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Schwann-cell differentiation in clonal cultures of the neural crest, as evidenced by the anti-Schwann cell myelin protein monoclonal antibody

(cell lineage/glial cells/peripheral nervous system/avian embryo/single-cell cultures)

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ABSTRACT **In the vertebrate embryo, Schwann cells lining the peripheral nerves originate from the neural crest (NC), a structure that also gives rise to ganglion satellite cells, most of the neurons of the peripheral nervous system, melanocytes, and part of the cranial mesenchyme. We have studied the emergence of the Schwann cell lineage *in vitro* in clonal cultures of quail mesencephalic NC cells by using the Schwann cell myelin protein antigen as an early and specific marker for myelinating and nonmyelinating cells. After 13–16 days in culture, numerous clones contained Schwann cell myelin protein-positive cells, sometimes isolated and sometimes associated with other NC-derived cell types. Detailed phenotypic analysis of the clones allowed us to infer the presence of differently committed Schwann-cell ancestors in the NC during the migration stage. In particular, we found evidence for the existence of a bipotent precursor of Schwann cells and nonneuronal satellite cells; a common precursor of neurons, satellite cells, and Schwann cells; and a pluripotent precursor of Schwann cells, satellite cells, neurons, and melanocytes. These founder cell types coexist in the NC with a committed Schwann cell progenitor of high-proliferative potential that differentiates *in vitro* in the absence of other peripheral cells and axons.**

With the exception of the neurons of certain cranial sensory ganglia, cell types that make up the peripheral nervous system (PNS) are derived from a transitory embryonic structure, the neural crest (NC). In the PNS, the glia comprises Schwann cells, which line the peripheral nerves and may or may not produce myelin, and satellite cells, which are associated with neuronal cell bodies in sensory and autonomic ganglia. In the enteric plexuses, satellite cells possess specific properties (1–3) that set them apart from the other categories of glia.

Schwann cells are usually defined by their close apposition to nerve fibers. It is known, in addition, that biochemical and functional interactions take place between axons and Schwann cells. *In vivo* studies have shown that neurites supply Schwann cells with mitogenic and myelinogenic signals (4–7). Studies of purified Schwann cells cultured *in vitro* have led to the demonstration that the axonal myelinogenic signal involves the production by the Schwann cell of a complex extracellular matrix that is necessary for both ensheathment and myelination of the nerve fibers (8–13).

Until recently, no obvious characteristic other than myelin production and their positions relative to the neurons permitted Schwann cells to be distinguished from satellite cells. Early in development, avian Schwann cells can now be distinguished from ganglion satellite cells, even in nonmyelinated nerves, by using the Schwann cell myelin protein (SMP) marker (3). The SMP antigen, a surface glycoprotein identified by SDS/PAGE under nonreducing conditions as a doublet of M_r 75,000–80,000, is expressed by all cells lining peripheral nerves from embryonic day (E) 6 onward in the quail, but not by satellite cells of either peripheral or enteric ganglia. In peripheral nerves, myelin is detectable only 6 days later on E12.

The SMP marker appears in cultures of dissociated NC cells (3), indicating that early migrating NC cells can express the Schwann-cell phenotype in the absence of other embryonic tissues. We have described (14) a culture system using a feeder layer of growth-inhibited 3T3 cells as a substrate that allows single explanted NC cells to grow and produce colonies in which virtually all NC-derived phenotypes are expressed. By using this system, we were able to demonstrate the existence of cells possessing various growth and differentiation capabilities in the mesencephalic NC removed from quail embryos during the migratory stage. Some NC cells are fully committed and able to give rise to only one cell type (e.g., neurons) whereas others yield a largely diversified progeny, thus indicating an inherent multipotentiality at the time of migration (14).

The feasibility of studying the developmental potentialities of individual NC cells prompted us to undertake the work reported here, which has consisted in the clonal analysis of the NC ancestors of Schwann cells by using the SMP marker to distinguish the latter from the other glial cells of the PNS. We were particularly interested in knowing if the presence of neurons is necessary for the emergence of the Schwann cell phenotype. In addition, we wanted to determine whether Schwann cells originate from committed precursors. The results of our investigation show that pluripotent precursors for Schwann cells and other cell types coexist with committed Schwann cell progenitors in the mesencephalic NC at the migratory stage.

MATERIALS AND METHODS

Culture methods and cloning procedures have been described in detail elsewhere (14). Briefly, the NC was removed from 9- to 13-somite quail embryos (E2) at the mesencephalic–metencephalic level, where it can be dissected without con-

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Abbreviations: NC, neural crest; SMP, Schwann cell myelin protein; PNS, peripheral nervous system; mAb, monoclonal antibody; TyrOHase, tyrosine hydroxylase; E, embryonic day; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

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tamination by cells of the neural tube (15, 16). NC cells were dissociated by enzymatic treatment and plated either as mass cultures (one NC per well in 4-well plates, Nunc) or as single cells (in Lab-Tek chamber slides, Miles). Individual cells were seeded, under microscopic control by using an elongated Pasteur pipet, on a feeder-layer of growth-inhibited mouse 3T3 fibroblasts, in a complex medium containing 10% (vol/vol) fetal calf serum and 2% (vol/vol) chicken embryo extract plus hormones and growth factors (14). Cultures were maintained for up to 16 days, half of the medium being changed every third day.

After detection of clones by means of the bisbenzimide nuclear stain (Hoechst 33342, Serva, Heidelberg), which enables the quail NC cell nuclei to be distinguished from mouse 3T3 cell nuclei, NC cells that had differentiated in the cultures were identified using various markers. Melanocytes possess their own marker, melanin, clearly visible with phase-contrast microscopy. The presence of cartilage, revealed by phase-contrast microscopy or Hoechst staining as dense cell aggregates, was confirmed by metachromaticism after toluidine blue staining. Neural cell types were identified with various antibodies and the indirect immunofluorescence technique. We first applied the anti-SMP mouse monoclonal antibody (mAb), which displays a strict cellular specificity for Schwann cells in avian PNS (3). Then, to assess the presence of neurons and adrenergic cells, the cultures were permeabilized with Triton-X100 [0.25% in isotonic phosphate-buffered saline (pH 7.4), 20 min] and treated with a mouse mAb directed against neurofilament proteins (Immunotech, Marseille, France) and a rabbit antiserum directed against tyrosine hydroxylase (TyrOHase) (Eugene Tech, Allendale, NJ) or a mouse mAb directed against TyrOHase (17) and a rabbit antiserum against vasoactive intestinal peptide [Cambridge Research Biochemicals (Cambridge, U.K.); for details, see ref. 14]. Finally, the cultures were incubated with mouse mAb HNK1 (18), which recognizes a carbohydrate epitope present on the majority of migrating NC cells and, later in development, on most neuronal and nonneuronal cells of the PNS, including Schwann cells and ganglion satellite cells (19–21). Because other mouse mAbs had already been applied to the cultures, HNK1 mAb could only be used to reveal NC cells that were negative for all the other markers. In this way it was possible to identify SMP^-, neurofilament protein-negative, $TyrOHase^-$, $HNK1^+$ cells as nonneuronal cells of a non-Schwann-cell type.

The secondary antibodies used were fluorescein isothiocyanate (FITC)- and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse (Cappel Laboratories) and FITC- and TRITC-conjugated goat anti-rabbit (Nordic, Tilburg, The Netherlands) immunoglobulins. In some mass cultures, double labeling was performed using anti-SMP with HNK1 directly conjugated to FITC.

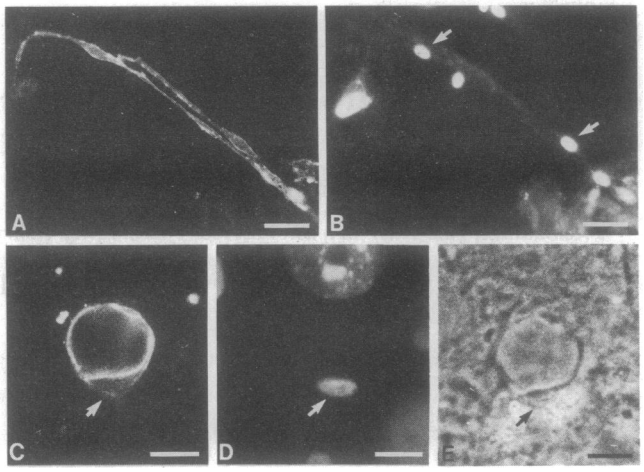

FIG. 1. Strongly anti-SMP-immunoreactive cells in clonal cultures of the NC. (*A*) Anti-SMP reactivity. (*B*) Same field with Hoechst staining. Elongated bipolar cells are brilliantly fluorescent and have a small ovoid nucleus (arrows). (Bars in *A* and *B* = 25 μm.) (*C*) Anti-SMP reactivity. (*D* and *E*) Hoechst staining and phase-contrast views, respectively, of the same field, showing a shell-shaped SMP^+ cell. The nucleus, visible in *D*, is not stained by the anti-SMP mAb (arrows). (Bars in *C–E* = 10 μm.)

For the analysis of cell proliferation, [^{3}H]thymidine (25 mCi/mmol; 1 Ci = 37 GBq; Amersham) was added to the medium at a final concentration of 1 μCi/ml. Twenty-four hours later, the cultures were fixed for 1 hr in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (pH 7.4). Autoradiography was performed, using Ilford K5 nuclear emulsion (Kodak) after immunocytochemistry.

RESULTS

Expression of SMP Immunoreactivity in Mass Cultures of Dissociated NC Cells. Mesencephalic NC cells grown on 3T3 cells expressed several identifiable phenotypes. As described (14), melanocytes, cartilage, and several $TyrOHase^+$ types of adrenergic cells as well as $TyrOHase^-$, vasoactive intestinal peptide-positive, or neurofilament protein-positive, non-adrenergic neurons differentiated after a few days in culture. In the present study, the following two types of nonneuronal cells could be distinguished: Schwann cells, which were revealed by the anti-SMP mAb, and non-Schwann (presumably satellite) cells, which were SMP^- and $HNK1^+$. Since satellite cells *in vivo* express the latter phenotype, we consider that at least some of the SMP^-, $HNK1^+$ cells in the

Table 1. Identification of various types of clones by their phenotypic composition

Type of clone	Cell type	Clones, no.	% of total clones
Nonneuronal			
Schwann cells	+/+ nonneuronal	22	13
Schwann cells	+/+ and −/+ nonneuronal	29	18
+ non-Schwann cells	+/+, −/+, and −/− nonneuronal	27	16.5
Non-Schwann cells	−/+ nonneuronal	11	7
	−/+ and −/− nonneuronal	8	5
Nonneuronal + neuronal	+/+ and −/+ nonneuronal and neuronal	61	37
Nonneuronal + melanocyte	+/+ and −/+ nonneuronal and melanocyte	1	1.5
	+/+, −/+, and −/− nonneuronal and melanocyte	2	
	−/+ and −/− nonneuronal and melanocyte	1	0.5
Nonneuronal + cartilage	+/+, −/+, and −/− nonneuronal and cartilage	2	1
	−/+ and −/− nonneuronal and cartilage	1	0.5

Symbols: +/+, SMP^+,$HNK1^+$; −/+, SMP^-,$HNK1^+$; −/−, SMP^-,$HNK1^-$. Phenotypes of neurons were neurofilament-positive, tyrosine hydroxylase-positive, or vasoactive intestinal peptide-positive.

cultures were of this cell type. However, it cannot be ruled out that some of these cells may have been undifferentiated NC cells. All SMP$^+$ cells were HNK1$^+$, as could be demonstrated with the latter mAb directly coupled to FITC (results not shown).

SMP immunoreactivity first appeared on day 8 of culture in a subpopulation of NC cells that increased in number as the cultures aged. At any given time, SMP$^+$ cells exhibited variable morphology and diverse intensities of immunostaining. In 13- to 16-day clonal cultures, the great majority of the cells that, in association with neurons, formed large cords around 3T3 cells, were faintly stained. A few elongated bipolar cells and rare shell-shaped cells fluoresced more brilliantly (Fig. 1).

Distribution of SMP$^+$ Cells in Clonal NC Cell Cultures. Six series of cultures were analyzed after 13–16 days *in vitro*. A total of 184 clones was obtained from 490 cells seeded, corresponding to a mean cloning efficiency of 38% (range, 25–57%). Ten clones, damaged during the working-up procedure, and nine clones, negative for all the markers tested, were not taken into account, since their derivation from a contaminating mesodermal cell could not be excluded.

SMP$^+$ cells differentiated in 87% of the cultures and were distributed among seven categories of clones, defined according to the other phenotypes with which they were associated (Table 1). As in our previous experiments (14), most of the clones (59.5%) were composed only of nonneuronal cells of one or more of the following three types: Schwann cells (SMP$^+$,HNK1$^+$), putative ganglion satellite cells (SMP$^-$, HNK1$^+$), and cells, whose identity remains undefined, devoid of these two markers. The proportion of the clones containing various combinations of these three kinds of cells is indicated in Table 1. (*i*) Thirteen percent of the clones were composed of a pure population of SMP-immunoreactive cells (Fig. 2 *A* and *B*). (*ii*) Thirty-four and a half percent of the clones were heterogeneous, containing both SMP$^+$,HNK1$^+$ and SMP$^-$, HNK1$^+$ cells, as shown in Fig. 2 *C* and *D*, and (*iii*) 12% were stained only by the HNK1 antibody.

Neuronal cells (adrenergic and/or nonadrenergic) were present in 37% of the clones (Fig. 2 *E* and *F*). They constituted a small population among large numbers of nonneuronal cells, which were always of both the SMP$^+$,HNK1$^+$ and SMP$^-$,HNK1$^+$ types. No melanocytes or cartilage cells differentiated in these clones.

A few melanocytes were detected in 2% of the clones that contained nonneuronal cells of different types: SMP$^+$,HNK1$^+$ cells, SMP$^-$,HNK1$^+$ cells, and cells negative for both markers (Fig. 2 *G* and *H*).

Finally, in three clones, formations of cartilage were observed. These were surrounded by nonneuronal cells that were HNK1$^+$ or HNK1$^-$ and, in two clones, SMP$^+$ cells were also present.

Proliferative Activity of SMP$^+$ Cells in NC Clonal Cultures. Most of the clones not only were phenotypically heterogeneous but also differed strikingly in the number of cells that they contained, ranging from 20 to more than 30,000 cells per clone, as described for 10-day clones (14). The distribution in size varied with the type of clone considered (Fig. 3). Homogeneous SMP$^+$ clones and clones with both neurons and nonneuronal cells constituted the largest populations. Of the nonneuronal precursors, direct progenitors of SMP$^+$ cells proliferated more intensely than did the precursors of SMP$^-$,HNK1$^+$ non-Schwann cells (Fig. 3).

Quantitative analysis of cell proliferation performed after a 24-hr pulse of [^{3}H]thymidine at the end of the clonal culture period indicated that cell multiplication in NC progeny, as a whole, declined between day 13 and day 16 of culture: in 70% of the 13-day clones (n = 20), 20–50% of the cells were labeled, whereas less than 20% were labeled in 16-day clones (97%; n = 107).

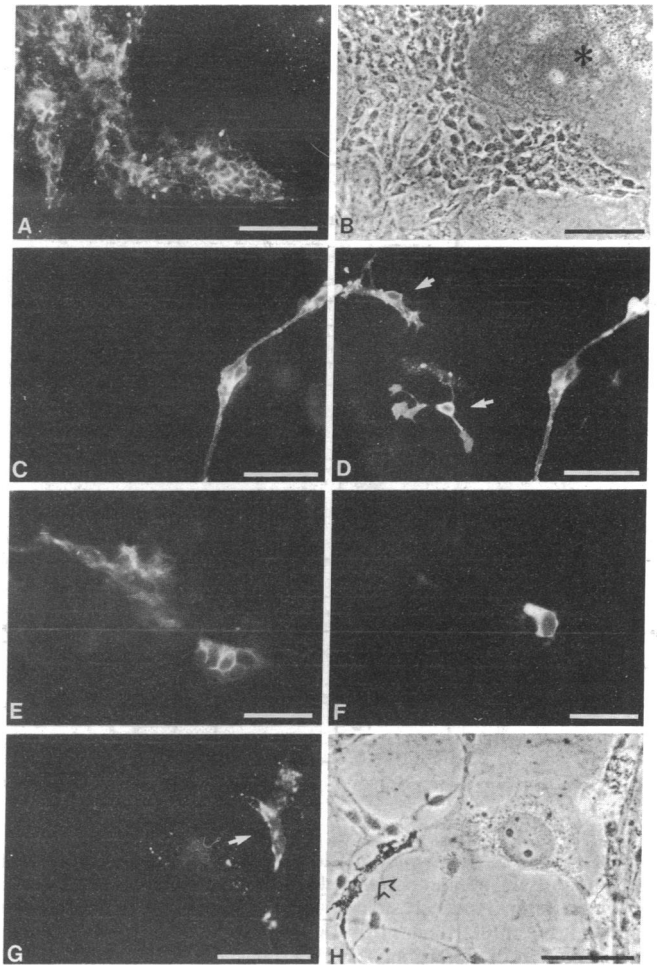

FIG. 2. Clones containing SMP$^+$ cells. (*A*, *C*, *E*, and *G*) Anti-SMP reactivity. (*B* and *H*) Phase-contrast. (*D*) mAb HNK1 reactivity. (*F*) Anti-TyrOHase reactivity. (*A* and *B*) Part of a clone containing only SMP$^+$ cells. Small NC-derived cells are fluorescent and surround large mouse 3T3 cells that are negative with anti-SMP mAb (*). Same field after staining with anti-SMP (*C*) followed by HNK1 (*D*) showing SMP$^+$,HNK1$^+$ cells and two SMP$^-$,HNK1$^+$ cells (arrows). Double labeling with anti-SMP mouse mAb and a rabbit antiserum to TyrOHase followed by FITC-conjugated goat anti-mouse and TRITC-conjugated goat anti-rabbit immunoglobulins showing a TyrOHase$^+$,SMP$^-$ cell (*F*) surrounded by SMP$^+$ TyrOHase$^-$ cells (*E*). (*G* and *H*) A clone containing SMP$^+$ cells (arrow in *G*) and a pigmented cell visible in the same field under phase-contrast microscopy (open arrow in *H*). (Bars = 50 μm, except in *E* and *F* where the bar = 25 μm.)

Most clones that contained neuronal and nonneuronal cells stopped growing between 13 and 16 days of culture; the proportion of cycling cells dropped from between 10 and 50% in 13-day clones to less than 1% in 16-day clones. No neurons were found to have incorporated [^{3}H]thymidine in clones of this type, demonstrating that they had been generated before day 12. Of all the categories of nonneuronal cells, the SMP$^+$ cell population usually contained the largest proportion of dividing cells. At the end of the culture period, percentages of labeled cells were higher in nonneuronal clones than in neuron-containing clones. In particular, at 16 days, a third of the entirely SMP$^+$ clones still contained up to 30% of cycling cells (Fig. 4). This indicates that a proliferative pool of SMP$^+$ cells remained after 2 weeks in clonal culture.

DISCUSSION

In this article we describe the differentiation of Schwann cells *in vitro* from their precursors in the NC. The identification of

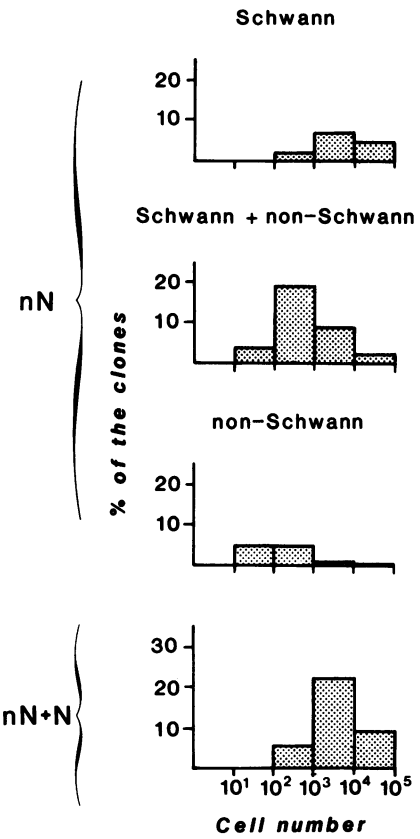

FIG. 3. Size distribution of clones according to the phenotypes they express. The number of cells in different types of clones was counted after Hoechst staining of 13- to 16-day cultures. The percentages of total clones (n = 160) falling into arbitrary size classes are shown in the histograms. N, neuronal; nN, nonneuronal.

the Schwann cell phenotype was based on immunoreaction with the anti-SMP mAb shown to react *in vivo* with myelinating and nonmyelinating Schwann cells lining peripheral nerves (3) and, in the central nervous system, with oligodendrocytes (22). In the embryo, SMP immunoreactivity appears earlier than any other known Schwann cell marker, since it is detectable on certain peripheral nerves at E6 in the quail (3). The earliest myelinating Schwann cell markers so far identified are the protein P_o and myelin basic protein, which are expressed in detectable amounts from E14 onward in chicken sciatic nerves (23). In mass cultures of quail NC cells removed from

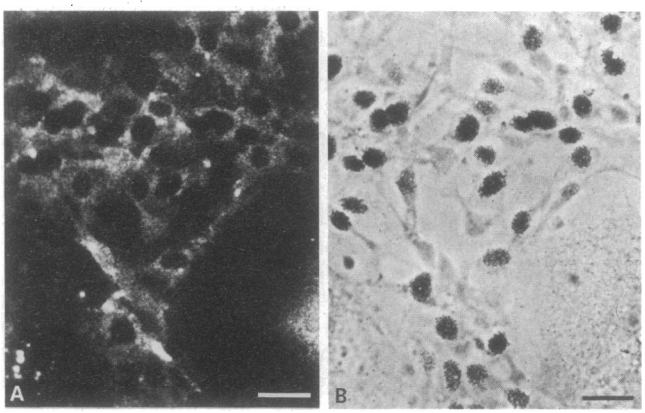

FIG. 4. Cell division in clones containing only Schwann cells. (*A*) Anti-SMP reactivity. (*B*) Phase-contrast micrograph. Part of a 16-day clone treated by anti-SMP followed by autoradiography to demonstrate [^{3}H]thymidine incorporation during the last 24 hr of culture. The nuclei of numerous SMP$^+$ cells are labeled. (Bars = 25 μm.)

embryos at the 10- to 13-somite stage (E2), the SMP antigen appears on a subset of cells after about 8 days (3).

Previous studies of Schwann cell development in culture have been carried out with differentiated cells removed from embryos or fetuses much later in ontogeny (for review, see ref. 24) and have dealt essentially with Schwann cell-specific growth factors and with the occurrence of differentiation traits related to the myelination process. Moreover, in most of these experiments, identification of the Schwann cell phenotype has relied on the coincident expression of Schwann cell-related antigens rather than on the use of a specific marker for this cell type (e.g., ref. 25). A case in point is the Ran1 antigen (26) expressed by rat Schwann cells and also by some rat sensory neurons and nerve cell lines (25).

In the present work, the early steps of Schwann cell differentiation, as shown by the anti-SMP mAb, were investigated in clonal cultures of mesencephalic NC cells. The culture method, as described (14), allows vigorous proliferation of individual NC cells as well as their differentiation into a large array of derivatives. Under these permissive conditions, identification of the phenotypic characters of the crest cell progeny enables one to define *a posteriori* the degree of commitment of the founder NC cell. We have so far identified several classes of more or less pluripotent precursors in the migrating NC. Here we describe the differentiation of SMP-immunoreactive cells in various classes of clones, a result that implies the existence of various types of Schwann cell precursors in the mesencephalic NC at the migrating stage.

We found that, in 13% of the clones, a pure population of SMP$^+$ cells differentiated from a single NC cell; the latter can, therefore, be considered a committed Schwann cell progenitor. Such progenitors gave rise to some of the largest clones observed after 13–16 days in culture, indicating that they have a large proliferative ability. Interestingly, these results show that early differentiation and proliferation of Schwann cells can occur independently of the presence of other cells of the PNS, particularly of neurons. Axons are crucial for the multiplication of Schwann cells taken at later stages of development from peripheral nerves or ganglia (27, 28). Neuron–Schwann cell interactions leading to myelin formation are mainly mediated by extracellular matrix components (e.g., ref. 11). In the culture system we have used, the 3T3 fibroblast feeder-layer played a critical role in the differentiation of Schwann cell precursors, since virtually no SMP$^+$ cells were found in mesencephalic NC cells cultured in their absence—i.e., on plastic or collagen-coated dishes (data not shown).

Among the several categories of clones that contained Schwann cells (see Table 1), the most common were those in which putative satellite cells and nonadrenergic and/or adrenergic neuronal cells were also present. This demonstrates unambiguously that the NC contains migrating bipotent precursors for neurons and nonneuronal cells. Evidence for such common precursors for neurons and Schwann cells has also been provided by labeling single premigratory trunk NC cells *in vivo* (29). In our clonal cultures, this type of precursor gave rise to numerous Schwann cells and to a limited number of neuronal cells, and it is striking that neither the intensity of SMP immunoreactivity nor the proliferative activity of Schwann cell progenitors (see comparative sizes of clones in Fig. 3) seemed to be increased by the presence of neurons. Preliminary data, using a rabbit antiserum to myelin basic protein (a gift of K. Mikoshiba, University of Osaka), indicate that some of the more brilliantly fluorescent SMP$^+$ cells contain cytoplasmic myelin basic protein, suggesting that further maturation of Schwann cells, and possibly myelination, can occur in these clones.

A third type of Schwann cell precursor is a bipotential precursor for SMP$^+$,HNK1$^+$, Schwann cells, and SMP$^-$, HNK1$^+$ nonneuronal nonpigmented cells. No specific

marker has so far been defined for ganglion satellite cells but, *in vivo*, they constitute a large population of SMP^-,$HNK1^+$ cells. Thus, in the absence of more specific markers, we consider that the SMP^-,$HNK1^+$ cells present in 13- to 16-day clones belong to the satellite cell type. mAb HNK1, prepared by immunizing a mouse with a human lymphoma cell line (18), has binding properties for migrating NC cells similar to those of mAb NC1 raised against quail ciliary ganglion cells (19–21). It has since turned out that the HNK1 epitope is carried by several surface molecules including molecules involved in cell adhesion—e.g., neural cell adhesion molecule (N-CAM) (30), neuron-glial cell adhesion molecule (Ng-CAM)/L1 (31), J1/cytotactin (32, 33)—and is thus not a lineage-related marker. However, in our system, it has proved to be useful since it is a permanent marker for NC-derived nonneuronal cells of ganglia and peripheral nerves—i.e., Schwann and satellite cells—plus a subpopulation of neuronal cells (20).

The fact that we also found $HNK1^-$ cells in some clones is not surprising, since this epitope is not carried *in vitro* by all nonneuronal ganglionic cells (ref. 34, and M. Barbu, personal communication). The diversity of the non-Schwann glial cell population is such that other differentiation markers are required to identify precisely satellite ganglion cells and enteric glia, both of which are SMP^-. However, our data show that common precursors for Schwann cells and non-Schwann satellite cells as well as committed progenitors for each of these cell types exist in the migratory NC. This challenges the common view that these two categories of cells correspond to a single cell type that assumes different morphological, biochemical, and functional properties according to its position relative to the neuron (35).

The presence of SMP^+ Schwann cells is not restricted to clones entirely composed of neural cells, since we observed some clones containing both pigment cells and Schwann cells. Similar results were reported *in vivo* (29), where some individual truncal NC cells were found to give rise both to descendants localized in the ventral roots of spinal nerves and to presumptive melanocytes under the lateral ectoderm. It is possible that such a bipotential precursor for Schwann cells and melanocytes can survive for a short time after migration in the embryo. This would account for the fact that peripheral nerves and dorsal root ganglia, until E5 in the quail, contain precursors that can undergo pigmentation under certain culture conditions (36, 37).

More surprisingly, we found a small percentage of founder NC cells that have the ability to give rise both to Schwann cells and to cartilage. This result and other data (A.B., E.D., and N.M.L.D., unpublished results) strongly suggest that the mesectodermal lineage may not be totally segregated from the neural lines as early as was proposed (38, 39).

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