Differentiation of catecholaminergic cells in cultures of embryonic avian sensory ganglia

(dorsal root ganglion/nodose ganglion/tyrosine hydroxylase/catecholamine synthesis/cell division)

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ABSTRACT From the results of previous studies in which developing peripheral ganglia from quail embryos were transplanted into younger chicken embryo hosts, we concluded that spinal and cranial sensory ganglia contain dormant precursors with autonomic potentialities. Here we describe the differentiation of these precursors in vitro, from dorsal root and nodose ganglion cell suspensions. Dorsal root ganglia were removed from quail embryos at 9 to 15 days of incubation, dissociated to single cells, and grown in tissue culture. The differentiation of cells with autonomic features was followed by monitoring properties associated with the adrenergic phenotype (absent from quail sensory ganglia during normal embryonic development). Provided that the medium was supplemented with chicken embryo extract, numerous cells displaying tyrosine hydroxylase immunoreactivity could be detected from day 4 onward. They possessed elongated multiple processes but appeared morphologically distinct from primary sensory neurons. The catalytic activity of tyrosine hydroxylase and of other enzymes required for catecholamine production was demonstrated in the cultures by glyoxylic acid-induced histofluorescence and by radiochemical measurement of the conversion of exogenous tyrosine to norepinephrine. A large proportion of tyrosine hydroxylase-positive cells were found to incorporate [3H]thymidine before and after differentiation. In contrast, recognizable sensory neurons never exhibited adrenergic properties and did not divide. Qualitatively similar results were obtained with cultures of dissociated nodose ganglia. These findings lend further weight to the assumption that latent autonomic precursors are included in the non-neuronal compartment of sensory ganglia.

Results of various experiments with the avian embryo in ovo, involving the use of cell markers, have enabled the identification of nearly all the component cells of the peripheral nervous system (PNS) to be traced back to a transient embryonic structure, the neural crest. The only exception concerns the neurons of certain cranial sensory ganglia (i.e., the distal part of the trigeminal ganglion and the geniculate, petrosal, and nodose ganglia, located respectively on nerves V, VII, IX, X), which derive from ectodermal placodes, only their glial cells being of neural crest origin. The crest is also the source of a number of other cell types that become distributed throughout the body in a variety of locations reached by their precursors after a phase of migration (see ref. 1 for review).

By means of the quail-chicken chimaera system it has been possible to establish a precise fate map of the neural crest derivatives on the neural primordium at premigratory stages and to demonstrate that the phenotypes expressed by the crest cells are, to a large extent, dictated by cues arising from the embryonic environments in which they become localized at the end of migration (2–4). The paramount importance of these local cues for crest cell differentiation is attested by the fact that they can elicit the expression of virtually all the phenotypes represented in the PNS in crest cell populations originating from any point on the neuraxis, even though a given region of crest normally yields a comparatively restricted set of cell types.

The pluripotentiality thus demonstrated at the level of the neural crest cell population is maintained in developing PNS ganglion cells, which are also acutely sensitive to the embryonic microenvironment. This has been shown by in vivo back-transplantation of quail autonomic (Remak, ciliary, and sympathetic) and sensory (spinal and nodose) ganglia into the neural crest migration pathways of younger chicken hosts (5–9). These experiments revealed that the non-neuronal cell population of all types of peripheral ganglia contains latent neuronal precursors capable of migrating, dividing, and participating again in the formation of ganglia and paraganglia in which they express phenotypes appropriate to the particular microenvironment that they have reached. One of the most striking results was the observation that dorsal root ganglia (DRG) and nodose ganglia, irrespective of their age when grafted into the host, always yielded autonomic neurons and adrenomedullary cells, some of which exhibited characteristic catecholaminergic traits. It is to be emphasized that both in quail and chicken, no cells with such properties have been detected in DRG or nodose ganglia during normal embryonic development (7, 10). The conclusion was reached that these autonomic phenotypes were expressed by progeny of dormant cells in the non-neuronal compartment of the ganglia (see ref. 11 for a discussion).

The present study was undertaken to obtain direct evidence for the existence of these autonomic precursors by eliciting their differentiation in tissue culture.

