Restriction of Neurogenic Ability during Neural Crest Cell Differentiation

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SUMMARY

Multipotent neural crest cells undergo developmental restrictions during embryogenesis and eventually give rise to the neurons and glia of the peripheral nervous system, melanocytes, and pheochromocytes. To understand how neuronal potential is restricted to a subpopulation of crest-derived cells, we have utilized sensitive markers of early neuronal differentiation to assess neurogenesis in crest-derived cell populations subjected to defined experimental conditions *in vitro* and *in vivo*. We describe environmental conditions that either (a) result in the irreversible loss of neurogenic potential over a characteristic time course or (b) maintain neurogenic potential among neural crest cells. © 1993 John Wiley & Sons, Inc.

Keywords: **neural crest, neurogenesis, developmental restrictions.**

INTRODUCTION

In vertebrates, peripheral neurons of most sensory and all autonomic and enteric ganglia are derived from the neural crest, a migratory population of embryonic stem cells that arises from the lateral edges of the neural plate. In addition to neurons, neural crest cells give rise to the nonneuronal cells of the peripheral nervous system, melanocytes, pheochromocytes, and skeletal derivatives of the branchial arches (see Weston, 1970 and LeDouarin, 1982 for reviews). It is now generally accepted that crest cells are multipotent early in development and become restricted later. Clonal analyses, both *in vivo* and *in vitro,* have unequivocally demonstrated that some early crest cells are multipotent progenitors capable of giving rise to as many as four different derivative cell types (Sieber-Blum and Cohen, 1980; Baroffio, Dupin, and LeDouarin, 1988; Bronner-Fraser and Fraser, 1988, 1989; Dupin, Baroffio, Dulac, Cameron-Curry, and LeDouarin, 1990; Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991). It should be noted, however, that each of these studies also describes clones that contain limited numbers of crest derivatives, including clones that contain a single type of derivative (e.g., sensory neurons). The significance of these more limited clones has been the subject of considerable speculation. Their occurrence suggests, but does not prove, the existence of clonal progenitors with restricted developmental abilities, and indicates that developmental restrictions occur early during the development of the neural crest lineage.

Consistent with this notion, analyses of the developmental abilities of crest cell *populations* also suggest that populations lacking certain developmental abilities arise early in development (Ciment and Weston, 1985). Similarly, late in development distinct, crest-derived cell populations have very limited developmental abilities, to the extreme of monopotency (Nichols, Kaplan, and Weston, 1977; Schweizer, Ayer-LeLievre, and LeDouarin, 1983). Application of cell-type specific markers has confirmed that phenotypic heterogeneity is established early within the crest-derived populations (Ciment and Weston, 1982; Barald, 1982; Girdlestone and Weston, 1985; Barbu,

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Ziller, Rong, and LeDouarin, 1986; Sieber-Blum, 1989). The functional significance of this early heterogeneity has been established in certain cases by observations that crest cell subpopulations identified or separated on the basis of expression of cell-type specific markers also exhibit restricted developmental abilities (Vogel and Weston, 1988; Maxwell, Forbes, and Christie, 1988; Barald, 1989).

The questions yet to be resolved are when and under what conditions developmental restrictions occur in neural crest-derived populations. In particular, it is not known if environmental factors influence directly the developmental potential of individual crest cells and their progeny, or conversely, if these factors act only later in a permissive fashion, that is, to allow specified subpopulations of cells to execute their developmental programs by facilitating the selective survival and/or subsequent differentiation.

To address this question, we have focused on the process by which neural crest cells decide to adopt neuronal versus nonneuronal fates. To this end, we have established a way to obtain populations of avian neural crest or crest-derived cells that either have or lack neurogenic potential. Moreover, we have shown that these populations can be experimentally manipulated *in vitro* to maintain or eliminate neurogenic ability, that crest cell populations with neurogenic ability will undergo neurogenic differentiation *in vitro* under the appropriate conditions, and that populations that lack neurogenic ability cannot be rescued by *in vivo* cues present along normal migration pathways in young embryos. Thus, we have been able to compare and study crest-derived populations that exhibit different features of neurogenesis/nonneurogenesis, namely populations that either (1) have neurogenic ability, (2) are undergoing neuronal differentiation, (3) are losing neurogenic ability, or (4) have lost neurogenic ability irreversibly. These crest-derived cell populations have also been used to characterize environmental cues that affect neurogenesis.

In this review we describe our current understanding of neural crest neurogenesis, based in large part on insights obtained using these crest-derived cell populations.

