FORMATION AND MALFORMATION OF THE ENTERIC NERVOUS SYSTEM

Cover: Chick chorioallantoic membrane showing extensive vascularization. Illustration from Malpighi, M. (1686): "*De Formatione Pulli in ovo*".

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FORMATION AND MALFORMATION OF THE ENTERIC NERVOUS SYSTEM

VORMING EN MISVORMING VAN DE DARMINNERVATIE

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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OP GEZAG VAN DE RECTOR MAGNIFICUS
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"He who sees things grow from the beginning will have the finest view of them."

Aristotle, Greek philosopher, 384-322 B.C.

K.V. 622, W.A. Mozart

Voor Hanne,
Aan mijn ouders.

CONTENTS

CHAPTER 4

THE FORMATION OF THE ENTERIC NERVOUS SYSTEM

CHAPTER 5

INTRODUCTION TO THE EXPERIMENTAL WORK

CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

CHAPTER 7

APPENDIX PAPERS

LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
AxCAM	Axon-associated Cell Adhesion Molecule
CAM	Cell Adhesion Molecule
E ..	Embryonic Day ..
ECM	Extracellular Matrix
ENS	Enteric Nervous System
GFAP	Glial Fibrillar Acidic Protein
HNK-1	Human Natural Killer cell (monoclonal antibody)
ls/ls	lethal spotted
L-CAM	Liver Cell Adhesion Molecule
MAG	Myelin-Associated Glycoprotein
NILE	Nerve growth factor Inducible Large External Glycoprotein
N-CAM	Neural Cell Adhesion Molecule
REDV	Arginine-Glutamine-Asparagine-Valine
RGD	Arginine-Glycine-Asparagine
SAM	Substrate Adhesion Molecule

CHAPTER 1

CONGENITAL MALFORMATIONS AND NEURAL CREST SYNDROMES

> *"We ought not to set them aside with idle thoughts or idle words about "curiosities" or "chances". Not one of them is without meaning; not one that might not become the beginning of excellent knowledge, if only we could answer the question--why is it rare or being rare, why did it in this instance happen?"*
>
> (James Paget, Lancet ii:1017,1882).

1.1 INTRODUCTION

The interest in birth defects has a long tradition in art and literature but is relatively new in science. From ancient times there has been continuous fascination with man's efforts to understand the form and meaning of abnormality. Primitive people had little regard for the wonders of the precise and exquisite processes of embryogenesis that result in the formation of a healthy child. But when prenatal development was disturbed and a monstrosity was born, their emotions were aroused and they reacted to such misfortune with admiration, awe or terror. These emotions often led to extreme measures: they either exterminated or adored the deformed and sometimes they did both. After killing a monster, they often made an image and set it up as an idol--god or demigod. Sculptures, carvings and drawings of malformed individuals by ancient peoples reflect early teratologic knowledge and interest in rare and unusual human beings.

Philosophy and religion were traditionally the sources for explanations, or rather speculations, how organisms develop. Plato assumed that the essence of all matter lies in the supernatural ideal forms and that living organisms are only pale shadows and imperfect specimens of these ideal forms. Aristotle rejected the concept of ideal forms but spoke of a final cause or a purpose for everything in nature: the living organism is its own final cause, determining its generation and development. Indeed, in Aristotle's view organisms have souls to manage the developmental process. The idea of final cause or purpose is reflected again and again in religion and myth.

The human need to understand origins and destinies, to see a purposeful design in nature, and ultimately, to grasp the principles behind life itself, as well as the growth and differentiation of individual organisms, has been a powerful force throughout history. Not until the advent of modern science, however, has there been a reasoned basis for speculation.

1.2 NATURAL SELECTION AND CONGENITAL MALFORMATIONS

The genotypes of organisms are adapted to their environment by the process of natural selection. This is illustrated by the fact that 50 percent of conceptuses contain abnormal chromosomes and about 90 percent of these are aborted spontaneously before pregnancy is diagnosed (Ohama, 1982). In general the process of natural selection results in normal human phenotypes. In four to seven percent of live births the natural selection process leads to the birth of a child with a genetic disorder. There are four groups of genetic disorders. First, there are single gene defects, conditions which can be traced through families and clearly defined as following dominant, recessive or sex-linked pattern of inheritance. Second, there are chromosome abnormalities, some of which can be related to specific clinical syndromes. The third group is comprised of congenital malformations, at least some of which have a strong genetic component; it is equally clear however, that environmental factors play a major role in the pathogenesis of many disorders which come under this heading. Finally, there is a group of common diseases in which genetic factors are thought to play a role, although for which the relative contribution of genetic and environment factors is still not clear.

Congenital malformations are structural defects present at birth. Some of them, such as cardiac defects and renal anomalies, may not become clinically apparent until years later. To a certain extent, congenital malformations comprise the less serious disorders of embryogenesis, those compatible with live birth. Holmes (1976) estimated that about two percent of newborns have a major malformation, which he defined as a malformation having either cosmetic or functional significance. A relatively comprehensive catalogue of recognizable patterns of congenital malformations has been presented by Jones (1988). The three types of genetic disorders, chromosomal, single gene (Mendelian), and multifactorial, can all produce multiple congenital anomalies. Chromosomal abnormalities commonly lead to multiple anomalies, single gene defects may lead to either single or multiple anomalies, and multifactorial traits usually involve only single anomalies but the ones they produce are frequent and important.

Congenital malformations have became more important in pediatric practice, probably because better postnatal care allows for survival of many malformed infants who would have died in years past. Congenital malformations constitute an extremely important cause of perinatal mortality, and remain a significant cause of illness, disability, and death throughout the early years of life (Hall et al., 1978). A compilation of the approximate incidence of congenital disorders is presented in table 1.

The origin of congenital malformations is a subject of intense interest, particularly for parents who have had a malformed child. They often want to know whether the developmental disorder represents a hereditary trait likely to affect subsequent offspring. It is frequently impossible to answer this question, but a few facts are known. Congenital anomalies arise either from genetic defects or from environmental factors in utero, such as maternal infections, drugs and ionizing radiation. Childs (1978) estimated that about 25 percent of congenital anomalies have a genetic basis, a few percent have an environmental cause, and the remaining from a

combination of both.

TABLE 1

INCIDENCE OF CONGENITAL DISORDERS IN MAN[a]

TYPE OF INHERITANCE	FREQUENCY/ 1000 POPULATION
Single gene disorders	
- Autosomal dominant	0.6-7.5
- Autosomal recessive	0.9-2.5
- X-linked recessive	0.9-7.0
Chromosome anomalies	5.6-6.8
Congenital malformations	19-22
- Anencephaly ± spina bifida	2-30
- Cardiac defects	6-8
- Cleft lip & palate	0.8
- Cleft palate	0.5
- Club foot	1-6
- Pyloric stenosis	2-14
- Hirschsprung's disease	0.2
- Miscellaneous	6.3
Total:	

[a] *Data are compilated from several studies (see Galjaard, 1980; Holmes, 1976; Weatherall, 1985).*

1.3 CONGENITAL MALFORMATIONS RELATED TO THE NEURAL CREST

Studying the development of chicken embryos, Wilhelm His (1831-1904) discovered a group of cells between the neural tube and the epidermal ectoderm, which he called the "Zwischenstrang" (1868). He presumed that at the end of neurulation these cells abandon their surface location, extend into the body, and eventually localize to form the sensory ganglia (see figure 1). These cells are now called neural crest cells because they form a transient crest on the dorsal surface of the neural tube. Analogous cell populations are present in all vertebrates, even when they do not form such a crest-like structure. The final localizations of neural crest cells in the embryo have been mapped (for review see Le Douarin, 1982; and see figure 2).

The neural crest is one of the most vulnerable structures during embryonic development and disturbed neural crest development has been related to several congenital malformations in man. Congenital malformations related to the neural crest, or neurocristopathies, primarily involve skeletal and mesenchymal tissues in the head,

the cardiac septum, the thymus, the enteric nervous system, the adrenal medulla, the neurons or Schwann cells of the autonomic nervous system, and pigment cells. Table 2 presents a number of neural crest syndromes: malformations, tumors, and a miscellaneous group. The neural crest syndromes are grouped according to the axial level of the neural crest. For detailed studies on neurocristopathies see Bolande (1974; 1981, 1984), Kissel et al. (1982), and Couly (1981), the latter especially for craniofacial neurocristopathies.

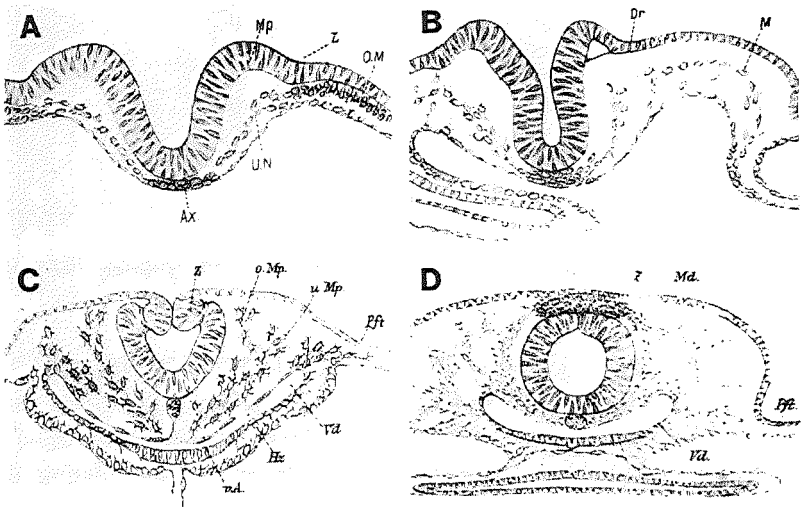


Figure 1: Illustrations from His (1868) depicting the formation and initial migration of neural crest cells (Z, Dr.) in relation to the neural plate (A: Mp.), neural tube (D: Md.), and epidermal ectoderm (C,D: Pft.) at four successive stages in the head of the 1 to 2-day chicken embryo. His regarded the neural crest as an individual primordium rather than a part of the neural plate or epidermal ectoderm. At early stages (A,B), however, His situated the neural crest too far laterally. (Ax. = Notochord; Vd. = foregut; Hz. and v.A. = heart and vascular elements; other abbreviations entail cranial endomesenchyme).

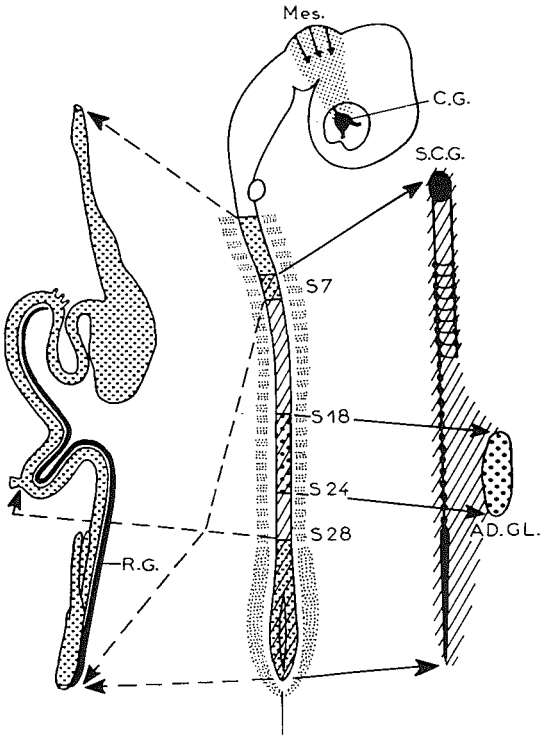


Figure 2: Final localizations of neural crest cells after their migration through the embryo. By exchanging the neural primordium of chicken embryos with that of quail embryos, Le Douarin mapped the destinies of neural crest cells from different axial levels. The vagal neural crest (opposite somites (S) 1 through 7) provides all the enteric ganglia. The lumbosacral neural crest gives rise to the ganglion of Remak (R.G.) and to some cells in enteric ganglia in the postumbilical bowel. The mesencephalic (Mes.) neural crest gives rise to the ciliary ganglion (C.G.). The sympathetic chain and plexuses derive from the neural crest caudal to S5, and the adrenomedullary cells (Ad. Gl.) originate from the level of S18-S24 (S.C.G. = superior cervical ganglion). (From Le Douarin, 1982).

TABLE 2

NEURAL CREST SYNDROMES

A. Cranial neural crest

I First and second branchial arch syndromes
- a. Waardenburg syndrome
- b. Deafmutism
- c. Cleft lip and cleft palate
- d. Treacher-Collins syndrome
- e. Pierre Robin sequence

II Third and Fourth arch syndromes
- a. Cardiac defects:
  - ventricular septal defect
  - common out-flow tract
- b. Thymic anomalies (DiGeorge syndrome):
- c. Congenital ENS malformations
- d. Goldenhar syndrome
- e. Sphrintzen syndrome
- f. CHARGE association

B. Truncal neural crest
- a. Neuroblastoma
- b. Pheochromocytoma
- c. Limb anomalies?

1.4 CONGENITAL MALFORMATIONS OF THE ENTERIC NERVOUS SYSTEM

Congenital malformations of the enteric nervous system (ENS) constitute a subgroup of congenital malformations of the neural crest. Bayliss and Starling (1899) recognized that: "The peristaltic contractions are true coordinated reflexes, started by mechanical stimulation of the intestine, and carried out by the local nervous mechanism (Auerbach's plexus). They are independent of the connection of the gut with the central nervous system. They travel only in one direction, from above downward, and are abolished on paralysing the local nervous apparatus...." Langley (1921) considered the "local nervous apparatus" or the enteric nervous system (ENS) as the third division of the nervous system. The ENS is thus a self-contained nervous system, the only system of that kind in the periphery (see figure 3).

Apart from the control of intestinal motility, the ENS serves important roles in gastrointestinal immunity, resorption and secretion (Gershon and Erde, 1981; Cooke, 1986; Felten et al., 1988; for a review of enteric neurophysiology see Wingate, 1986).

Disturbances in these functions are encountered in congenital malformations in which the ENS is totally or partially absent. The absence of the ENS is most clearly shown by disturbances in colonic motility that result in megacolon (Hirschsprung's disease).

1.5 AIM OF THE THESIS

The subject of this thesis is one group of congenital malformations related to the vagal neural crest: congenital ENS malformations. The aim of this thesis is to clarify pathogenetic mechanisms of congenital ENS malformations. To this end, ENS formation in chicken and murine embryos was studied experimentally, and the results were related to the pathology of human ENS malformations and the associated malformations.

The experimental data enabled a comparison between formation and malformation of the murine ENS. The combination of the experimental data and the literature enabled a classification of ENS malformations and associated anomalies based on developmental biological criteria.

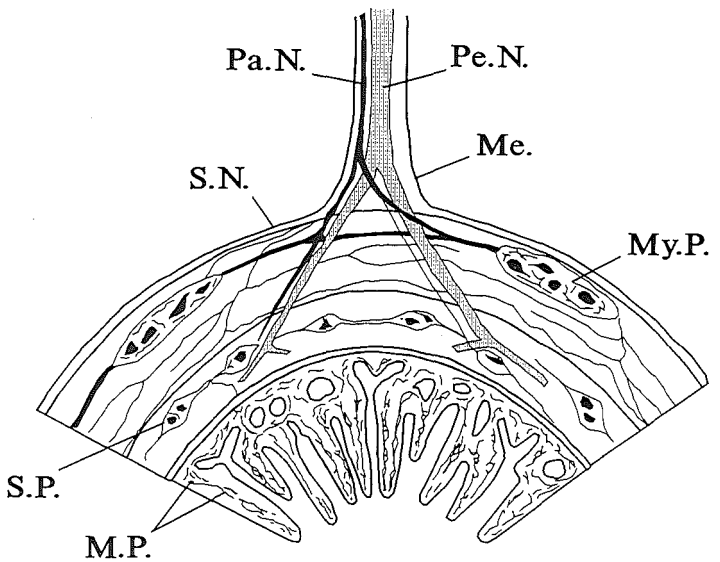


Figure 3: Diagram of the arrangement of the enteric nervous system in a transverse section of the bowel. Me. = mesentery; M.P. = mucosal plexus; My. P. = myenteric plexus; Pa. N. = paravascular nerve; Pe.N. = perivascular nerve; S.N. subserous nerve; S.P. = submucous plexus. (From Furness and Costa, 1980).

CHAPTER 2

CONGENITAL MALFORMATION OF THE ENTERIC NERVOUS SYSTEM

2.1 HISTORICAL SURVEY

When diseases are first described they are usually defined by their clinical and pathological manifestations. Occasionally, increased knowledge of a particular disease reveals, however, that the original description did not refer to a disease, but to a complex of symptoms resulting from several pathogenetic mechanisms. Congenital megacolon (Hirschsprung's disease) is a typical example of such a disease.

Harald Hirschsprung (1830-1916) was the senior physician at Denmark's oldest children's hospital, Queen Louise Children's Hospital, Copenhagen, from 1870 to 1904. In a lecture to "der Geselschaft für Kinderheilkunde" in Berlin (1886), he described two boys, who both presented with a characteristic clinical picture: severe defecation difficulty from birth onwards, increasing abdominal distension, a gradually declining general condition eventually leading to death at the age of 11 and 7 months respectively. The autopsies revealed pronounced dilatation and hypertrophy of the colon in both. The lecture was published in the "Jahrbuch für Kinderheilkunde" under the title "Stuhlträgheit Neugeborener in folge von Dilatation und Hypertrophie des Colons" (Hirschsprung, 1887).

