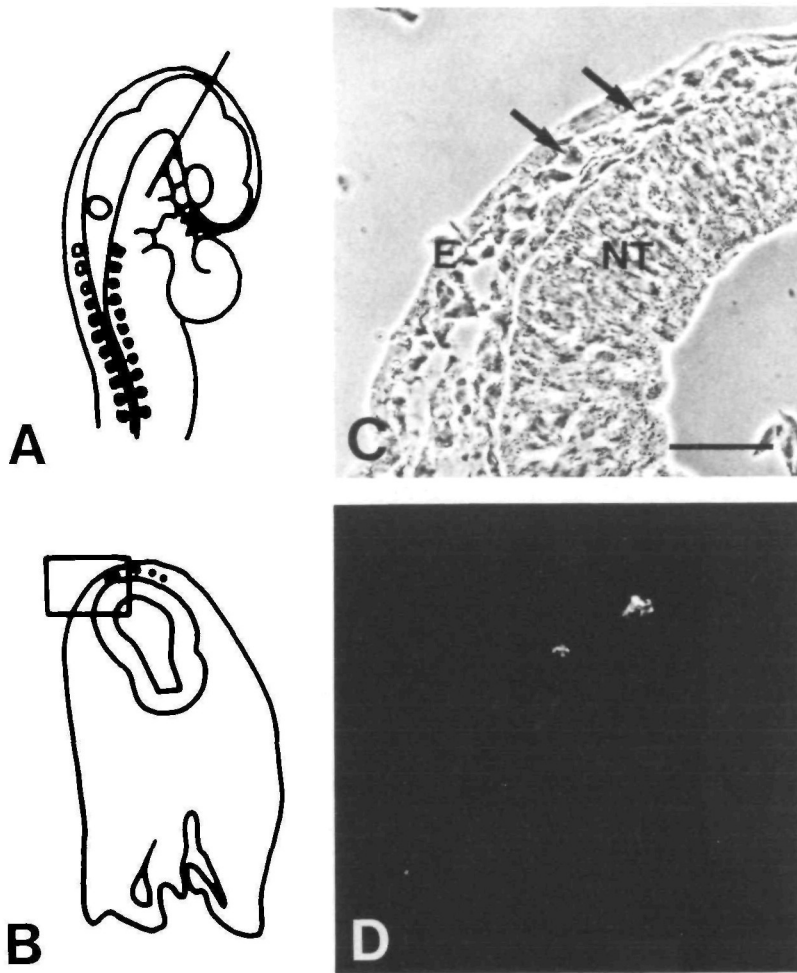


Fig. 8. Phase-contrast and immunofluorescence micrographs of a section of the mesencephalic region at stage 16, stained by indirect immunofluorescence with MEBL-1. (A) Axial levels corresponding to B-D can be seen. (B) Schematic diagram of a section; the square indicates an area from photomicrographs C-D. (C) Phase-contrast micrograph. (D) Immunofluorescence micrograph. (C, D) MEBL-1-positive precursors appeared first in mesenchyme between the mesencephalic neural tube (NT) and ectoderm (E) at this stage (arrows in "C"). Bar=50 μ m.

potential of neural crest cells is restricted step by step during migration or after they have reached their destination (Weston, 1988; Anderson, 1989; Le Douarin, 1990). The site of restriction of multipotential neural crest cells that become melanocytes in normal development poses a question. From the present results on the trunk, neural crest cells that subsequently differentiate into melanocytes in situ, remain in the trunk neural tube up to at least stages 17-18 and differentiate into no other neural crest derivatives in situ. Although the strict developmental potential of these cells should be examined in vitro, they possibly may be committed to some extent in the neural tube to become melanogenic. If this is the case, the temporal segregation of neural crest cells with melanogenic potential in the neural tube would be a possibility. MEBL-1-positive cells at vagal and cephalic levels appeared above the neural tube after stage 16, a much later time than that of the emigration of neural crest cells that become other neural crest derivatives (Figs 7-9; Noden, 1975). Thus, the above possibility of temporal segregation may apply to the vagal and cephalic neural crest cells.

