Proc. Nat. Acad. Sci. USA
Vol. 72, No. 2, pp. 728–732, February 1975

Cholinergic Differentiation of Presumptive Adrenergic Neuroblasts in Interspecific Chimeras after Heterotopic Transplantations

(neural crest/neurons/avian embryos)

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Communicated by François Jacob, November 6, 1974

ABSTRACT **The levels of the neural axis from which parasympathetic and orthosympathetic neurons and adrenomedullary cells are derived under normal developmental conditions were determined in avian embryos by a biological labeling technique. The technique is based on nuclear differences between two species of birds, the chick and the quail. In quail interphase nuclei a part of the chromatin is condensed in large heterochromatic masses associated with the nucleolus, while in the chick, DNA is evenly dispersed in the nucleoplasm. These characteristics provide a stable nuclear marker that can be used to study cell migrations and differentiation in chimeric embryos resulting from the association of quail and chick tissues. Isotopic and heterotopic transplantations of quail neural primordium into chick before the outset of neural crest cell migration show that the autonomic ortho- and parasympathetic neuroblasts are not determined to differentiate into cholinergic or adrenergic neurons when they begin to migrate. The neurotransmitter synthesized by crest autonomic neuroblasts depends on the microenvironment in which crest cells become localized at the term of their migration. The splanchnic mesoderm induces presumptive adrenergic cells to become fully differentiated cholinergic neurons.**

The neural crest, a transitory embryonic structure, gives rise to a variety of physiological cell types. It originates early in embryonic life after closure of the neural tube, and its cells, rapidly dispersed throughout the body, differentiate into various tissues. During their migration, neural crest cells show no specific characteristics that could distinguish them from the cells among which they move. These facts make it difficult to analyze the neural crest derivatives and explain why their evolution is always a controversial subject. There are questions especially about the mechanisms that control determination and differentiation of this apparently homogeneous structure into various cell types as well as the precise pattern of their migration throughout the developing body.

By deletion experiments of the neural crest before the outset of migration, it has been demonstrated that the neurons of the autonomous nervous system and the adrenomedullary paraganglia derive from this structure (see ref. 1 for references). However, the precise level of the neural axis from which they originate was controversial, especially concerning the enteric parasympathetic ganglia (see refs. 2 and 3 for references).

A natural cell-marking technique devised by one of us (4) has been applied to the problem of neural crest cell migration. This technique is based on differences in the structure of the interphase nucleus between two species of birds, the japanese quail (*Coturnix coturnix japonica* T. and S.) and the chick (*Gallus gallus* L.). In the quail, an important part of the chromatin is condensed during interphase, either in a single central mass or in several heterochromatic masses associated with the nucleolar RNA. In the chick the chromatin is dispersed in a fine network. When quail cells are associated with chick cells either in organotypic cultures *in vitro* or by graft *in ovo*, the cells from each species retain indefinitely their characteristics and can be identified in the chimera. In a first step, isochronic and isotopic grafts of fragments of the quail neural primordium into chick embryos have been done in order to study the normal fate of crest cells originating from the various levels of the neural axis. It was demonstrated that the enteric ganglia derive exclusively from two regions of the neural axis located at the *vagal* (from somite 1 to 7) and the *lombosacral* (behind the 28th somite) levels of neural tube. The *cervico-dorsal* crest, corresponding to somite 8 to 28, does not participate in the histogenesis of cholinergic enteric ganglia, but gives rise to orthosympathetic chains and plexuses (2). From the level of the neural axis located between the 18th and 24th somites originate the adrenomedullary paraganglia (*adrenomedullary* area of the neural crest) (5) (Fig. 1). This being established, it was interesting to investigate whether this regionalization of the neural crest corresponds to an early determination of the autonomic neuroblasts. To that end heterotopic transplantations of the *adrenomedullary* region of the quail neural crest have been made into the *vagal* area of a chick (6). In this case, presumptive adrenergic neuroblasts of the dorsal region migrate into the intestine and give rise to normally located and morphologically developed Auerbach's and Meissner's plexuses enteric ganglia. These cells that in normal development are not attracted by splanchnic structures are led by a preferential pathway to the developing gut when grafted at the hind brain level. Will the presumptive adrenergic neuroblasts, which thus become ectopically localized in the gut mesenchymal environment, differentiate according to their normal fate or will they give rise to functional cholinergic neurons, analogous to enteric neurons normally originating from the *vagal* and *lombosacral* neural crest? In the present work, a histochemical and physiological approach to this problem is reported. The presence of a cholinergic innervation in the digestive tract can be tested by physiological assays in chick embryo. The sensitivity to acetylcholine of the gut develops on the seventh day of incubation; the response is qualitatively the same as that of the adult, but increases throughout embryonic development (7–9).