RESULTS AND DISCUSSION

Neurogenic Potential Is Progressively Lost *IN VIVO*

The ability of sensory and autonomic ganglion populations to give rise to new neurons diminishes during development. Young dorsal root (DRG) and nodose sensory ganglia contain undifferentiated cells that can give rise to sensory neurons *in vitro* (Rohrer, Henke-Fahle, El-Sharkawy, Lux, and Thoenen, 1985; Rohrer and Thoenen, 1987; Ernsberger and Rohrer, 1988) and *in vivo* (LeLievre, Schweizer, Ziller, and LeDouarin, 1980; Schweizer et al., 1983; Ayer-LeLievre, and LeDouarin, 1982). In contrast, nonneuronal ganglion cells isolated from older embryos do not have the ability to give rise to sensory neurons (Ayer-LeLievre and LeDouarin, 1982; Rohrer et al., 1985, 1986). Apparently, nonneuronal cells in sensory ganglia retain their ability to give rise to catecholaminergic cells late in development (Newgreen and Jones, 1975; Schweizer et al., 1983; Xue, Smith, and LeDouarin, 1985); however, nonneuronal cells from autonomic ganglia never give rise to sensory neurons (LeLievre et al., 1980; but see Duff, Langtimm, Richardson, and Sieber-Blum, 1991). In young sympathetic ganglia, cells that express differentiated neuronal and catecholaminergic traits can divide and generate additional sympathetic neurons (Rothman, Gershon, and Holtzer, 1978; Rohrer and Thoenen, 1987; Ernsberger, Edgar, and Rohrer, 1989; DiCicco-Bloom, Townes-Anderson, and Black, 1990). Sympathetic neurons from older ganglia lose this proliferative ability. Thus, the neurogenic ability of neural crest-derived ganglion populations declines during development, regardless of whether new neurons arise from undifferentiated precursors (sensory neurons) or from cells expressing some neuronal traits (sympathetic neurons).

Neurogenic Ability Is Also Progressively Lost *IN VITRO*

To begin to identify developmental signals that influence the loss of neurogenic potential by neural crest cell populations in avian embryos, we have exploited tissue culture, immunocytochemical, and *in ovo* microsurgical techniques. First, we have shown that cells that express several neuronal traits arise in cultures of quail neural crest cells (Ciment and Weston, 1982; Girdlestone and Weston, 1985; Vogel and Weston, 1988). To account for the kinetics of neuronal appearance, the "birthdays" of neuronal cells, and the loss of neurogenic ability from crest cell populations under various *in vitro* conditions, we proposed that neurons arise from a limited number of neurogenic precursors (Vogel and Weston, 1988). Second, we have demonstrated that neurogenic potential is progressively diminished in crest cell populations when cell dispersal is delayed, and lost if the delay exceeds 24 h (Vogel and Weston, 1988); the loss of neurogenic potential appears to be irreversible, as neurogenic precursors in these populations cannot be rescued by culture conditions known to support neuronal differentiation, or by back-transplantation onto the neural crest migratory pathway of young embryos (Vogel and Weston, in preparation). Third, we have begun to identify molecules that

influence neuronal differentiation in neural crest cell cultures.

When embryonic day (E) 2 quail neural tubes are explanted onto a nonadhesive substratum, clusters of neural crest cells form after 18–24 h (primary culture) (Glimelius and Weston, 1981; Loring, Glimelius, Erickson, and Weston, 1981). These neural crest cell clusters (24-h clusters) can be dissected off the neural tube and placed onto tissue culture plastic, where the cells disperse and differentiate (secondary culture). Cells that express a neuron-specific glycolipid recognized by the monoclonal antibody A2B5 (Eisenbarth, Walsh, and Nirenberg, 1979) appear after 1 day in secondary culture; by 4 days in secondary culture, 15%–30% of the neural crest cells are $A2B5^+$ [Fig. 1(A); Girdlestone and Weston, 1985; Vogel and Weston, 1988]. These $A2B5^+$ cells are postmitotic, have long processes, and express several other neuronal markers (Girdlestone and Weston, 1985; Vogel and Weston, 1988). After 5 days in secondary culture, the percentage of $A2B5^+$ cells decreases, presumably because $A2B5^-$ neural crest cells continue to proliferate and few or no new $A2B5^+$ cells are added (Girdlestone and Weston, 1985).

