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Phenotypic plasticity of Schwann cells and enteric glial cells in response to the microenvironment

(neural crest/peripheral nerves/quail-chicken marker/gut mesenchyme)

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ABSTRACT **We produced earlier a monoclonal antibody against Schwann cell myelin protein (SMP), a glycoprotein expressed on Schwann cells (SC) but not on satellite cells of the ganglia or enteric glial cells. We now studied whether SMP expression is environmentally regulated in the different compartments of the peripheral nervous system. Quail neural-crest cells from either mes-metencephalic, vagal, or truncal levels of the neuraxis were heterochronically associated with gut wall, skin, or muscle tissues from embryonic day (E) 7 to E11 chickens. Coculture of these chimeric organs revealed that as in normal development glial cells, characterized by HNK1 immunoreactivity and the quail nuclear marker, expressed the SMP phenotype exclusively in skin and muscle, failing to do so in gut. However, when SMP$^+$ SC from quail sciatic nerves were cocultured with chicken gut, these cells rapidly lost their initial SMP immunoreactivity. In contrast, when associated with muscle and skin, SC remained SMP$^+$, even in the complete absence of neuronal cells. Enteric plexuses from E8 to E15 quail gut express SMP$^+$ and laminin when withdrawn from the intestinal-mesenchyme environment. These results show that SMP can be expressed by enteric glial cells and that the SC SMP phenotype is strongly inhibited by the gut-wall environment. Moreover, these results strongly suggest that these two types of glial cells belong to the same lineage and that their terminal phenotype is modulated through cell-to-cell interactions.**

The glial elements of the peripheral nervous system (PNS) are derived from a common transient embryonic structure, the neural crest. After a migratory phase throughout the developing embryo, the neural-crest cells become distributed in four main classes of ganglia—sensory, sympathetic, parasympathetic, and enteric—within which they differentiate into a variety of neuronal cell types associated with the so-called satellite glial cells. Moreover, the fate of many neural-crest cells is to associate with the network of peripheral nerves, where they yield the two categories of Schwann cells (SC), those generating a myelin sheath and those that are unmyelinating (for review, see ref. 1). The gut is densely innervated through the two myenteric (Auerbach's) and submucosal (Meisner's) plexuses that are mainly derived from a relatively small supply of neural-crest cells of vagal origin; a small contribution also comes from the lumbosacral neural crest (2, 3).

The enteric nervous system differs from other sections of the PNS by several characteristics, not the least of which concerns glia (4–8). Beside being devoid of myelin, the enteric glial cells (EGC), whether in ganglia or lining interconnective nerves, display features similar to those of the astrocytes of the central nervous system rather than to the SC or satellite cells of the PNS. Enteric plexuses appear as compact structures, surrounded by a basal lamina, in which nerve fibers and neuronal cell bodies are lined with ramified and intermingled glial processes. However, glial ensheathments comparable to those formed by satellite cells and SC in PNS ganglia and nerves are never seen in the gut. Moreover, EGC synthesize neither individual basal lamina containing laminin and fibrillary collagen nor any of the identified myelin components that SC do. EGC are, in contrast, characterized by a high level of glial fibrillary acidic protein (9).

We know that differentiation of the neural-crest cell populations arising from the various levels of the neural axis strongly depends upon environmental influences exerted by the tissues reached at the end of their migration (10, 11). This result was particularly evident for the adrenergic phenotype, which is totally suppressed in the gut at all developmental stages in birds (11) and which exists only transiently in early embryonic life in the mammalian gut (12, 13).

In the present work, we studied the influence of various tissue environments on the phenotype expressed by embryonic glial cells. Our approach was 2-fold. (*i*) We subjected undifferentiated quail crest cells from different levels of the neuraxis to the environment of the gut wall and of muscle and skin taken from chicken embryos from midincubation time onward. (*ii*) The tissue microenvironment of differentiating glial cells isolated from enteric plexuses and from peripheral nerves was experimentally modified. The criterion used to discriminate the SC from the EGC phenotype was expression of Schwann cell myelin protein (SMP), previously characterized in birds *in vivo* and *in vivo* by a monoclonal antibody (mAb) as an early and specific marker of SC (14, 15).