The present study shows that MEBL-1 antigens are expressed in the mesenchyme over the neural tube prior

to dorsolateral migration, indicating that the micro-environment above the neural tube, after about stage 16 in cephalic level and stage 18-19 in vagal and wing bud levels, may influence the differentiation of migratory neural crest cells with melanogenic potential into precursors of melanocytes. This is supported by studies on the promotion of melanogenesis above the neural tube (Loring et al., 1981, 1982; Weston et al., 1988).

Melanogenesis has been observed in the cultured dorsal root ganglion (Nichols et al., 1977; Ciment et al., 1986). However, cells of the dorsal root ganglion do not express MEBL-1 antigens and they do not subsequently differentiate into melanocytes in situ (data not shown). The reason for this may be that oligopotential neural crest cells migrate to form the dorsal root ganglion but a microenvironment appropriate for melanogenic differentiation is not available in the dorsal root ganglion (Sieber-Blum, 1989a).

It is unknown at present whether all MEBL-1-positive cells differentiate into melanocytes. It was shown by immunoelectron microscopy that some MEBL-1-positive cells do not possess a healthy intracellular structure (unpublished data). Programmed cell death in the melanogenic cell lineage derived from neural crest cells would thus appear a possibility.

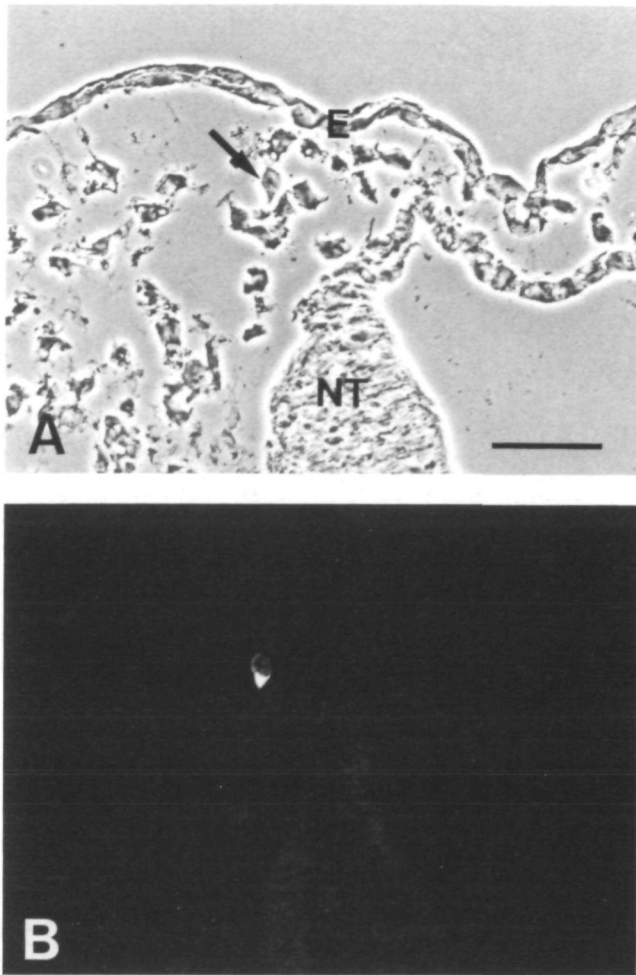


Fig. 9. Phase-contrast (A) and immunofluorescence (B) micrographs of a section of the vagal region at stage 18, stained by indirect immunofluorescence with MEBL-1. (A, B) A MEBL-1-positive precursor first appeared in mesenchyme between the posterior rhombencephalic neural tube (NT) and ectoderm (E) at this stage (an arrow in "A"). Bar=50 μ m.

Characteristic of MEBL-1 antigens in the melanocyte lineage

The MEBL-1 antigens are expressed very early in melanogenic neural crest cells before distinct melanosome structures are formed and a DOPA-positive reaction can be histochemically detected. Expression of the MEBL-1 antigens was noted quite early compared with the appearance of a molecular marker in other neural crest cell lineages, such as the expression of Schwann cell myelin protein in Schwann cells or SSEA-1 and substance P in sensory neurons derived possibly from a common progenitor cell along with melanocytes (Dulac et al., 1988; Sieber-Blum, 1989b; New and Mudge, 1986).