To determine whether new neurons continue to differentiate in older neural crest cell cultures, we used the A2B5 antibody and complement to immunoablate existing neurons (Vogel and Weston, 1988). At 1 day after immunoablation, we stained the cultures with A2B5 to assess the ability of the remaining neural crest cells to give rise to neurons. When $A2B5^+$ cells are immunoablated from young (1–3 days) secondary cultures, the surviving $A2B5^-$ neural crest cells give rise to large numbers of $A2B5^+$ cells, reaching 60%–100% of the values in paired, untreated control cultures. In contrast, $A2B5^-$ neural crest cells in older (days 6–7) cultures give rise to very few or no new neurons, even if the cultures are allowed to "recover" for 5 days after the immunoablation (Vogel and Weston, 1988). We concluded that neural crest cell populations progressively lose neurogenic potential with time *in vitro*, and that this developmental restriction is likely to reflect the depletion of a subpopulation of neurogenic precursors. These results did not allow us to determine if the developmental repertoire of neurogenic precursors is limited to neurons or if it includes other neural crest derivatives.

Neurogenic Potential of Neural Crest Cell Populations *IN VITRO* Can Be Controlled by Manipulating the Timing of Cellular Dispersal

As described above, 15%–30% of the cells in secondary cultures derived from 24-h neural crest cell clusters express neuronal markers (Fig. 1), and if these neurons are immunoablated within the first few days of secondary culture, new neurons probably differentiate from a subpopulation of the remaining $A2B5^-$ cells. In contrast, when neural crest cell dispersal in primary cultures is delayed for 8 (32-h clusters) or 24 (48-h clusters) h by maintaining the neural tubes on a nonadhesive substratum, few neurons differentiate in the resulting secondary cultures [Fig. 1 (A, C, E) Girdlestone and Weston, 1985; Vogel and Weston, 1988]. Moreover, cultures derived from 32- or 48-h neural crest cell clusters contain few or no neurogenic precursors, since when the few existing $A2B5^+$ cells are immunoablated, they not replaced with new neurons.

To test whether the loss of neurogenic potential by neural crest cell populations maintained as clusters is correlated with delayed dispersal and does not result from prolonged interaction with the neural tube, we manipulated the dispersal of neural crest cells in the absence of the neural tube. When 24-h (neurogenic) neural crest cell clusters are removed from the neural tube and placed on a nonadhesive substratum to prevent dispersal for 8 or 24 h, few neurons differentiate in the cultures derived from these clusters, and immunoablated $A2B5^+$ cells are not replaced (Vogel and Weston, 1988). Thus, the loss of neurogenic potential in 32- and 48-h neural crest cell clusters is not a result of prolonged interaction with the neural tube.

Recently, Weston (1991) has proposed that delayed dispersal may act similarly *in vivo* to affect neural crest cell neurogenesis and subsequent patterns of gangliogenesis. Thus, sensory and sympathetic neurons arise from neurogenic precursors within the crest cell population that disperses promptly along the medial pathway between, or in the rostral halves of the somites. These cells localize in the nascent dorsal root ganglia and sympathetic chains where interactions between differentiating neurons and nonneuronal crest cells facilitate gangliogenesis. In contrast, dispersal is delayed for the crest cell population that initially resides adjacent to the caudal half-somite. These neural crest cells, which follow the lateral pathway, never undergo neurogenesis, but give rise to nonneuronal derivatives (melanocytes and perhaps Schwann cells) in the skin. The neurogenic precursors in this population of neural crest cells may either die, or lose neurogenic potential.

Neurotrophic Factors Influence Neural Crest Cell Differentiation

Punctual dispersal by neural crest cells may ensure that they are exposed to environmental cues that promote the survival and/or differentiation of neurogenic precursors. Alternatively, this process may reduce contact-mediated signals between adjacent neural crest cells. To identify such developmental signals, we have used two approaches. First, to determine whether the neurogenic potential of 32- and 48-h neural crest cell clusters can be rescued by the *in vivo* environment, we have back-transplanted these quail clusters onto the neural crest migratory pathway of young chicken embryos. Second, we have exposed quail neural crest cells *in vitro* to neurotro-