Combination of SMP immunoreactivity with the evidence brought by the quail nuclear marker allows visualization of the phenotype expressed by glial-cell progenitors placed in the situations described above. Whatever the source of glial cells, their capability to express SMP was abolished in the gut environment; moreover, EGC can synthesize SMP when withdrawn from the gut environment.

MATERIALS AND METHODS

Experiments were done on Japanese quail and White Leghorn chicken embryos. Eggs were incubated at 38°C in a humidified atmosphere.

Associations of Quail Neural Primordium and Peripheral Nerves with E6–E11 Chicken Tissues. The mes-metencephalic neural crest and the vagal and truncal parts of neural tubes were removed from quail embryos at the 12-somite stage, according to described procedures (16, 17). Sciatic nerves and brachial plexuses were removed under sterile conditions from 10-, 12-, and 15-day quail embryos (E10, E12, and E15) and partly dissociated in 0.03% collagenase (Cooper Biomed-

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Abbreviations: SC, Schwann cells; mAb, monoclonal antibody; SMP, Schwann cell myelin protein; EGC, enteric glial cells; PNS, peripheral nervous system; CA, catecholamine; E with number, embryonic day; CAM, chicken chorioallantoic membrane.

ical)/0.25% trypsin (Difco)/Tyrode's solution without Ca^{2+} or Mg^{2+}. Quail peripheral nerves, neural crests, or neural tubes were inserted into a slit made in segments of postumbilical gut fragments of limb and vertebral muscle and of dorsal skin from E6 to E11 chickens.

Chimeric explants were then cultured *in vitro* for 7–12 days in Dulbecco's modified Eagle's medium (GIBCO)/15% fetal calf serum (Biological Industries, Beth Haemek, Israel)/2% chicken embryo extract or on the chicken chorioallantoic membrane (CAM) grafts. The *in vitro* "organotypic" cultures were done at the air–medium interface on top of a Millipore filter placed on a metal grid (18).

Cell Cultures. Myenteric plexuses were isolated from quail ceca at E15 and from quail gizzards at E8, E10, E11, and E13 after 2 hr of incubation in Tyrode's solution/1% collagenase. The nerves belonging to the extrinsic innervation of the gut together with the external serosa were carefully removed from bowel fragments before collagenase treatment. Mesenchymal cells always remained associated with the enteric plexuses in variable quantities, depending on the type of experiment. The enteric plexus and associated mesodermal cells were then cultured either as small explants or after further dissociation into single cells by enzymatic digestion (with Tyrode's solution lacking Ca^{2+} and Mg^{2+}/0.25% trypsin). Different culture conditions were used, as indicated in Table 1.

Immunocytochemistry. Chimeric explants or cell cultures were fixed for 1 hr in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline. Explants were subsequently frozen at −80°C for cryostat sectioning. Sections (10–15 μm) were deposited on poly(lysine)-coated slides (Sigma). Indirect immunofluorescence double-staining was performed on tissue sections and cell cultures. Slides and cell cultures were first incubated with the mAbs HNK1 (21) and anti-SMP (14) of the IgM and IgG1 isotypes, respectively, and then subjected to fluorescein- and tetramethyl/rhodamine/isothiocyanate-conjugated goat anti-mouse IgG and IgM (Southern Biotechnology Associates, Birmingham, AL). Antilaminin rabbit antiserum was purchased at Sigma. The quail nuclear marker was identified after staining tissue sections with Hoechst nuclear dye (Hoechst 33342, Serva).

RESULTS

SMP immunoreactivity of the extrinsic nerves innervating the digestive tract disappears sharply as they enter the gut; Auerbach's and Meissner's plexuses, as well as the nerves interconnecting them, are SMP negative (Fig. 1). Therefore, the SMP phenotype can be used to discriminate SC from EGC. In contrast, HNK1 immunoreactivity is common to all the glial cells and most neurons in the PNS.