METHODS AND RESULTS

Two experimental series were done: one is a control, consisting in the isotopic and isochronic graft of a quail neural primordium into the chick, in two different regions of the

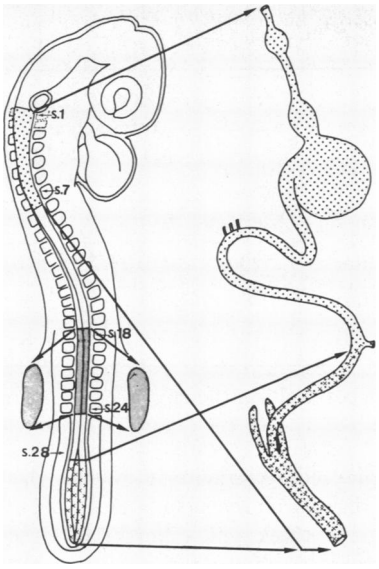


FIG. 1. Diagram showing the anterior and posterior levels of the embryonic neural axis from which the enteric ganglion cells originate, as demonstrated by isotopic and isochronic transplantations of quail neural tube into chick embryo (2). The neuroblasts arising from the anterior level (between 1 and 7 somites) colonize the whole gut. Those that come from the posterior level located behind the 28th somite contribute only to the formation of the ganglia of the post-umbilical gut. The neural crest of the cervical and dorsal region (8–28 somites) does not participate in the formation of enteric ganglia but gives rise to adrenergic orthosympathetic neurons and to adrenomedullary cells, the latter coming from the precise level of somite 18 to 24 (6).

neural axis: (*i*) the *adrenomedullary* level (from somites 18 to 24); embryos are operated at the 24- to 26-somite stage (experiment 1, Fig. 2). (*ii*) The *vagal* level (i.e., the posterior rhombencephalon) corresponding to somites 1 to 7 (experiment 2, Fig. 2); embryos in this case are at the 7- to 12-somite stage. In the second experimental series, heterotopic transplantations of fragments of quail neural primordia into the chick are performed. The *adrenomedullary* area (somites 18 to 24) of 24- to 26-somite quails is transplanted at the *vagal* (somites 1 to 7) level of 7- to 12-somite chicks (experiment 3, Fig. 2). Since the initiation of crest cell migration proceeds craniocaudally, following the closure of the neural tube, donor and host embryos must necessarily be at different developmental stages in order that crest cells have not begun migrating at the time of intervention in either the host or the graft at the selected levels of the neural axis. In a first step the neural tube and associated neural crest of the chick are removed by microsurgery in the egg. Endoderm, notochord, and somatic mesenchyme are left *in situ*. The selected portion of quail neural primordium is isolated from the various surrounding structures by incubation of the adequate transverse strip of the quail embryo in 0.1% trypsin in solution in Ca^{2+},-Mg^{2+}-free Tyrode solution. Contamination of the neural primordium by non-neural cells is obviated by this procedure. In a second step, the dissociated quail neural tube is grafted into the appropriate space in the host chick embryo and the incubation is continued up to 7–18 days.