MATERIALS AND METHODS

Culture of Quail Spinal Ganglia. DRG were removed from Japanese quail embryos at ages ranging from 9 to 15 days of incubation (E9–E15), over a length encompassing thoracic and lumbosacral levels of the rachis (i.e., between the last pair of ribs and the sacral vertebrae). Particular care was taken to avoid contamination by remnants of the sympathetic chains, which were always removed prior to dissection of the DRG. Approximately 100 pooled DRG were dissociated to single cells by agitation in Ca2+/-Mg2+-free phosphate-buffered saline, containing 0.25% trypsin (GIBCO), twice for 10 min at 37°C. Cells were pelleted by centrifugation at 1000 x g for 10 min and suspended in complete culture medium, consisting of Eagle’s minimum essential medium and 10% heat-inactivated horse serum (GIBCO), supplemented (unless otherwise stated) with 2.5S nerve growth factor (NGF).

Abbreviations: PNS, peripheral nervous system; DRG, dorsal root ganglion or ganglia; TyrOHa, tyrosine hydroxylase; CEE, chicken embryo extract; En, embryonic day n; NGF, nerve growth factor.
immunochemistry and Cytoskeleton. Tyrosine hydroxylase (TyrOHase; tyrosine 3-monooxygenase, EC 1.14.16.2), the first enzyme in the pathway leading from tyrosine to the catecholamines, was revealed immunocytochemically with an antiserum (kindly provided by A. Berod) directed against bovine TyrOHase and which does not crossreact with dopamine-β-hydroxyxylase (12). Cultures were fixed with 4% paraformaldehyde in Ca2+/Mg2+-free phosphate-buffered saline and incubated with the primary antibody at a dilution of 1:200. Antigen was detected by indirect fluorescence, using fluorescein-labeled goat anti-rabbit immunoglobulins (Nordic, Tilburg, The Netherlands).

Endogenous catecholamines were detected in cultured cells by the glyoxylic acid technique (13).

Radiochemical Assay of Catecholamine Production. Ganglion cell cultures were incubated for 4 hr in medium containing 25 μM L-[side-chain-2,3-3H]tyrosine [Amersham; specific activity 2–10 Ci/mmol (1 Ci = 37 GBq)] and the quantity of radioactive catecholamines synthesized was determined by HPLC analysis as described (14).

DNA Synthesis in Cultured Cells: Combined Immunocytochemistry and Autoradiography. Cultures of DRG were grown in the presence of [6-3H]thymidine (Centre d'Etudes Nucléaires, Saclay, France; specific activity 29 Ci/mmole) for various periods of time. The radioactive precursor was added as a solution (0.1 mCi/ml) in complete culture medium, the final concentration being adjusted according to the age of the cultures as follows: day 0–3, 0.5 μCi/ml; day 4–9, 2 μCi/ml. Except for some experiments in which a 3-hr pulse of [3H]thymidine was given, the indicated amounts of precursor were added every day until the end of the desired period of incorporation. The cultures then were rinsed three times in phosphate-buffered saline, fixed, and treated for TyrOHase immunocytochemistry as described above. After a further wash in phosphate-buffered saline, the cultures were processed for autoradiography using K3 emulsion (llford, Saint-Priest, France) and an exposure time of 1 week (2 weeks for the pulse experiments) at 4°C.

RESULTS

DRG Cultures. Morphological evolution of ganglion cells in culture. Freshly dissociated DRG cells displayed an obvious heterogeneity in size, with diameters ranging from 3 to 15 μm (Fig. 1A). In medium devoid of CEE but containing NGF, neurites had grown out from the large and medium-sized cells within 24 hr of plating (Fig. 1B). Cells of non-neuronal aspect were also present, but their growth was slow. The neuronal cell bodies, initially ovoid and phase-bright, subsequently flattened down upon the substrate. When the cultures were started in medium enriched by the addition of 10% CEE, nerve-fiber regeneration proceeded as before, but

non-neuronal cells grew much more rapidly and approached confluence after 5–6 days (Fig. 1C).

Appearance of adrenergic cells in vitro and characterization of their phenotype. At daily intervals, DRG cultures were treated for immunocytochemistry with antibody to TyrOHase. Irrespective of the culture conditions, no response was ever observed during the first 48–72 hr after plating. However, at later times, the results were highly dependent on the composition of the medium. In the presence of 10% CEE and exogenous NGF, 4-day cultures contained several hundred cells displaying various degrees of TyrOHase immunoreactivity. The number of positive cells subsequently increased, reaching a maximum between 6 and 9 days. The time course illustrated in Fig. 2 was established using DRG from E10 quails. Although not yet analyzed so extensively, E15 DRG were found to give quantitatively very similar results (in 3 determinations, 1727 ± 27 TyrOHase-positive cells were counted after 10 days in culture).

In marked contrast, when CEE was left out of the NGF-supplemented medium, there was a 96–100% reduction in the number of TyrOHase-immunoreactive cells that differentiat-
ed. On the other hand, provided CEE was present, omission of exogenous NGF affected neither the survival of primary sensory neurons (suggesting that NGF-like factors are con-
tained in CEE) nor the development of adrenergic cells. The results that follow exclusively concern cultures grown in "complete" medium: 80% Eagle's minimal essential medium/10% horse serum/10% CEE/NGF (20 ng/ml).
Fig. 2. Development of TyrOHase-positive cells in vitro as a function of time. Each culture dish initially contained $7 \times 10^4$ cells from E10 quail DRG. The symbols represent the means ± SEM of the total number of immunoreactive cells per dish that were identifiable at the indicated times. The number of cultures examined at each time point is given in parentheses.

In 4-day cultures, TyrOHase-positive cells occurred singly or in small clusters. However, by 6 days, the aggregates were usually much larger (Fig. 3), and it was not uncommon to find occasional groups of over 100 strongly immunoreactive cells. Although a few TyrOHase-positive cells had an irregularly shaped cell body with numerous, ramified outgrowths or dendritic structures, the majority were small and rounded, with multiple, long, branching processes. Large sensory neurons were invariably TyrOHase-negative (Fig. 4).

To determine whether the TyrOHase immunoreactivity corresponded to functional enzyme, we first applied the glyoxylic acid technique for the detection of intracellular catecholamines. From 4 days onward, an extremely dense network of brightly fluorescent, highly ramifying fibres was detected in all DRG cultures tested in this way. On the other hand, cell bodies were generally much less conspicuous than they were after immunocytochemical processing (Fig. 5).

The presence of catecholamine-synthesizing cells in DRG cultures was confirmed in a series of experiments in which their ability to convert exogenous tyrosine to catecholamines was quantified by radiochemical analysis. Undetectable in cultures that had been grown for <4 days, significant amounts of radioactive catecholamines could be measured in older cultures after a 4-hr incubation with $[^3H]$tyrosine (Fig. 6). The labeled product was identified as norepinephrine by HPLC. This finding implies that, in addition to TyrOHase, dopa decarboxylase and dopamine-$eta$-hydroxylase were also functional in the cultures. The increase in the ability to synthesize catecholamines that occurred between days 4 and

Fig. 3. Fluorescence photomicrograph of a group of small TyrOHase-positive cells in a 9-day culture of dissociated DRG from E10 quail. (Bar = 20 $\mu$m.)

Fig. 4. Fluorescence (A) and phase-contrast (B) photomicrographs of a 9-day culture of E10 quail DRG. Three small, TyrOHase-positive cells (two are indicated by outlined arrows) surround a large, TyrOHase-negative sensory neuron (solid arrows). TyrOHase-positive cells are often hard to distinguish by phase-contrast microscopy. (Bar = 20 $\mu$m.)

Fig. 5. Glyoxylic acid-induced histofluorescence revealing endogenous catecholamines in cell bodies (arrows) and fibers of a 9-day culture of dissociated DRG from E10 quail. (Bar = 20 $\mu$m.)
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dine present
Dissociated E9
culture (Fig. 7). Proportionately
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TyrOHase-positive population
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TyrOHase-positive and exposed
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Incorporation
[3H]thymidine
for various periods before being
fixed and processed for immunocytochemistry. Subsequent
autoradiography revealed that a significant proportion of
TyrOHase-positive cells were always labeled, irrespective of
the time at which the radioactive precursor had been added.
The highest percentage of labeled cells (44% of the
TyrOHase-positive population) was observed at 4 days,
when [3H]thymidine had been present from the start of the
culture (Fig. 7). Proportionately fewer TyrOHase-immuno-
reactive cells were found to have incorporated the DNA
precursor at later stages (16% between days 4 and 6 and 19%
between days 6 and 9).

That the multiplication occurring from day 4 onward
involved TyrOHase-positive cells themselves in addition to
their precursors was indicated by the results of pulse-labeling
experiments in which immunocytochemistry was carried out
only 3 hr after the addition of [3H]thymidine to the medium.
It was found that, at days 4 and 6 respectively, ≈25% and 10%
of the TyrOHase-immunoreactive cells (presumably already
adrenergic when exposed to the precursor) had incorporated
radioisotope.

In addition to TyrOHase-positive cells, numerous non-
neuronal cells (fibroblasts and glia) were labeled whenever
[3H]thymidine was introduced into the culture medium.
However, silver grains were never seen over the nuclei of the
TyrOHase-negative neurons.

**Nodose Ganglion Cultures.** Experiments were also con-
ducted with nodose ganglia in order to ascertain whether a
cranial sensory ganglion, of mixed neural crest/placodal
origin, would develop in the same way as DRG when grown
in dissociated cell culture. The results were qualitatively
similar to those described above. Several series of dissoci-
cated cultures of E9–E11 nodose ganglia were prepared and
monitored for the presence of adrenergic cells after 9 days in
vitro. A small number (20–40 per culture) of TyrOHase-
immunoreactive cells were found; they displayed a multipol-
ar morphology comparable to that of the TyrOHase-positive
cells that differentiated in DRG cultures. Glyoxylic acid
treatment revealed catecholamine-containing fibers, and low
but significant levels of synthesis of catecholamines from
[3H]tyrosine were also observed. When [3H]thymidine was
present throughout the culture period, about half the
TyrOHase-containing cells were found to be labeled.

**CONCLUSIONS AND DISCUSSION**

The results show that the development of numerous cells
exhibiting adrenergic characteristics can be triggered in
cultures of dissociated cell suspensions of quail embryonic
DRG taken at E9–E15. Cells similar in appearance, but much
fewer in number, also differentiated in cultured E9–E11
nodose ganglia.

The ages of the ganglia used in these studies were chosen
to ensure that sensory neuron precursors were incontrovert-
ibly postmitotic. In chicken embryo DRG, precursors of the
large lateroventral and the small mediodorsal neurons under-
go their terminal mitoses between E4.5 and E6.5 and between
E4.5 and E7.5, respectively (15). In the chicken nodose
ganglion, the neurons are virtually all postmitotic by E8 (16).
The maturation pattern is essentially similar in the quail (ref.
8 and unpublished data).

In contrast to frequent practice in the culture of developing
nervous tissue, we made efforts not to eliminate but to
maintain the non-neuronal ganglion cells and stimulate their
proliferation by the addition of serum and high concen-
trations of CEE. This approach was dictated by the inference,
based on the results of extensive studies in vivo, that cells
with autonomic-type potentialities are included in the non-
neuronal cell population of the ganglion (11, 17). This
assumption is supported by the data presented here. The
TyrOHase immunoreactivity that developed in cultured,
dissociated sensory ganglion cells was restricted to a minor
population (in the case of the DRG, ≈2% of the number of
cells initially plated) that, although heterogeneous in size and
shape and possessing long processes, appeared morpholog-
ically distinct from typical primary sensory neurons. Fur-
thermore, the results of the [3H]thymidine-incorporation
experiments, while confirming the postmitotic status of the
TyrOHase-negative sensory neurons, showed that, in con-

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**Fig. 6.** Synthesis and accumulation of catecholamine by cultures of E10 quail DRG. The conversion of [3H]tyrosine to [3H]norepi-
nephrine by sister cultures from two separate platings (● and △) was
assayed after different times in vitro. The points indicate the quantity
of norepinephrine produced per culture during a 4-hr incubation.

9 paralleled the increase in the number of TyrOHase-positive
cells over the same period.

**Adrenergic expression and cell division.** One characteristic
of the sensory neurons of the DRG is their withdrawal from
the cell cycle early in embryogenesis (between E4 and E8 in
the chicken and the quail; refs. 8 and 15). In an attempt to
discriminate between these postmitotic neurons and the
newly appearing TyrOHase-positive cell population, a series
of experiments was performed in which DRG cultures were
exposed to [3H]thymidine for various periods before being
fixed and processed for immunocytochemistry. Subsequent
autoradiography revealed that a significant proportion of
TyrOHase-positive cells were always labeled, irrespective of
the time at which the radioactive precursor had been added.
The highest percentage of labeled cells (44% of the
TyrOHase-positive population) was observed at 4 days,
when [3H]thymidine had been present from the start of the
culture (Fig. 7). Proportionately fewer TyrOHase-immuno-

**Fig. 7.** Incorporation of [3H]thymidine by TyrOHase-positive
cells. Dissociated E9 DRG were grown for 4 days, with [3H]thymi-
dine present throughout. Cultures were treated with anti-TyrOHase
and processed for autoradiography at the end of the fourth day. Three
of the five intensely immunoreactive cells seen in this field have a
radioactively labeled nucleus. (Bar = 20 μm.)
trast, a large proportion of the cells that expressed the adrenergic phenotype in culture divided before or after differentiating.

These in vitro studies extend earlier in vivo observations (see ref. 1) in that identification of catecholaminergic cells, based entirely on formaldehyde-induced fluorescence in the previous experiments, is here founded on more comprehensive criteria—i.e., TyrOHase immunoreactivity, catecholamine histochemistry, and ability to synthesize norepinephrine. It should be stressed that the functional expression of catecholamine-synthesizing enzymes by certain quail sensory ganglion cells in culture distinguishes adrenergic differentiation in this system from incomplete manifestations of the catecholaminergic phenotype that have been described in various uncharacteristic situations in vivo and in vitro (18–20).

The validity of our results depends crucially on the absence of contamination of the DRG preparations by neurons from the adjacent sympathetic ganglion chains. The possibility of such contamination can be discounted for the following reasons: ganglia were dissected to discard the extreme lateroventral region of the DRG; the first detectable cytochemical and biochemical signs of adrenergic differentiation in dissociated DRG cells did not appear before 3 or 4 days in vitro, whereas dissociated sympathetic ganglion cells displayed glyoxylic acid-induced fluorescence and were able to convert [3H]tyrosine to catecholamines from the moment they were put into culture (unpublished results). The anatomical location of avian nodose ganglia precludes their contamination by cells of the sympathetic nervous system.

Our choice of catecholamine-related properties as markers for autonomic, as opposed to sensory, ganglion cells appears justified in the light of our inability to observe detectable levels of the same features in DRG or nodose ganglia during development in ovo (7, 10). This is also in agreement with the results of an earlier in vivo study in which chicken sensory ganglia were found to contain no fluorescent cells even after L-dopa loading (21), although it contrasts with recent findings in the rat, where a few adrenergic neurons were shown to occur in the nodose ganglion (22–24) and in certain specific DRG (23–25). In the quail, therefore, the potentially adrenergic precursors located in sensory ganglia are all seemingly dormant under normal developmental circumstances, their latent properties materializing only when they are exposed experimentally to appropriate environmental conditions. Factors promoting adrenergic differentiation are associated with precise regions of the young embryo, notably the neural tube, notochord, and somitic mesenchyme (26–29). The findings reported here indicate that they are present also in an extract of whole embryos and are distinct from NGF.

The results of our investigation into the relationships between DNA synthesis and the expression of adrenergic properties revealed that ~50% of the TyrOHase-positive cells appearing at day 4 in cultured DRG had incorporated [3H]thymidine at some time during the previous 96 hr. Although prior multiplication in vitro is thus apparently not an absolute prerequisite for the differentiation of the precursors, it is plausible that at least some of the TyrOHase-positive cells that do not divide before expressing the catecholaminergic phenotype do multiply once they have differentiated. Our data show that cells already displaying catecholaminergic properties can incorporate [3H]thymidine during a brief exposure to the precursor, presumably in preparation for division (although DNA repair might account for some of the labeling). A similar phenomenon has been described during normal development of the sympathetic nervous system (30).

Retrotransplantation of avian peripheral ganglia has provided valuable data on the segregation of cell lineages in neural crest and its derivatives. The analysis in vitro of phenomena brought to light by these in vivo experiments opens new possibilities for understanding some of the mechanisms underlying the emergence of phenotypic diversity during ontogeny of the PNS.

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