Association of Quail Neural-Crest Cells with Gut Wall, Skin, and Muscle from E7–E11 Chicken Embryos. To follow the differentiation of glial progenitors in various microenvironments, isolated mes-metencephalic neural crest or segments of vagal and truncal neural tubes, including the premigratory neural-crest cells, were associated with pieces of chicken postumbilical gut and with skin or muscle fragments. The tissues were then cultured for 12 days *in vitro* or on the CAM. We note that at E7–E11 the chicken gut had already received its own supply of neural-crest cells.

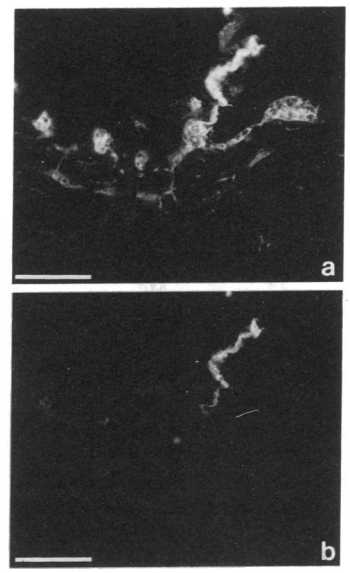

FIG. 1. Extrinsic and intrinsic innervation of the gut wall from adult quail. (*a*) HNK1 immunoreactivity. (*b*) Anti-SMP immunoreactivity. The extrinsic nerve is SMP^+, $HNK1^+$ until fibers enter the enteric wall. Intramural plexuses of Auerbach and Meissner are SMP^-, $HNK1^+$. (Bar = 50 μm.)

Phenotypic analysis of the chimeric explants is reported in Table 2. After 12 days of *in vitro* or CAM culture, the mesenchyme of the gut wall became thicker, and villosity-like structures were generated. In the associations where a piece of neural tube was included in the explant, a large mass of quail central nervous tissue developed in apposition to the chicken mesenchyme. The gut wall of the chicken embryos had a strong proliferative effect on quail neural-crest cells. These cells colonized the whole host tissue and formed pure quail or mixed quail/chicken ganglionic structures with interconnecting strands, as in normal intramural plexuses (Fig. 2 *a–c*); the chimeric explants also resembled normal gut in being totally devoid of SMP^+ cells. In contrast, the central nervous tissue that developed from the neural tube contained elements with oligodendrocyte-like morphology of the SMP phenotype. The $HNK1^+$ SMP^- enteric plexuses seen in the explants were similar whether quail-crest cells originated from either the cephalic, vagal, or trunk levels of the neural axis and whatever the age of host tissue (E7 or E11).

Table 1. Culture conditions of isolated enteric plexuses

Culture	Stage of chicken donor embryo	Ex/DiC	Medium	Substratum	Time in culture, days	Cultures examined, no.
Cecal plexuses						
Abundant mesodermal cells	E15	Ex/DiC	DMEM/10% FCS/2% CEE	3T3 feeder cells*	3–9	48
Rare mesodermal cells	E15	Ex/DiC	DMEM/10% FCS/2% CEE	3T3 feeder cells*	7	4
Gizzard plexuses	E8–E13	Ex	BBM, SFRI4, DMEM/10% FCS	Plastic, laminin, fibronectin, and 3T3 feeder cells*	5–12	3–8 for each experiment

Ex, explant; DiC, dissociated cells; BBM, basic Brazeau medium (19) SFRI4 (Berganton, France); CEE, chicken embryo extract; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium.
*Mitomycin growth-inhibited 3T3 fibroblasts (see ref. 20 for culture methods).

Table 2. Differentiation of glia from quail neural-crest cells associated with different chicken tissues in chicken chorioallantoic membrane grafts and in *in vitro* culture

Host tissue			Quail explant	Quail cells	
Nature	Stage	No.	nature	HNK1$^+$	SMP$^+$
Gut	E7	2*	Mc	++	0
	E7	2*	Vc	++	0
	E7	3*	Tc	++	0
	E11	2	Vc	++	0
Skin	E7	1*	Mc	+	+
	E7	3*	Tc	+	+
	E11	2	Vc	+	+
Muscle	E7	2*	Mc	+	+
	E7	2*	Tc	+	+

Mc, mes-metencephalic neural crest; Vc, vagal neural crest; Tc, truncal neural crest; No., number of cultures; +, positive cells; ++, many positive cells.
*CAM-graft-associated.