Immunolocalization of the MEBL-1 antigens under electron and light microscopes indicated that the antigens are restricted to the cell membrane and (pre)melanosomes of melanocytes and their precursors (Fig. 10). Two MEBL-1 antigens were identified by

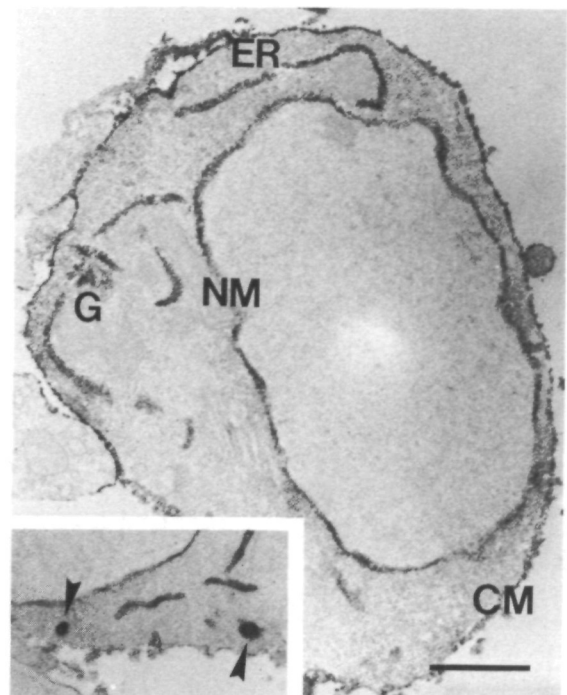


Fig. 10. Immunoelectron micrographs of ultrathin sections of a precursor of a melanocyte in the trunk at stage 24, stained by indirect immunoperoxidase method with MEBL-1. Cell membrane (CM), in addition to nuclear membrane (NM) and in a portion of the endoplasmic reticulum (ER) and Golgi apparatus (G), is strongly stained. Immunoreactive premelanosome-like granules (arrowheads) are present in a small number in the cytoplasm (inset). Bar=0.25 μ m.

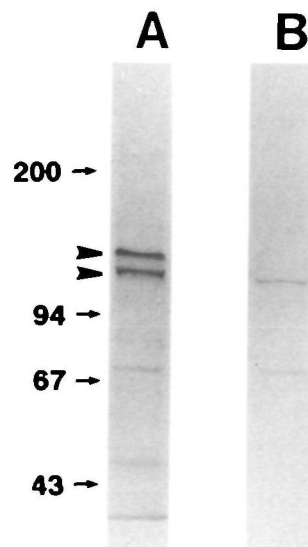


Fig. 11. Immunoblotting analysis of MEBL-1 antigens in dorsal skin at E8. MEBL-1 (A), fresh culture medium (B). Possible antigens ($135 \times 10^3 M_r$ and $115 \times 10^3 M_r$) are shown by arrowheads. Numbers indicate relative molecular masses $\times 10^3$.

western blotting (Fig. 11) and immunochemically shown to be similar glycoproteins (unpublished data). One of these was restricted to the cell membrane and the other to (pre)melanosomes (unpublished data). Thus, it is of considerable interest that similar antigens

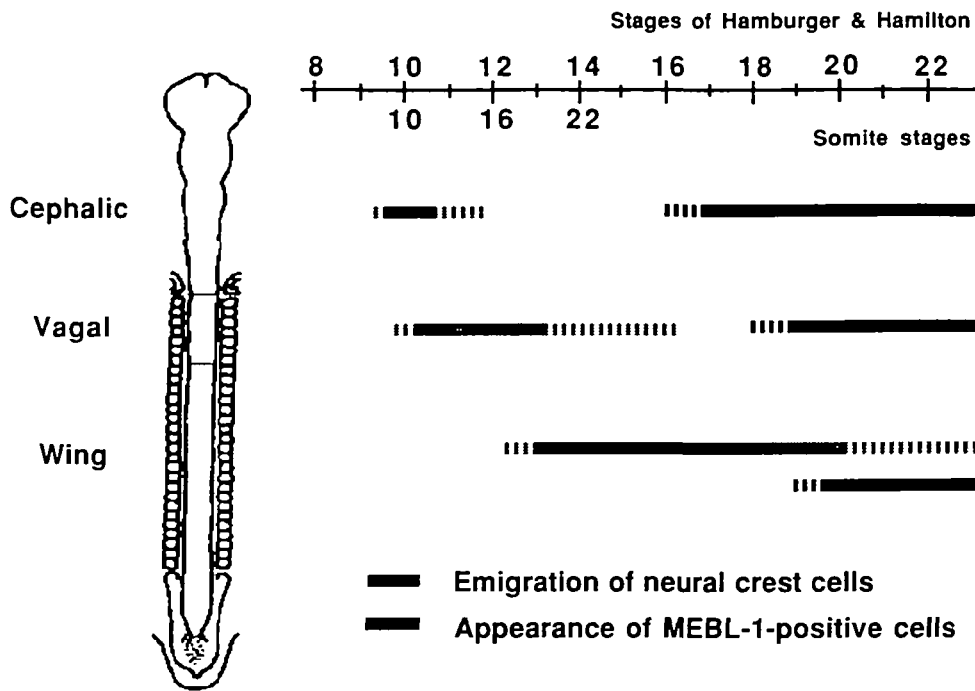


Fig. 12. Summary of the timing of appearance of MEBL-1-positive precursors of melanocytes along the cephalocaudal axis. The timing of emigration of neural crest cells is indicated based on data from Noden (1975), Thiery et al. (1982), Duband and Thiery (1982) and Serbedzija et al. (1989). In the trunk region, neural crest cells that subsequently differentiate into melanocytes can be clearly seen to have emigrated from the neural tube after stages 17-18 (Fig. 5, 13). Precursors of melanocytes at cephalic and vagal levels appeared above the dorsal side of the neural tube at a time much later than that of the emigration of other neural crest-derived precursors.

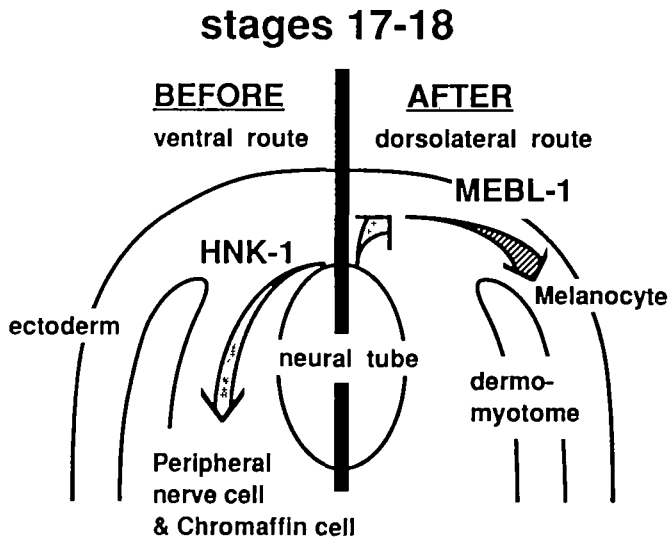


Fig. 13. Emigration, differentiation and migration of melanogenic neural crest cells at the level of a wing bud. Neural crest cells that finally differentiate into melanocytes begin to emigrate from the neural tube at stages 17-18, acquire MEBL-1 antigenicity, lose HNK-1 antigenicity and then migrate along the dorsolateral route while most neural crest cells, which finally differentiate into peripheral nerve and chromaffin cells, emigrate before stage 17, migrate along the ventral route and retain HNK-1 antigenicity during migration.

specific for melanocyte lineage are expressed in cell membrane and premelanosome-like granules of the precursors of melanocytes at an early stage of differentiation (Bagnara et al., 1979).

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