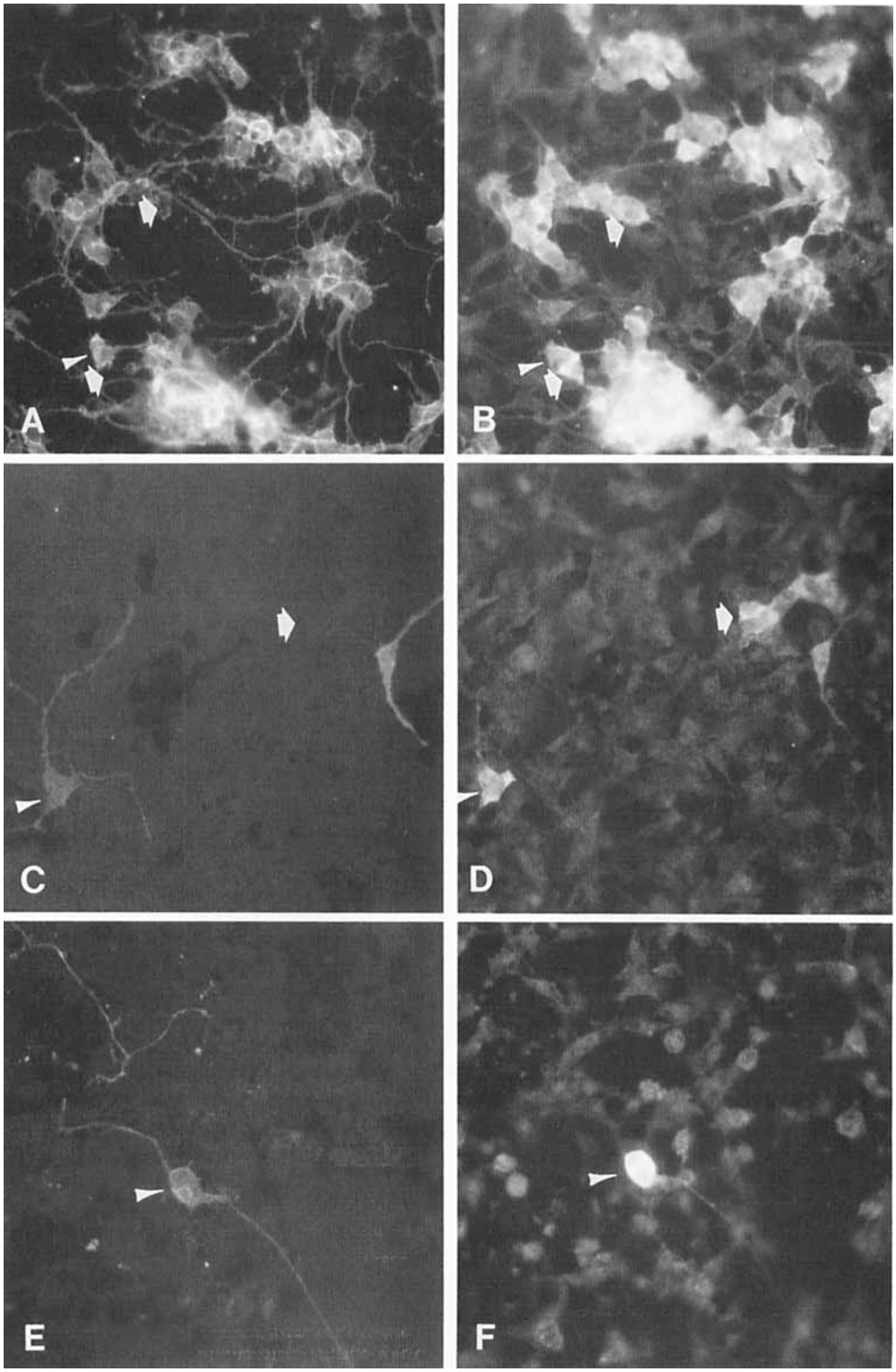

Figure 1 Neurogenic potential is lost when neural crest cell dispersal is delayed *in vitro.* Photomicrographs of A2B5$^+$ cells (arrowheads) in cultures of (A) 24-h, (C) 32-h, and (E) 48-h quail neural crest cell clusters, after 4 days in secondary culture. The same cultures were also stained for Anti-Hu immunoreactivity, and Anti-Hu$^+$ cells are shown in (B), (D), and (F) corresponding to the fields shown in (A), (C), and (E). Arrows point to cells that are Anti-Hu$^+$ and A2B5$^-$.

phic factors that support the survival of neural crest-derived neurons later in development.