Control explants, in which quail neural-crest cells were associated with chicken muscle and skin, respectively, produced dermal and epidermal structures, including feather buds and striated fibers. The neural-crest-derived HNK1$^+$ quail cells were less numerous than in the gut and were found scattered in the chicken tissues. All explants included a subpopulation of cells double-labeled with HNK1 and anti-SMP mAbs. SMP-positive cells sometimes formed myelin-like structures around the quail neuronal processes (Fig. 2 *d–e*).

Associations of Differentiating SC with Gut Wall, Skin, and Muscle from E6–E7 and E10 Chicken Embryos. Peripheral nerves were dissected out from quail embryos and partly dissociated before their association to favor the immigration of SC into the chicken tissues. After 7 days of coculture, grafted SC were distinguished from quail fibroblasts as HNK1$^+$ cells carrying the quail nuclear marker. These cells were found dispersed in the host tissue and around the quail explant. In the gut they were sometimes integrated in host enteric plexuses. Although numerous quail HNK1$^+$ cells were present in the chicken-gut mesenchyme, no SMP$^+$ cells were detected (Fig. 3 *a* and *b*), except in one case where a very faint SMP immunoreactivity was seen in the original quail explant. In contrast, quail SMP$^+$ cells still exist in skin or muscle explants (Fig. 3 *c–e*) in spite of the lack in such tissues of any neurons of host (chicken) or donor (quail) origin. Results were similar, whatever the stage of host and quail tissues.

***In Vitro* Culture of EGC.** Explants or cell suspensions of cecal plexuses taken from E15 quails were cultured together with most of the mesodermal gut wall or after isolation of the myenteric plexus (see Table 1 for culture conditions). HNK1$^+$ cells were abundant in all these cultures. Double-labeled HNK1$^+$, SMP$^+$ cells appeared from 4 days of culture onward; the number of positive cells and immunostaining intensity increased with culture time (Fig. 4). The mesenchymal cells accompanying the plexuses rapidly divided and spread around the rather compact ganglionic masses. SMP$^+$ cells were found in roughly similar numbers in explants of equivalent ages in culture, whatever the amount of mesenchymal cells was initially. Distribution of the SMP$^+$ cells varied; they were isolated or in small groups within the area of migratory cells around the initial explant or still along neurites and in contact with neuronal cell bodies.

The gizzard plexuses were removed at earlier developmental stages (E8–E13) and were cultured as explants on different substrates in the virtual absence of mesodermal cells. Three types of medium were used, DMEM/10% fetal calf serum, the serum-free medium SFRI4, or the fully defined medium BBM. The SMP phenotype developed in all cases. Some gizzard plexuses cultured in DMEM/10% fetal calf serum and in SFRI4 were treated with an antilaminin antiserum and