At the time of Hirschsprung's publication, the clinical and pathological picture of congenital megacolon appeared to be unknown to the medical world. Later it was realized that there were earlier reports on similar cases. There is no doubt, however, that Hirschsprung was the first to give an accurate description of both the clinical picture and the pathology. Hirschsprung himself pointed out that is was remarkable that a disease with such pronounced symptoms and such obvious pathological manifestations had not been the object of discussion much earlier, "*but*", he wrote, "*possibly it will be the case here as often before that attention only needs to be drawn to this in order that cases will be found with greater frequency*" (Hirschsprung, 1890). Between 1886 and 1904 Hirschsprung reported ten other cases (Hirschsprung, 1904).

Jayle (1909) called attention to a description dating back to 1691. Frederik Ruysch (1638-1731), a professor of anatomy, surgery and botany at Amsterdam, reported a case with "Enormis Intestini Colo Dilatatio" in his book "Observationum Anatomico-Chirurgicarum Centuria" (Observation XCII). He described the clinical history of a five-year-old girl that showed an enormous dilatation of the colon at post mortem examination.

The literature on Hirschsprung's disease was dominated by case reports until the late 1940s. The discussion focused on two main themes: pathogenesis and treatment.

Several different pathogenetic mechanisms were suggested, but they all lacked systematic pathological studies. Several methods of treatment were proposed as well, but they were unsuccessful due to the same reason: fallacious pathological data. For a discussion of pathogenetic mechanisms see Madsen (1964). It was Ehrenpreis (1947) who proved that all existing pathogenetic theories were wrong, and who suggested that the inability of the colon to empty itself was a primary factor in Hirschsprung's disease.

A new era in the history of Hirschsprung's disease started when Whitehouse and Kernohan (1948) and Zuelzer and Wilson (1948) discovered that the wall of the distal colon lacked ganglion cells. They also found hypertrophic nerve trunks in the same bowel segment. Proximal to the aganglionic bowel segment, there was a transition zone with variable length containing sporadic ganglion cells and fewer nerve trunks.

Swenson and Bill (1948) were the first to report appropriate surgical treatment for Hirschsprung's disease. Using radiographic and manometric methods, they reported that the distal colon was continuously contracted and lacked normal peristalsis. They logically deduced that the obstruction problem should be resolved when normal bowel was brought as low in the rectum as possible, e.g. by resecting or bypassing the contracted bowel. They developed a surgical procedure in dogs involving anastomosis of normal bowel to the anus with subsequent retention of continence. The essential features of the operation are eversion of the closed rectal stump through the anus, and a precise anastomosis of the pulled-trough normal colon to the everted rectum, performed perineally. At the conclusion of the procedure, the levators pull the anastomosis up above the anus. This anal pull-through technique was then applied successfully in a human patient. The therapeutical success obtained in this way indicated that the underlying pathologic defect was indeed the cause of the dilated colon.

After the demonstration that the level of aganglionosis of the submucous plexus corresponds to that of the myenteric plexus, the safer suction mucosal biopsies supplanted full-thickness biopsies (Gherardi, 1960; Aldridge and Campbell, 1968). Rectal suction biopsies can be performed at the bedside without anesthesia or sedation, even in the neonatal patient (Dobbins and Bill, 1965). But the immature ganglion cells of the submucous plexus in the infant are less distinctive than the ganglion cells in older patients or those of the myenteric plexus, and can be mimicked by macrophages, fibroblasts, smooth muscle cells, Schwann cells, and even lymphocytes (Ariel et al., 1983; Campbell and Noblett, 1969; Yunis et al., 1976). Thus it was often necessary to examine many sections before arriving at a conclusion.

Using the acetylcholinesterase (AChE) staining method of Karnovsky and Roots (1964), Meier-Ruge and co-workers (1972) demonstrated that an increase in AChE activity in the lamina propria and muscularis mucosae in suction biopsies of the rectal mucosa is pathognomonic for Hirschsprung's disease. However, the demonstration of AChE activity in cryostat sections may be difficult to interpret, for there may be excessive mucosal hemorrhage with excessive red blood cell AChE activity (Lake et al., 1978; Meier-Ruge, 1982). Thus, the identification of enteric neurons remains important

in the diagnostic process.

The visualization of extrinsic nerve fibers and enteric neurons and glial cells was facilitated by the advent of immunocytochemical techniques. Jessen and Mirsky (1980) demonstrated that enteric glial cells in normal bowel could be visualized using antibodies against glial fibrillar acidic protein (GFAP). Pobert and co-workers (1981) visualized enteric neurons using monoclonal antibodies raised against the neurotransmitters, substance-P and vasoactive intestinal polypeptide. Ferri and co-workers (1982) reported the presence of S-100 protein in the glial component in the human enteric nervous system.

Klück and co-workers (1984) visualized the hypertrophic nerve fibers in Hirschsprung's disease using monoclonal antibodies directed against neurofilaments. Subsequently, other workers used antibodies to visualize the cell bodies of enteric neurons (neurofilaments, neuron-specific enolase) or enteric glial or Schwann cells (GFAP and S100) (Bishop et al., 1985; Vinores and May, 1985; Hall et al., 1985). The use of monoclonal antibodies in the diagnosis of congenital ENS malformations is reviewed by Tibboel et al. (1987).

2.2 CLASSIFICATION OF CONGENITAL MALFORMATIONS OF THE ENTERIC NERVOUS SYSTEM

Several classifications of congenital aganglionosis have been proposed in the past. From clinical and pathological observations Ravitch (1958) perceived that Hirschsprung's disease was not the underlying pathological substrate in a number of patients with congenital megacolon. He therefore proposed a division between Hirschsprung's disease and pseudo-Hirschsprung's disease. Ehrenpreis (1966) preferred to speak about "Hirschsprung's disease and allied disorders".

Another classification of Hirschsprung's disease is based on the distance from the internal anal sphincter encompassed by the aganglionosis, and distinguishes four classes (Boley, 1978; Kleinhaus, 1978). The first is an ultrashort segment, which involves only the anus and the part of the rectum below the pelvic floor. The second is the short segment or "classic" type, involving the anus, rectum, and a part of the sigmoid colon. The third class, the long-segment aganglionosis, involves the colon proximal to the sigmoid colon, whereas the fourth class involves the entire colon.

Howard and Garret (1984) considered Hirschsprung's disease and allied disorders as variations of neuronal dysplasia (which is abnormal tissue development). Neuronal dysplasia refers to differentiation disturbances; a significant number of congenital ENS malformations probably involves disturbances in the migration and homing of neural crest cells, rather than disturbances in terminal neuronal differentiation. Furthermore, it can be questioned whether aplasia (which is the absence of a tissue) of the enteric nervous system (e.g. aganglionosis) is a subtype of dysplasia.

An attempt to classify congenital ENS malformations and the result is presented in table 3. The classification is based on phases in the formation of the ENS, distinct

morphologic features of the pathological specimens, the pattern of inheritance, and partly on clinical presentations.

TABLE 3

CLASSIFICATION OF CONGENITAL MALFORMATIONS OF THE ENTERIC NERVOUS SYSTEM

1. DEVELOPMENTAL DISTURBANCES

1.1. Migration disturbance of vagal neural crest cells

Total intestinal aganglionosis (single gene defect)

a. autosomal recessive
b. autosomal recessive with kidney abnormalities

1.2. Proliferation disturbance of neural crest cells

Neuronal intestinal dysplasia

a. with neurofibromatosis (single gene defect)
b. with MEA IIb (single gene defect)
c. intestinal hyperganglionosis
   Type A: with hypoplasia of sympathetic innervation
   Type B: with normal sympathetic innervation

1.3. Disorders of the enteric mesenchyme

1.3.1. Total colonic aganglionosis (sometimes with small intestinal aganglionosis)
   a. Zuelzer-Wilson disease (multifactorial inheritance)
   b. associated with neuroblastoma (chromosome abnormality)

1.3.2. Hirschsprung's disease
   a. with first and second branchial arch syndromes
   b. with third and fourth branchial arch syndromes
   c. with dysautonomia
   d. with regional anomalies
   e. without the above (multifactorial inheritance)

1.3.3. Zonal aganglionosis

1.4. Differentiation disturbance of enteric neurons

Intestinal pseudo-obstruction

a. with mental retardation
b. with other neurologic abnormalities
c. isolated to the myenteric plexus, without the above

2. DEGENERATIVE VISCERAL NEUROPATHIES

2.1 Recessive with intranuclear inclusions
2.2 Recessive, with mental retardation and calcification of the basal ganglia
2.3 Dominant with neither of the above

2.3 TOTAL INTESTINAL AGANGLIONOSIS

Total intestinal aganglionosis is a rare congenital malformation of the ENS, characterized by the absence of enteric neurons from the entire intestinal tract. Detailed histochemical studies are not available. With one exception, all the reported cases (see table 4) share a fatal outcome; by performing myotomies, Ziegler and co-workers (1987) accomplished survival for over 18 months. Histochemical studies show normal acetylcholinesterase activity in small-intestinal and colonic lamina propria (Caniano et al., 1985).

There is a high incidence of familial occurrence in the reported cases. Although complete pedigrees are not available, it appears that approximately 50 percent of siblings are affected, in whom the length of the aganglionic segment is the same. The available evidence suggests that total intestinal aganglionosis is inherited as an autosomal recessive trait (MacKinnon and Cohen, 1977).

There are no reports of anomalies associated with total intestinal aganglionosis, apart from the report of DiLorenzo and co-workers (1985), describing the occurrence of renal dysplasias.

TABLE 4

CASES OF TOTAL INTESTINAL AGANGLIONOSIS

	REPORT	AGANGLIONIC SEGMENT
1	Bodian et al., 1951	jejunum-anus #
2	Lee, 1955	duodenum-anus
3	Boggs and Kidd, 1958	jejunum-anus ##
4	ibid.	jejunum anus ##
5	Bodian and Carter, 1963	unknown #
6	Walker et al., 1966	lower duodenum-anus
7	Ahmed et al., 1971	duodenum-anus
8	Talwalker, 1976	jejunum-anus
9	ibid.	jejunum-anus
10	ibid.	jejunum-anus
11	MacKinnon and Cohen, 1977	pylorus-anus
12	Walther and Kootstra, 1981	pylorus-anus
13	DiLorenzo et al., 1985	lower duodenum-anus *
14	ibid.	lower duodenum-anus **
15	ibid.	duodenum-anus ***
16	Caniano et al., 1985	lower duodenum-anus
17	Rudin et al., 1986	duodenum-anus
18	ibid.	lower duodenum-anus
19	ibid.	duodenum-anus
20	Ziegler et al., 1987	jejunum-anus

*# and ## siblings; * left renal agenesis; ** subcapsular microcysts in both kidneys; one of a twin; *** dysplastic left kidney and right renal cysts.*

2.4 INTESTINAL HYPERGANGLIONOSIS

Intestinal hyperganglionosis, or "neuronal intestinal dysplasia", is characterized by an above-average number of neurons in the submucous and myenteric plexuses and the presence of hyperplastic parasympathetic nerve trunks (Meier-Ruge, 1971; Howard and Garret, 1984). Initially the abnormalities were thought to be confined to the colon, but later it was found that they can occur in the entire bowel.

There are two types of neuronal intestinal dysplasia: type A, characterized by hypoplasia or aplasia of the sympathetic innervation, accounts for 15 percent of the cases. The early clinical presentation is characterized by acute onset of severe constipation, diarrhea and ulcerative colitis. Type B, characterized by normal sympathetic innervation, comprises 70 percent of the cases. Type B presents late and chronic. The remaining 15 percent of the cases comprises a combination of both types. Neuronal intestinal dysplasia has been recognized with increasing frequency in the last few years. Fadda and co-workers (1983) claim that it may occur as frequently as Hirschsprung's disease, but there is no sufficient proof for this statement.

Neuronal intestinal dysplasia has been reported in concurrence with Hirschsprung's disease (Gullotta and Straaten, 1970; Lassmann and Wurnig, 1973; Puri et al., 1977; Briner et al., 1986), and also with neurofibromatosis and multiple endocrine neoplasia syndrome type IIB (Staple et al., 1964; Phat et al., 1980; Saul et al., 1982; Demos et al., 1983; Feinstat et al., 1984).

2.5 ZUELZER-WILSON DISEASE

Zuelzer-Wilson disease or total colonic aganglionosis is characterized by the absence of enteric neurons in the entire colon (Zuelzer and Wilson, 1948). Meier-Ruge and co-workers (1972) reported that AChE-positive nerve fibers decreased exponentially toward the oral side from the rectum to the splenic flexure.

Given the incidence of classical Hirschsprung's disease (1:5,000), the incidence of Zuelzer-Wilson disease can be extrapolated as 1 in 50,000 to 1 in 100,000. Noteworthy examples of familial occurrence of Zuelzer-Wilson disease are those reported by Zuelzer and Wilson, and the one described by Richardson and Brown (1962), in which six out of seven brothers by three different fathers were affected by this disease. At present, no definite conclusions concerning the mode of inheritance of Zuelzer-Wilson disease can be drawn, except that, in contrast to classical Hirschsprung's disease, the disorder occurs as often in boys and in girls.

From isolated case reports it can be concluded that Zuelzer-Wilson disease may concur with other malformations. Chatten and Voorhess (1967) described familial neuroblastoma in which one sibling presented with total colonic aganglionosis. Michna and co-workers (1988) reported multifocal neuroblastoma with total colonic aganglionosis.

In up to 31 percent of the cases, Zuelzer-Wilson disease is complicated by enterocolitis (Kleinhaus et al., 1979; Careskey et al., 1982). It might well be that the

enterocolitis is due to a lack of nervous regulation of intestinal immunity and secretion (cf. Felten et al., 1988).

2.6 HIRSCHSPRUNG'S DISEASE

Hirschsprung's disease is the best described of the congenital ENS malformations. The disease is defined as the absence of enteric neurons and the presence of hypertrophic nerve trunks in the distal bowel beginning with and including the internal anal sphincter. The aganglionosis extends over varying distances proximally but always includes the anus and at least part of the rectum.

At the ultrastructural level, neurites of intrinsic neurons are absent throughout the aganglionic bowel segment; close to the transitional zone, however, abnormal nerve fibers are present, surrounded by a hypertrophic perineurium that contains increased amounts of collagen (Baumgarten et al., 1973). The ultramorphology of supporting glial cells reveals features of immature, undifferentiated Schwann cells. Schwann-cell units inside these bundles are mono- or oligoaxonal.

The monoclonal antineurofilament antibody 2F11 visualized all extrinsic nerve fibers in Hirschsprung's disease (Klück et al., 1984). Kawana and co-workers (1988) showed that the extrinsic, hypertrophic nerve fibers are associated with GFAP-positive supportive cells. Intrinsic nerve trunks are positive for S100 but negative for GFAP.

2.6.1 INCIDENCE

The majority of early statements on the incidence of Hirschsprung's disease only mention that Hirschsprung's disease is a rare disease. The incidence rates in these early reports range from 1 in 2,000 to 1 in 12,000.

Detailed studies from Denmark, the British Isles, and British Columbia indicate that the incidence of Hirschsprung's disease approximates 1 in every 5,000 newborns (table 5; Madsen, 1964; Passarge, 1967; Goldberg, 1984; Spouge and Baird, 1985).

TABLE 5

STUDIES OF THE INCIDENCE OF HIRSCHSPRUNG'S DISEASE

Report	Incidence	Area
Althoff, 1962	1 in 12,000	Bremen
Bodian and Carter, 1963	1 in 2,000-10,000	British Isles
Madsen, 1964	1 in 4,700	Denmark
Passarge, 1967	1 in 5,000	Cincinnatti
Orr and Scobie, 1983	1 in 4,500	Southeast Scotland
Ikeda and Goto, 1984	1 in 4,697	Japan
Goldberg, 1984	1 in 5,682	Baltimore
Spouge and Baird, 1985	1 in 4,417	British Columbia
Global average:	1 in 5,000	

2.6.2 FAMILIAL OCCURRENCE OF HIRSCHSPRUNG'S DISEASE

A positive family history exists in 3.6 percent of all cases (females 7.2 percent; males 2.6 percent; see table 6). If Zuelzer-Wilson disease is included, the incidence of familial occurrence rises to 21 percent (Swenson et al., 1973; Kleinhaus et al., 1979). Two-generation transmission (from parent to child) is well recorded in the literature (Madsen, 1964), and even three-generation transmission has been reported (Lipson and Harvey, 1987). A nine-generation family with presumptive Hirschsprung's disease had associated Waardenburg syndrome, congenital deafness and Down's syndrome (Cohen and Gadd, 1982).

As a generation of treated and surviving patients grow to maturity and reproductive age, genetic counselling is indicated, if only to provide early and prompt surgical care and treatment of the disease (Carter et al., 1981). The actual mode of inheritance will also become clearer. Genetic counselling should also take into account the Mendelian inheritance of some syndromes associated with Hirschsprung's disease (discussed below). Non-syndromic cases of Hirschsprung's disease are thought to inherit in a sex-modified multifactorial mode (Passarge, 1983). This term implies that the threshold of genes needed for expression of the trait in one sex is lower than in the other.

2.6.3 CONGENITAL ANOMALIES ASSOCIATED WITH HIRSCHSPRUNG'S DISEASE

The importance of anomalies associated with Hirschsprung's disease is obvious to some physicians, but this is not properly reflected in the literature. The combinations of anomalies and malformations are virtually infinite and detailed knowledge of most of these combinations is of limited use to the practising pediatrician or pediatric surgeon. However, awareness of certain of these associations may alert the physician to look for defects that are not immediately obvious at birth. Syndrome delineation enables preliminary sorting of disorders for proper study of pathogenesis.