The developmental potential of neural crest-derived cells isolated from quail embryos can be tested by back-transplanting them onto the neural crest migratory pathway of young (E2) chicken embryos (Erickson, Tosney, and Weston, 1980; LeLievre et al., 1980). The quail cells can be identified in the resulting chimeras by a characteristic nuclear structure (LeDouarin, 1973), and an array of cell-type specific markers of differentiation can be used to determine the phenotypes of transplanted cells. We have exploited this technique to test the developmental potential of 24-, 32-, and 48-h quail neural crest cell clusters. We back-transplanted clusters onto the medial pathway at the trunk level of E2 chickens, allowed the chimeras to develop an additional 7–9 days, and used an antiserum against the 160 kD neurofilament (NF) protein to determine the phenotypes (neuronal versus non-neuronal) of the graft-derived cells in serial sections of the chimeras. The 24-h neural crest cell clusters give rise to NF^+ and NF^- quail cells in the dorsal root and sympathetic ganglia, NF^- cells in the dorsal and ventral roots, NF^- cells in the adrenal gland, and NF^- melanocytes in the feather germs (Vogel and Weston, in preparation). In contrast, 48-h neural crest cell clusters give rise to very few or no NF^+ cells *in vivo;* we observed NF^- quail cells in ganglia and in the dorsal and ventral roots, and many NF^- melanocytes in the feather germs. Although 32-h neural crest cell clusters have lost neurogenic potential by *in vitro* criteria (Vogel and Weston, 1988), they can give rise to NF^+ cells in the dorsal root and sympathetic ganglia when back-transplanted onto the medial pathway (Vogel and Weston, in preparation). We conclude that 24-h neural crest cell clusters have the same developmental potential as premigratory neural crest populations, and that when dispersal of these cells is delayed for 24 h *in vitro,* neurogenic potential is irreversibly lost.

Because 32-h neural crest cell clusters contain neurogenic precursors that can be rescued by the *in vivo* environment, they provide an excellent population on which to test molecules that might influence neurogenesis. Nerve growth factor (NGF), which supports the survival of crest-derived sympathetic and sensory neurons (reviewed by Levi-Montalcini, 1987), does not increase the number of neurons that differentiate in cultures of quail neural crest cells (Vogel and Weston, 1990). This is consistent with other reports that NGF acts only to support the survival of neurons after they have contacted their targets and does not influence neurogenesis among neural crest cells *in vitro* (Ziller, Dupin, Brazeau, Paulin, and LeDouarin, 1983; Christie, Forbes, and Maxwell, 1987; Kalcheim and Gendreau, 1988; Sieber-Blum, 1991).

More recently, we have examined the effects of neurotrophin-3 (NT-3), another member of the NGF family of polypeptide neurotrophic factors (Hohn, Leibrock, Bailey, and Barde, 1990), on neurogenesis among cultured quail neural crest cells. In addition to its traditional neurotrophic effects on the survival of older sensory neurons, NT-3 also increases the number of NF^+ cells in cultures of young (E4.5) chicken dorsal root ganglia by a mechanism other than enhanced survival of existing neurons (Wright, Vogel, and Davies, 1992). To determine whether NT-3 could act earlier to increase neurogenesis among the neural crest cell precursor population for dorsal root ganglia, we exposed clusters of quail neural crest cells to NT-3 *in vitro* and identified neurons in the cultures with the monoclonal antibody A2B5. NT-3 increases the proportions of $A2B5^+$ cells in cultures of 24- and 32-h quail neural crest cell clusters, but has no effect on neurogenesis in cultures of 48-h clusters. The effect of NT-3 on neuronal differentiation in cultures of 32-h neural crest cell clusters is particularly striking (Fig. 2). Although NT-3 has little or no effect on the survival of early neurons (Wright et al., 1992), our present results with neural crest cell cultures do not allow us to determine whether NT-3 promotes proliferation of committed neuronal precursors or biases differentiation of uncommitted neural crest cells along the neurogenic pathway.

Brain-derived neurotrophic factor (BDNF) has also been reported to support the survival of neural crest cells *in vivo* (Kalcheim, Barde, Thoenen, and LeDouarin, 1987), and promote the survival and/or differentiation of neurons among neural crest cells *in vitro* (Kalcheim and Gendreau, 1988; Sieber-Blum, 1991). These data, and our results with NT-3, challenge the traditional role of neurotrophic factors as molecules that support the survival and growth of peripheral neurons only after they have contacted their targets.

Identification of Early Neurogenic Neural Crest Cells

As described above, we can account for neurogenic ability by the presence of a developmentally restricted subpopulation of neural crest-derived cells (Vogel and Weston, 1988, see above). If this hypothesis were correct, then it should be possible to identify neurogenic precursors directly with appropriate cell-type specific markers. Such markers would facilitate study not only of the identified subpopulation, but also of the environmental factors that affect the appearance and subsequent differentiation of neurogenic precursors.

We have recently described a differential immunological screening protocol that appears to allow us to identify such markers for neurogenic precursors (Marusich and Weston, 1992). We have shown that sera obtained from patients that present with *both* small-cell lung carcinoma (SCLC) and subacute sensory neuropathy (Graus, Cordon-Cardo, and Posner, 1985; Graus, Elkon, Cordon-Cardo, and Posner, 1986; Budde-Steffen, Anderson, Rosenblum, and Posner, 1988; Dalmau, Furneaux, Gralla, Kris, and Posner, 1990) reveals cellular immunoreactivity (Anti-Hu-IR), which is expressed in a transient population of crest-derived cells that lack neuronal morphology and fail to express other neuron-specific markers such as A2B5 or neurofilament immunoreactivities. We have concluded that these Anti-Hu^+ "nonneuronal" crest-derived cells are either neurogenic precursors or

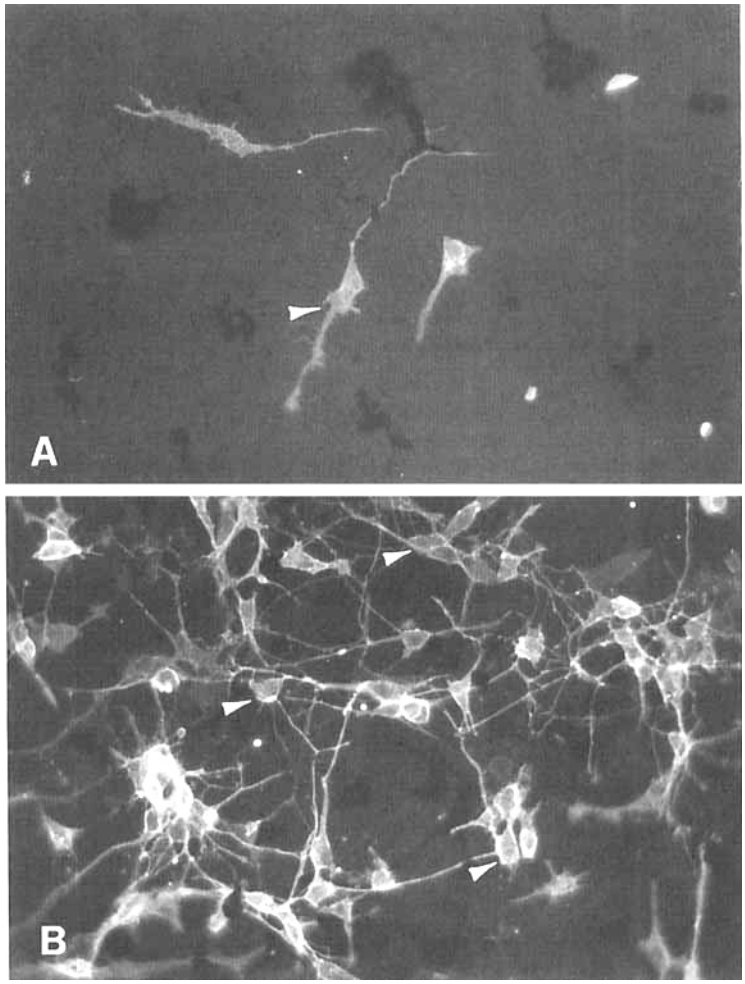

Figure 2 Neurotrophin-3 increases the number of neurons (A2B5$^+$ cells) in neural crest cell cultures. Photomicrographs of A2B5$^+$ cells (arrowheads) in cultures of 32-h quail neural crest cell clusters after 4 days in secondary culture in control (A) or NT-3-containing (B) medium.

immature neurons. In humans, Anti-Hu antibodies specifically label all SCLC cells, the neuroendocrine Kulchitsky cells of the lung (from which SCLCs are thought to arise), and all neurons of the central and peripheral nervous systems (Graus et al., 1985, 1986; Budde-Steffen et al., 1988; Dalmau et al., 1990).

Thus, in addition to the neuronal expression, Anti-Hu-IR is expressed in a subpopulation of crest-derived cells early in development. Some cells within the nascent dorsal root ganglia, for example, exhibit Anti-Hu-IR at day 3 of embryonic development. To determine more precisely when Anti-Hu-IR appeared relative to other established neuronal markers, and to establish its validity as a marker of neurogenic crest cells, we utilized the neurogenic and nonneurogenic populations of crest-derived cells that have been described above.

We predicted that, if Anti-Hu identifies neurogenic neural crest cells, Anti-Hu-IR would be detected in apparently nonneuronal crest cells only in crest cell populations known to have neurogenic ability. Nonneuronal crest cells were operationally defined as crest-derived cells lacking neuronal morphology and lacking expression of established neuron-specific traits such as A2B5, neurofilament, or neurofilament-associated protein immunoreactivities (Marusich and Weston, 1992). This prediction was confirmed to be the case, as Anti-Hu$^+$ "nonneuronal" cells were observed in crest cell cultures known to have neurogenic ability, that is primary cultures of neural tube outgrowth and secondary cultures of 24-h neural crest cell clusters. In contrast, few or no Anti-Hu$^+$ cells were observed in crest cell cultures known to lack neurogenic ability, that is, those derived from 32- or 48-h clusters [Fig. 1(B, D, F); Fig. 3; see also Marusich and Weston, 1992]. Similarly, young DRG (<E7) known to have neurogenic ability contain Anti-Hu-IR$^+$ nonneuronal cells, whereas older DRG that lack neurogenic ability correspondingly lack such cells (Marusich and Weston, 1992). It is particularly striking that the time course of decline in Anti-Hu-IR$^+$ nonneuronal DRG cells closely approximates the previously reported time course in decline of neurogenic ability in DRG. A similar decline is observed in secondary cultures of 24-h

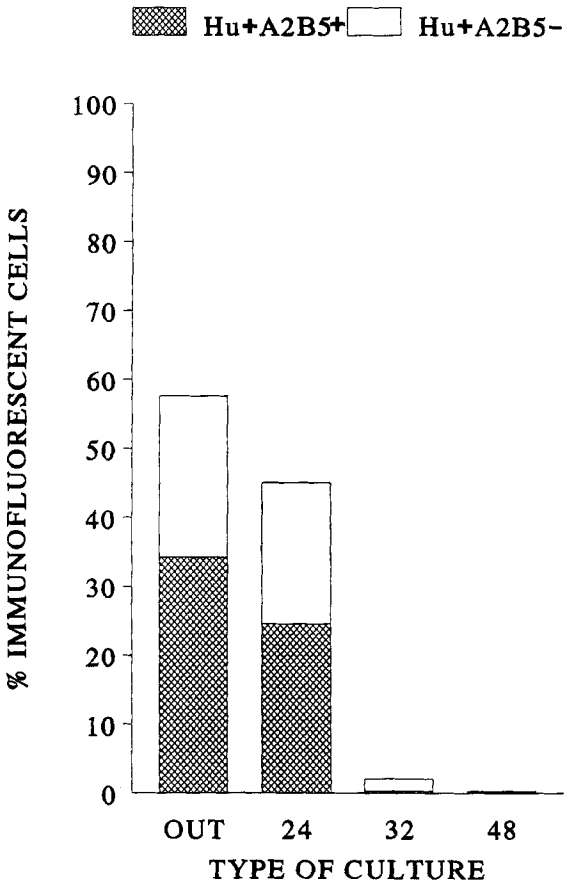

Figure 3 The proportion of Anti-Hu$^+$ cells decreases when neural crest cell dispersal is delayed *in vitro*. Primary cultures of neural crest cell outgrowth (OUT), and secondary cultures of 24-h, 32-h, and 48-h neural crest cell clusters were stained for A2B5 and Anti-Hu immunoreactivity after 4 days *in vitro*. The proportions of immunofluorescent cells were determined as described in Vogel and Weston (1988). Each bar represents an average of values obtained from three cultures.

neural crest cell clusters. In both cases, the subpopulation of Anti-Hu-IR$^+$ nonneuronal cells is transient, and appears to be replaced by Anti-Hu-IR$^+$ neuronal cells. We have suggested that the Anti-Hu nonneuronal cells are either neurogenic precursors or immature neurons that eventually express other neuronal traits.

Although it is clear that Anti-Hu-IR is expressed prior to other neuronal markers, our results do not suggest a role for this protein. Anti-Hu-IR is localized both within the nucleus and in the cytoplasm of the neuronal cell body, and is associated with molecules of approximately 40 kD relative molecular weight, properties again held in common with the human antigen(s). Recently, molecular genetic analysis of the human antigen has provided some insight into the role of the Hu protein, as the human gene (HuD) that encodes the Anti-Hu antigen has now been described (Szabo et al., 1991). Knowledge of the predicted amino acid sequence of HuD has provided a fundamentally important insight regarding the possible role of HuD in neurogenesis. Thus, HuD shows strong similarity to the *Drosophila* proteins *Elav* (51% homology) and sex-lethal (42% homology). It is significant that *Elav* is a neuron-specific protein that has been reported to be the earliest neuron-specific trait expressed during *Drosophila* neurogenesis (Robinow, Campos, Yao, and White, 1988; Robinow and White 1991). Moreover, genetic analysis has shown that expression of *Elav* is required for normal development and maintenance of the *Drosophila* nervous system (Jimenez and Campos-Ortega, 1987). An additional feature of interest is that both *Elav* and sex-lethal, as well as HuD, contain ribonucleoprotein consensus sequences, indicating that they are RNA binding proteins. Thus, it is possible that HuD may regulate the processing of mRNAs in early neurogenic cells. In this regard, the recent elucidation of the mechanism of action of sex-lethal provides an outstanding example of how regulation of mRNA processing can control cellular differentiation in a stable inherited pattern (Baker, 1989). Indeed, sex-lethal has been referred to as a master regulatory gene (Wolfner, 1988), and it is possible that HuD may play a similar role in mammalian neurogenesis. Our characterization of Anti-Hu-IR as a precocious indicator of neural crest cell neurogenesis suggests that an avian HuD-like protein may play an important role in neurogenesis of neural crest cells.

CONCLUSIONS

To understand mechanisms by which developmental restrictions occur among neural crest cells, we have exploited the existence of crest-derived populations that exhibit different features of neurogenesis/nonneurogenesis. These populations have allowed us to identify environmental conditions that promote or attenuate neurogenic potential among avian neural crest cells. In addition, we have identified an early marker of putative neurogenic cells, which may represent a regulatory protein involved in neuronal differentiation.

Clearly, to analyze the role of environmental cues in the loss of neurogenic potential among neural crest cells requires identification of specific signal molecules and receptors for these signals. Thus, potentially responsive crest-derived populations that express particular receptors for growth and survival factors can be identified and exposed to relevant signals. Because several members of the NGF family of neurotrophic factors support the survival of crest-derived neurons, these factors and their corresponding tyrosine kinase (*trk*) receptors are good candidates for signals and signal receptors that are relevant to neural crest cell development. Indeed, both BDNF and NT-3 influence neurogen-

esis among neural crest cells (Kalcheim and Gendreau, 1988; Sieber-Blum, 1991; Vogel and Weston, in preparation), and an analysis of *trk-B* and *trk-C* (Lamballe, Klein, and Barbacid, 1991; Soppet et al., 1991; Squinto et al., 1991) receptor expression during neural crest ontogeny would identify potentially responsive populations. Several experimental approaches could be used to pursue this analysis. Genetic and molecular analyses of signals and signal receptors in the mouse have allowed researchers to identify environmental cues that influence the development of a number of nonneurogenic neural crest derivatives, including melanocytes and skeletal structures (Morrison-Graham and Weston, 1989; Nishikawa et al., 1991; Morrison-Graham, Schatteman, Bork, Bowen-Pope, and Weston, 1992). Antisense oligonucleotides directed against specific mRNAs can be used to perturb expression of signal molecules and signal receptors in cultured vertebrate cells (Represa, Leon, Miner, and Giraldez, 1991; Wright et al., submitted).

Although clonal analyses of neural crest cells isolated or labelled at different stages of migration and localization (Bronner-Fraser and Fraser, 1989; Fraser and Bronner-Fraser, 1991; Duff et al., 1991) provide useful information about the developmental potential of neural crest-derived cells, they do not address the state of *commitment* of these cells. To test the commitment of an individual cell, one must be able to identify cells known to have a particular fate during normal development and then expose them to a different environment. A cell that is committed will retain its original fate, regardless of the new environment (Slack, 1983). Such an analysis of neural crest cell commitment is now possible in the zebrafish embryo (Wood, Hodsdon, Raible, Weston, and Eisen, submitted), where the commitment of individual motor neurons to particular patterns of axon outgrowth has already been tested (Eisen, 1991). Advances in genetics, molecular biology, and embryo manipulations should allow us to dissect further the mechanisms by which neural crest cells become restricted to particular fates during development.

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