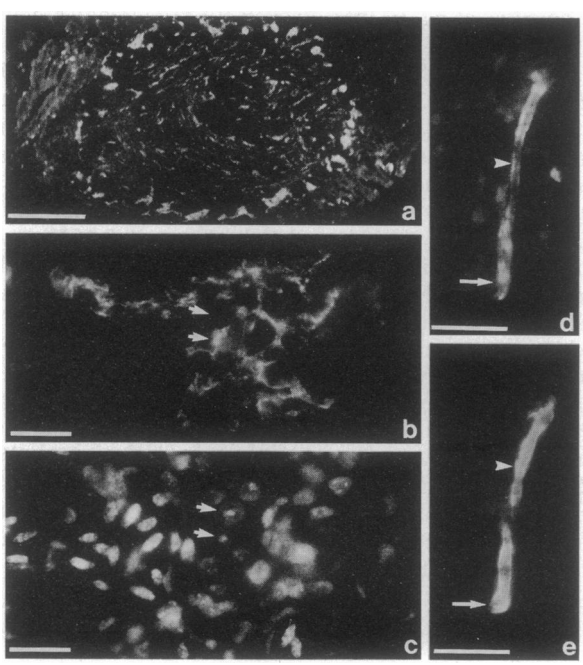

FIG. 2. Association of quail mesencephalic neural crest with gut wall from E7 chicken embryo. (*a*) After 12 days as CAM graft the mesenchyme of the gut wall developed villosity-like structures disposed here in an inside-outside configuration. HNK1 stained the neural-cell population. (Bar = 1 mm.) (*b*) HNK1 immunoreactivity of ganglion. All cells are SMP$^-$. (*c*) Mixed quail/chicken ganglionic structure in the gut wall detected by Hoechst nuclear dye. Arrows, quail nucleolus. (Bar = 50 μm.) Association of quail mesencephalic neural crest with skin from E7 chicken embryo, cultured 12 days as CAM graft. (*d*) SMP immunostaining. (*e*) HNKI immunostaining. Structure composed of a quail neuronal cell body (arrow) HNK1$^+$SMP$^-$ and a myelin-like structure HNK1$^+$SMP$^+$ (arrowhead). (Bar = 100 μm.)

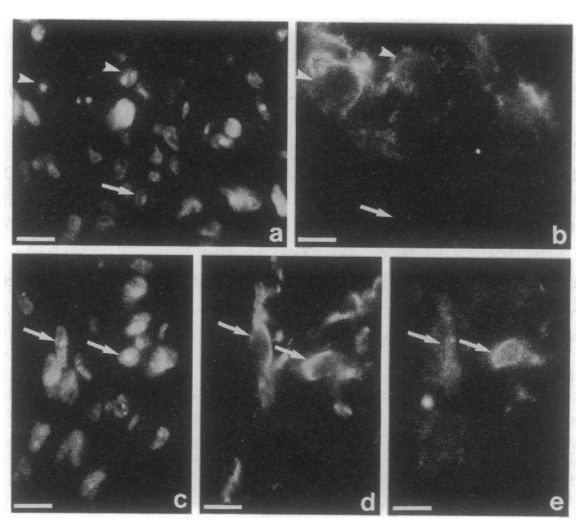

FIG. 3. (*a* and *b*) Association of peripheral nerves from E10 quail embryo with gut wall from E6 chicken embryo. The explant was maintained 7 days *in vitro*. (*a*) Hoechst nuclear dye. (*b*) HNK1 immunostaining. Two populations of quail cells can be recognized: fibroblasts (HNK1$^-$, arrow) and SC (HNK1$^+$, arrowheads). None of them are SMP$^+$. (*c–e*) Association of peripheral nerve from E10 quail embryo with skin from E6 chicken embryo. Several quail cells (arrows) visualized after nuclear staining (*c*) are double-labeled with HNK1 (*d*) and anti-SMP (*e*) mAbs. (Bar = 10 μm.)

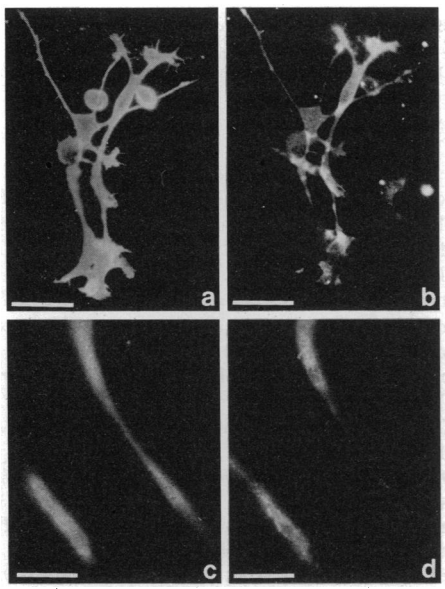

FIG. 4. *In vitro* culture of dissociated enteric cells from E15 quail embryo on a 3T3 cell feeder layer. After 5 days in culture, EGC are double-labeled with HNK1 (*a*) and anti-SMP mAbs (*b*). Some SMP^+ cells (*c*) are double-labeled with antilaminin antiserum in a spotty pattern (*d*). (Bar = 25 μm.)

exhibited conspicuous immunoreactivity, appearing in a spotty pattern on many SMP^+ cells (Fig. 4).

DISCUSSION

Our goal was to study the developmental relationships between the various neural-crest-derived cell types of the PNS. This communication concerns the SC lining the peripheral nerves and EGC. The work was expedited by the anti-SMP mAb, which identifies a glycoprotein specifically expressed at the SC surface, whether the cells are or are not myelinating in the PNS. Moreover, SMP can be detected *in vivo* from E5–6 onward in quail and chicken embryos; it is constitutively expressed by cultured SC even in the absence of neurons, and it appears in the differentiating neural crest in culture. In contrast, as demonstrated in our previous work (14), enteric plexuses are totally devoid of SMP^+ cells in embryos and adult birds. The quail/chicken chimera system showed that, despite the exclusive contribution of vagal and lumbosacral crest cells to formation of the enteric nervous system (2, 3, 22–24), EGC can arise from any neuraxis level, provided that their precursors find the appropriate environmental conditions to differentiate. Moreover, clonal cultures have shown the existence of a high percentage of SC progenitors in the cephalic neural crest, some fully committed and others still in a multipotential state (25).

We asked whether it was possible to determine, by SMP expression, if EGC and SC belong to two distinct lineages, segregating early from a putative common neural-crest ancestor. Alternatively, EGC precursors could be either partly or totally converted into SC when transferred in the appropriate microenvironment. In the latter case, one would assume that these cells arise from a common precursor and that the gut mesenchyme behaves as a nonpermissive (or an inhibitory) environment for SMP-encoding gene expression. In fact, SMP expression is not the only feature that distinguishes SC from EGC—e.g., the synthesis of a laminin-containing basal lamina is a specific trait of SC differentiation both *in vivo* and *in vitro* (26, 27), whereas enteric plexuses are devoid of basal lamina and also of fibrillar collagen (4, 5). Beside SMP we have also used laminin as a marker to distinguish SC from EGC here. In contrast some other markers often used for EGC (essentially in rat) such as glial fibrillary acidic protein, Ran 2, and S100 are also expressed by a subpopulation of SC and, therefore, cannot be used to distinguish the two glial-cell categories (28–30).

The quail/chicken chimera system enabled us to experimentally change the microenvironment of SC and EGC and to then recognize the cells from the tissue with which they had been associated.

As a first step, we carried out heterochronic associations of quail neural-crest cells with various chicken tissues—one of them was the gut, which was putatively inhibitory for the SC SMP phenotype, and other tissues included skin and muscle, in which SC differentiate when they become innervated. Both types of tissues were taken from the chicken at similar developmental stages E7 and E11. Various levels of the neuraxis, previously demonstrated to produce SC (11, 25), were involved in these experiments. The results agreed completely with the situation seen in normal development: EGC developing from the crest within gut explants did not express the SC SMP phenotype, from whichever neuraxis level the crest cells were derived. $HNK1^+$ quail cells, however, were abundant in these explants and contributed, together with chicken neural-crest cells, to enteric plexuses. In contrast, all skin and muscle explants in which neural-crest cells could be detected by their nucleolar marker and their HNK1 immunoreactivity contained a significant number of $HNK1^+$, SMP^+ cells.

Therefore, neural-crest cells experimentally associated with older embryonic tissues can invade them and participate in their innervation, although at a later stage than in normal development. As far as the SMP phenotype is concerned, its environmental association or exclusion is the same as seen in normal development. A similar inhibitory effect of the gut wall has been demonstrated on the differentiation of catecholamine (CA)-expressing cells in avian and mammals (11–13, 31, 32). The chicken hindgut taken at E5 and associated with either vagal or truncal neural-crest cells *in vitro* was found to inhibit CA synthesis in enteric neurons just as in normal development of the enteric digestive tract in birds (11, 31). CA-expressing cells exist transiently in the embryonic gut of the mouse; whether they die, remain quiescent, or are directed toward another transmitter synthesis, is not yet clearly established (32).

