Lineage of neurons and glia in chick dorsal root ganglia: analysis in vivo with a recombinant retrovirus

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Summary

We used retrovirus-mediated gene transfer to study the lineage of neural crest cells in chick embryos. Individual crest cells were infected before they migrated from the neural tube, and their clonal progeny were subsequently revealed in sensory ganglia and associated structures by a histochemical stain for the viral gene product (lacZ). We found that crest cells were multipotential in several respects. (1) Many clones contained both ventrolateral (VL) and dorsomedial (DM) neurons, which had been suggested to be lineally distinct. (2) Many clones contained both large and small neurons, which are known to innervate distinct targets. (3) Many clones contained multiple glial subtypes, e.g. both Schwann cells, which ensheathe axons, and satellite cells, which ensheath neuronal somata. (4) Many clones contained both neurons and glial cells. On the other hand, a sizeable minority of clones was homogenous, e.g. they contained only neurons or only glial cells – suggesting that some progenitors may be, or become, restricted in potential. Finally, this study provides the first opportunity to compare directly the two methods currently available for tracing cell lineage in vertebrate embryos, retroviral infection and tracer injection: our results and those of Bronner-Fraser and Fraser (1989), who used the latter method, provide complementary but consistent views of crest lineage.

Key words: neural crest, lineage, retrovirus, β-galactosidase, neuron, glia, sensory ganglia.

Introduction

A central question in development is how heritable programs of gene expression and intercellular signalling mechanisms, i.e. lineage and environment, interact to determine phenotype. For neurobiologists, a favorite system in which to study these determinants is the neural crest. The neural crest is a rostrocaudally oriented aggregate of cells that arises from the dorsal margin of the neural tube. Crest cells migrate along defined routes to form the elements of the peripheral nervous system, e.g. sensory, autonomic and enteric neurons, plus Schwann and satellite cells, as well as several non-neural structures, e.g. melanocytes, craniofacial mesenchyme and some cardiac septa (LeDouarin, 1983). When blocks of crest are transplanted to novel locations in vivo, they frequently follow migratory routes and express phenotypes characteristic of the host site, suggesting that crest cells are multipotential and that cues along the migratory path influence phenotypic choices (LeDouarin, 1986). Studies in which crest cells or derivatives are maintained in defined conditions in vitro have provided insight into what some of these cues might be (Weston, 1986; Anderson, 1989; Landis, 1990). Furthermore, in some of the studies in vitro, individual cells have been followed as they divide, permitting a direct demonstration that crest cells are multipotential (e.g. Sieber-Blum et al. 1980; Barroio et al. 1988). However, it has been difficult to mark individual crest cells in embryos in ways that permit later identification of their progeny. Therefore, comparatively few data are available on the range of phenotypes that clonally related cells actually acquire in vivo.

Recently, two methods have been developed that permit tracing of cell lineage in vivo. One is a method of retrovirus-mediated gene transfer, in which a recombinant retrovirus bearing the E. Coli β-galactosidase (lacZ) gene infects a progenitor; the provirus integrates into the cell's genome and is inherited by its progeny, which can then be identified by a histochemical stain for lacZ (Sanes et al. 1986; Price et al. 1987; Sanes, 1989; we refer to the gene as lacZ and the enzyme as lacZ). Here, we have applied this method to the neural crest of the chick embryo. Focusing on dorsal root ganglia (DRGs) and their associated nerves and roots, we show that a
single progenitor can produce multiple types of neurons, multiple types of glia, and/or both neurons and glia (see Frank and Sanes, 1989, for a brief account).

The other method for analyzing lineage in vivo is to inject a single cell with a dye, using a micropipette. Then, until the dye fades or is diluted excessively, it serves to mark descendants of the injected cell. Originally devised to label the large blastomeres of invertebrates (Weisblat et al. 1978) and vertebrates (Jacobson and Hirose, 1978), this method was subsequently adapted to permit marking of small somatic cells within complex tissues (Wets and Fraser, 1988; Holt et al. 1988). Bronner-Fraser and Fraser (1988, 1989) have applied this method to the neural crest of the chick trunk, injecting cells at the same stages that we infected them, and analyzing several crest derivatives including DRGs. Together, their study and ours provide the first opportunity to compare results obtained with both methods in the same system.

Materials and methods

Injection of viruses

The preparation of the two types of virus used, LZ10 and LZ12, has been described by Galileo et al. (1990). LZ10 encodes a freely soluble gag-lacZ fusion protein, whereas the lacZ in LZ12 is fused to a nuclear-localizing signal from a viral antigen. Thus, the cytoplasm of LZ10-infected cells is more intensely stained than the nucleus, whereas the opposite is true for LZ12-infected cells. Chicken eggs (White Leghorn, SPAFAS, Roanoke, IL) were incubated at 37°C. A viral concentrate containing either or both viral types was pressure-injected into the neural tube as detailed by Leber et al. (1990) and was observed to spread rostrocaudally throughout the tube. The injection volume, ~0.5 μl, corresponded to ~10^4 virions.

Histology

After a variable period of incubation, embryos were perfused through the heart with cold fixative (0.4 % glutaraldehyde and 2.0 % formaldehyde in 150 mM NaCl, 15 mM sodium phosphate, pH 7.4 [PBS]), and then left in fixative for 1 h. The limbs and head were removed, and the carcasses were eviscerated and rinsed in several changes of PBS for 1 h at room temperature. They were then stained overnight for lacZ in 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), rinsed in PBS, and refixed in 2.0 % glutaraldehyde plus 2.0 % formaldehyde (details in Galileo et al. 1990). The spinal cord and attached nerves and ganglia were exposed by a complete ventral laminectomy and scored for the presence of lacZ-positive cells. A total of 120 embryos contained labeled crest derivatives, and these were processed in one of two ways. (1) Three or more spinal segments of the body (including spinal cord, spinal column, axial muscles, sensory and sympathetic ganglia, and spinal nerves) were removed and serially sectioned in the transverse plane at 20 μm on a freezing microtome (Frank et al. 1980). Sections were counterstained with neutral red and coverslipped in Mowiol (Calbiochem), in which the X-gal reaction product is stable for at least a year. (2) Individual labeled DRGs with attached nerves were embedded in Polybed 812 (Polysciences), drawn en bloc as whole mounts, then sectioned serially at 10–20 μm and mounted in immersion oil. Each frozen or plastic section was scored for the presence of lacZ-positive cells, which were classified according to their location, viral phenotype and type.

Staging

Animals were staged by the criteria of Hamburger and Hamilton (1951). Approximate correspondence between stages and days of embryonic development (E) was as follows: 1–5, E0; 6–10, E1; 11–16, E2; 17–21, E3; 22–24 E4,E5; 25–29, E6; 30–31, E7; 32–33, E8; 34–35, E9; 36, E10; 37, E11; 38, E12; 39, E13; 40, E14; 41, E15; 42, E16; 43, E17; 44, E18; and 45, E19.

Results

The neural tube of the embryonic chick trunk closes dorsally in a rostral-to-caudal wave, beginning ~stage 10. Crest cells appear at the dorsal margin of the tube soon after closure, and migrate away from the tube between ~stages 14 and 21. Migrating cells coalesce to form the rudiments of DRGs by ~stage 20. Within each DRG, cell divisions continue for several days; neurons are born until ~stage 30 and glia until at least stage 36 (Hamburger and Levi-Montalcini, 1949; Pannese, 1969; Weston, 1970; Tosney, 1978; Carr and Simpson, 1978; Loring and Erickson, 1987; Lallier and Bronner-Fraser, 1988; Serbedzija et al. 1989).

To mark crest progenitors before they migrated, we injected retrovirus into the neural tube between stages 11 and 19. After allowing the embryos to develop to stages 31–45, we fixed and stained them with X-gal to visualize the progeny of infected cells. The positions of labeled cells in the spinal cord, ganglia and nerves were noted on schematic diagrams, and tissues with labeled cells were reconstructed from serial sections. Two examples of the resulting reconstructions are shown in Fig. 1A and 1B.

We also injected virus into and outside of the neural tube in a series of stage 21–22 embryos. No labeled cells were found in the ganglia of these animals. Together with data presented below, this result suggests that cells in the first series were infected when they were still associated with the neural tube, rather than after they migrated.

Definition of a clone

Before proceeding with an analysis of the cell types that

Fig. 1. Distribution of lacZ-positive cells in embryos injected with both retroviral strains (LZ10 and LZ12) at stage 17 and serially reconstructed at stage 31(A) or 34(B). Filled symbols indicate cytoplasmic lacZ-positive (LZ 10-infected) cells, and hatched symbols indicate nuclear lacZ-positive (LZ12-infected) cells. Sections through three ganglia in B are illustrated in C–E. Of the 14 labeled DRGs in the two embryos shown, the five indicated by arrowheads were 'isolated' and used for clonal analysis (see text). In an additional three pairs of ganglia (*), one member of each pair (partially filled in) contained <10 % as many labeled cells as the other; each pair probably represents a single clone, but they were not included in the lineage analysis. The embryo in B had many more labeled ganglia than was usual. Bars are 50 μm for C–E.
were labeled, we needed to establish what constituted a clone. In many parts of the developing embryo, clonal siblings do not migrate extensively, so in retroviral studies of cell lineage, a cluster of labeled cells is usually presumed to be a single clone (Sanes, 1989). In the neural crest, however, there is extensive migration of cells away from the neural tube, and we were initially uncertain whether the descendants of a single neural crest cell would remain sufficiently restricted in space to be interpreted as members of a single clone. We therefore injected small numbers of virions and restricted our analysis to structures in the proximity of the spinal cord: sensory and sympathetic ganglia, dorsal and ventral roots, and spinal nerves.

Within this area, we found that groups of labeled cells were often restricted to a single, isolated ganglion; that is, neither the contralateral nor the immediately rostral or caudal ipsilateral ganglia contained any labeled cells. For example, in 21 embryos with \( \leq 3 \) labeled ganglia each, 33 of the 42 labeled ganglia were 'isolated' in this sense. This result indicates that many neural crest clones must be confined to a single segment (excluding cells that may have migrated to more distant targets). Similar restrictions on the rostrocaudal migration of neural crest cells to sensory ganglia have been observed using chick-quail chimeras (Yip, 1986; Teillet et al. 1987).

Labeled cells confined to an isolated ganglion might, however, be members of more than one clone, although the probability of such an event is low. To calculate the expected probability, we made reasonable assumptions about the number of crest cells infected per embryo, based on the fact that the embryos from which we obtained data contained an average of 3.2 ganglia with labeled cells out of 40 ganglia examined per embryo (considering each viral phenotype separately; see below). Given these assumptions, the probability that an isolated ganglion contained \( > 1 \) clone is calculated to be \( \leq 5 \% \) (see Appendix). In addition, we attempted to determine this probability experimentally. We injected a set of embryos with a mixture of two types of virus, which label infected cells differently: the X-gal reaction product is primarily restricted to the cytoplasm for one type of virus (LZ10) and to the nucleus for the other (LZ12; see Methods). The two viral stocks were prepared and titrated separately, then approximately equal numbers of virions were mixed, diluted appropriately and injected into the neural tube together. If the labeled cells within an isolated ganglion were members of more than one clone, then in some cases, one should observe mixtures of nucleus- and cytoplasm-positive cells. Fig. 1A and B illustrate two examples of this test and representative sections showing the viral phenotypes in three of the labeled DRGs are shown in Fig. 1C–E. Of the 14 labeled ganglia in these two embryos, none contained mixtures of the two viral phenotypes. Overall, in 60 embryos injected with both viral types, 42 isolated ganglia had cells with only the cytoplasmic phenotype, 38 contained cells with only the nuclear phenotype, and 2 contained a mixture of both. Both double-labeled ganglia were from embryos with an unusually high number of labeled DRGs. Assuming that both double-labeled ganglia represent double clones, then twice this number (because approx. half of all double clones would contain only one phenotype), or \( 4/82 = 4.9 \% \) of isolated ganglia contain two clones. Based on these theoretical considerations and empirical results, we restricted our analysis to groups of lacZ-positive cells that were confined to a single isolated ganglion and/or its associated nerves, i.e. cases in which neither the contralateral nor the adjacent ipsilateral ganglia (or nerves) contained lacZ-positive cells. Although this restriction probably led us to exclude some clones, and some cells (e.g. in the neural tube) in other clones, we believe that \( < 5 \% \) of the clusters that met our criteria were polyclones.

**Distribution of clonal relatives**

A total of 123 clones met the criteria specified above, and were used for lineage analysis. Labeled cells were restricted to the DRG in 86 of these clones (Fig. 2A), and to spinal nerves and/or roots in another 7. In 30 clones, however, labeled cells were present both within a ganglion and its associated nerves and/or roots (Fig. 2B and C). Thus, progeny of a single crest cell frequently (24% of clones) contribute both to a ganglion and to extraganglionic structures.

Only four of the clones that met our criteria contained cells in sympathetic ganglia, and none of these contained labeled cells in DRGs. This may be a consequence of the stage at which virus was injected, since crest cells that populate sympathetic ganglia migrate away from the neural tube before those that populate sensory ganglia (Serbedzija et al. 1989). These clones may be further under-represented because our definition of a clone excluded cases in which labeled cells spread beyond a single segment, whereas sympathetic cells are known to migrate further along the neuraxis than sensory neurons (Yip, 1986). The small number of clones containing sympathetic cells precluded a meaningful analysis, and they were not considered further.

We also asked whether labeled cells in the spinal cord were associated with labeled crest derivatives. The spinal cord was serially reconstructed along with the ganglia for 44 clones. In ten (23%) of these, labeled spinal cord cells were located in the same segment as the labeled ganglion. For example, one of six isolated DRGs in Fig. 1 is adjacent to spinal cord cells of the appropriate viral phenotype. Although the clonal relationship of the ganglionic and spinal cord cells was not confirmed, the high incidence of co-labeling suggests that the progeny of a single neural tube cell may populate both targets.

In addition to isolated ganglia, labeled DRG pairs were also frequently observed, either contralaterally or immediately adjacent ipsilaterally. In many such cases, the pair of ganglia contained both viral phenotypes, indicating that the pair contained at least two clones. In a sizeable subset of labeled pairs, however, (three illustrated in the two examples in Fig. 1), over 10 times more neurons were labeled in one ganglion than in the
other and the few neurons in the second ganglion were located on the side nearest the first (Fig. 1A and B). In the double label experiments, the viral phenotype in each of eight such 'asymmetric' pairs was uniform. This pattern suggests that one crest cell in the neural tube may give rise to progeny occupying two adjacent ganglia. However, because both on statistical grounds and in the double label experiments two adjacent ganglia are more likely to contain a polyclone than is a single ganglion, we excluded all labeled pairs from subsequent phenotypic analysis.

**Classification of labeled cells**

We divided lacZ-positive cell types in ganglia and nerves into three groups. As labeled by the cytoplasmic virus, sensory neurons had darkly stained cytoplasm, pale round nuclei and, often, one or more stained processes (Fig. 3A–C). Their somata were usually round or, in younger embryos, bipolar, and lay immediately adjacent to other labeled or unlabeled neurons within a DRG. In contrast, Schwann cells were usually located within the spinal nerves or roots. Their somata and nuclei were elongated and were oriented longitudinally in the nerve (Fig. 3D and E). These cells often occurred in clusters or rows. No other lacZ-positive cell type (for example perineurial cells) was seen in the nerves or roots. Occasionally, cells with a similar morphology were seen within a DRG, probably associated with intraganglionic axon fascicles; they were also classified as Schwann cells. A third type of cell was located within the DRG but was neither a neuron nor a Schwann cell. All cells in this class had smaller nuclei than neurons and their stained cytoplasm was angular. Some were flattened against the edge of the DRG and appeared to be associated with its sheath; others (Fig. 3F–H) ensheathed sensory neurons and were clearly satellite cells. Still others were irregular in shape and may have been interstitial cells (see Pannese, 1969). Although these cells form a heterogeneous group, we classified them all as ganglionic non-neuronal cells.

Crest cells labeled with the nuclear virus were divided into the same three categories. Sensory neurons had round, darkly stained nuclei surrounded by a ring of unstained or lightly stained cytoplasm. A unique feature of neurons stained with the nuclear virus was a perinuclear dot (the Golgi apparatus?) of reaction product (Fig. 3I and J). All labeled nuclei in the spinal roots and nerve were elongate and oriented longitudinally (Fig. 3K) and, like Schwann cells, they often occurred in clusters or rows. Because Schwann cells were the only cells in this location stained with the cytoplasmic virus, all such nuclei were classified as belonging to Schwann cells. The most difficult cells to identify were the ganglionic non-neuronal cells. Their nuclei were distinguished from the nuclei of neurons by their small size and irregular or elongate shape (Fig. 3L–N). Some of these cells were also identifiable by the irregular shape of their cytoplasm, visualized either by a counterstain or Nomarski optics. In some cases, however, the distinction between ganglionic non-neuronal cells and small neurons was not convincing, and these clones were excluded from an analysis of phenotype.

**Clonal relationships of neurons and glia**

Using the criteria given above, we classified the cells that constituted our population of 123 clones. Table 1 shows the combinations of cell types found and the numbers of cells per clone; some examples are drawn in Fig. 2. The existence of multiple cell types within a clone was common; 45% of the clones contained both neurons and non-neuronal cells. Therefore, many neural crest cells were pluripotent at the time that they were labeled. Neuron-only clones were also quite common, however, making up 43% of the total. In contrast, only 12% contained non-neuronal cells exclusively.

Consistent with this distribution of clonal types, the total number of neurons in the 118 clones for which
Fig. 3. Morphology of neural crest derivatives labeled with cytoplasmic (LZ10; A–H) or nuclear (LZ12; I–N) viral strains. (A–C, I, J) neurons; (D, E, K) Schwann cells; (F–H and L–N) ganglionic non-neuronal cells. A non-neuronal nucleus is labeled in J (arrow) and neurons neighbor satellite cells in G and H. Bar is 50 μm for all parts.

Counts were possible was 3149, substantially larger than the number of ganglionic non-neuronal cells (946) or Schwann cells (881). On average, clones that contained only neurons not only had fewer cells but also had fewer neurons than did clones of mixed phenotype (Fig. 4). Thus, neuron-only clones contained an average of 11.9 neurons each, and 8 of 53 contained only a single neuron, whereas mixed clones contained an average of 48.7 neurons and only 1 of these 55 clones contained fewer than three neurons. However, not all of the neuron-only clones were small; 17% had more than 18 neurons, requiring a minimum of five rounds of cell division without producing a single non-neuronal cell.

The implications of these results for possible restrictions of cell fate are considered in the Discussion.

Within the interval of stages 11–19, the stage at which embryos were injected made little difference in the sizes and types of clones obtained. For example, the number of cells per clone was 49.9 ± 9.3 in embryos injected before stage 16 (mean ± s.e., n = 42 clones), and 39.9 ± 11.8 in embryos injected after stage 17 (n = 33). The difference is in the expected direction, but is not statistically significant. In addition, clones that contained both neurons and glial cells appeared with similar frequency in early- and late-injected embryos. These results suggest that at least some properties of
Table 1. Cellular composition of clones in DRG and associated nerves

<table>
<thead>
<tr>
<th>No. (% of clones)</th>
<th>DRG neurons</th>
<th>DRG non-neuronal cells</th>
<th>Schwann cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>53 (43%)</td>
<td>11.9±2.4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25 (20%)</td>
<td>44.6±10.2</td>
<td>17.9±5.7</td>
<td>-</td>
</tr>
<tr>
<td>23 (19%)</td>
<td>60.2±9.6</td>
<td>20.3±5.4</td>
<td>28.7±10.2</td>
</tr>
<tr>
<td>7 (6%)</td>
<td>25.7±13.7</td>
<td>-</td>
<td>6.0±2.5</td>
</tr>
<tr>
<td>2 (2%)</td>
<td>-</td>
<td>11.5±3.5</td>
<td>-</td>
</tr>
<tr>
<td>7 (6%)</td>
<td>-</td>
<td>-</td>
<td>12.7±1.8</td>
</tr>
<tr>
<td>6 (5%)</td>
<td>-</td>
<td>14.0±4.0</td>
<td>25.7±16.4</td>
</tr>
</tbody>
</table>

The first column gives the number and percent of clones that contained each combination of cell types. Columns 2–4 show average number of cells (±S.E.) of each type in each category of clone. Schwann cells within the ganglion are included in column 4, not in column 3. Methods of analysis are detailed in Results. Only cells in DRG and associated nerves and roots are counted; these clones may also have contained uncounted members in other structures.

crest progenitors change little over the day-long interval that our injections spanned.

Clonal relationships of DM and VL neurons
An obvious difference among sensory neurons in developing avian DRGs is their separation into two spatially distinct subpopulations. Neurons located in the ventrolateral (VL) region of the ganglion have large somata and their cytoplasm is relatively light when stained for Nissl substance. Neurons in the dorsomedial (DM) region, in contrast, are smaller and have relatively dense cytoplasm (Hamburger and Levi-Montalcini, 1949). Because these populations differ in their time of development and immunochemical characteristics, it has been suggested that they may represent distinct lineages (see Discussion). To address this possibility, we fixed a group of injected embryos at stages 31–34, when the DM/VL distinction is most clear (e.g. Fig. 6A; at later stages, the boundary between the two populations is less obvious). Of the 33 clones in this series, 32 contained sensory neurons that could be unambiguously classified as belonging to one population or the other. None of these clones contained only DM neurons, one third contained only VL neurons and the remaining two thirds contained neurons in both the DM and the VL regions (Table 2 and Fig. 5). Thus a single progenitor can (but does not always) give rise to both DM and VL neurons.

A striking feature of the arrangement of neurons in these clones was that they were often arranged in strings that extended from the DM pole, where the dorsal root enters the ganglion, to the VL pole, where the spinal nerve exits (Fig. 5B). The position and orientation of these strings mirror the dorsal migratory pathway of crest cells leaving the neural tube (Loring and Erickson, 1987), suggesting that the progeny of a crest cell could be left behind as the progenitor migrates. If this pathway permitted migration only away from the neural tube, then a crest cell dividing infrequently as it migrated might pass through the DM region without leaving any progeny there, and none of its VL progeny could migrate back into the DM region. Clones containing only VL neurons could be generated in this way. On the other hand, a migratory crest cell that was actively dividing while passing through the DM region would leave progeny in both DM and VL regions. Consistent with this interpretation, the DM/VL clones were 10 times as large as the VL-only clones (Table 2). The absence of any DM-only clones shows that at least one cell in every clone migrates past the DM region.

Clonal relationships of mature neuronal subtypes
As they mature, sensory neurons innervate a variety of...
targets and come to subserve diverse modalities including touch, proprioception, thermoception and pain. Each type has its own unique constellation of properties, including neurotransmitter content, spectrum of soma sizes and distribution within the ganglion (e.g. Brown, 1981; Harper and Lawson, 1985; Lee et al. 1986), any or all of which could be influenced by a cell’s lineage. Unfortunately, we were not able to address directly the question of whether all neurons within a clone project to the same peripheral target: axons were not labeled well enough to trace them to the periphery, and too few clones were present in each embryo to permit retrograde labeling from individual targets or immunohistochemical labeling of ganglia. However, three separate observations provided indirect evidence that clonal relatives are not restricted to a single transmitter phenotype, or to a single target.

First, at stages when the DM/VL distinction is clear, certain peptide-containing neurons are located exclusively within the DM population. For example, Fig. 6A illustrates the distribution of cells immunopositive for substance P; all are located in the DM region (see also Fontaine-Perus et al. 1985; New and Mudge, 1988). As noted above, none of the clones that we observed was limited to this region, although over half of them included it. This result suggests (although it does not prove) that cells within a clone are diverse in transmitter phenotype and therefore in function.

Second, large differences in neuronal size arise in both DM and VL regions of DRGs as embryos mature, and the functional properties of a mature neuron are better correlated with its size than with its position in the ganglion. For example, muscle spindle afferents and low threshold cutaneous mechanoreceptors are generally large, while nociceptive neurons are small, yet both DM and VL regions contain cells of both types (e.g. Honig, 1982; Cameron et al. 1986). It is therefore of interest that, at stages 44–45, shortly before hatching, single clones often contained both large and small neurons (Fig. 6B), implying that they are likely to include neurons of diverse functional types. Furthermore, both large and small cells often occurred within loose clusters of lacZ-positive cells. In that neurons presumably remain clustered because they were produced after migration was nearly complete, the coexistence of large and small clonal relatives within a restricted area implies that both DM and VL neurons can eventually mature into either small or large neurons.

Finally, we frequently observed small groups of contiguous lacZ-positive neurons, which presumably represent the products of a progenitor’s final divisions (e.g. Fig. 6C and D). In frogs, similar clusters have been revealed by late-stage labeling with [3H]thymidine and suggested to be clonal siblings (Mendelson and Frank, 1990). Individual neurons within these clusters were shown directly to project to different peripheral targets. If, indeed, the two methods mark similar clusters, then products of the final intraganglionic mitoses project to different peripheral targets.

Clonal relationships of glial types

As noted earlier, several different types of lacZ-positive non-neuronal cells were identifiable within the clones that we analyzed, including satellite cells, interstitial cells, capsule cells and Schwann cells. As shown in Table 1, 40% of all the clones containing any type of non-neuronal cell contained both Schwann cells and ganglionic non-neuronal cells. In addition, Schwann cells were found in various combinations of ganglion, root and nerve. For example, Fig. 2C shows a clone containing Schwann cells in the peripheral nerve and DRG but not in either root, while the Schwann cells in
Neural crest lineage

Fig. 6. (A) Section through a DRG at stage 34, stained with antisera to substance P. Many substance P-positive cells are present in the DM region, but none in the VL region. The print was overexposed to render substance P-negative cells visible, thereby illustrating that VL neurons are larger than DM neurons. An intraganglionic nerve (N) runs between the two regions. (B) Neighboring large and small lacZ-positive neurons in the DM region of DRG, from a clone analyzed at stage 41. (C,D) Small clusters of clonally related neurons in the DRG, at stages 44-45. Bars are 50 μm in A, 20 μm in B and D, and 100 μm in C.

Fig. 2B are in the dorsal root and in the DM region of the DRG. Finally, some clones contained multiple types of ganglionic non-neuronal cells, such as satellite and interstitial cells. Thus, we observed no obvious restrictions on the combinations of glial cells within individual clones.

Discussion

We have used a retroviral marking method to study cell lineage in the chick neural crest. The fact that crest cells migrate long distances, and often disperse as they migrate, exacerbated the problem of determining which groups of lacZ-positive cells were clonally related. However, we were able to circumvent this problem by using a dilute suspension of virus (to mark few progenitors), by using a pair of retroviruses that confer distinguishable phenotypes upon infected cells (Galileo et al. 1990), and by analyzing only cell groups that were confined to one side of a single segment with no labeled cells in surrounding segments. This latter criterion probably led us to underestimate true clonal size in two ways: by excluding from analysis some clones that spanned multiple segments, and by excluding cells that had either remained in the neural tube or migrated far
from the vertebral column. Despite these limitations, however, we were able to demonstrate a considerable degree of multipotentiality in the progenitors that give rise to DRGs and peripheral nerves.

**Progenitors of neurons and glia**

Numerous studies have shown that many cells from the neural crest or its derivatives can acquire novel phenotypes when transplanted to novel sites in vivo or exposed to novel conditions in vitro. These studies demonstrate that the environment influences the phenotypic choices made by crest and crest-derived cells, and that at least some such cells are multipotential (LeDouarin, 1983, 1986; Anderson, 1989). However, our study and that of Bronner-Fraser and Fraser (1988, 1989, discussed below) are the first to ask whether individual crest cells give rise to more than one cell type during the course of normal development.

Perhaps our most striking result was that nearly 40% of the clones that we analyzed contained both neurons and glial cells within a single DRG. This result is not unexpected, given the evidence for multipotentiality obtained under experimental conditions. Furthermore, a common progenitor for neurons and glia has been demonstrated to exist in several regions of the central nervous system, including retina (Turner and Cepko, 1987; Holt et al. 1988; Wets and Fraser, 1988), spinal cord (Leber et al. 1990) and optic tectum (Galileo et al. 1990), although perhaps not in cerebral cortex (Luskin et al. 1988). However, neurons and glia have not yet been reported to coexist within single crest-derived clones in vitro (Sieber-Blum et al. 1980; Sieber-Blum, 1989a; Barrofio et al. 1988), and there have been some reasons to suspect that these cell types would have separate progenitors in vivo. For example, the neurons in some cranial sensory ganglia (e.g. the nodose and geniculate) are derived from the placodes, whereas satellite and Schwann cells arise from the crest (Ayer-LeLievre and LeDouarin, 1982; D'Amico-Martel and Noden, 1983); in these ganglia, neurons and glia clearly have separate progenitors. In addition, immunolabeling, back transplantation and cell culture studies have all suggested that some committed neuronal (see below) and glial (Girdlestone and Weston, 1985; Bhattacharyya et al. 1990) precursors exist in the crest. Nonetheless, our results demonstrate unequivocally that individual progenitors can give rise both to sensory neurons and to the glial cells that ensheathe them.

The intermingling of clonally related neurons and glia highlights the question of how cells exposed to an apparently similar environment choose between two (or more) fates. One possibility is that the choice is made stochastically; i.e. that each newly generated cell has the capacity to adopt either a neuronal or a glial fate. In this scheme, environmental factors are thought of as influencing the probabilities of phenotypic choice rather than imposing strict commitments. Such a model appears to explain many aspects of hematopoiesis (Till and McCulloch, 1980; Hall and Watt, 1989), and has been invoked to explain some aspects of neural crest lineage (Anderson, 1989), but available data are insufficient to test it critically. Other possibilities, not inconsistent with the first, are that intrinsic clocks in crest cells or temporal changes in the ganglionic environment affect phenotypic choice. It is already known that most neurons in the DRG are born before most glia (e.g. Carr and Simpson, 1978). Taken together with our demonstration that progenitors are multipotential, this result suggests that an early withdrawal from the mitotic cycle could predispose cells to become neurons, while late withdrawal makes cells more likely to become glia. Also consistent with sequential models is the result that few clones (8/123 or 6.5%) contained ganglionic non-neuronal cells but no neurons, as expected if most non-neuronal cells arise from progenitors that have previously given rise to neurons. Even some of the ‘neuron-less’ clones might, in fact, have once contained neurons – about half of all sensory neurons produced die between stages 24 and 37 (Hamburger et al. 1981), and we analyzed no ganglia before stage 31. (See Leber et al. 1990, for evidence that neuronal cell death is not itself a lineage-dependent phenomenon.)

Less obviously consistent with the notion of a common progenitor for neurons and glia was the high frequency (43%) of clones that contained sensory neurons but no detectable glia. This result is unlikely to reflect a random binary choice, given the size of these clones and the overall ratio of neurons to glia of ~2:1 in our population. For example, 9 of the 53 ‘pure’ neuronal clones contained >18 neurons, while the odds of finding even a single clone of 19 neurons if phenotypic choices are random and two thirds of the cells are neurons is (2/3)^19 or <1 in 2000. However, there are several tenable explanations for this result. First, when a newly generated cell becomes committed, it might have a high probability of becoming a postmitotic neuron, and a low probability of becoming a glialblast, which would then continue dividing to produce many glia. A clone composed of many neurons and many glia could then reflect a series of highly probable ‘neurogenic’ decisions but only a single ‘gliogenic’ decision. The odds of finding a pure neuronal clone in such a system would be correspondingly higher. Second, sensory neurons might arise from two types of progenitors – some that also produce glia, and others that are committed to the production of neurons. In fact, several monoclonal antibodies recognize subpopulations of premigratory crest cells that are thought to be committed to a neurogenic program (e.g. Ciment and Weston, 1982; Maxwell et al. 1988; Vogel and Weston, 1988; Barald, 1989). Specifically, Sieber-Blum (1989a, b) has used an antibody specific for chick sensory neurons, SSEA-1, to suggest that two populations of ‘sensory precursors’ may be present in cultures of crest; one that appears to be committed to a neuronal phenotype, and one that can divide to produce both neurons and glia.

Finally, fate might be progressively restricted, such that initially bipotential progenitors eventually produce separate sets of neuronal and glial precursors. Such restrictions have been documented in several lineages,
including those of the hematopoietic system (Till and McCulloch, 1980), skin and extraembryonic membranes (Sanes et al. 1986). Further comparisons of neurons that do or do not have glial relatives could help to distinguish among these and other alternatives.

**Progenitors of neuronal and glial subtypes**

In that individual crest cells can produce both neurons and glia, it might seem obvious that multiple neuronal or glial subtypes would arise from a common progenitor. However, many cases are known in which neuronal or glial subtypes arise from separate progenitors (e.g. Raff, 1989; Fraser et al. 1990). In the crest, LeDouarin and colleagues have provided strong but indirect evidence that separate progenitors produce sympathetic, enteric and sensory neurons (LeLievre et al. 1980; LeDouarin, 1986; Fontaine-Perus et al. 1988), even though all three progenitors may produce glia as well.

In this context, it was interesting to find several combinations of neurons or glia in the same clones.

First, many clones contained both VL and DM neurons. These two populations differ in many respects. During the middle third of the embryonic period, DM neurons are smaller and more closely packed than VL neurons, with a fairly sharp boundary separating the groups from each other (Hamburger and Levi-Montalcini, 1949). In addition, on average, VL neurons arise, differentiate and undergo naturally occurring cell death 1–2 days earlier than DM neurons (Carr and Simpson, 1978; Hamburger et al. 1981). The two populations also differ chemically: for example, many DM neurons but few VL neurons contain substance P and myelin-associated glycoprotein (Omlin et al. 1985; Fontaine-Perus et al. 1985; New and Mudge, 1986).

In several of these respects, DM neurons resemble the crest-derived neurons of cranial ganglia whereas VL neurons resemble the lineally distinct placodally derived neurons (D’Amico-Martel, 1982; Fontaine-Perus et al. 1985). For these reasons, it has been suggested that VL and DM neurons might arise from distinct lineages (LeLievre et al. 1980; Fontaine-Perus et al. 1985; Sieber-Blum, 1989a). Indeed, the frequent occurrence of clones (11 of 32) containing VL but not DM neurons raises the possibility that some committed VL progenitors exist. However, these clones are small and their composition might be due merely to their small size. In contrast, the more frequent occurrence of clones (21 of 32) containing both VL and DM neurons provides direct evidence that at least some progenitors are uncommitted in this respect, and suggests the existence of regionally varying environmental influences within ganglia.

Second, our results suggest that clonally related neurons project to multiple targets and have multiple neurochemical phenotypes. Here, the evidence is indirect, and rests on the dissimilarity of clonal and functional topographies. For example, individual clones often contain highly clustered neurons and/or both large and small neurons, whereas neurons that project to a single target type (e.g. muscle spindles or Pacinian corpuscles) are usually widely dispersed in a ganglion but fairly homogeneous in size (e.g. Brown, 1981; Honig, 1982; Harper and Lawson, 1985; Cameron et al. 1986; Lee et al. 1986). Also, many clones contain both DM and VL neurons, whereas several antibodies to neuropeptide transmitters (e.g. New and Mudge, 1986; Du et al. 1987) stain subsets of DM neurons only.

The notion that transmitter phenotype is a lineage-independent property is consistent with a large body of evidence showing that target-derived factors influence transmitter choice (Landis, 1990).

Finally, many ganglia contained both satellite cells, which ensheath neuronal somata, and Schwann cells, which ensheath axons. These cell types are morphologically and immunochemically distinct (e.g. Dulac et al. 1988; Bhattacharyya et al. 1990). In addition, Schweitzer et al. (1983) found that older DRGs gave rise to Schwann cells but not to satellite cells when transplanted into younger embryos. Because these cell types arose within a common ganglionic environment, they might have arisen from separate progenitors.

However, our results support the alternative (and prevailing) view that local environmental cues, e.g. contact with axons versus cell bodies, influence the choice between a Schwann cell or satellite cell phenotype. The absence of perineurial cells from glia-containing clones supports our previous conclusion (Bunge et al. 1989) that perineurium is of mesenchymal (fibroblastic) rather than neural crest (Schwann cell) origin.

**Injection or infection**

Two methods are now available to label progenitors for lineage analysis in vertebrates: intracellular microinjection of a dye and injection with a recombinant retrovirus. To a considerable extent, the two methods have complementary strengths and weaknesses. (a) Injection permits identification and targeting of particular progenitors, whereas virions infect cells more or less at random. (b) Clonal relationships are unambiguous following injection, whereas it is impossible to rule out that some clusters of virally marked cells are polyclones. (c) The provirus is a heritable, non-diluting label, whereas injected tracers are diluted as cells grow and divide. (d) Virions can infect some cells that are difficult to reach with a microelectrode, or too small to be impaled without injury.

Given these differences, it is gratifying that the two methods have given generally consistent results, now that they have both been applied to the same tissue. For example, we and Bronner-Fraser and Fraser (1989) both found evidence for multipotential progenitors, we both found labeled cells in DRGs associated with labeled cells in the spinal cord, and we both found many clones that spanned both a ganglion and a nerve root. Perhaps more revealing, however, is that the differences between our results are largely explicable in terms of the complementary strengths of the two methods. (a) Because they could establish clonal relationships throughout the embryo, Bronner-Fraser and Fraser were able to include three crest derivatives that we had to exclude – melanocytes, adrenal
chromaffin cells and metanephric mesenchyme. They found that these cell types could all occur in combination with sensory neurons, demonstrating a degree of multipotentiality that we were unable to ascertain. (b) On the other hand, we were able to discern phenotypic and positional differences within clones that Bronner-Fraser and Fraser did not detect. Thus, we distinguished VL from DM cells at stages 31–34, large from small neurons at stages 40–45 and neurons from intraganglionic glia at both times. These distinctions are absent and/or undetectable at embryonic day 4 (~stage 24), when problems of dilution forced Bronner-Fraser and Fraser to terminate their experiments. (c) Because we had to restrict our analysis to isolated ganglia, we probably rejected bilateral or multisegmental clones that Bronner-Fraser and Fraser included. This biased our sample toward smaller or later-arising clones, and may explain why we found fewer sympathetic and spinal cord cells in our sensory neuron-containing clones than they did. (d) However, as expected from the later stage of analysis, the clones we studied were larger, on average, than those of Bronner-Fraser and Fraser, giving us a more complete view of genealogical patterns within the DRG. Thus the clones that Bronner-Fraser and Fraser studied contained an average of 27 cells in all tissues, with only 1 of 30 clones (3%) having over 55 cells, whereas our clones contained an average of 46 cells within the DRG and its nerves alone, with 21 of 123 (17%) having over 55 cells.

In summary, theory and practice agree that the injection method is best suited for assaying complete clonal boundaries at early stages (see also Fraser et al. 1990), whereas the retroviral method permits more complete analysis of definitive cell positions, numbers and phenotypes after development is complete (see also Gray et al. 1990). In some tissues, such as neural crest, a complete genealogical analysis may require application of both methods; or, ultimately, development of a new method that combines the strengths of the two now available.

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Appendix

With the assumption that all the progeny from a single neural crest progenitor occupy a single ganglion, one can compute the probability that one of the labeled ganglia contains more than one clone (see Feller, 1957). The probability that an embryo with m labeled ganglia contains k labeled ganglia is given by the formula:

$$P_{m,k} = \binom{n}{k} \sum_{i=0}^{k} \left( -1 \right)^i \binom{k}{i} \left( \frac{k-i}{n} \right)^m$$

$$= \sum_{m=k}^{\infty} \prod_{m=k}^{\infty} \sum_{m=k}^{\infty} \prod_{m=k}^{\infty} \left( \frac{k-i}{n} \right)^m$$

where

- k = number of labeled ganglia
- m = number of clones (i.e. number of infected crest progenitors)
- n = total number of ganglia in each embryo.

The probability that any individual ganglion in an embryo with k labeled ganglia contains more than one clone is then

$$P_{k} = \frac{\sum_{m=k+1}^{\infty} \prod_{m=k}^{\infty} \left( \frac{k-i}{n} \right)^m}{\prod_{m=k}^{\infty} \left( \frac{k-i}{n} \right)^m}$$

$$= \frac{\sum_{m=k}^{\infty} \prod_{m=k}^{\infty} \left( \frac{k-i}{n} \right)^m}{\prod_{m=k}^{\infty} \left( \frac{k-i}{n} \right)^m}$$

$$= \frac{\sum_{m=k}^{\infty} \prod_{m=k}^{\infty} \left( \frac{k-i}{n} \right)^m}{\prod_{m=k}^{\infty} \left( \frac{k-i}{n} \right)^m}$$

These series converge rapidly for reasonable estimates of Pi as long as the fraction of labeled ganglia (k/n) is small. If one assumes a Poisson distribution for Pi with an average of 4 clones per embryo (conservatively based on the average of 3.2 labeled ganglia in our material) then for an embryo with 4 of 40 labeled ganglia, P_{4} = 4.5% and for an embryo with 8 of 40 labeled ganglia, P_{8} = 4.2%. Even with the unlikely assumption that the probability of any number of clones is equally likely (i.e. Pi = 1), then for 4 of 40 labeled ganglia, P_{4} = 5.75% and for 8 of 40 labeled ganglia, P_{8} = 7.8%.

References


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