Histology and Histochemistry. The digestive tract is dissected and cut into fragments (Table 1) which are fixed either

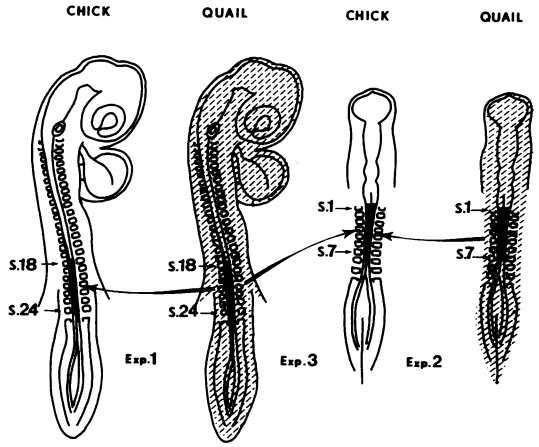


FIG. 2. Transplantations of quail neural tube and crest into chick embryos. A fragment of neural primordium is surgically removed at a precise level of the neural axis in a chick, and a quail neural tube and crest isolated by trypsinization is grafted into the chick in the groove resulting from the operation. *Exp. 1:* Isotopic and isochronic transplantation of the *adrenomedullary* region of the neural tube (somites 18–24) at 24- to 26-somite stages. *Exp. 2:* Isotopic and isochronic graft to the *vagal* neural primordium (somites 1–7) at 7- to 12-somite stage. *Exp. 3:* Heterotopic and heterochronic transplantation of the *adrenomedullary* neural tube and crest of a 24- to 26-somite quail into the *vagal* area of a 7- to 12-somite chick.

in Zenker's or in Bouin's fluid, and embedded in wax. Since the distinction of quail and chick cells is based on the chromatin pattern of the interphase nucleus, the Feulgen–Rossenbeck technique for DNA is used as routine, after Zenker's fixation (Fig. 3). The neural differentiation of quail cells aggregates present in the host gut is controlled by silver impregnation according to Tinel (10) after fixation in Bouin's fluid. In this case, serial sections of tissue are alternately placed on two different slides. One is stained by Tinel's method, the other is post-fixed in Zenker's fluid, and stained by Feulgen-Rossenbeck's technique. By this procedure it is

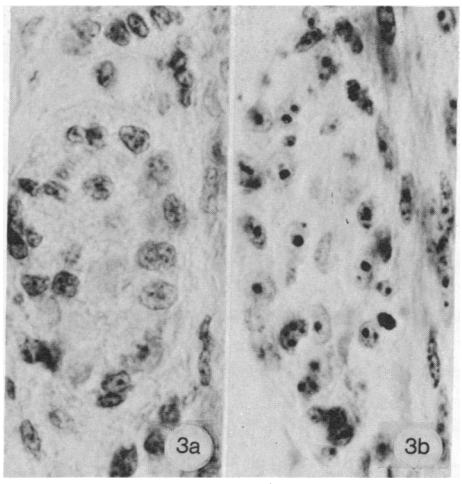


FIG. 3. Enteric ganglia of the Auerbach's plexus in the duodenum of 17-day chick (a) and 16-day quail (b) embryos. Feulgen–Rossenbeck's staining shows one or two large clumps of nucleolar heterochromatin in quail nuclei, while in the chick the chromatin is evenly distributed in the nucleoplasm with small dispersed chromocenters. ×900.

TABLE 1. *Presence of quail ganglion cells in the host gut after orthotopic and heterotopic transplantations of quail neural tube and crest into chick embryos at the vagal and the adrenomedullary levels*

Experimental series				No. of embryos observed	Presence of quail ganglion cells in Auerbach's and Meissner's plexuses in different parts of digestive tract of host				
Level of graft in chick	Level of quail neural primordium	Stage of host	Stage of donor		A*	B*	C*	D*	E*
From 18–24 somites	From 18–24 somites	24–26 Somites	24–26 Somites	20	0	0	0	0	0
From 1–7 somites	From 1–7 somites	7–12 Somites	7–12 Somites	8	8	8	8	8	8
From 1–7 somites	From 18–24 somites	7–12 Somites	24–26 Somites	16	13	13	8	0	0

* A, esophagus, proventriculus, and gizzard; B, duodenum; C, preumbilical ileum; D, postumbilical ileum; E, ceca and large intestine.

possible to see whether ganglionic cells belong to the quail or chick species.

Biogenic amine content of the neurons is evidenced by the formol vapor-induced fluorescence procedure according to Falck (11). This technique is applied to fragments of digestive tract of 15- to 18-day-old chick embryo hosts and also to trunk regions of 9-day-old chicks that have received an isotopic graft of the *adrenomedullary* part of quail neural tube. In some cases, the procedure is followed by the Feulgen–Rossenbeck's reaction in order to identify the chick or quail species of fluorescent cells.

Physiological Assays. The gizzard and duodenal loop associated with the right branch of the vagus nerve are dissected from 15- to 17-day-old embryos in Tyrode solution at 37°. The preparation is transferred into a perspex chamber with a device that makes it possible to stimulate the nerve while the gizzard and duodenal loop are bathed in oxygenated Tyrode solution. The gizzard and the distal end of the duodenal loop are pinned on the bottom of the bathing chamber and the contractions of the ascending limb of the loop are recorded by an R.C.A. 5734 electromechanical transducer valve connected to a Tektronix 502 A oscilloscope. The nerves are stimulated at 50 min^{-1} by application of rectangular current pulses (15 V, 20 msec) between two platinum electrodes fixed in the lateral frame of the chamber. Acetylcholine is added to the medium at 10^{-8}–10^{-4} M final concentrations. Pretreatment by atropine (10^{-4} M) lasts 45 min. Between the assays the preparation is perfused at a flow rate of 2.5 ml/min.

The results obtained after isotopic and heterotopic transplantations of quail neural primordia into chicks in the different experimental series are summarized in Table 1. *In isotopic and isochronic grafts of the adrenomedullary area of the neural primordium* (experiment 1, Fig. 2), the autonomic neuroblasts differentiate into adrenomedullary cells and adrenergic neurons of the orthosympathetic ganglion chains and of the aortic plexus, as shown by the application of formol vapor-induced fluorescence and Feulgen–Rossenbeck associated techniques (Fig. 4). The crest cells migrate very near the dorsal mesentery but do not colonize the wall gut where the ganglia are entirely made up of chick cells (Fig. 5).

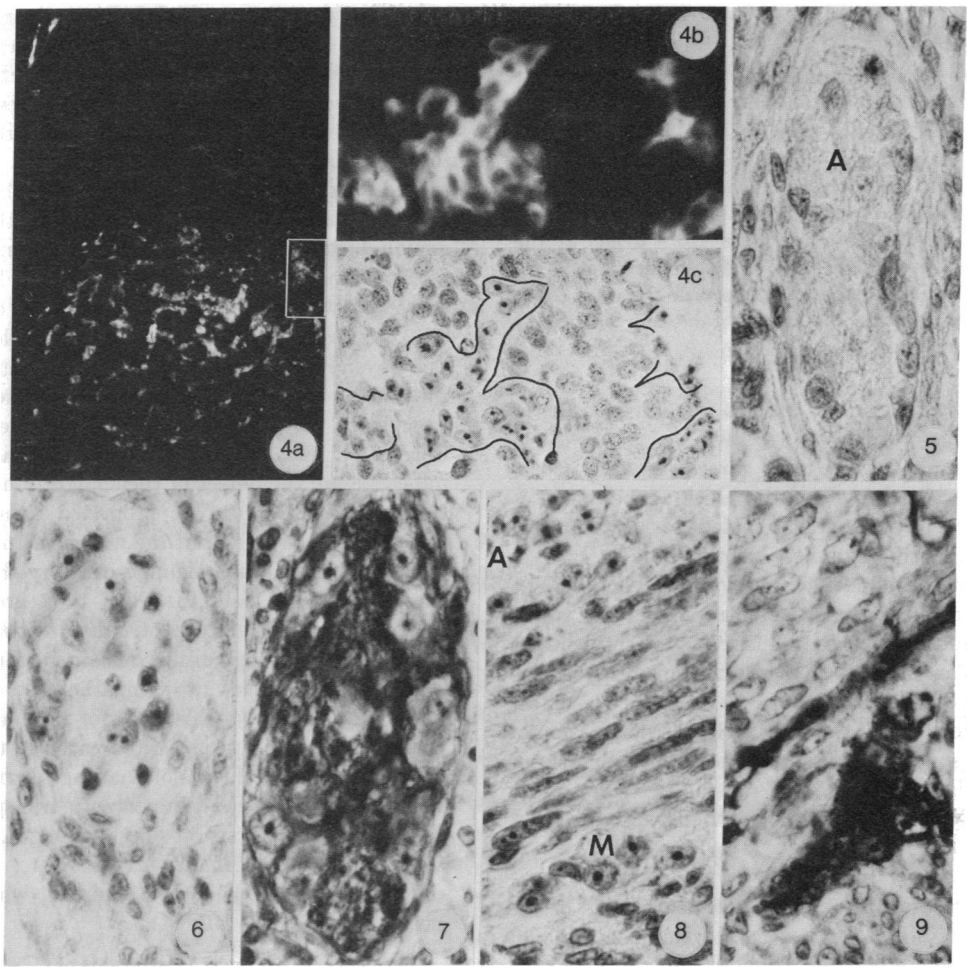
In isotopic and isochronic grafts of the vagal region of the neural crest (experiment 2, Fig. 2), quail cells are found in the enteric ganglia of the host in the whole gut. In the best cases, the ganglia are entirely made up of quail cells in the preumbilical gut (Fig. 6) and formed by a mixture of quail and chick cells in the postumbilical gut. In the latter region, the enteric ganglion cells originate from both the *vagal* and the *lumbosacral* regions of the neural crest (2). The quail cells that invade the chick gut differentiate into normal ganglionic neurons, i.e., silver-positive (Fig. 7) and negative as to content in fluorogenic amine.

Heterotopic transplantation of quail adrenomedullary neural primordium at the vagal level of a chick neural axis (experiment 3, Fig. 2). In 13 out of 16 cases observed, the enteric ganglia of the host preumbilical gut are made up of quail neurons (Figs. 8 and 9). However, quail ganglionic cells do not migrate into the postumbilical region of the digestive tract, as observed after isotopic transplantations at the *vagal* level (experiment 2, Fig. 2). The preumbilical gut is innervated by branches of the pneumogastric nerve which is, however, thinner than in normal embryos of the same stage or than in chick embryos receiving an isotopic graft of the *vagal* neural tube.

Fragment B (Table 1) of the gut in four cases has been submitted to the formol vapor-induced fluorescence Feulgen–Rossenbeck associated technique. No fluorogenic amine has been found in the enteric ganglia that were made up of quail cells. This indicates that quail neuroblasts that colonize the gut under these experimental conditions do not differentiate into catecholaminergic neurons, according to their presumptive fate.

The physiological properties of duodenal innervation were tested first in control embryos. The experimental conditions make it possible to record the mechanical activity of duodenal loop for several hours. For the nerve stimulation to be effective, it must last at least 1 min. Fig. 10a shows a typical response, which consists of an increase of the tension. A positive inotropic effect is also frequently observed. The response is qualitatively the same whatever the stage of development of the embryos (15–18 days of incubation) but its amplitude increases with age. In most cases the positive tonotropic effect slows down several minutes after the stimulation stops. When several stimulation series separated by 15-min resting periods are applied, the inotropic effect decreases but the positive tonotropic effect persists. Sensitivity to acetylcholine is seen from 10^{-6} M final concentration (Fig. 10b). The time required to return to normal mechanical activity can vary from one embryo to another between 30 and 90 min. Pretreatment with atropine inhibits the effects of both nerve stimulation (Fig. 10c) and acetylcholine.

These results lead us to investigate the effect of nerve stimulation on mechanical activity of the duodenum in 17-



FIGS. 4–9. *Fig. 4.* Transverse section of a 9-day chick host after isotopic and isochronic graft of *adrenomedullary* neural primordium at 24-somite stage (Exp. 1). The formol vapor-induced fluorescence technique shows that the adrenomedullary cells and orthosympathetic fibers contain fluorogenic amines (a–b). Post-staining of the same slide by Feulgen–Rossenbeck's method reveals that fluorescent cells belong to quail species (c). a, ×90; b and c, ×470. *Fig. 5.* Transverse section in the duodenum of a 13-day chick embryo into which the *adrenomedullary* neural primordium of a quail has been grafted isotopically (Exp. 1) at 24-somite stage. The enteric ganglia are of host origin. A, ganglion of Auerbach's plexus. Feulgen–Rossenbeck's staining. ×900. *Fig. 6.* Transverse section in the duodenum of a 17-day chick embryo into which the vagal neural primordium of a quail has been grafted at 9-somite stage (Exp. 2). The enteric ganglia of the preumbilical gut are of quail origin. Feulgen–Rossenbeck's staining. ×900. *Fig. 7.* Silver impregnation according to Tinel applied on the duodenum of the same embryo. Quail ganglion cells in Auerbach's plexus. ×900. *Fig. 8.* Heterotopic transplantation of a quail adrenomedullary neural primordium at the vagal level of a chick (Exp. 3). Auerbach's (A) and Meissner's (M) plexuses ganglia made up of quail cells in the duodenum of the chick host. Feulgen–Rossenbeck's staining. ×900. *Fig. 9.* Same experiment as in Fig. 8. Tinel's procedure showing that quail cells differentiate into neurons. ×900.

day-old grafted embryos. Younger embryos were not used for the physiological assays because of the retardation in embryonic development usually observed in operated embryos.

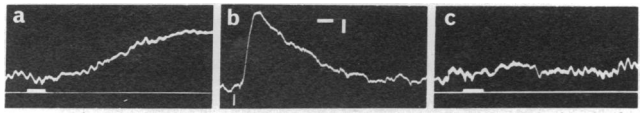


FIG. 10. Mechanical activity of the ascending limb of the duodenal loop in normal 17-day-old chick embryo. Calibration (indicated in b): vertical scale, 5 mg; horizontal scale, 1 min. (a) Vagus stimulation (large part of the baseline); a progressive positive tonotropic effect develops. (b) Acetylcholine administration (10^{-5} g/ml) at the time marked by a vertical line; note the rapid appearance of the tonotropic effect. (c) Lack of effect of nerve stimulation (large part of the baseline) in atropinized preparation.

After isotopic and isochronic graft of the *vagal* region of the neural crest (experiment 2, Fig. 2), the results of either nerve stimulation (Fig. 11a) or acetylcholine administration (Fig. 11b) are in good agreement with those obtained in controls,

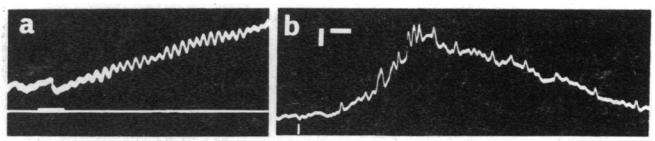


FIG. 11. Mechanical activity of the ascending limb of the duodenal loop in 17-day-old grafted embryo (isotopic and isochronic graft: Exp. 2, Fig. 2). Calibration (indicated in b): vertical scale, 5 mg; horizontal scale: 1 min. (a) Nerve stimulation (large part of the baseline); positive inotropic and tonotropic effects are observed. (b) Acetylcholine administration (10^{-5} g/ml) at the time marked by a vertical line.

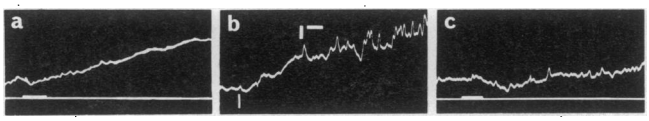


FIG. 12. Mechanical activity of the ascending limb of the duodenal loop in 17-day-old grafted embryo (heterotropic graft: Exp. 3, Fig. 2). Calibration (indicated in b): vertical scale, 5 mg; horizontal scale, 1 min. (a) Nerve stimulation (large part of the baseline). (b) Acetylcholine administration (10^{-5} g/ml) at the time marked by a vertical line. (c) Lack of effect of nerve stimulation (large part of the baseline) in atropinized preparation. Note that these results are similar to those obtained in normal or isotopically grafted embryos.

and show that the graft does not perturb the development of the duodenal loop innervation.

After the heterotopic graft of quail *adrenomedullary* neural primordium at the vagal level of the chick (experiment 3, Fig. 2), two cases have been submitted to physiological assays and subsequently examined histologically to control the quail origin of the duodenal innervation (Fig. 8). The nerve stimulation results in positive inotropic and tonotropic effects (Fig. 12a) and in both cases sensitivity to acetylcholine is found to be similar to controls (Fig. 12b). This response has been observed several times, and the decrease of the effect after the stimulations develops very slowly. Atropinization of the preparations suppressed the responses to both acetylcholine and stimulation (Fig. 12c), as in the two series (control and isotopically grafted embryos) previously studied.

DISCUSSION

The present investigation shows that the neuroblasts of the dorsal neural crest, which in normal development do not participate in the wall gut innervation, can migrate into the splanchnic mesoderm and differentiate into normally organized Auerbach's and Meissner's plexuses ganglia, when transplanted at the *vagal* level at an early stage.

It is established that the expression of sympathetic nerve traits in cells of neural crest origin requires the presence of somitic mesoderm (12, 13). Thus, the question arose whether the presumptive adrenergic neurons that colonize the gut under experimental conditions are able to give rise to normally differentiated cholinergic enteric neurons. Indeed, during their dorsoventral migration, the ganglioblasts from the dorsal crest transplanted at the *vagal* level are in contact for a while with the anterior somites. But contrary to the orthosympathetic neuroblasts of the trunk, which remain in the dorsolateral mesoderm to constitute the sympathetic chains and plexuses, they proceed on their migration and become ventrally localized when the dorsal neural primordium is transplanted at the *vagal* level. Both silver impregnation and the Falck technique for detection of catecholamines have been applied to the intestines of chick embryos in which the quail *adrenomedullary* neural crest has been transplanted at the *vagal* level. They have shown, first, that the quail cells that colonize the gut have differentiated into neurons and, second, that they do not contain detectable catecholamines. This indicates that their contact with the somitic mesenchyme during the migration process was not efficient in promoting their adrenergic differentiation. Either it was too short or it was exerted too early in the cell differentiating process. On the contrary, the neurons that colonize the gut show the physiological characteristics of normal cholinergic neurons of the parasympathetic intestinal innervation. It can be concluded that the presumptive adrenergic ganglioblasts originating from the dorsal neural crest are not fully determined before the outset of their migration. Their differentiation into neurons can be promoted either in dorsal or in splanchnic mesoderm, but the nature of the neurotransmitter they elaborate (catecholamine or acetylcholine) depends on the microenvironment in which they are finally localized. When they migrate into the trunk *adrenomedullary* region, all the autonomic ganglioblasts remain in the dorsal mesoderm and then differentiate into sympathoblasts for the more dorsal ones and adrenomedullary cells for the more ventral ones. If the dorsal crest is transplanted early at the *vagal* level, the developmental conditions of pharyngeal and cervical morphogenesis (6) result in an important "transport" of crest cells towards the lateroventral sides of the gut. Thereafter, in the splanchnic mesodermal environment, the ganglioblasts differentiate into cholinergic neurons, which are able to innervate the wall gut muscle.

This work was supported by grants of C.N.R.S. and D.G.R.S.T.

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