The obvious second step of this investigation was to see whether regulation of the SMP phenotype could be modified in SC and EGC subpopulations at a stage when the differentiation process is already well underway. For this purpose, cells dissociated from E10–E15 quail peripheral nerves, mainly composed of SC expressing both the HNK1 and SMP markers, were associated with similar substrates, as in the previous experiments (i.e., E6–E7–E10 chicken postumbilical gut, dorsal skin, and muscle). After 7 days of culture these cells, recognizable by the quail nuclear marker, still constitutively expressed the SMP antigen in skin and muscle, although they were not associated with neurons. In contrast, SMP expression was dramatically affected when SC were confronted with the enteric mesenchyme. In the gut environment quail cells either scattered in the gut wall, or, participating in the constitution of host ganglia, were $HNK1^+$, SMP^- like the chicken elements of the enteric nervous system. The radical changes seen in SMP expression can be interpreted as complete inhibition of SMP gene expression in SC exposed to the gut-wall microenvironment. This view was further confirmed by short-term *in vitro* cultures of EGC done in a variety of media, including the fully defined basic Brazeau medium (19) and on various substrates. Whatever the conditions, the SMP phenotype was found to appear in most EGC after only 96 hr in culture. It is striking that, particularly in basic Brazeau medium, which

does not favor cell proliferation, the number of $HNK1^+$ cells remained virtually stable while the cells acquired the SMP phenotype. This result strongly argues for a plasticity of the peripheral glial-cell phenotype, rather than of the emergence from enteric plexuses of another population of previously resting SC-committed precursors. The explanted EGC, even without any mesenchymal substrate, started also to produce laminin, an extracellular matrix component absent in the glial cells of the gut but characteristic of SC basal lamina, in addition to initiating SMP synthesis. In the lethal spotted mutant (*ls/ls*), intramural SC lining extrinsic nerves and still exhibiting some ultrastructural features of SC together with elevated glial fibrillary acidic protein content have been described (33). Interestingly, this mutation is characterized by abnormally high levels of extracellular matrix-associated proteins synthesized by the enteric mesenchyme, which might be crucial for SC differentiation (34, 35).

The switch in glial-cell phenotype, seen when enteric plexuses were cultured with a certain amount of mesenchyme from the gut wall, revealed that the tissue interactions occurring between mesodermal and neural-crest cells and responsible for the EGC differentiation can occur only when the three-dimensional arrangement of intestinal tissues is intact. Similar observations were made before, when neural-crest cells were cocultured with dissociated gut mesenchymal cells. In contrast to the strong inhibitory effect exerted by the gut wall on CA synthesis in enteric ganglia, the culture conditions permitted some level of CA differentiation in neural-crest-derived neurons (16).

In answer to the initial question, therefore, the two types of peripheral glial cells (SC and EGC) concerned in this investigation exhibit, at least during development, a large range of phenotypic plasticity manifested by their ability or inability to synthesize SMP and laminin. Expression of these molecules depends highly upon environmental influences. Glial cells differentiating from the neural crest seem able to constitutively express SMP, as they do in cultured neural-crest cells. The gut mesenchyme, in contrast, turns off SMP/laminin gene expression. This inhibition must constantly be exerted upon the EGC because, when withdrawn from gut environment, EGC, even at the end of incubation, can reacquire their capacity for SMP/laminin synthesis.

A similar phenotypic plasticity to environmental cues has been shown between myelinating and nonmyelinating SC (36, 37); this phenomenon led to the conclusion that both kinds of cells (SC and EGC) originate from the same precursor oriented or not oriented for myelin synthesis by a still undefined membrane signal related to the ensheathed-axon diameter. The SMP phenotype, which appears from 5 to 6 days before myelination onset, is therefore shared by both SC types and their common precursor. From our work, SC and also enteric glia can be assumed to belong to the same lineage; their segregation occurs in the gut under a strong inhibitory signal to SC differentiation from the gut wall. The commitment to both cell types seems reversible with respect to the studied phenotypes.

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