The incidence of anomalies associated with Hirschsprung's disease varies from 2.5 to 35 percent (table 6). The wide range is due to the diligence with which they are sought and the manner in which they are reported. Some reports show an increase in additional malformations, while others show only an increase in Down's syndrome and anomalies of the genitourinary tract (Boley et al., 1978; Passarge, 1967; Warkany, 1971). In recent years, the number of (case-) reports on anomalies associated with Hirschsprung's disease has grown considerably.

The anomalies associated with Hirschsprung's disease were classified according to developmental-biological criteria (table 7). One group includes neural crest malformations occurring in other systems or malformations more distant in the gastrointestinal tract [over one third of the conditions with megacolon have associated

or identifiable neural crest components (Saul and Stevenson, 1988)]. Another group includes regional anomalies that are thought to occur secondarily to either the development of megacolon (e.g. megaloureter, hydronephrosis) or its perforation in utero (e.g. colonic, anal and rectal stenosis, and atresia) or postoperatively for corrective surgery (rectal and anal stenosis and atresia).

Association of Hirschsprung's Disease with Down's Syndrome

Down's syndrome is the most common single syndrome in patients with Hirschsprung's disease. The prevalence of Down's syndrome in studies of Hirschsprung's disease varies from 0.6 to 9.5 percent (Table 6), but an association between the two is consistent. In the general population the incidence of Down's syndrome is one in every 600 newborns. Thus the incidence of Down's syndrome in patients with Hirschsprung's disease is about 3.5 to 59.4 times higher than in the general population.

TABLE 6

FAMILIAL OCCURRENCE AND INCIDENCE OF ANOMALIES ASSOCIATED WITH HIRSCHSPRUNG'S DISEASE

	Familial Occurrence (%)	Associated Anomalies (%)	Down's Syndrome (%)
Althoff, 1962			1.5
Bodian and Carter, 1963			1.5
Madsen, 1964			0.6
Gordon et al. 1966			1.8
Emanuel et al., 1965			1.0
Hoffmann and Rehbein, 1966			2.3
Graivier and Sieber, 1966		3.6	
Passarge, 1967	3.6	21	9.5
Aubresby, 1970		6	
Ehrenpreis, 1970	2.4	<5	
Bethenod, 1973		9	
Swenson, 1973	7.8	11	
Rickham, 1978	6.0	21	
Angerpointner, 1981		9.3	
Kaiser and Bettex, 1982			5.0
Goldberg, 1984			9.0
Klein, 1984		35	
Ikeda and Goto, 1984		11.1	
Spouge and Baird, 1985		30	3.2
Ikeda and Goto, 1986			2.9
Polley and Coran, 1986		26	

Association of Hirschsprung's Disease with First and Second Arch Syndromes

The first and second arch syndromes comprise a group of disorders in which anatomical abnormalities are found in the tissues that derive from the first and second branchial arches. The first and second arch syndromes include: 1) Waardenburg syndrome (deafness, heterochromia, a white forelock and various other anatomical changes); 2) congenital deafmutism (middle ear malformations, absent organ of Corti); 3) cleft lip and cleft palate; 4) the Treacher-Collins syndrome or mandibulofacial dysostosis (notching of the lower eyelid, poorly developed eyelashes, deafness, mandibular agenesis, and abnormalities of the malar bones); 5) the Pierre Robin sequence (hypoplasia of the mandible with glossoptosis).

TABLE 7

ADDITIONAL ANOMALIES IN HIRSCHSPRUNG'S DISEASE

	Spouge & Baird, 1985	Ikeda & Goto, 1986
Neural Crest related anomalies		
Sensorineural	6.7	
Deafness	2.2	
Cardiovascular	5.6	2.1
Skeletal and limb anomalies	3.4	
Cleft palate	1.1	0.6
Other anomalies		
Down's syndrome	2.8	2.9
Microcephaly	1.1	
Mental Retardation	0.5	
Inguinal hernia	2.8	0.7
Small bowel atresia	1.1	0.2
Duodenal atresia		0.1
Genital reproductive tract	2.8	
Undescended testes	1.7	
Regional anomalies		
Urinary tract	2.2	0.2
Rectal stenosis	1.7	
Anal stenosis	1.1	
Imperforate anus	1.1	0.2
Colonic atresia	0.6	

Waardenburg syndrome with deafness occurs with a frequency of 2 cases per 100,000 and represents 2 to 5% of cases of profound childhood deafness (Fraser, 1976; Brown and Chung, 1967). Waardenburg syndrome is inherited in an autosomal dominant mode with a varying penetrance and expression. Waardenburg syndrome has such

variable manifestations that diagnosis is often difficult. Even in cases of profound childhood deafness with pigment anomalies, heterogeneity of the Waardenburg syndrome may be indicated by individual patients of families without the characteristic displacement of the inner canthi (Arias, 1971). It is not clear whether this variability reflects multiple alleles or different major genes for the Waardenburg phenotype or factors in the genetic and environmental background.

There are several reports of an association of Hirschsprung's disease with congenital deafness (McKusick, 1973; Lowry, 1975; Skinner and Irvine, 1973; Weinberg et al., 1977; Branski et al., 1979; Shah et al., 1981; Currie et al., 1986). McKusick was the first to report the association between Waardenburg syndrome and Hirschsprung's disease.

Cleft palate also occurs more frequently in patients with Hirschsprung's disease in the normal liveborn population (Spouge and Baird, 1985; Goldberg and Sphrintzen, 1981; Brunoni et al., 1983). There are no case reports on an association of Treacher-Collins syndrome or Pierre Robin sequence with Hirschsprung's disease.

Association of Hirschsprung' disease with Third and Fourth Branchial Arch Syndromes

The incidence of cardiac anomalies varies from 2.1 to 5.6 percent of patients with Hirschsprung's disease (Spouge and Baird, 1985; Ikeda and Goto, 1986). Spouge and Baird (1985) reported that particular atrioseptal defect occurs frequently (2.2 percent of Hirschsprung cases), which is 18 times the frequency in the liveborn population in British Columbia. The difference is statistically significant. They also reported that ventricular septal defect occurs in 1.7 percent of Hirschsprung cases, several times the rate in the British Columbia liveborn population, consequently a non-random association. Because of the known relationship between these cardiac anomalies and Down's syndrome, the Down cases were removed from the study group and the rates recalculated. Both atrioseptal defect and ventricular septal defect still occurred significantly more frequently in Hirschsprung's disease cases.

Association of Hirschsprung's Disease with Trunk Neural Crest Syndromes

Skeletal and limb anomalies occur in 3.4 percent of Hirschsprung cases (Laurence, 1975; Reynolds et al, 1983; Santos et al., 1988; Toriello et al., 1988; Ikeda and Goto, 1986). Polydactyly occurs more often than expected and is a non-random association (Spouge and Baird, 1985).

Some patients with Hirschsprung's disease suffer from a failure of the autonomic control of ventilation during sleep - this is called Ondine's curse. There is a decrease in ventilation during sleep and this does not correspond to the decrease in metabolic activity. This decrease occurs predominantly during non-rapid eye movement or quiet sleep. The neurophysiologic mechanisms responsible for this change in ventilation are poorly defined. In a number of case reports congenital failure of autonomic ventilation

is associated with Hirschsprung's disease (Haddad et al., 1978; Bower and Adkins, 1980; Stern et al., 1981; Hamilton and Bodurtha, 1989). Recently a patient was admitted in the Sophia Children's Hospital, Rotterdam, who suffered from Hirschsprung's disease, congenital central hypoventilation and neuroblastoma (unpublished observation).

The association of Hirschsprung's disease and neuroblastoma is rare; it might occur by chance, but there might be a causal relation between the two diseases. Hope and co-workers (1965) first reported the concurrence: a two-month-old girl who had aganglionosis of the distal colon and a thoracic neuroblastoma. Two other cases have been reported in the literature (Chatten and Voorhess, 1967; Gaisie et al., 1979). It is of interest that the interstitial deletion of chromosome 13q, which is frequently associated with neuroblastoma, was also found in a patient with both disorders (Lamont et al., 1989).

Another truncal neurocristopathy, pheochromocytoma, has also been reported in conjunction with Hirschsprung's disease (Shocket and Pelok, 1957; Duffly et al., 1962).

Association of Hirschsprung's Disease with Regional Anomalies

Megaloureter, hydronephrosis, and megalocystis are included in the group of associated regional malformations. It might well be that these anomalies result from local bowel dilatation, since these urinary tract anomalies routinely disappear after corrective bowel surgery (Boley et al., 1978). Colonic, rectal, and anal atresia (and stenosis) possibly result from the secondary dilatation of the bowel, producing local perforation and scarring while in utero (Bughaighis and Emery, 1971). Moreover, postpartum corrective surgery for Hirschsprung's disease can lead to complications of rectal or anal stenosis. Both anal and rectal lesions may then be coded under the diagnosis of imperforate anus.

2.7 COLONIC HYPOGANGLIONOSIS

Hypoganglionosis of the colon is characterized by a below-average number of enteric neurons and nerve fibers in the myenteric plexus (Meier-Ruge, 1971). Hypoganglionosis is well described in the transition zone between normal and aganglionic bowel of patients with Hirschsprung's disease (Gherardi, 1960; Walker et al., 1966; Garret et al., 1969; Meier-Ruge, 1969). The occurrence of hypoganglionosis without Hirschsprung's disease is questioned by several authors; the hypoganglionosis could be secondary to megacolon. A few case reports have been published (Bentley, 1964; Ehrenpreis, 1966). A conclusive diagnosis can only be made on morphometrical criteria.

2.8 ZONAL AGANGLIONOSIS

The existence of ganglionic bowel segments in aganglionic bowels, which is called zonal aganglionosis, is often questioned (Swenson, 1959; for review see Yunis, 1983). In the

few reports available, zonal aganglionosis is found to affect various segments, single or double, of the colon and rectum (Tiffin et al., 1940; Haney et al., 1982; Tagushi et al., 1983). There are nine case reports of single zonal aganglionosis in the literature and four of double zonal aganglionosis.

2.9 INTESTINAL PSEUDO-OBSTRUCTION

Intestinal pseudo-obstruction is characterized by functional obstruction of the ganglionic bowel. Intestinal pseudo-obstruction has been associated with "immaturity" of enteric neurons (Bughaighis and Emery, 1971; Tanner et al., 1976; Erdohazi, 1974). Normally, enteric ganglia contain both mature and immature neurons at birth (Smith, 1968). In cases of intestinal pseudo-obstruction, there are only few mature neurons or none at all. Immunocytochemical studies using anti-neurofilament antibodies showed a lack of immunoreactive nerve fibers in the bowel (Klück et al., 1986).

2.10 DEGENERATIVE ENTERIC NEUROPATHIES

Three different degenerative enteric neuropathies have been described to date. Neuronal intranuclear inclusion disease was first described in 1978 in two siblings who presented with symptoms of intestinal pseudo-obstruction and who were also found to have diffuse neurologic abnormalities, evidence of mild autonomic insufficiency, and denervation hypersensitivity of the pupillary and esophageal smooth muscles (Schuffler et al., 1978). Schuffler suggested that the disease is familial with an autosomal mode of inheritance.

A second type of degenerative enteric neuropathy was reported in 4 siblings of a sibship of 16 (Cockel et al., 1973). The children had episodes of pseudo-obstruction and malabsorption, mental retardation, and calcification of the basal ganglia. This disorder is thought to inherit in a autosomal recessive mode.

A third type of degenerative enteric neuropathy, with a dominant mode of inheritance, has been reported in two families (Roy et al., 1980; Mayer et al., 1988). Patients in both reports had intestinal pseudo-obstruction affecting predominantly the small intestine, without evidence of central, autonomic, or peripheral nervous system involvement.

2.11 SPONTANEOUS ANIMAL MODELS FOR CONGENITAL ENS MALFORMATIONS

Studies directed toward improvement of understanding of the pathophysiology of Hirschsprung's disease have been performed on animal models in which aganglionosis is congenital. Spontaneous occurrence of aganglionosis has been reported in mice, rats, and horses. Absence of enteric neurons in the colon and ileum has been reported in white foals of overo spotted horses. The foals are generally normal at birth, but they do not defecate. Signs of colic occur between 5 and 124 hours after birth, leading to death at 23 to 132 hours (Hultgren, 1982). The characteristics of the murine models, and the similarities and differences with the human disease are the subject of this section.

Congenital megacolon in mice was first reported by a group in Australia and later in America. Derrick and St. George-Grambauer (1957) reported megacolon in mice with characteristics similar to Hirschsprung's disease. Bielschowski and Schofield (1960) described megacolon related to genetic factors and associated with deficiency of hair pigmentation characteristic of the piebald (S/S) NYZ strain. Lane (1966) reported two mutant mouse strains in which autosomal recessive genes resulted in spots of reduced pigmentation in the fur and reduction of the number of enteric neurons in the distal bowel. She called one strain 'piebald lethal' and the other 'lethal spotting'. Recently, a new dominant spotting mutation that produces megacolon and was called dominant megacolon (Dom) arose in the Mouse Mutant Stock Center of the Jackson laboratory (Lane and Liu, 1984). In heterozygotes the Dom gene produces white spotting and a deficiency of enteric neurons in the colon; in homozygotes, the Dom gene is lethal prior to 13 days of gestation.

In the piebald-lethal (Sl) strain, the homozygotes (Sl/Sl) are white-coated except for patches of black pigment; they invariably develop megacolon. These homozygotes tend to develop megacolon early in life, and often die from diarrhea and enterocolitis before breeding age is attained. The establishment of colonies of this stock for study is therefore difficult.

The lethal spotting strain (ls) physically resembles the piebald lethal strain except that the ears and tail are less pigmented. These homozygotes tend to survive longer than the sl/sl strain and are usually better to breed for several months before dying of the effects of megacolon. Cross-breeding of homozygotes produces litters in which all members develop aganglionosis in the hindgut. Abdominal distension and gross megacolon become obvious in most animals by 2 to 3 weeks of age.

The aganglionic zone in ls/ls mice is not denervated (Bolande, 1975; Rothman and Gershon, 1984; Meijers et al., unpublished observations) as it does receive many nerve fibers. The aganglionic zone is innervated by noradrenergic nerve fibers, which are exclusively derived from neurons whose cell bodies lie outside the bowel (Payette et al., 1987). The distal bowel thus receives an extrinsic innervation. It is therefore possible that the distal bowel excludes both neural crest cells and processes of intrinsic enteric neurons.

The ls/ls mouse has been used in developmental and physiological studies (for review see Gershon and Rothman, 1984; Wood, 1979).

2.12 CONCLUSION

Congenital ENS malformations were classified according to phases in ENS formation, and pathological and genetic data were updated and summarized. The anomalies associated with Hirschsprung's disease were rubricated according to developmental biological criteria.

The malformations associated with Hirschsprung's disease indicate that Hirschsprung's disease results from a number of different pathogenetic mechanisms. A

notable number of the associated malformations is related to other neural crest syndromes. The occurrence of particular neural crest lesions in association with Hirschsprung's disease provides information about the timing of the developmental failure. Different pathogenetic mechanisms for congenital aganglionosis in the distal colon are also encountered in mammals.

It is proposed that congenital ENS malformations result from disturbances in the migration, proliferation, homing and differentiation of enteric neural crest cells. Chapter 3 deals with morphoregulatory molecules which are involved in these processes, while chapter 4 describes these molecules in relation to the development of the ENS.

CHAPTER 3

THE HNK-1 FAMILY OF MORPHOREGULATORY MOLECULES

3.1 INTRODUCTION

Knowledge of embryology facilitates the understanding of the pathogenesis of congenital malformations. Ever since the early studies by Holtfreter (1944), Moscona (1952), and others, demonstrating tissue- and species-specific sorting out of embryonic cells, it has been assumed that specialized adhesive properties of cells play a key role in morphogenesis. Recent progress in defining the molecules that mediate these adhesion properties, so called morphoregulatory molecules, provides the opportunity to analyze morphogenetic processes in molecular and causal terms.

Morphoregulatory molecules are molecules whose gene expression affects cell patterning and tissue signalling through mechanochemical effects on cell linkage, shape and movement. At present several classes of morphoregulatory molecules have been identified: a) cell adhesion molecules (CAMs), which are involved in the adhesion of cells to one another (see table 8); b) extracellular matrix (ECM) molecules, which are involved in the three-dimensional positioning of cells; and c) substrate adhesion molecules (SAMs), which are involved in the attachment of cells to ECM molecules.

3.2 HNK-1 AND OTHER CARBOHYDRATE MOIETIES DEFINE FAMILIES OF MORPHOREGULATORY MOLECULES

A carbohydrate moiety that is recognized by monoclonal antibody HNK-1 defines a family of morphoregulatory molecules and a family of CAMs (see table 9; Kruse et al., 1984). The HNK-1 epitope is also present on uncommon glycolipids from human peripheral nerves and embryonic fetal brain (Ilyas et al., 1984; Chou et al., 1985, 1986; Noronha et al., 1986; Schwarting et al., 1987). The glycolipids were characterized as sulfate-3-glucuronyl paraglobosides and sulfate-3-glucuronyl neolactohexasosyl ceramides (Ariga et al., 1987; Chou et al., 1986). In this thesis the HNK-1 bearing glycolipids are not discussed since there is little knowledge concerning their role in development.

The HNK-1 antibody was originally raised against the human T cell lymphoma cell line (HSB2) (Abo and Balch, 1981), and found to cross-react with nervous tissue (Schüller-Petrovic et al., 1983). The epitope has been characterized as a sulfated

glucuronic acid (Chou et al., 1986). The same epitope is recognized by other monoclonal antibodies (see table 10).

Keilhauer and co-workers (1985) postulated that the HNK-1 epitope was involved in cell-cell interactions. Their postulate was based on observations that Fab fragments of L2 antibodies (similar to the HNK-1 antibody) interfere with neuron-astrocyte and astrocyte-astrocyte adhesion. Riopelle and co-workers (1986) reported that HNK-1 antibodies inhibit neurite outgrowth in vitro. Since antibodies do not only cover the epitope they are directed against, but can also sterically hamper the function of the adhesive domains, direct demonstration of the HNK-1 moiety for cell-cell interactions was necessary.

TABLE 8

MAJOR CELL ADHESION MOLECULES ON VERTEBRATE NEURAL AND NONNEURAL CELLS

Molecule	Species	MW	Distribution
		CALCIUM DEPENDENT ADHESION	
E-Cadherin	Mouse	124	Preimplantation embryos
Uvomorulin	Mouse	120	Epithelia
L-CAM	Chick	124	Adherens junctions
Cell CAM120/80	Human	120	
Arc-1	Canine	130	
P-Cadherin	Chick	118	Trophoblast, lung, heart, intestine
N-Cadherin	Mouse	127	Embryonic mesoderm
N-cal CAM	Chick	125	Neural ectoderm
A-CAM	Chick	135	Nervous system, muscle, heart, lens, adherens junctions
		CALCIUM INDEPENDENT ADHESION	
Cell CAM	Rat	105,110	Liver and other epithelia
N-CAM	Various		
Neural N-CAM-H		180-220	Embryonic mesoderm,ectoderm
Neural N-CAM-L		180	Nervous system, glia
		140	
		120	
Muscle N-CAM		155	Embryonic and adult muscle
		140	
		125	

Künemund and co-workers (1988) demonstrated the role of the HNK-1 carbohydrate moiety, without the attached protein backbone, in cell-cell and cell-substrate interaction. They tested several carbohydrates to gain information about the requirements underlying the HNK-1-dependent adhesion. Of all compounds tested, only sulfatide and heparin interfered with cell interactions in a similar manner as the HNK-1 carbohydrate-containing compounds. Both compounds are structurally related to the HNK-1 epitope in that they contain sulfate groups at the 2' or 3' hydroxyl groups of hexose sugars. The sulfate group appeared to be the decisive factor in ligand activity. Removal of the sulfate group from the HNK-1 glycolipid or sulfatide led to complete disappearance of the inhibitions of cell adhesion. It is possible that the HNK-1 carbohydrate is responsible for binding of morphoregulatory molecules to laminin (personal communication Dr V. Künemund).

Canning and Stern (1988) showed that the HNK-1 antibody identifies tissues involved in the process of mesodermic induction. Before induction, HNK-1 recognizes the 'inducing' tissue (the hypoblast) and reveals a mosaic pattern in the responding tissue (the epiblast); after primitive streak formation, the epiblast displays an anteroposterior gradient of HNK-1 expression. At the end of gastrulation, the primitive streak region loses its HNK-1 immunoreactivity. HNK-1 expression is next seen in the cells of the forming notochord and in cranial neural crest cells.

In view of the existence of the multiple HNK-1 antigens during embryogenesis and adult life, the possibility to visualize specifically migrating neural crest cells in avian embryos with the use of HNK-1 or related antibodies (Tucker et al., 1984; Vincent et al., 1983; Vincent and Thiery, 1984) might seem surprising. Although HNK-1 reactivity is not restricted to neural crest cells in avian embryos, the staining of other antigenic cell lineages does not overlap topographically or temporally with the distribution of the crest cells. Tucker and co-workers (1988a) described the expression of the HNK-1 epitope in early vertebrate neurogenesis. Neural crest cells do not become HNK-1 positive until they leave the neuroepithelium and start to migrate. Injection of HNK-1 antibodies lateral to the mesencephalic neural tube perturbs cranial neural crest cell migration (Bronner-Fraser, 1987).

Evidence is accumulating that the HNK-1 family of morphoregulatory molecules is not unique and that a system of carbohydrate epitopes affecting the function of adhesion molecules may be widespread. Another family of neural cell adhesion molecules described recently is characterized by the L3 carbohydrate epitope (Kurcherer et al., 1987). The L3 family includes some members of the HNK-1 family. The L3 epitope resembles the HNK-1 epitope in that the carbohydrate-carrying moieties are N-glycosidically linked and regulated independently of the protein backbone core. There appears to be a partial overlap between the HNK-1 and L3 families, L3 being expressed by the adhesion molecule on glia (AMOG), L1 and MAG, and by other as yet unidentified molecules of the nervous system, but not by N-CAM or J1.

There is increasing evidence that cell adhesion and recognition in vertebrate

TABLE 9

HNK-1 FAMILY OF MORPHOREGULATORY MOLECULES

CELL ADHESION MOLECULES		
Neural N-CAM	Chick	Kruse et al., 1984
Ng-CAM	Chick	
L1	Mouse	Kruse et al., 1984
F11	Chick	Rahtjen et al., 1987
G4	Chick	Rahtjen et al., 1984
F3	Mouse	Gennari et al., 1989
P0	Mouse	
MAG	Mouse	Quarles et al., 1985
EXTRACELLULARMATRIX MOLECULES		
Cytotactin	Chick	Grumet et al., 1985
J1	Mouse	Kruse et al., 1985
CBP	Chick	Hoffman and Edelman,1987
CELL SURFACE RECEPTORS FOR EXTRACELLULAR MATRIX MOLECULES		
Integrin		Pesheva et al,, 1987
α subunit	Chick	
β subunit	Chick	

TABLE 10

MONOCLONAL ANTIBODIES WITH HNK-1 SPECIFICITY

ANTIBODY	AUTHOR	IMMUNOGEN
HNK-1	Abo & Balch, 1981	HSB-2 cells
NC1	Vincent & Thiery, 1983	Quail retina cells (E7)
L2	Rathjen & Schachner, 1984	Forebrain of adult mice
	Kruse et al. 1984	Enriched membrane fractions of adult mouse brain
4F4	Schwarting et al. 1987	Rat forebrain E15-E17
clone 105	Edelman et al., 1984	
IgM	Patients with plasmacell dyscrasias and polyneuropathy	

development is mediated in part by cell surface oligosaccharides. Early proposals for an involvement of oligosaccharides in neural adhesion and recognition derived from the detection of a high degree of heterogeneity in cell surface oligosaccharides of neural cells and from the discovery of cell surface glycosyl transferases capable to interact with cell surface oligosaccharides (Roth et al. 1971). In addition to the 68-kD laminin receptor and integrin, cell-surface galactosyltransferases have been identified as a laminin receptor (Runyan et al., 1986; 1988). This finding points at a role for cell-surface oligosaccharides in cell adhesion and recognition. The cell-surface galactosyl transferases act as receptors for extracellular lactosaminoglycan-type oligosaccharides, which presumably terminate in N-acetylglucosamine (GlcNAc) residues (Shur and Hall, 1982; Shur, 1982). Similar lactosaminoglycan oligosaccharide chains have been found on laminin (Arumugham et al., 1986).

3.3 A CELL ADHESION MOLECULE OF THE HNK-1 FAMILY: N-CAM

The HNK-1 carbohydrate moiety is present on subsets of CAMs, such as the Neural Cell Adhesion Molecule (N-CAM), the Myelin Associated Glycoprotein (MAG) and L1 (see table 9). N-CAM is the major calcium-independent CAM in vertebrate tissues and promotes cell-cell adhesion via homophilic interactions. Modifications in the structure of N-CAM contribute to multiple developmental functions. The diversity in N-CAM structure is achieved in at least three ways.

First, N-CAM exists in several polypeptide forms that are generated by tissue-specific splicing of RNA molecules derived from a single gene (Barthels et al., 1987; Cunningham et al., 1987). Alternative splicing generates a muscle-specific form of N-CAM that exhibits a variant extracellular polypeptide sequence (Dickson et al., 1987). The three neural N-CAM polypeptides, N-CAM-180, N-CAM-140, and N-CAM-120, have identical amino-terminal domains (Cunningham et al., 1987). N-CAM-180 and N-CAM-140 are integral membrane proteins, whereas N-CAM-120 is attached to membranes by a phospholipase C-sensitive phospholipid anchorage (He et al., 1986) and can be released spontaneously. Functional differences in the two integral membrane polypeptide forms derive from variations in their cytoplasmic domains (Pollerberg et al., 1987). The extracellular domain of N-CAM contains repeated segments that resemble immunoglobulin variable regions (Cunningham et al.,1987). N-CAM, Thy-1, and several other potential neural CAMs have therefore been incorporated into the immunoglobulin gene superfamily (Williams, 1987).

Secondly, N-CAM polypeptides can be further diversified by posttranslational modifications. The most striking of these is an alteration in the length of alpha-2,8 linked sialic acid homopolymer chains (Finne et al., 1983). The highly sialylated (H) form of N-CAM contains about 30 percent sialic acid, and the low (L) form about 10 percent. The degree of sialylation appears to modify the binding properties of N-CAM since vesicles containing N-CAM-H aggregate more slowly than those containing N-CAM-L (Hoffman and Edelman, 1983; Sadoul et al., 1983). N-CAM molecules are also

heterogeneous with respect to the expression of the HNK-1 epitope. The location of the HNK-1 epitope on N-CAM has been mapped to the third loop on the N-CAM (Cole and Schachner, 1987).

Thirdly, N-CAM binding is modified by association with proteoglycans. The amino-terminal 65 kDa fragment of N-CAM contains a binding site for heparan sulfate that is near to, but distinct from the homophilic binding domain (Cole et al., 1986). N-CAM-mediated cell-cell adhesion appears to be stabilized by the interaction of heparan sulfate with this binding site. In contrast, the binding of heparin inhibits N-CAM-mediated cell adhesion. These findings raise the possibility that other general CAMs have multiple binding sites.

The diversity in N-CAM polypeptide structure correlates with distinct stages of neuronal differentiation. N-CAM-140 and N-CAM-120 are expressed from the time of neural tube formation, whereas N-CAM-180 first appears on postmitotic and postmigratory cells (Pollerberg et al., 1986). Moreover, N-CAM-L predominates at early stages of neural tube formation (Sunshine et al., 1987), and the expression of N-CAM-H is first detected during neuronal differentiation. Thus the enhanced migration of neuroblasts and the extension of axons may result, in part, from changes in the adhesive properties of neuronal surfaces associated with a transition in the state of N-CAM sialylation. Neuronal cells gradually reexpress N-CAM-L during late development and postnatal development after the establishment of final synaptic connectivity (Rutishauser, 1986).

3.4 TISSUE-SPECIFIC CELL ADHESION MOLECULES OF THE HNK-1 FAMILY

The increasing number of distinct axon-associated glycoproteins implicated in fiber-fiber interaction suggests that this group of molecules constitutes a sizeable class of tissue-specific CAMs; they are therefore collectively called Axon-associated CAMs (AxCAMs) (Rutishauser and Jessel, 1988). Many of the individual members of this group have been named according to the monoclonal antibodies originally used to detect them. The populations of tissue-specific CAMs are also heterogeneous with respect to the expression of the HNK-1 epitope (Kruse et al., 1984; Poltorak et al., 1987).

Several distinct AxCAMs have been described: L1 in rat and mouse, and G4, F11 and neurofascin in chicken, and several other molecules that are restricted to axonal subsets and most likely play roles in axon-axon interactions (Rathjen and Schachner, 1984; Rathjen et al., 1987). L1 expression can be induced by nerve growth factor (NGF) in cultured pheochromocytoma PC12 cells. It has been characterized independently and termed NILE (NGF-inducible large external) glycoprotein on this basis (Salton et al., 1983, Stallcup and Beasly, 1985). The L1-like molecule in chicken has been termed Ng-CAM on the original assumption that its primary function was to mediate the adhesion of neurons to glial cells (Grumet and Edelman, 1984). Both Ng-CAM and G4 appear to be similar or identical to the 8D9 antigen (Langenauer and Lemmon, 1987).

The best structurally characterized AxCAMs are chicken G4, F11 and neurofascin

proteins. Tryptic mapping and limited sequence analysis of proteolytic fragments of these transmembrane glycoproteins indicate that they are structurally distinct, although there appear to be similarities in molecular structure and in their orientation in membranes (Wolff et al. 1987). Each of these molecules can be detected as several distinct glycoprotein sequences with molecular masses in the range of 130-190 kDa, together with minor components in the range of 60-80 kDa.

Another set of tissue-specific CAMs function primarily in the adhesive interactions of glial cells. The best characterized of these is MAG, which is also a member of the HNK-1 family (Poltorak et al., 1987).

3.5 EXTRACELLULAR MATRIX MOLECULES OF THE HNK-1 FAMILY

Extracellular matrices are made up of an insoluble meshwork of proteins and carbohydrates that is produced by cells and that fills most of the intercellular spaces. The ECM in different locations in the body consists of different combinations of collagens, proteoglycans, elastin, hyaluronic acid, and various glycoproteins such as fibronectin and laminin. Cell-substratum adhesion is mediated by complexes of ECM molecules, associated with specific receptors at the cell surface. Elucidation of the amino acid sequence of the cell-attachment domain in the ECM molecule fibronectin, and its duplication with synthetic peptides, established the sequence Arg-Gly-Asp (RGD) as the essential structure recognized by cells in fibronectin (Pierschbacher et al., 1982). This RGD sequence is the cell recognition site of a surprising number of ECM and platelet adhesion molecules. Vitronectin, type I collagen, fibrinogen, von Willebrand factor, cytotactin, osteopontin, and cytotactin each contain one or more RGD sequences, and their interactions with cells can be inhibited with RGD-containing peptides (for review see Buck and Horwitz, 1987)

ECM molecules containing the HNK-1 epitope have also been described, e.g. cytotactin and cytotactin binding proteoglycan (CBP) (Grumet et al., 1985; Hoffman and Edelman, 1987).

Cytotactin was formerly called myotendinous antigen (Chiquet and Fambrough, 1984), and is probably similar or identical to tenascin (Grumet et al., 1985), hexabrachion (Vaughan et al., 1987), and J1 (Kruse et al., 1985). Electron-microscopical analyses of cytotactin revealed a six-armed structure with a central core (Erickson and Inglesias, 1984; Hoffman et al., 1988). Biochemical analyses suggest that cytotactin is a disulfide-linked oligomer (Grumet et al., 1985). Although the polypeptide subunits of cytotactin that are expressed during development in different organs vary in molecular mass, all of these polypeptides are derived from one single gene (Jones et al., 1988). The functional domains of cytotactin were mapped by Friedlander and co-workers (1988). Hoffman and co-workers (1988) reported the location of the HNK-1 epitope on cytotactin molecules.

Three recent reports indicate that tenascin and cytotactin have distribution patterns that are closely related to the areas where neural crest cells migrate (Mackie

et al., 1988; Tan et al., 1987; Bronner-Fraser, 1988). Cytotactin is localized in the rostral half of sclerotomes at about the time of neural crest invasion. Cytotactin binding proteoglycan is initially diffusely distributed throughout the sclerotome but becomes restricted to the caudal half after the appearance of cytotactin and invasion of neural crest cells in the rostral half. These distributions are crest cell independent. Aufderheide and co-workers (1988) reported the distribution of tenascin during bowel development.

3.6 HNK-1 BEARING CELL SURFACE RECEPTORS FOR EXTRACELLULAR MATRIX MOLECULES

Despite the similar cell attachment sequences in the various ECM molecules, cells can recognize them individually. This specificity is provided by a number of cell-surface receptors, each of which is capable of recognizing only a single RGD-containing protein ligand, or in some cases only a few ligands. On the basis of structural and functional similarities, numerous investigators have suggested that the cell surface receptors for ECM molecules belong to one supergene family of cell surface receptors: integrins (Hynes, 1987).

One of the first molecules to be identified and characterized extensively as the cellular receptor for ECM molecules (fibronectin) was the CSAT antigen or integrin (Neff et al., 1982; Greve and Gottlieb, 1982). The HNK-1 epitope is also present on both the α and β subunit of avian integrin (Pesheva et al., 1987). Among the members of the integrin family, avian integrin is somewhat anomalous in that it consists of at least three subunits. Avian integrin may be a promiscuous heterotrimer or -dimer capable of binding several ligands, or it may be a mixture of heterodimers each with a common beta-subunit with which the CSAT and JG22 monoclonal antibodies react. Injection of CSAT or JG22 perturbs cranial neural crest cell migration in vivo (Bronner-Fraser, 1985; 1986b).

3.7 OTHER FAMILIES OF MORPHOREGULATORY MOLECULES

3.7.1 CALCIUM-DEPENDENT CELL ADHESION: CADHERINS

Three members of the cadherin gene family have been characterized to date: E-cadherin, N-cadherin, and P-cadherin, named after the tissues from which they were first isolated: epithelial (E), neural (N), and placental (P) tissues (Table 8; Nagafuchi et al., 1987; Nose et al., 1987; Takeichi et al., 1987; for review see Takeichi, 1988).

Three lines of evidence indicate that cadherins actually participate in cell-cell binding. 1) Transfection of fibroblasts with cDNA clones encoding E-cadherin or its chicken homolog, L-CAM, produces calcium-dependent adhesive properties that can be inhibited by anti-E-cadherin or anti L-CAM antibodies (Edelman et al., 1987). 2) Soluble E-cadherin fragments inhibit the adhesion of epithelial cells (Wheelock et al., 1987). 3) Anti-cadherin antibodies disrupt epithelial and other early embryonic tissues

(Hyafil et al., 1983; Duband et al., 1987). These findings are consistent with a homophilic mechanism of cadherin binding.

Analysis of the primary sequence of the cadherins provides a possible basis for the calcium-dependency of cadherin-mediated cell adhesion. The extracellular domain of E-cadherin contains conserved sequences that resemble the presumptive calcium-binding domains of other proteins (Ringwald et al., 1987). In the absence of calcium there appears to be a conformational change in the structure of the cadherins that renders them susceptible to proteolytic degradation (Hyafil et al., 1983; Yoshida-Noro et al., 1984).

The occurrence of calcium-dependent aggregation of some cells and tissues that do not express any of the three identified cadherins, points at other, not yet identified members of the vertebrate cadherin gene family. Moreover, cadherins may be expressed by embryonic cells in invertebrates. The Drosophila lethal (2) giant larvae gene encodes a 127-130 kDa protein (Jacob et al., 1987; Lutzelschwab et al., 1987) that is homologous to E-cadherin and many adhesive proteins. Deletions of this gene result in malignant transformation of neuroblasts, raising the possibility that cadherins are involved in the regulation of cell proliferation and differentiation as well as cell adhesion.

3.7.2 FIBRONECTINS

Two major forms of fibronectin have been distinguished: a soluble dimeric form that is synthesized by hepatocytes and then secreted into the bloodstream (plasma fibronectin), and a dimeric or cross-linked multimeric form, made by many cell types including fibroblasts and epithelial cells, which after secretion can be deposited as long insoluble fibrils in the ECM (cellular fibronectin).

Despite their heterogeneity, all fibronectin subunits show a common modular organization in their primary sequence, which contains a series of homologous repeating units, the so-called homology types I, II and III (about 40, 60 and 90 amino acids in length, respectively (Petersen et al., 1983). In addition to these structural similarities, the various forms of fibronectin subunits share common functional properties that correspond to domains for binding other ECM macromolecules and the cell surface. Each subunit bears two binding sites for heparin, two for fibrin, one for collagen, and at least one site for binding to the cell surface (reviewed by Ruoslahti, 1988; Dufour et al., 1988b).

Several additional cell-adhesion sites have been mapped along fibronectin molecules. First, a binding site located upstream of the RGD binding site has been identified by deletion and site-directed mutagenesis (Obara et al., 1988). Secondly, two other binding sites have been mapped in a distant region of the fibronectin molecule. A tetrapeptide Arg-Glu-Asp-Val (REDV) maps to the C-terminal portion of the type III connecting sequence (IIICS). The other site is located in the N terminal portion of the IIICS region and may function in an additive manner with the REDV binding site (Humphries et al., 1988).

In vivo, the migration of neural crest cells is correlated with the presence of fibronectin (Thiery and Duband, 1986; Brauer and Markwald, 1988). In vitro, fibronectins constitute the most suitable substrate for displacement of neural crest cells (Newgreen et al., 1982; Rovasio et al., 1983; Tucker and Erickson, 1984; for review see Dufour et al., 1988). Direct interaction between fibronectin and its 140 kDa receptor, integrin, is required for neural crest cell migration both in vitro and in vivo (Rovasio et al., 1983; Boucaut et al., 1984; Bronner-Fraser, 1986b; Duband et al., 1986). In addition, RGD-containing decapeptides used as competitors, can perturb neural crest cell migration both in vivo and in vitro, indicating that, besides its role in cell attachment and spreading, this sequence is also involved in cell motility (Boucaut et al., 1984).

3.8 CONCLUSION

In this chapter the literature concerning some morphoregulatory molecules of the HNK-1 family was reviewed. Most known molecules of the HNK-1 family are related to neural crest cell determination, individualization, migration, and homing. In chapter 4, the expression of these morphoregulatory molecules will be related to morphogenetic events that are involved in the formation of the ENS.

CHAPTER 4: THE FORMATION OF THE ENTERIC NERVOUS SYSTEM

4.1 INTRODUCTION

The nonneural tissues of the avian bowel (i.e. endoderm lining, muscle, loose connective tissue) derive from endoderm and mesodermic cells. The tubular bowel is formed by lateral infolding of the endoderm and the splanchnic mesodermal epithelium at both ends (for figure see under 4.5). The resulting bowel tube, therefore consists of a lumen surrounded by concentric endoderm and splanchnic epithelia. The mesenchymal cells of the bowel arise by a process called de-epithelialization, during which some of the cells migrate out of the outer epithelium, and form a loose mesenchyme between the outer and the endoderm epithelium. These mesodermically-derived splanchnic mesenchymal cells later give rise to the muscle and other connective tissue elements of the bowel.

4.2 ORIGIN OF ENTERIC NEURONS

The origin of enteric neurons has long been a vexed question. The older literature encompassing the derivation of enteric neurons has been reviewed by Abel (1912) and VanCampenhout (1930). Over the years, several sources have been suggested for enteric neurons: for instance, endoderm (Masson and Berger, 1923) and the local mesoderm (Camus, 1921; Tello, 1924, 1925; Keuning, 1944; 1948). This work has been reviewed by Andrew (1971).

It is now well established that enteric neurons derive from the neural crest, but there is no consensus of opinion about the level of the neural axis from which they derive. Neural crest ablation studies indicate that enteric neurons derive from the vagal neural crest (Yntema and Hammond, 1954). A vagal neural crest source for enteric neurons is also supported by experiments in which the vagal neural primordium of chicken embryos was exchanged with the quail neural primordium (Le Douarin and Teillet, 1973). In these experiments, quail enteric neurons were found along the entire bowel.

In an analogous experiment, Le Douarin and Teillet (1973) transplanted the quail neural primordium to the lumbosacral neural primordium of chicken embryos. After the incubation period, they observed some quail enteric neurons up till the umbilicus. They concluded that the sacral neural crest was the source of enteric neurons in the hindgut. The sacral neural crest cells, originating from behind the 28th somite level give also rise to Remak's ganglion but they are not found in the hindgut before the seventh day of incubation (E7).

To determine the role of the sacral neural crest cells more precisely, Allan and

Newgreen (1980) explanted bowel segments at various developmental stages and cultured these on the chorioallantoic membrane. They were not able to visualize neural crest cells at the time of explantation and argued that the neurons observed after one week culture would have been derived from neural crest cells that were present in the explant. They observed that neural crest cells colonize the bowel in a craniocaudal direction. Their experiments could not exclude that sacral neural crest cells colonize the hindgut in vivo, since they removed the bowel explants relatively early, thus preventing the colonization of sacral neural crest cells by E7.

Appendix paper 5 reports experiments that allowed for the colonization by sacral neural crest cells, but prevented the craniocaudal migration of vagal neural crest cells. We transected the embryonic bowel in front of the vanguard of migrating neural crest cells at early stages of development. The bowel was left in situ and the eggs were incubated until E12. We did not find enteric neurons in the hindgut. In conclusion: experimental data indicate that in avian embryos the sacral neural crest on its own is not able to give rise to a normal ENS.

4.3 INDIVIDUALIZATION OF NEURAL CREST CELLS

Figure 4 shows the map of a primitive three layered chicken embryo. The region of ectoderm that gives rise to the neural crest is characterized by the expression of the following cell adhesion molecules: E-cadherin (L-CAM), N-CAM and N-cadherin (Thiery et al., 1984). E-cadherin is lost permanently from neural crest cell ancestors during early neurogenesis (Thiery et al., 1985). The neural crest cells that begin their migration do not express N-cadherin or N-CAM. They are thus demarcated from the overlying ectoderm (which expresses E-cadherin) and from the neural tube (which expresses N-cadherin). Neural crest cells maintain this situation during migration. The reduction in the expression of CAMs coincides with a reduction of intercellular junctions, such as gap and tight junctions, in premigratory neural crest cells (Revel and Brown, 1975). The neural crest cells acquire a mesenchymal form with numerous probing filopodia (Newgreen and Gibbins, 1982; Tosney, 1982) that are able to interact with the ECM components besides those of the basal lamina. During the migratory phase, N-CAM is reduced at the surface of neural crest cells (Thiery et al., 1982) while N-cadherin expression is undetectable during neural crest cell migration, but both CAMs are reexpressed in most sites of neural crest cell aggregation (Hatta and Takeichi, 1986; Hatta et al., 1987; and see figure 5).

4.4 CHARACTERISTICS OF VAGAL NEURAL CREST CELLS

The vagal neural crest contains other cell populations than caudal levels of the neural crest. For instance, when truncal neural crest from a quail embryo is grafted to the vagal region of a chicken embryo, quail enteric neurons are found in ganglia in the

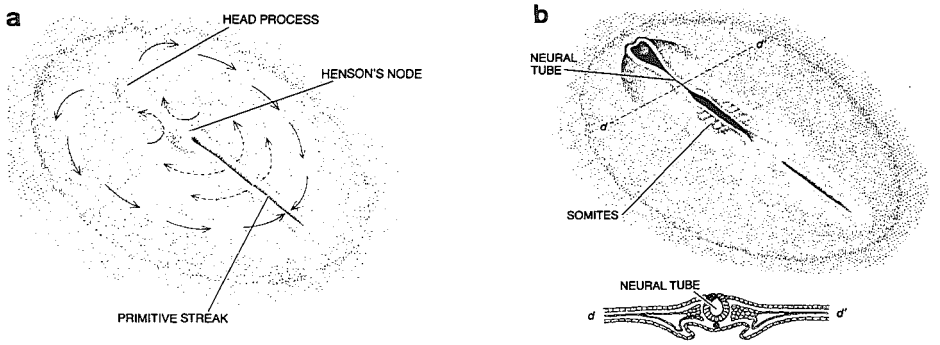
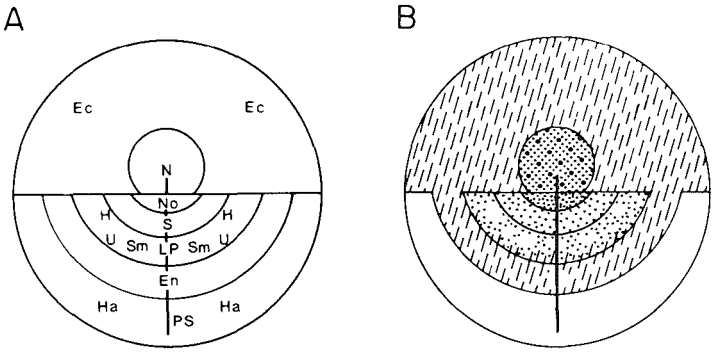


Figure 4: (I) Key morphogenetic events in the early development of the chicken embryo are gastrulation (A) and neurulation (B). In late gastrulation cells from the top layer of the blastoderm converge toward the central groove (solid arrows) called the primitive streak; movements of the mesoblast (middle layer) cells are shown by broken arrows (A). In primary (neural) induction, a key event, interactions between mesoderm and endoderm result in the formation of the neural plate. The plate folds to form the neural tube; soon afterward mesodermal cells segregate to form the segmented precursors called somites (B).



Fate map based of the blastodisc (Vakaet, 1984) showing areas of cells that will give rise to different tissues (indicated by letters; see below for designations). (B) Map of cells that will express CAMs. The distribution of N-CAM (dotted area) or L-CAM (slashed area) in tissues at 5-14 days as determined by immunofluorescence staining is mapped back onto the blastodisc fate map. Cells that will give rise to the urinary tract (U) express both L-CAM and N-CAM. Smooth muscle (Sm) and hemangioblastic (Ha) tissues express neither N-CAM nor L-CAM; areas giving rise to these tissues are blank on this map. Ng-CAM is represented on neuroectoderm by (•) symbols; it is seen only after three days. The vertical bar represent the primitive streak (PS). Other abbreviations: Ec = intraembryonic and extraembryonic ectoderm; En = endoderm; H = heart; LP = lateral plate (splanchno-somatopleural mesoderm); N = nervous system; No = precordal and chordamesoderm; S = somite.

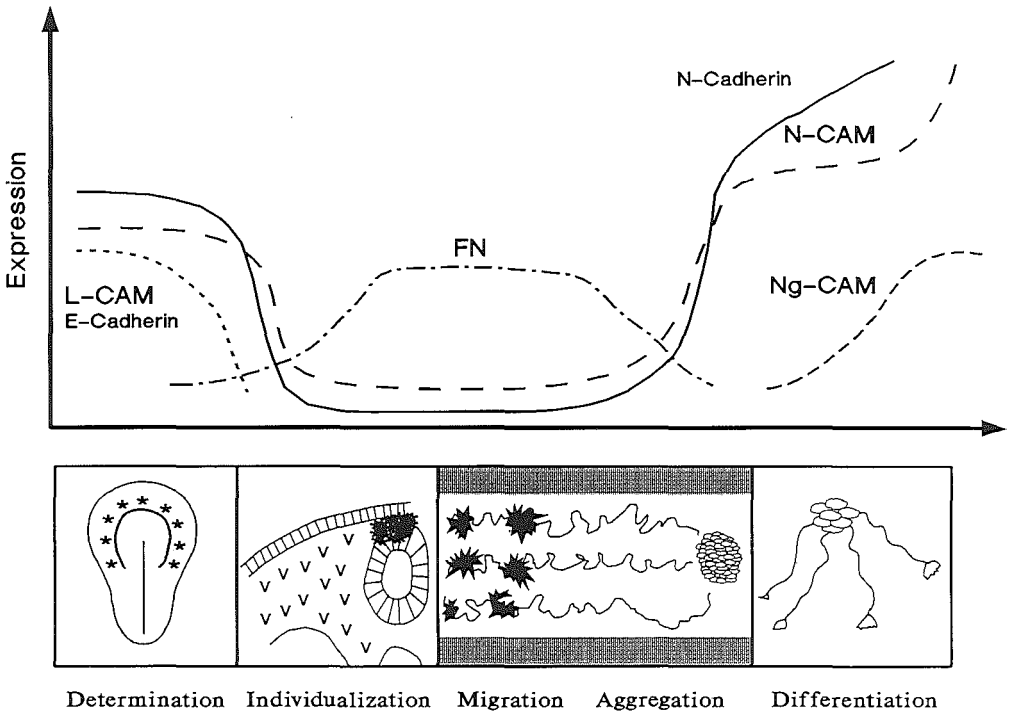


Figure 5: Diagram summarizing neural crest cell appearance, migration and differentiation as related to the expression of morphoregulatory molecules. Prior to neural induction, presumptive neural crest cells express both L-CAM (E-cadherin), N-cadherin and N-CAM. At the time of their separation from the neural tube, neural crest cells first lose the adhesion mediated by L-CAM and then progressively that mediated by N-CAM and N-cadherin to acquire much greater adhesivity to fibronectin during their migration. When neural crest cells aggregate into ganglion primordia, the ratio of adhesion to fibronectin versus N-CAM and N-cadherin is inverted. The aggregation phase is soon followed by differentiation into neurons by the appearance of Ng-CAM and neurofilaments.

preumbilical bowel. Some quail cells are found in the postumbilical bowel, but they are exclusively melanocytes and never participate in ganglion formation (Le Douarin and Teillet, 1974). Such melanocytes do not appear in the bowel following isotopic and isochronic transplantation of quail vagal neural crest into the chicken embryo, nor in the bowel of normal quail embryos. These melanocytes are always found in two regions: the one lining the inner surface of the circular smooth muscle layer, the other in contact with the outer surface of the circular smooth muscle layer. While presumptive neural tissue needs precise conditions for differentiation, melanocytes do not appear to have such requirements.

On the other hand, when vagal neural crest cells from quail embryos are

transplanted to the region of somites 18-24, they migrate massively to the adrenal gland, but some colonize the bowel (Le Douarin and Teillet, 1974). Quail cells are never found in the bowel after orthotopic transplantation of truncal neural primordium. Therefore, chemical attractions must play a role in the final localization of neural crest cells.

Vagal neural crest cells colonize the ventrolateral region of the pharynx and have a wide range of developmental capabilities: some of them give rise to mesenchymal derivatives, others differentiate into Schwann cells, carotid body cells, calcitonin-producing cells and (enteric) neurons (Hammond and Yntema, 1964; Johnston, 1966; Le Douarin and Le Lievre, 1971; Le Douarin et al., 1972; Pearse et al., 1973; Le Douarin et al., 1974; Le Lievre and Le Douarin, 1973, 1974; Le Lievre, 1974; Le Douarin and Teillet, 1973; Ciment and Weston, 1983; Kirby, 1983; Bockman and Kirby, 1985).

Although the migration and fates of neural crest cells have been mapped with great precision, little is known about the potentials of individual cells or the processes involved in their diversification. At the time they begin migration, all neural crest cells look alike and there are few markers that distinguish any characterized subsets. A clonal analysis of neural crest cells is necessary to determine whether neural crest cells are multipotential or restricted in their differentiation. Two approaches are interesting in this respect: in vitro culture of neural crest cells on 3T3 feeder cells (Baroffio et al., 1988) and the injection of vital fluorescent dyes into single dorsal cells in the neural tube (Bronner-Fraser and Fraser, 1988).

4.5 MIGRATION OF VAGAL NEURAL CREST CELLS

Chicken neural crest cells begin their migration first at the level of the mesencephalon (Holmdahl, 1928; DiVirgilio et al., 1967; Noden, 1975, 1978; Newgreen and Thiery, 1980; Duband and Thiery, 1982; Tosney, 1982). The first neural crest cells leave the mesencephalon as the folds touch, whereas in other cranial regions, the neural crest is still continuous with the epidermis neural fold, when apposition is nearly complete (Tosney, 1982). This process extends more rostrally, and more caudally from the rostral rhombencephalon through the trunk region (Holmdahl, 1928; Bancroft and Bellairs, 1976; Tosney, 1978; Chevallier, 1972; Thiery et al., 1982).

The first postotic cranial and trunk level neural crest cells migrate not until the neural folds have fused completely and the epidermal ectoderm has separated from the neural anlage (Bancroft and Bellairs, 1976; Thiery et al., 1982; Tosney, 1978). The wave of onset of neural crest dispersion at somitic levels does not exactly parallel the wave of segmentation of the somites, but is more closely related to the epithelio-mesenchymal transformation of the sclerotome. The time span between the first and the last cells to emigrate from the same axial level varies from about 4-6 hours in cranial levels to over 24 hours at trunk levels.

Neural crest cells from the level of somites 1 to 5 give rise to enteric neurons, whereas those neural crest cells originating at the level of somites 6 and 7 also

differentiate into sensory and sympathetic neurons. Figure 6 shows the migration pathways in the vagal region of the chicken embryo (Thiery et al. 1982).

HNK-1 positive vagal neural crest cells that give rise to the cephalic and branchial skeleton, the dermis and other connective tissues, loose the HNK-1 epitope, whereas HNK-1 positive vagal neural crest cells that colonize the developing bowel retain their reactivity as they become organized in the enteric ganglia. Most HNK-1 positive neural crest cells migrate in a transient space between the ectoderm and the somites. Others can also be found along the ventral aspect of the myotome. These two streams lead neural crest cells to the vicinity of the splanchnopleure, near the visceral arches. While most neural crest cells accumulate in the visceral arches and contribute to the mesectodermal derivatives of the neck and to cranial sensory ganglia, a few neural crest cells are seen between the endoderm and the splanchnopleural mesoderm at the time of the formation of the foregut. Noden (1983) found that the primordium of the entire length of the esophagus and crop is localized beneath the cranial occipital somites at the neurula stage.

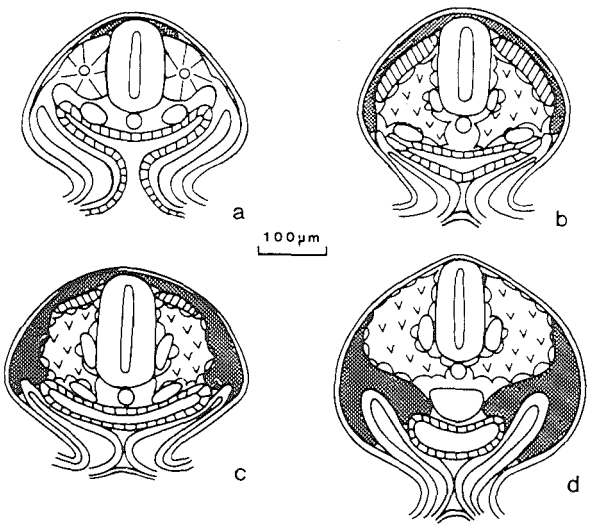


Figure 6: Vagal neural crest cell migration pathways. (a) 11-somite stage: neural crest cells initiate their migration under the ectoderm. (b) 14-somite stage: Within 3hr, neural crest cells occupy a narrow space between the dissociated somite and ectoderm. The apparent rate of migration is close to 100 um/hr. (c) 18-somite stage: Neural crest cells have reached the apex of the gut and become subdivided into two streams. (d) 28-somite stage: The last neural crest cells are separated from the neural tube by the expanding sclerotome. The number of crest cells increases rapidly, while they penetrate between the splanchnopleural mesoderm and the endoderm (the two components of the gut) and between the somatopleural mesoderm and ectoderm. From Thiery et al., 1982.

4.6 ENTERIC NEURAL CREST CELL MIGRATION

The progression of neural crest cells within the bowel follows the wave of de-epithelialization of the splanchnopleural mesoderm. At least in the foregut, neural crest cells remain close to the serosa. Caudal to the yolk stalk neural crest cells encounter the differentiating smooth muscle layer and become distributed on both sides (see figure 7).

It is important to note that the bowel is elongating rapidly when neural crest cells progress more caudally. Neural crest cell colonization of the bowel may result both from an active migration and passive displacement. We observed that enteric neural crest cells proliferate during migration, and continue to do so after aggregation (Appendix paper 9). It seems that the vanguard of migrating neural crest cells proliferates intensely and is mainly responsible for the progression of crest cells (Duband et al., 1985).

4.7 HOMING OF ENTERIC NEURAL CREST CELLS

There is no obvious reason for the arrest of neural crest cell migration, since the ECM does not disappear and no morphological barriers are interposed ahead of them. As assessed by the presence of N-CAM on their surface, neural crest cells aggregate immediately behind the leading edge of migration and they even start growing neurites (Crossin et al., 1985; Duband et al., 1985; Aoyama et al., 1985).

We studied the homing of neural crest cells in the hindgut. Aneuronal bowel was isolated and cultured on the chorioallantoic membrane, either on its own, or in combination with the neural primordium. When aneuronal bowel was cultured on its own, HNK-1 immunocytochemistry revealed a layer of HNK-1 immunoreactive mesenchymal cells at the site where the submucous plexus would have been. Neural crest cell colonization changed the pattern of HNK-1 immunoreactivity completely; HNK-1 immunoreactive neural crest cells and enteric neurons were observed, whereas the mesenchymal cells had lost the HNK-1 epitope. We surmise that HNK-1 immunoreactive mesenchymal cells constitute the target for migrating neural crest cells, and that HNK-1 antigen(s) present on the neural crest cells interact with the HNK-1 antigen(s) of the subpopulation of mesenchymal cells. Preliminary findings indicate that the mesenchymal cells express one 42 kDa and one 44 kDa glycoprotein. The HNK-1 immunoreactive mesenchymal cells probably derive from the splanchnopleural ectoderm.

4.8 EFFECT OF THE ENTERIC MICROENVIRONMENT ON NEURAL CREST CELL PHENOTYPES

There is a sequential development of different neuronal phenotypes. Enteric neural crest cells are able to express transiently a catecholaminergic phenotype, although they do not do so in vivo. This ability can be revealed by dissociating embryonic bowel and culturing the enteric neural crest cells in vitro, outside the enteric microenvironment. Both in birds and mammals, the expression of the catecholaminergic phenotype is lost upon the

arrival of sympathetic nerves in the bowel (Rothman et al. 1987).

Cochard and Le Douarin (1984) and Gershon and co-workers (1984) reviewed the development of diverse differentiated enteric neuronal phenotypes. Enteric neurons with cholinergic properties appear first (Cantino, 1970; Keller, 1976; Smith et al., 1977) followed by presumed precursor neurons which take up serotonin (Epstein et al., 1980; Gershon et al., 1980) and then by peptide-immunoreactive neurons (Sundler et al., 1979; Fontaine-Perus et al., 1981; Saffrey et al., 1982) and adrenergic nerves (Bennet and Cobb, 1969; Enemar et al., 1965). It might well be that the enteric mesenchyme determines the region-specific phenotypes of neural crest cells. Perris and co-workers (1988) reported that at the axial level local embryonic matrices also determine region-specific phenotypes in neural crest cells.

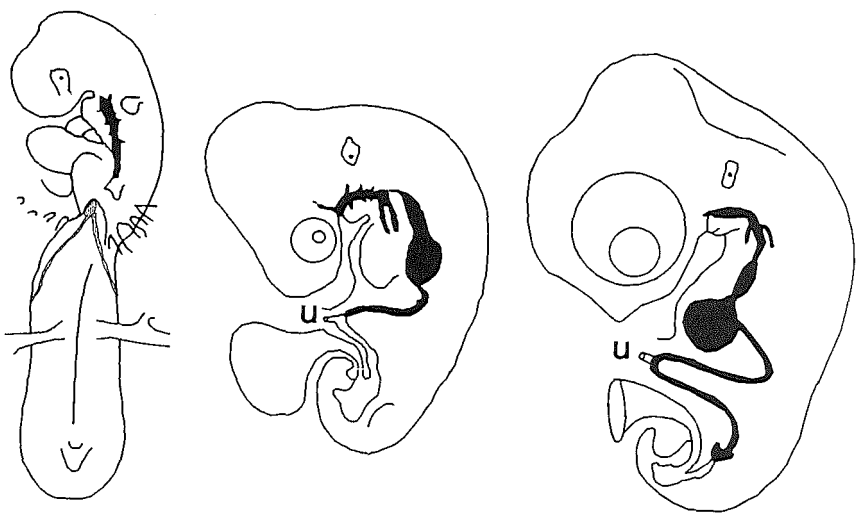


Figure 7: Colonization of the bowel by vagal neural crest cells. Black areas correspond to the levels reached by migrating neural crest cells. (a) Stage 16 (E3): neural crest cells are distributed mainly in the dorsal part of the gut at the level of the developing visceral arches. (b) Stage 26 (E5): neural crest cells reach the umbilicus. (c) Stage 28 (E6): vagal neural crest cells colonize the cecal appendages. u = umbilicus.

Both caudocranial and craniocaudal expression sequences have been reported for acetylcholinesterase activity (Keller, 1976), uptake of radioactively labelled serotonin (Gershon et al., 1980), and for vasoactive intestinal peptide- (Saffrey, 1982), and

neurofilament immunoreactivity (Payette et al., 1984). In contrast, single craniocaudal expression sequences have been reported for vasoactive intestinal peptide, substance P, Met-enkephalin, choline acetyl transferase (Fontaine-Perus et al., 1981; Saffrey et al., 1982; Smith et al., 1977).

4.9 ORIGIN AND DIFFERENTIATION OF THE GANGLIONATED NERVE OF REMAK

The ganglionated nerve of Remak, a structure peculiar to birds, is a nerve trunk belonging to the autonomic nervous system, running parallel to the bowel, within the mesentery and the mesorectum. First described by Remak in 1847 as the "nervus intestinalis", it extends from the duodenal-jejunal junction, where it is connected with the coeliac plexus, to the cloaca, where it joins with the pelvic plexus (Nolf, 1934). In addition, the posterior region connects with the aortic plexus and the paravertebral sympathetic trunks (Van Campenhout, 1931; Jones, 1942; Hammond and Yntema, 1947; Browne, 1953). It appears at E4 in the chick in the mesorectum (His, 1897; Kuntz, 1910) when no ganglion cells are yet present in the mid- and hindgut (Abel, 1912; Van Campenhout, 1931).

From studies in quail-chick chimeras it was concluded that the neurons of Remak's ganglion derive from the lumbosacral neural crest located behind the level of the 28th somite (Teillet, 1978). These data are in agreement with the findings of Yntema and Hammond (1953 and 1955), who showed that extirpation of the lumbosacral neural crest resulted in deficient formation of the ganglion of Remak and the pelvic plexus.

In quail-chicken chimeras, the early primordium of the ganglion of Remak contains some cells which have migratory capabilities and invade the hindgut wall where they participate in formation of enteric ganglia. However, the contribution of the lumbosacral neural crest to the enteric ganglia, via the ganglion of Remak, is not significant.

4.10 COMPARISON OF ENS FORMATION IN VARIOUS SPECIES

Recently Copenhaver and Taghert (1989) described the development of the ENS in *Manduca Sexta* (the moth). In the moth the ENS supplies the innervation to the visceral musculature and includes two small ganglia and more distributed groups of neurons that are positioned along the surface of the intestinal tract. Previous studies have shown that most enteric neurons in insects are derived from specific zones within the foregut epithelium (Schoeller, 1964; Campos-Ortega and Hartenstein, 1985; Penzlin, 1985). Between 40 and 65 per cent of embryogenesis the cells of this packet of foregut epithelium become progressively dispersed by a sequence of migratory events: an initial, slow phase of migration that is circumferentially directed around the foregut, and a rapid, dispersing phase by which the enteric plexus cells achieve their mature distributions across the foregut and midgut surface (Copenhaver and Taghert, 1989).

Experimental data indicate that in avian species, too, there are different phases in neural crest cell colonization of the bowel. Vagal neural crest cells reach the region of the branchial arches via migratory pathways that are rich in fibronectin (Thiery and Duband, 1986). Enteric neural crest cells are able to express transiently a catecholaminergic phenotype, although they do not do so in vivo. This ability can be revealed by dissociating the bowel and growing the enteric neural crest cells in vitro, outside the enteric microenvironment. Both in avians and mammals, the expression of the catecholaminergic phenotype is lost upon the arrival of sympathetic nerves in the bowel (Rothman et al., 1987). Neural crest cell migration through the bowel seems not to depend on fibronectin (Tucker et al., 1986). This suggests that different mechanisms control neural crest cell migration at the axial level and through the bowel. We observed that enteric neural crest cells proliferate during migration, and continue to do so after aggregation (*Appendix paper 8*). Aggregated neural crest cells express NF triplet proteins and neuron-specific enzymes (neuron specific enolase, AChE, choline acetyltransferase) and various neurotransmitters (serotonin, Vasoactive Intestinal Peptide (VIP), acetylcholine). Moreover, neural crest cells differentiate into enteric glial cells as is shown by GFAP immunoreactivity.

It has been suggested that ENS formation in birds differs from that in mammals. In chickens, the foregut is colonized long before the hindgut, but Rothman and Gershon (1982) suggested that in mice foregut and hindgut are colonized simultaneously at E9. Our findings (described in appendix paper 3) argue against this supposed difference in ENS formation, and consequently also against the supposition that it takes longer for murine neural crest cells to acquire the differentiated neuronal phenotype.

Several multigene families seem to regulate vertebrate development (for review see Dressler and Gruss, 1988; Duboule et al., 1989). One of these multigene families comprises the homeobox-containing genes (for review see Gehring, 1987). The expression of several of these homeo-box containing genes has been detected in neural crest cells (for review see Holland and Hogan, 1988a). Recently, Holland and Hogan (1988b) reported Hox-2.1 homeo-gene transcripts in autonomic ganglia derived from the neural crest. Gaunt et al (1989) suggested that homeo-gene transcripts may provide positional cues in neural crest cells. In support of this notion is the report of Robert and co-workers (1989) and Hill and co-workers (1989) who cloned the Hox-7 gene and found that this gene is expressed in the neural crest, developing limb bud, and visceral arches of E9.5 embryos. In older embryos, the gene is expressed in regions of the face that are derived from the neural crest and in the interdigital mesenchymal tissues in both the fore- and hind limbs. In addition, another homeobox-containing gene Hox-1.4 is expressed in enteric mesenchyme. Wolgemuth and co-workers (1989) reported that overexpression of the Hox-1.4 gene in transgenic mice is correlated with the development of megacolon.

The development of the ENS in other vertebrate species has barely been characterized. With regard to rats, the lack of markers for migrating neural crest cells

necessitated the use of organotypic tissue culture for growing embryonic bowel (Ito et al., 1977). AChE-positive enteric neurons were detected after 5 days organotypic tissue culture of dissociated distal E12 rat colon. Kamagata and Donahoe (1985) found AChE-positive enteric neurons in situ in the small bowel of E13.5 embryos, but they did not observe AChE positive neurons in the colon until E15.5. Enteric neural crest cells also transiently express the enzyme TH (from E11.5 to E13.5) (Teitelman et al., 1979). These TH-positive cells are predominantly located in the distal esophagus, the stomach, and the proximal small bowel (Cochard et al., 1978). The TH expression coincides with a CA phenotype.

In guinea pigs, neuritic islands were first recognized in the outer gut mesenchyme of the small bowel at E25 (Gershon et al, 1981a). Before the neuritic islands could be recognized, there were already (neural crest?) cells that specifically take up ^{3}H-5HT at E20. Neuronal cells that synthesized ^{3}H-acetylcholine from ^{3}H-choline, or specifically bound opiates were detected at E25 (Gintzler et al., 1980).

The characterization of the development of the human ENS is still incomplete due to the scarcity of fetal material. Using Bodian's technique of silver impregnation, Hüther (1954) observed both a craniocaudal and a caudocranial sequence of the staining of enteric neurons; he observed neuroblasts in the rectosigmoid from the 7th week of gestation. In contrast, Okamoto and Ueda (1967) only observed a craniocaudal sequence of neuroblasts, using a similar technique. The acquisition of a mature neuronal phenotype occurred in a similar way as in mice (Bryant et al, 1982; Griffith and Burnstock, 1983; Tam and Lister, 1986).

4.11 CONCLUSION

In this chapter the literature concerning ENS formation was reviewed. The experimental evidence indicates that enteric neurons derive from the vagal neural crest. The migration process of vagal neural crest cell to and through the developing bowel is mediated by morphoregulatory molecules, such as N-CAM, E-cadherin, N-cadherin, fibronectin. Within the bowel, migrating neural crest cells recognize and adhere to some specialized mesenchymal cells, hereby initiating aggregation. The morphoregulatory molecules of the HNK-1 family that are present on migrating neural crest cells have to be defined. Neural crest cell colonization occurs in distinct phases, and this colonization pattern is encountered in diverse vertebrate species (chick, mouse, rat and man); likewise, neural crest cell differentiation in diverse species follows one general pattern to acquire a mature neuronal phenotype.

CHAPTER 5: INTRODUCTION TO THE EXPERIMENTAL WORK

5.1 INTRODUCTION TO APPENDIX PAPERS

To clarify pathogenetic mechanisms of congenital malformations of the ENS, the formation of the ENS was investigated in chicken and murine embryos. The experimental work was concentrated on several aspects of the interaction between neural crest cells and the enteric microenvironment. We investigated (a) whether enteric neural crest cells are sensitive to ischemia; (b) whether the migration substrates in aneuronal bowel persist when neural crest cell colonization is postponed; (c) when neural crest cells colonize various parts of murine bowel; (d) when and where the ls/ls gene affects neural crest cell colonization; (e) which axial level of the neuraxis provides precursor cells for enteric neurons; (f) how neural crest cells are directed to the future sites of the enteric ganglia; (g) whether neural crest cells proliferate during, migration and homing in the bowel.

Hirschsprung's disease has a heterogeneous etiology that has been related to genetic, chromosomal, and other yet unknown factors. Family studies suggest genetic factors in the etiology of Hirschsprung's disease but there is little evidence of simple Mendelian inheritance. It seems that Hirschsprung's disease is an example of sex-modified multifactorial inheritance.

The importance of environmental factors in the etiology of intestinal atresia and stenosis has been extensively studied (Louw, 1952, 1964; Nixon, 1955). Experimental and clinicopathologic observations support the view that atresia and stenosis may result from intestinal ischemia due to a uterine vascular accident (e.g. volvulus or intussusception).

An ischemic cause was also sought for Hirschsprung's disease in the early 1960s. Pathologic evidence of vascular involvement was provided by Lister (1966). He described changes in the blood vessels to the transitional zone in Hirschsprung's disease, resembling those found in periarteritis. Microscopy revealed hamartomatous changes in the wall of the vessels, sometimes with an obliterative endarteritis, which possibly might have caused impairment of the vascular supply to the afflicted bowel segment.

Earlier experimental work of Cannon and Burket (1913) indicated that the ENS is more sensitive to ischemia than the other tissues of the bowel wall. This supposition was tested experimentally by a number of other investigators (Hukuhara et al., 1961; DeVilliers, 1966; Okamoto et al., 1967; Pompino, 1969). Their studies did not result in a consensus of opinion concerning the sensitivity of enteric neurons to intestinal ischemia. Earlam (1972, 1985) proposed a theory that explained that the development of aganglionosis, stenosis, and atresia depended on the degree of ischemia. This theory

rests on the assumption that fetal enteric neurons or their precursor neural crest cells are as sensitive to ischemia as their adult analogues.

We investigated experimentally the effect of ischemia on enteric neurons or their precursor neural crest cells by ligating mesenteric vessels in chicken embryos. The experiments are described in *appendix paper 1*. We found that ischemia of the small bowel resulted in stenosis or atresia, but not in a selective disappearance of enteric neurons. To extrapolate these findings to the effect of ischemia on neurons in the hindgut, we compared tissue sections of patients with small-bowel atresia and colonic atresia. Extensive ischemic changes were found in the bowel wall in both types of atresia, but no selective disappearance of enteric neurons was found. We concluded that ischemia does not result in a selective disappearance of enteric neurons.

Webster (1973) compared ENS development in the mutant piebald lethal mouse strain and control mice. He concluded that in piebald lethal mice neural crest cells migrate slower than the bowel elongates. This implies that when neural crest cells reach the hindgut, they encounter bowel wall in advanced stages of differentiation. The differentiated target organ would impede neural crest cell colonization, leaving the distal hindgut aganglionic.

We tested this premise experimentally in chicken embryos, by coculturing the neural primordium of E2 chicken embryos with aneuronal bowel of different stages of development. The various aneuronal bowel segments were obtained by growing explants of aneuronal E4 bowel for different periods. These experiments are described in *appendix paper 2*. We found that neural crest cells colonized aneuronal bowel, even if the aneuronal bowel segment was well differentiated. This implies that the migration substrates in the bowel persist when neural crest cell colonization is delayed.

The formation of enteric ganglia has been most extensively studied in avian embryos (chapter 4). There are few reports on ENS development in higher vertebrates. Some authors suggest important differences in ENS development in avian and mammalian embryos. Therefore, we investigated neural crest cell colonization of murine bowel. These experiments are described in *appendix paper 3*. We found that there are three distinct phases in neural crest colonization of murine bowel. At an early embryonic stage (E9) a major part of bowel is colonized (phase I). During E10 to E13 the innervated bowel segment elongates. This elongation is not due to craniocaudal migration of neural crest cells, but to a combination of neural crest cell proliferation and longitudinal growth of the bowel (phase II). During E13 and E14, neural crest cells colonize the remaining distal 2 mm of the colon (phase III).

We studied the development of aganglionosis in mice by comparing neural crest cell colonization of the bowel in mutant ls/ls and control mice. These experiments are described in *appendix paper 4*. We found that in ls/ls mice neural crest cell colonization of the bowel proceeds as in control mice until E13. We did not obtain data that show that neural crest cells of ls/ls mice migrate at a lower speed. Only colonization phase III was defective, however. It might well be that neural crest cell migration through the

bowel requires different subsets of morphoregulatory molecules in the various bowel segments and that in ls/ls mice the subset for the colonization of the distal colon is defective.

In our experiments described in appendix paper 3 we were not able to show whether sacral neural crest cells colonize the hindgut during phase III. The contribution of the sacral neural crest to enteric neurons in the hindgut is also controversial in avian embryos. To determine whether the sacral neural crest is involved in avian ENS development, we prevented craniocaudal migration of vagal neural crest cells by transecting postumbilical bowel in ovo at early stages of development, thus allowing for colonization by sacral neural crest cells until E12. These experiments are described in *appendix paper 5*. Immunohistological and ultrastructural investigations did not reveal enteric neurons in the postumbilical bowel and therefore we concluded that enteric neurons only derive from the vagal neural crest. Immunohistological investigation using HNK-1 antibodies of bowel specimen that had been transected at E4, did not reveal enteric neurons, but a layer of HNK-1 positive cells at the site where the submucous plexus was to develop, and HNK-1 positive neuron-free, myenteric plexuses.

In view of the adhesive properties of the HNK-1 epitope (chapter 2) we investigated the role of the layer of HNK-1 immunoreactive cells. We grafted postumbilical bowel segments of E4 embryos to the chorioallantoic membrane. After one week culture the layer of HNK-1 immunoreactive cells was present at the same site as after bowel transection. The HNK-1 positive neuron-free, myenteric plexuses were not present. The role of the HNK-1 immunoreactive enteric mesenchyme was investigated in cocultures of aneuronal embryonic bowel and the vagal neural primordium. These experiments are described in *appendix paper 6*. We found that the HNK-1 immunoreactive enteric mesenchymal cells had disappeared after coculture with the vagal neural primordium, and that submucous enteric ganglia had formed. We surmise that these cells constitute the target for migrating enteric neural crest cells.

We wondered whether the HNK-1 positive neuron-free, myenteric plexuses also could be populated by neural crest cells. Again we used the chorioallantoic membrane as the culture environment to coculture neural primordium from the vagal region and aneuronal bowel from an embryo of 12 days of development that had undergone bowel transection at day 4. These experiments are described in *appendix paper 7*. We found that quail neural crest cells were more invasive compared to the chicken counterparts. Quail neural crest cells were found at the site of the myenteric plexus in 50 percent of the cocultures whereas chicken neural crest cells did not colonize this region at all.

Since the number of neural crest cells that enter the bowel is relatively small (1000 to 10,000), a great deal of proliferation must occur during the formation of the enteric ganglia to expand the population of colonizing neural crest cells. This premise was substantiated by pulse labelling E4 to E8 chicken embryos with radioactive thymidine. These experiments are described in *appendix paper 8*. By combining the immunocytochemical detection of neural crest cells with the visualization of tritiated

thymidine by radioautography, we demonstrated that neural crest cells proliferate during their migration through the bowel and even for a certain period after plexus formation.

5.2 CULTURE OF EMBRYONIC BOWEL ON THE CHORIOALLANTOIC MEMBRANE

The use of the chorioallantoic membrane of the developing chick as a substratum for grafts originated with Murphy and Rous (1912). The chorioallantoic membrane is formed at E4-E5 by fusion of the somatic mesoderm of the chorion with the vascularized splanchnic mesoderm of the allantois. The chorioallantoic membrane and its vessels in the intact egg spread over the surface of the yolk sac and on E6 or E7 cover it completely. In our experiments with chicken embryos we frequently used the chorioallantoic membrane as the environment to culture rudiments of embryonic bowel. Since the chorioallantoic membrane does not contain putative sources for enteric neurons, it is a suitable environment to culture aneuronal bowel.

5.3 CULTURE OF EMBRYONIC BOWEL IN THE RENAL SUBCAPSULAR SPACE

We found that the chorioallantoic membrane of chicken embryos does not provide the optimal environment for the culture of embryonic murine bowel. Therefore, we used the renal subcapsular space as culture environment. The renal subcapsular space is a suitable environment for the growth and differentiation of organ rudiments of murine embryos (Svajger et al., 1986). The renal capsule consists of an outer layer of fibrous connective tissue covering loose connective tissue adjacent to the renal parenchyma. The renal capsule contains small acetylcholinesterase positive nerve fibers, but there are no putative sources of enteric neurons in this structure. Therefore the renal subcapsular space is suited to culture aneuronal bowel of murine embryos.

5.4 CONCLUSION

The experiments described in appendix papers 1 and 2 provided data that refute two current pathogenetic theories of Hirschsprung's disease, the ischemic and the slow-migration theory. Appendix papers 3 and 4 described ENS formation in normal mice in relation to ENS malformation in a mutant mouse strain. Appendix papers 5 through 9 provided insights in ENS formation in chicken embryos; we proved that enteric neurons derive from the vagal neural crest only, and we identified that the enteric mesenchyme in aneuronal bowel is involved in the homing of neural crest cells, a process mediated by morphoregulatory molecules of the HNK-1 family. The data concerning ENS formation in chicken and murine embryos provided the developmental biological basis for the classification of ENS malformations in man (see chapter 2).

CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 FORMATION OF THE ENTERIC NERVOUS SYSTEM

Our experimental data combined with the literature data indicate that enteric neurons derive from the vagal neural crest. The vagal neural crest consists of a heterogeneous group of cells; only a minority of these cells give rise to enteric neurons, while the other vagal neural crest cells participate in the formation of heart and thymus. These embryological data provide insights in pathogenetic mechanisms of syndromic cases of Hirschsprung's disease.

The molecular nature of the mechanism that mediates enteric neural crest cell migration is unknown. Using anti-fibronectin antibodies, Tucker and co-workers (1986) were not able to show defined migration pathways in the bowel. The presence of areas in the bowel that are rich in fibronectins containing the CS1 binding sequence might be correlated with more motile and invasive properties of enteric neural crest cells (Dufour et al., 1988). One could speculate that enteric neural crest cells respond to fibronectins in the bowel because they have specific receptors for the CS1 binding site. Programmed fluctuations in the splicing of the IIICS region of fibronectin may therefore play a crucial role in path-finding mechanisms in the bowel.

There are no data about the molecular nature of the homing of cranial neural crest cells and the vagal neural crest cells in the thymic region, heart, carotid body and the bowel. There are few data about the homing of trunk neural crest cells (Aoyama et al., 1985; Lallier and Bronner-Fraser, 1988). We obtained evidence that the local enteric mesenchyme plays a role in the homing of neural crest cells at the future sites of enteric ganglia (appendix paper 6). This specialized mesenchyme expresses morphoregulatory molecules of the HNK-1 family; our provisional results suggest that at least two of such polypeptides, with apparent molecular weights of 42 and 44 kD respectively, are involved (Personal communication Th.M. Luider).

Kruse and co-workers (1984) proposed that neural CAMs are "presenters" of functionally important carbohydrate structures, such as HNK-1/L2, L3 and L4. The existence of a carbohydrate-based system that regulates protein function may help to explain why previous attempts to find regional protein differences in the early chicken embryo have largely proved fruitless. On the basis of this concept, Slack (1980) proposed that the structure of glycoconjugates at cell surfaces could form an "epigenetic coding"

system to specify positional information. In support of this hypothesis, Thorpe and co-workers (1988) have shown that antibodies directed against specific sugar moieties reveal tissue-specific patterns during development. Moreover, Canning and Stern (1989) suggested that the carbohydrate moiety that is recognized by the HNK-1 antibody plays a role in gastrulation.

It is tempting to speculate that the HNK-1 epitope has a specialized function in conjunction with carbohydrates on multifunctional glycoproteins, as has been suggested for the hormone chorionic gonadotropin (Calvo and Ryan, 1985). This hormone binds with its protein backbone to a receptor different from the receptor for the hormone's carbohydrate moiety that is necessary for activation of adenyl cyclase. Enzyme activation, however, occurs only when protein backbone and carbohydrate are simultaneously bound to the cell surface, possibly inducing a crosslinking of the two receptors.

Cell recognition mediated by cell surface oligosaccharides is likely to involve complementary receptors that function as carbohydrate-binding proteins. A variety of membrane-bound and soluble carbohydrate-binding proteins (lectins) has been isolated from vertebrate tissues (Drickamer, 1987; Barondes, 1984). The most prevalent class of lectins exhibit specificity for terminal B-galactoside linkages on cell surface carbohydrates.

Two carbohydrate-binding proteins on developing neural cells have been characterized, that are likely candidate molecules for the interaction with lactosyl oligosaccharides: soluble B-galactoside-binding lectins with molecular masses of 14.5 and 29 kDa, termed RL-14.5 and RL-29 (Dodd and Jessel, 1986). In sensory neurons, RL-14.5 and RL-29 are expressed selectively in the cell bodies and terminals of the subset of sensory neurons that express lactosyl oligosaccharides (Regan et al., 1986). The developmental coincidence in lectin and oligosaccharide expression together with their characterized ligand function therefore provides a possible mechanisms for initiating or enhancing adhesive interactions between developing neuronal cells.

6.2 MALFORMATION OF THE ENTERIC NERVOUS SYSTEM

The data concerning ENS formation provided the basis for the classification of ENS malformations as described in chapter 2. Putative pathogenetic mechanisms that can lead to congenital ENS malformations are described below.

a) Failure of vagal neural crest cells to colonize the pharyngeal region of the bowel results in total intestinal aganglionosis. This extremely rare ENS malformation is thought to inherit in an autosomal recessive mode. This particular gene is probably essential for enteric neural crest cell migration, since there is only one report of associated malformations (DiLorenzo et al., 1985). The gene could code for a receptor for ECM molecules, or could regulate the tissue specific modification of ECM molecules.

b) There is a distinct phase during which enteric neural crest cells proliferate considerably. A defect in the down- regulation of the proliferation of enteric neural crest

cells could lead to increased numbers of enteric neurons and glial cells, a phenomenon seen in neuronal intestinal dysplasia and intestinal neurofibromatosis. As the enteric mesenchyme can stimulate the proliferation of subsets of neuroepithelial cells (Rothman et al., 1987), it might well be that both diseases result from a defective enteric microenvironment.

c) Neural crest cell colonization of the distal ileum and the colon is disturbed in Zuelzer-Wilson disease. The absence of hypertrophic nerve fibers in the aganglionic bowel segments indicates that Zuelzer-Wilson disease is not a variation of Hirschsprung's disease, but a separate entity.

d) It might well be that the homing of neural crest cells in the distal colon is disturbed in Hirschsprung's disease. The fact that extrinsic nerve fibers innervate the aganglionic bowel segment indicates that the enteric microenvironment is suited for axonal sprouting. This also indicates that enteric neural crest cell migration differs from enteric axonal sprouting, although similarities between the two processes have been reported (Bronner-Fraser and Lallier, 1988).

The developmental studies with the mouse models for aganglionosis point at interesting phenomenon with respect to the role of the enteric microenvironment in neural crest cell migration. Jacobs-Cohen and co-workers (1987) reported that in organotypic tissue culture neural crest cells could not colonize the distal colon of ls/ls mice. Their conclusions are undermined, however, by a lack of appropriate controls. The failure of neural crest cells to colonize the distal hindgut seemed to be associated with an accumulation of ECM molecules in the distal bowel (Payette et al., 1988). Abnormalities have also been found in the hindgut of adult ls/ls mice (Tennyson et al., 1986). It is of interest that Vaos and Lister (1987) reported an excessive amount of elastin and collagen fibers in the aganglionic segment of the bowel of patients with Hirschsprung's disease.

The coincidence of Hirschsprung's disease with other neural crest malformations, such as first and second arch syndromes, third and fourth branchial arch syndromes, and limb anomalies, points at which moment the developmental failure occurs. A homing disturbance is also possible in cases of zonal aganglionosis.

The association of Hirschsprung's disease and Down's syndrome (trisomy 21) is difficult to explain. Which mechanism can explain how an increase in dosage of a set of mostly normal genes can cause such profound abnormalities? Cell-cell recognition might well be such a dosage-dependent event in morphogenesis. In support of this concept is the finding of an enhanced adhesiveness of cultured fetal cardiac and lung fibroblasts in patients with trisomy 21 (Wright et al., 1984). It might well be that Hirschsprung's disease in patients with trisomy 21 is the result of an increased adhesiveness of neural crest cells in proximal bowel segments.

Other genetic data show that several gene defects are related to Hirschsprung's disease. One or more genes on chromosome 21; one gene on chromosome 9 (Waardenburg syndrome; Westerveld et al. 1976), one on chromosome 13 (Lamont et

al., 1989). It will be interesting to investigate how the different genes at different loci can lead to a similar malformation.

e) There is a sequential development of different neuronal phenotypes in neural crest cells after homing and aggregation in the enteric microenvironment. Disturbance in the acquisition of the mature enteric phenotype could lead to an abnormally functioning ENS in the presence of ganglionic bowel; this might be the case in intestinal pseudo-obstruction. In support of this presumption is the report of the absence of VIP-positive enteric neurons (Koch et al., 1988).

6.3 FUTURE PERSPECTIVES

Neuropathological studies are required to describe congenital ENS malformations in more detail, particularly intestinal pseudo-obstruction and chronic constipation. Developmental studies on ENS formation in humans are required as well. The use of monoclonal antibodies or DNA probes as markers for neural crest cells will facilitate these studies.

Future clinical-genetical studies are required to determine the incidence of neural crest syndromes or other malformations that are associated with Hirschsprung's disease. It is essential to collect fibroblasts and DNA from families in which ENS malformations occur frequently. The analysis of restriction-fragment-length polymorphisms (RFLPs) in these families might shed a light on the genes that are involved in ENS formation.

It is likely that genes will be identified that regulate the expression of morphoregulatory molecules and determine the position of neural crest cells in the developing bowel. Candidates are the homeo-box containing genes, several genes that code for zinc-finger proteins such as Krox-20 (Wilkinson et al., 1989), and several proto-oncogenes (e.g. *int*-1 and *int*-2; Nusse, 1988). Injection of synthetic mRNA or antibodies corresponding to two different frog homeobox genes can disrupt the morphogenesis of embryonic somites or derivatives of the neural crest respectively (Harvey and melton, 1988; Cho et al., 1988). It might well be that homeo-gene transcripts provide positional cues in neural crest cells since the course of differentiation in these cells seems to be specified not simply by their site of origin in the central nervous system, but instead by environmental cues encountered after migration (Le Douarin, 1982). A promising experimental approach to study the function of such genes is provided by the quail-chick chimera model. Quail neural crest cells infected with the candidate genes and subsequently injected into the chicken host will behave as chicken neural crest cells. An heat-inducible promoter region flanking the gene will enable to switch on the genes at any moment during development.

The identification of developmental genes in animals, combined with the progress made in defining morphoregulatory molecules, will provide the framework for further studies into the pathogenetic mechanisms of congenital malformations.

REFERENCES

Abel, W. (1912): Further observations on the development of the sympathetic nervous system in the chick. J. Anat. Physiol. 47:35-71.

Abo, T., and C.M. Balch (1981): A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J. Immunol. 127:1024-1029.

Ahmed, S., S.J. Cohen, and S.I. Jacobs (1971): Total intestinal aganglionosis presenting as duodenal obstruction. Arch. Dis. Child. 46:868-896.

Allan, I.J., and D.F. Newgreen (1980): The origin and differentiation of enteric neurons of the intestine of the chick embryo. Am. J. Anat. 157:137-154.

Aldridge, R.T., and P.E Campbell (1968): Ganglion cell distribution in the normal rectum and anal canal. A basis for the diagnosis of Hirschsprung's disease by anorectal biopsy. J. Pediatr. Surg. 3:475-490.

Althoff, W. (1962): Zur Genetik der Hirschsprungschen Krankheit. Z. Menschliche Vererbungs-Konstitutionslehre 36:314-340.

Andrew, A. (1971): The origin of intramural ganglia. IV The origin of enteric ganglia: a critical review and discussion of the present state of the problem. J. Anat. 108:169-184.

Angerpointner, Th., W. Radtke, and J.D. Murken (1981): Catamnestic investigations in children with malformations of the gastrointestinal tract and the abdominal wall. Z. Kinderchir. 32:129-144.

Aoyama, H., A. Delouvee, and J.P. Thiery (1985): Cell adhesion mechanisms in gangliogenesis studied in avian embryo and in a model system. Cell Differentiation 17:247-260.

Arias, S. (1971): Genetic heterogeneity in the Waardenburg syndrome. Birth Def. Orig. Art. Ser. 7:87-101.

Ariel, I., I. Vinograd, O.Z. Lernau, S. Nissan, and E. Rosenmann (1983): Rectal mucosal biopsy in aganglionosis and allied conditions. Hum. Pathol. 14:991-995.

Ariga, T., T. Kohriyama, L. Freddos, N. Latov, M. Saito, K. Kon, S. Ando, M. Suzuki, M.E. Hemling, K.L. Rinehart, S. Kusunoko, and R.K. Yu (1987): Characterization of sulfated glucuronic acid containing glycolipids reacting with IgM M-proteins in patients with neuropathy. J. Biol. Chem. 262:848-853.

Arumugham, R.G., T.C.C-Y. Hsieh, M.L. Tanzer, and R.L. Laine (1986): Structures of the asparagine-linked sugars of laminin. Biochim. Biophys. Acta 883:112-126.

Aubresby, P. (1970): Symposium sur les résultats du traitement de la maladie de Hirschsprung. Ann. Chir. Infantile 11:5-8.

Aufderheide, E., and P. Ekblom (1988): Tenascin during gut development: appearance in the mesenchyme, shift in molecular forms, and dependence of epithelial-mesenchymal interactions. J. Cell Biol. 107:2341-2349.

Bancroft, M., and R. Bellairs (1976): The neural crest of the trunk region of the chick embryo studied by SEM and TEM. Zoon 4:73-85.

Baroffio, A., E. Dupin, and N.M. Le Douarin (1988): Clone-forming ability and differentiation potential of migratory neural crest cells. Proc. Natl. Acad. Sci. USA 85;5325-5329.

Barondes, S.H. (1984): Soluble lectins: a new class of extracellular proteins. Science (Washington DC): 223:1259-1264.

Barthel, D., M-J Santoni, W. Wille, C. Ruppert, J-C Chaix, M-R Hirch, J.C. Fontecillacamps, and C. Goridis (1987): Isolation and nucleotide sequence of mouse NCAM cDNA that codes for a Mr 79,000 polypeptide without a membrane-spanning region. EMBO J. 6:907-914.

Baumgarten, H.G., A.F. Holstein, and F. Stelzner (1973): Nervous elements in the human colon of Hirschsprung's disease. Virchows Arch. Abt. A Path. Anat. 358:113-136.

Bayliss, W.M., and E.H Starling (1899): The movements and innervation of the small intestine. J. Physiol. 24:99-143.

Bentley, J.F.R. (1964): Some new observations on megacolon in infancy and childhood with special reference to the management of megasigmoid and megarectum. Dis. Colon Rectum 7:462-470.

Bennet, T., and J.L.S. Cobb, (1971): Studies on the avian gizzard: the development of the gizzard and its innervation. Z. Zellforsch. Mikrosk. Anat. 98:599-621.

Bethenod, M., M. Hermier, C. Sellem, and A. Thomas (1973): Semeiologie clinique du megacolon. A partir de 136 observations. Pediatrie 28:623-629.

Bielschowski, M., and G.C. Schofield (1960): Studies on the inheritance and neurohistology of megacolon in mice. Proc. Univ. Otago Med. School 38:14-15.

Bishop, A.E., F. Carlei, V. Lee, J. Trojanowski, P.G. Marangos, D. Dahl, and J.M. Polak (1985): Combined immunostaining of neurofilaments, neuron specific enolase, GFAP, and S100. A possible means for assessing the morphological and functional status of the enteric nervous system. Histochemistry 82:93-97.

Bockman, D.E., and M.L. Kirby (1985): Neural crest interactions in the development of the immune system. J. Immunol. 135:766s-768s.

Bodian, M., F.D. Stephens, and B.C.H. Ward (1949): Hirschsprung's disease and idiopathic megacolon. Lancet i:6-11.

Bodian, M., C.O. Carter and B.C.H. Ward (1951): Hirschsprung's disease. Lancet i:302-309.

Bodian, M., and C.O. Carter (1963): A family study of Hirschsprung's disease. Ann. Hum. Genet. 16:261-277.

Boggs, J.D., and J.M. Kidd (1958): Congenital abnormalities of intestinal innervation. Pediatrics 21:261-265.

Bolande, R.P. (1974): The neurocristopathies. A unifying concept of disease arising in neural crest maldevelopment. Hum. Pathol. 5:409-429.

Bolande, R.P. (1975): Hirschsprung's disease, aganglionic or hypoganglionic megacolon. Am. J. Pathol. 79:189-192.

Bolande, R.P. (1981): Neurofibromatosis: the quintessential neurocristopathy: pathogenetic concepts and relationships. Adv. Neurol. 29:67-75.

Bolande, R.P. (1984): Models and concepts derived from human teratogenesis and oncogenesis in early life. J. Histochem. Cytochem. 32: 878-884.

Boley, S.J., G. Dinari, and M. Cohen (1978): Hirschsprung's disease in the newborn. Clin. Perinatol. 5:45-60.

Boucaut, J.C., T. Darribiere, T.J. Poole, H. Aoyama, K.M. Yamada, and J.P. Thiery (1984): Biologically active synthetic peptides as probes of embryonic development: a competitive inhibitor of fibronectin function inhibits gastrulations in amphibian embryos and neural crest cell migration in avian embryos. J. Cell Biol. 99:1822-1830.

Bower, R.J., and J.C. Adkins (1980): Ondine's curse and neurocristopathy. Clin. Pediatr. 19:665-668.

Branski, D., N.R. Dennis, J.M. Neale, and L.J. Brooks (1979): Hirschsprung's disease and Waardenburg's syndrome. Pediatrics 63:803-805.

Brauer, P.R., and R.R. Markwald (1988): Specific configurations of fibronectin containing particles correlate with pathways taken by neural crest cells at two axial levels. Anat. Rec. 222:69-82.

Briner, J., H.W. Oswald, J. Hirsig, and M. Lehner (1986): Neuronal intestinal dysplasia - Clinical and histochemical findings and its association with Hirschsprung's disease. Z. Kinderchir. 41:282-286.

Bronner-Fraser, M. (1985): Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. J. Cell Biol. 101:610-617.

Bronner-Fraser, M. (1986a): Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev. Biol. 115:44-55.

Bronner-Fraser, M. (1986b): An antibody to a receptor for fibronectin and laminin perturbs neural crest development in vivo. Dev. Biol. 117:528-536.

Bronner-Fraser, M. (1987): Perturbation of cranial neural crest migration by the HNK-1 antibody. Dev. Biol. 123:321-331.

Bronner-Fraser, M., and T. Lallier (1988): A monoclonal antibody against a laminin-heparan sulfate proteoglycan complex perturbs cranial neural crest migration in vivo. J. Cell Biol. 106:1321-1329.

Bronner-Fraser, M. (1988): Distribution and function of tenascin during cranial neural crest development in the chick. J. Neurosci. Res. 21:135-147.

Bronner-Fraser, M., and S.E. Fraser (1988): Cell lineage analysis reveals multipotency of some avian neural crest cells. Nature (London): 335:161-164.

Brown, K.S., and C.S. Chung (1967): Observations on the epidemiology, genetics and pathogenesis of deafness in children and lower mammals. Audiology 10:234-256.

Browne, M.J. (1953): A study of the sacral autonomic nerves in a chick and a human embryo. Anat. Rec. 116:189-203.

Brunoni, D., R. Joffe, L.M.S. Farah, and A.J.B., Cunha (1983): Hirschsprung megacolon, cleft lip and palate, mental retardation and minor congenital malformations. J. Clin. Dysmorphol. 1:20-22.

Bryant, M.G., A.M.J. Buchan, M. Gregor, M.A. Ghatei, J.M. Polak, and S.R. Bloom (1982): Development of intestinal regulatory peptides in the human fetus. Gastroenterology 83:47-54.

Buck, C.A., and A.F. Horwitz (1987): Cell surface receptors for extracellular matrix molecules. Ann. Rev. Cell Biol. 3:179-205.

Bughaighis, A.G. and J.L. Emery (1971): Functional obstruction of the intestine due to neurological immaturity. Progr. Pediatr. Surg. 3:37-52.

Calvo, F.O., and R.J. Ryan (1985): Inhibition of adenyl cyclase activity in rat corpora luteal tisue by glycopeptides of human chorionic gonadotropin and the a-subunit of human chorionic gonadotropin. Biochemistry 24:1953-1959.

Campbell, P.E., and H.R. Noblett (1969): Experience with rectal suction biopsy in the diagnosis of Hirschsprung's disease. J. Pediatr. Surg. 4:410-415.

Campos-Ortega, J.A., and V. Hartenstein (1985): *The embryonic development of Drosophila melanogaster.* Springer-Verlag, Heidelberg.

Camus, R. (1921): Über die Entwicklung des sympatischen Nervensystems beim Frosch. Arch. Mikrosk. Anat. Entw. Mech. 82:1-52.

Cantino, D. (1970): An histochemical study of the nerve supply to the developing alimentary tract. Experientia 26:766-767.

Caniano, D., H.S. Ormsbee III, W. Polito, C.C. Sun, F.C. Barone, and J.L. Hill (1985): Total intestinal aganglionosis. J. Pediatr. Surg. 20:456-460.

Canning, D.R., and C.D. Stern (1988): Changes in the expression of the carbohydrate epitope HNK-1 associated with mesoderm induction in the chick embryo. Development 104:643-655.

Cannon, W.B., and I.R. Burket (1913): The endurance of anemia by nerve cells in the myenteric plexus. Am. J. Physiol. 32: 347-357.

Careskey, J.M., T.R. Weber, and J.L. Grosfeld (1982): Total colonic aganglionosis. Am. J. Surgery 143:160-167,

Carter, C.O., K. Evans, and V. Hickman (1981): Children of those treated for Hirschsprung's disease. J. Med. Genet. 18:87-90.

Chatten, J., and M.L. Voorhess (1967): Familial neuroblastoma. Report of a kindred with multiple disorders, including neuroblastomas in four siblings. New Engl. J. Med. 277:1230-1236.

Chévalier, A. (1972): Localisation et duree des potentialites medullo-surrenaliennes des cretes neurales chez l'embryon de poulet. J. Embryol. Exp. Morphol. 27:603-614.

Chiquet, M., and D.M. Fambrough (1984): Chick myotendinous antigen. I. A novel extracellular glycoprotein complex consisting of large disulfide-linked subunits. J. Cell Biol. 98:1937-1946.

Childs, B. (1978): Priorities for research on birth defects. In: *Birth defects: Proceedings of the fifth international conference.* Eds. J.W. Littlefield, and J. DeGrouchy. Excerpta Medica, Amsterdam.

Cho, K.W.Y., J. Goetz, C.V.E. Wright, A. Fritz, J. Hardwicke, and E.M. De Robertis (1988): Differential utilization of the same reading frame in a *Xenopus* homeobox gene encodes two related proteins sharing the same DNA-binding specificity. EMBO J. 7:2139-2149.

Chou, K.H., A.A. Ilyas, J.E. Evans, R.H. Quarles, and F.B. Jungalwala (1985): Structure of a glycolipid reacting with monoclonal IgM in neuropathy and with HNK-1. Biochem. Biophys. Res. Commun. 128:383-388.

Chou, D.K.H., A.A. Ilyas, J.E. Evans, C. Costello, R.H. Quarles, and F.B. Jungalwala (1986): Structure of sulfated glucuronyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. J. Biol. Chem. 261:11717-11725.

Christensen, J., M.J. Stiles, G.A. Rick, and J. Sutherland (1984): Comparative anatomy of the myenteric plexus of the distal colon in eight mammals. Gastroenterology 86:706-713.

Ciment, G., and J.A. Weston (1983): Enteric neurogenesis by neural crest-derived branchial arch mesenchymal cells. Nature (London): 305:424-427.

Cochard, P., M. Goldstein, and I.B. Black (1978): Ontogenetic appearance and disappearance of tyrosine hydroxylase and catecholamines in the rat embryo. Proc. Natl Acad. Sci. USA 75:2986-2990.

Cockel, R., E.E. Hill, D.I. Rushton et al. (1973): Familial steatorrhoea with calcification of the basal ganglia and mental retardation. Q. J. Med. 168:771-783.

Cohen, I.T., and M.A. Gadd (1982): Hirschsprung's disease in a kindred: a possible clue to the genetics of the disease. J. Pediatr. Surg. 17:632-634.

Cole, G., A. Loewy, and L. Glaser (1986): Neuronal cell adhesion depends on interactions of N-CAM with heparin-like molecules. Nature (London): 320:445-447.

Cole, G., and M. Schachner (1987): Localization of the L2 monoclonal antibody binding site on chicken neural cell adhesion molecule (NCAM): and evidence for its role in NCAM-mediated cell adhesion. Neurosci. Lett. 78:227-232.

Cooke, H.J. (1986): Neurobiology of the intestinal mucosa. Gastroenterology 90:1057-1081.

Copenhaver, P.F., and P.H. Taghert (1989): Development of the enteric nervous system in the moth. II. Sterotyped cell migration precedes the differentiation of embryonic neurons. Dev. Biol. 131:85-101.

Couly, G.F. (1981): Les neurocristopathies du bourgeon nasofrontal humain: les syndromes ethmoidiens (hypo et hyperseptoethmoidismes): Revue Stomatol. Chir. Maxillofac. 82:213-225.

Crossin, K.L., C.M. Chuong, and G.M. Edelman (1985): Expression sequences of cell adhesion molecules. Proc. Natl. Acad. Sci. USA 82:6942-6946.

Cunningham, B.A., J.J. Hemperley, B.A. Murray, E.A. Prediger, R. Brackenbury, and G. M Edelman (1987): Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science (Washington DC) 236:799-803.

Currie, A.B.M., M.Haddad, M. Honeyman, and S-A. