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IN VIVO SEGREGATION OF NEURONAL CELLS FROM NEURAL CREST CELL POPULATION

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A BSTRAK

Pemisahan sel-sel syaraf dari sel-sel neural crest pada embrio ayam telah dipelajari dengan memakai pewarna ganda immuno-sitokimia. Monoklonal antibodi HNK-1 dipakai sebagai pewarna khusus sel-sel neural crest, sedang nomoklonal antibodi neurofilamen dipakai untuk menditeksi terbentuknya sel-sel syaraf dari populasi sel diatas. Berbeda dari hasil Girdlestone dan Weston (1985) *in vitro* yang menemukan bahwa neuron sensori terbentuk lebih dulu dari neuron sympatis, pada penelitian ini pemisahan neuron-neuron sensori (dorsal root ganglia) dan neuron-neuron sympatis terjadi pada waktu yang sama yang terlihat sejak stadium 30-somit, pada saat mana belum terbentuk neurotrasmitter neuron sympatis. Hasil ini menunjukkan bahwa (se-kurang-kurangnya pada ganglia sympatis) ciri-ciri yang umum dari sel-sel syaraf tampak mendahului ciri- ciri khusus, seperti terbentuknya neurotransmitter. Hasil ini juga menyokong teori pemisahan dua garis turunan sel yang diusulkan oleh Le Douarin pada tahun 1986.

INTRODUCTION

For many years, it has been believed that the neural crest is pluripotent, homogenous population of cells wich will differentiate into any of several derivatives when encountering the appropriate environmental cues (Le Douarin and Teillet, 1974). Therefore, under this hypothesis, neural crest cell differentiation depends on the influence of the environment. However, it has been show recently *in vivo* that the neural crest is not an homogenous population of cells. The presence of neural crest subpopulation at early migratory stages and even before individualizing from the neural tube have been directly demonstrated by monoclonal antibodies (Vincent and Thiery, 1984; Barbu, *et al.*, 1986) and by acetylcholine esterase activity (Chochard and Cotley, 1983). Taken together, these data point to preexisting differences among the neural crest cells before their migration.

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In vitro studies have also shown that the neural crest is a heterogenous population of cells. Using a lipophilic fluorescent dye, merocyanine 540, as a probe for developmentally regulated plasma membrane components of early migrating trunk neural crest cells in cultures, it was demonstrated that three different types of clonal progeny are descendants of distinct subpopulations of progenitor cells (Sieber-Blum and Sieber, 1984).

Phase contrast and immunocytochemical studies using antibodies against fibroblast intermediate filament protein, vimentine, and against neurofilament prtein revealed that only mesenchymal cells and no neuronal cells were detected in one day cultures of premigratory mesencephalic and truncal neural crest. However, two population, stellate (mesenchymal) and neuronal cells, appeared in 2 day cultures (Ziller, *et al.*, 1984). So, after two days in culture, neuronal cells segregate from the mesenchymal-like neural crest cells.

Heterogeneity of neural crest cell population was also shown by Girdlestone and Weston (1985) *in vitro* using monoclonal antibodies A2B5 and R24 which recognize gangliosides specific for neuronal and glial precursors, respectively. When neural crest cell cleusters isolated from one day neural tube explants were cultured, they found about 1% of cells were already positive to A2B5 after one day. The A2B5 positive cells appeared to encrease after a few days. The early subpopulation of A2B5 cells exhibited unipolar or bipolar morphology while the later subpopulation exhibited small stellate morphology. None of these A2B5 positive cells bind detectable levels of R24.

Furthermore, using autoradiography to detect the cells that undergo mitosis, they obtained a second subpopulation of A2B5 cells derived from the negatively labelled cells of the early population which had divided at least once. The first subpopulation was assumed to be the precursor of sensory neurons while the second subpopulation was assumed to be the precursor of sympathetic neurons (Girdlestone and Weston, 1985). This result means that neural crest cells segregate to sensory neurons before they segregate to sympathetic neurons.

Based on the above *in vitro* and *in vivo* studies, neural crest cell sub-populations found at premigratory stages or even before emergence from the neural tube are probably the precursors of neurons and nonneuronal cells. As a consequence, precursors of neuronal cells will differentiate into sensory or sympathetic neurons when they encounter the appropriate cues, while the nonneuronal precursors will differentiate into melanocytes or glial cells. Some nonneuronal cells are able to undergo melanoggenesis when their interaction whit neurons is prevented. For instance, sensory ganglia of 4-day avian embryos which contain precursors of glial cells produced melanocytes when they were cultured *in vitro* (Nichols and Weston, 1977;Nichols, et al., 1977). Immunocytochemical studies also showed that monoclonal antibody R24 against human melanoma cells also binds to cultured crest-derived melanocytes, as well as to nonneuronal cells from avian embryonic spinal ganglia, indicating a common ancestry (Girdlestone and Weston, 1985).

If segregaation of neuronal cells *in vivo* follows the segregation pattern found *in vitro* by Girdlestone and Weston (1985) as described above, then the sensory neurons will differentiate from a common precursor cell before the sympathetic neurons differentiate. Up to-now, however, there is no information about the segregation of these neuronal types *in vivo*. The present study set out to fill theis gap in information about the in vivo segregation of neurons by using a specific neuronal marker.

MATERIALS AND METHODS

White leghorn straain (Ohio State University Poultry Science Dept) chick embryos, incubated in a Leahy forced draft incubator at 38° C and sacrificed at 30, 35, and 40 somite stage (Hamburger and Hamilton, 1951) were used in this study to trace the differentation of neuronal cells from migrating neural crest cells at trunk region. The studies were carried out at the 11^{th} to 15^{th} level of somites for each stage of the embryo using indirect immunofluorescence methods.

a. Histological procedures

The eggs that had been incubated for 2.5, 3 and 3.5 days in order to get embryos of 30, 35 and 40 somite stages, respectively, were fixed with Zenker's

fixative for 2-2.5 hours. Each embryo studied was carefully staged by counting the somites, and the posterior region was discarded by cutting the embryo at the middle level of the 16th somite. Following washing in running water for 30 minutes, the embryos were dehydrated trhough a series of alcohol for 10 minutes each, cleared with xylene, and infiltrated and embedded in paraffin. During the dehydration, the embryos were also washed with 70% alcohol containing several drops of a saturated solution of iodine in 95% alcohol for 2-3 minutes to remove $HgCl_2$ crystals from tissues that had been exposed to Zenker's fixative. The embryos then were serially sectioned at 7 um on a Leitz rotary microtome in saggital plane. The sections were mounted on albuminized slides, and dried overnight on a heated slide warmer prior to staining.

b. Immunofluorescent staining

In this study, double immunocytochemical staining was carried out, in which cells derived from the neural crest were labelled with monoclonal antibody to HNK-1 (Becton Dickinson), and with monoclonal antibody against neurofilament medium (NF) subunit (gifts of DR. V. Lee, University of Pennsylvania, Philadelphia, PA). After deparaffinizing in xylene and hydrating through a series of alcohols, serial sections from one half of each embryo were incubated with a detergent, 0.4% nonidet P40 in phosphate buffered saline (PBS), for 15 minutes, then washed in PBS for a total of 15 minutes with 3 changes. After that, section were stained with undiluted NF antibody for 1 hour, washed in 2 changes PBS, and then stained with the second primary undiluted HNK-1 antibody for 2 hours. Following 3 washes of PBS of 5 minutes each, the binding of the antibodies was visualized by incubating the sections for 30 minutes in a mixture of Rhodamine conjugated goat antimouse IgM(Cooper Biomedical) and Fluorescein-conjugated goat anti mouse IgG (Cooper Biomedical) which had been diluted 1:50 in PBS containing 3% Bovine Serum Albumin (BSA). Al of these immunofluorescent stainings were carried out in a humidified petridish at room temperature. After being rinsed in PBS for a total of 15 minutes, the sections were mounted with glycerin-PBS (9:1) containing 4% (pH 8) N-propyl gallate to retard quenching (Giloh and Sedat, 1982), and viewed with a Leitz Dialux-20 epifluorescence microscope. With the appropriate Ploempak filter, the HNK-1 labeled neural crest cells appeared as bright green fluorescent cells, while the neural crest cells that exhibited neurofilament-positive staining

appeared as red fluorescent cells. The labelled cells were photographed with T-max 400 high speed film (Kodak) using 2 minute exposures. As a control, sections were stained with non specific mouse serum instead of primary antisera and run in parallel.

RESULTS AND DISCUSSION

Migration of neural crest cells along their route on ventral pathway toward the dorsal aorta was studied with HNK-1 monoclonal antibody, while their neuronal differentiation was identified with 160-kD components of neurofilament triplet as a marker of neuronal cells (Payette *et al.*,1984). This double labelled method is possible to carry out because the monoclonal antibody HNK-1 is mouse IgM, while the monoclonal antibodyNF is mouse IgG. They can be distinguished using secondary antibody fluorescein-conjugated goat anti mouse IgM and rhodamine-conjugated goat anti mouse IgG, respectively.

HNK-1 antibody selectively labels neural crest cells, as well as some cells of the neural tube as also seen by Rickmann *et al.* (1985) and Bronner Fraser (1986). HNK-1 staining appears as bright fluorescence on the surface of the cells, while the NF staining appears as non fibrillar imunoreactivity in the cytoplasm surrounding the nonfluorescent nucleus. A similar appearance for NF staining was also shown by Payette *et al.* (1984) in vagal neural crest-derived precursors of enteric neurons. Both HNK-1 and NF show quite similar staining in neural crest cells, however, their specificity can be seen *in situ* control as shown in figure 1. This fogure shows that NF antibody stains the cells in the neural tube that are not stained by HNK-1 antibody. On the nother hand, HNK-1 antibody stains the neural crest cells that are also stained by NF antibody.

From serial sections of three embryos for each stage studied showed that during their migration, most of neural crest cells migrate through sclerotome mesenchymal cells in the anterior part of each somite. Neural crest cells appears to be oriented in the dorsoventral plane and aligned in files or streams of cells (Fig.2)

Neurofilament staining showed that as early as 30-somite stage, some cells were positive for Nf staining at dorsal region of the dorsal aorta, apparent

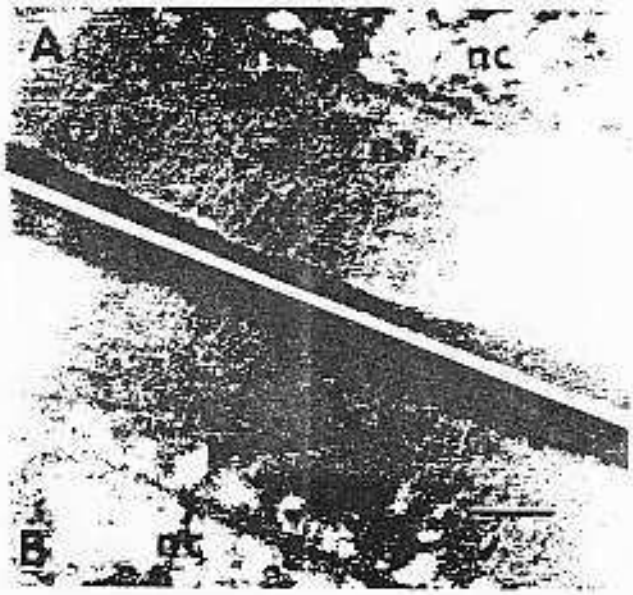

Figure 1. Photograph of a ventral section of dorsal region of 35-somite stage chick embryo.
a. HNK-1 staining shows that neural crest (nc) are stained. No cel in the neural tube is stained.
b. NF staining of the same section as in a. antibody stains not only the neural crest cells but also cells along the neural tube which probably are motor neurons.

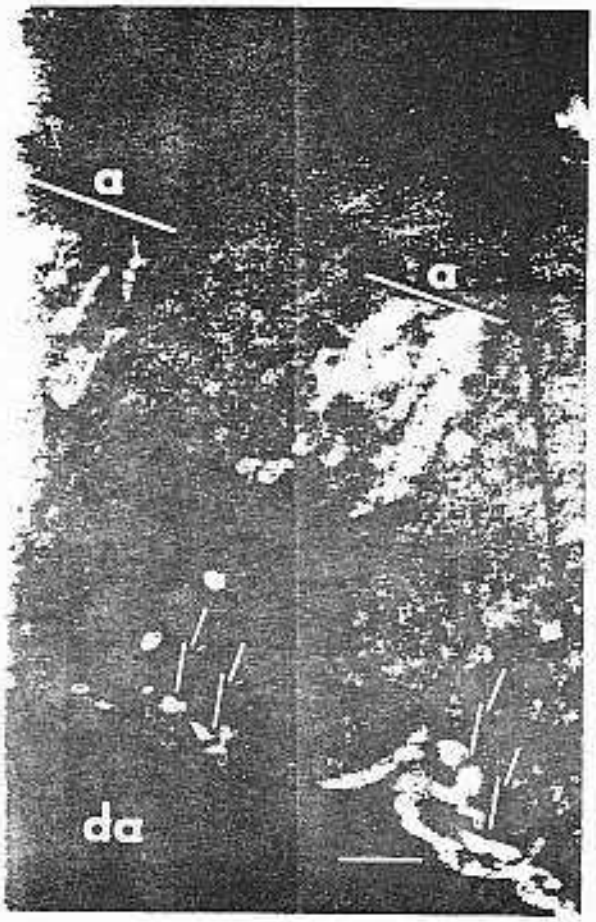

Figure2. NHK-1 staining of a sagittal section of a 30-somite stage at level of 11th and 12th somites (left to right) showing migration of the neural crest at anterior part (a) of each somite. Some of neural crest cells (arrows) have reached the ventral border at dorsal to the dorsal aorta (da).

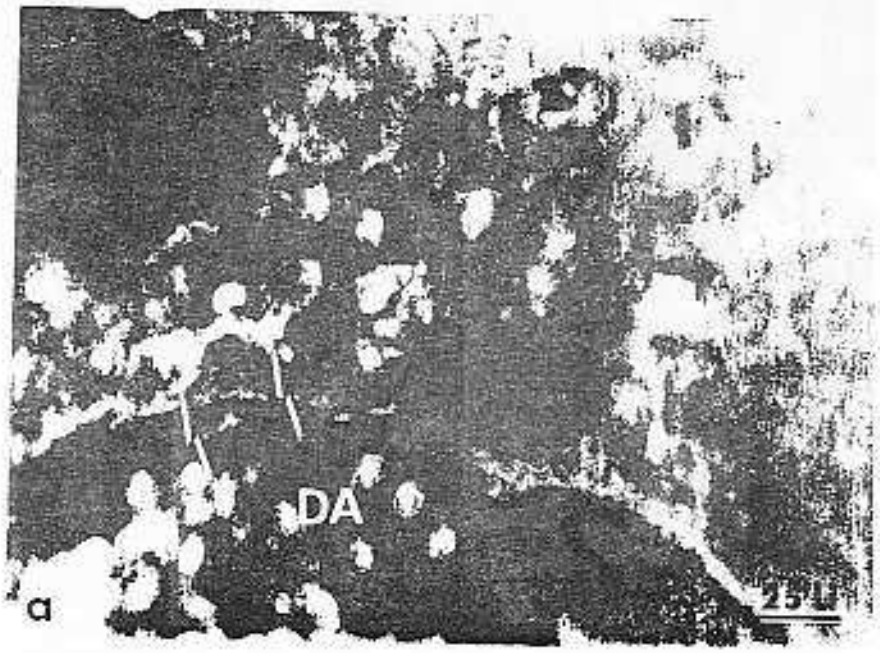

Figure 3 a.

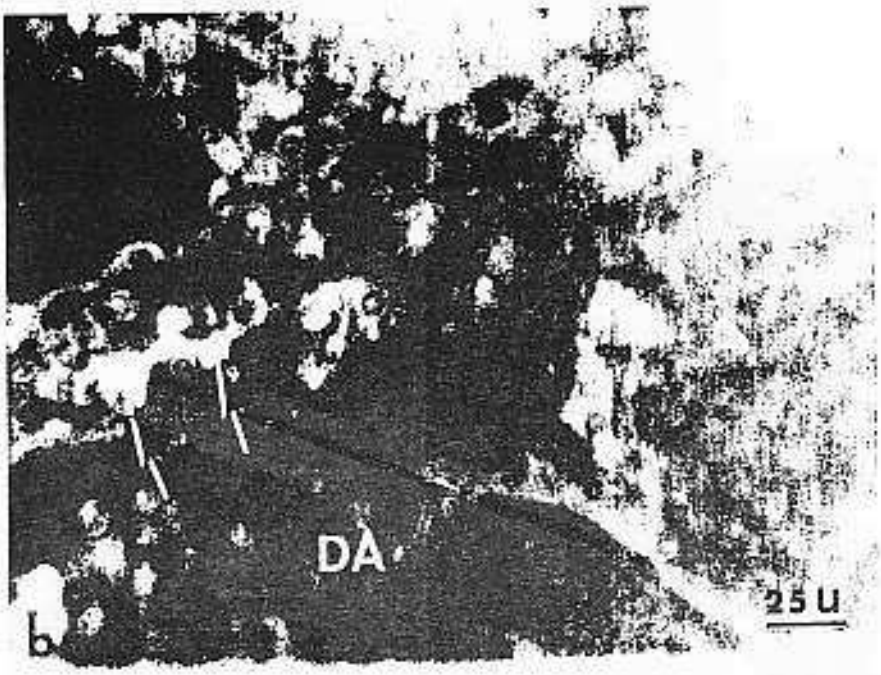

Figure 3 b.

Figure 3. Photographs of a sagittal section of a 30-somite stage at 11th somite level.

a. HNK-1 stained cells that have reached the dorsal region of the dorsal aorta (da).

b. NF staining of the same section as in a showing that some of these cells are also positive for NF immunoreactivity (arrows) while some of them are negatively stained (arrow heads)

precursors of sympathetic ganglia (fig.3) and within mesenchymal cells, apparent precursors of dorsal root ganglia (DRG) at level of 11^{th}-13^{th} somites (not shown). The simultaneous expression of NF immunoreactivity at both DRG and sympathetic ganglia precursors is clearly shown in 35-somite stage and older embryos in which more HNK-1 cells become NF positive cells. Lallier and Bronner-Fraser (1988) also observed NF staining within the rostral half of somite 15^{th} at stage 18-19 (Hamburger and Hamilton, 1951) which is equivalent to the 35- somite stage used in this study.

The presence of the NF cells at the same stage in DRG and sympathetic ganglia rudiments contrasts with the *in vitro* results of Girdlestone and Weston (1985) who showed a stepwise pattern of neuronal segregation from neural crest cultures. They found that neuronal cells which were assumed to be sympathetic precursors differentiated after the precursors of sensory neurons had been differentiated from neural crest population. However, it seems likely that the result in the present study support the dual cell line segregation model of Le Douarin (1986). She proposed that two types of precursors, sensory and autonomic, arise from a common progenitor during neural crest cell migration, and their terminal differentation take place under the influence of environmental cues. These precursors which have different survival requirements are present within DRG and sympathetic ganglia rudiments. The sensory precursors are able to survive and differentiate only in ganglia located in close proximity to CNS, while the autonomic precursors remain alive within developing DRG at least until hatching as shown by back transplantation of quail embryonic sensory ganglia into the chick neural crest migration (Le Douarin, 1986). The sensory precursors which are included in sympathetic ganglia rudiments will die due to lack of CNS influence. Therefore, this model points to the differentiation of a general neuronal characteristic namely neurofilament immunoreactivity, preceding specialized neuronal characteristic such as neurotransmitter, a possibility borne out by the present study.

CONCLUSSION

From this study it can be concluded that :

1. NF cells are present in presumptive sympathetic and dorsal root ganglia at the same stage.

2. The general neuronal characteristic, namely neurofilamen immuno reactivity, preceds the specialized neuronal

3. This study support the dual cell line segregation model proposed by Le Douarin

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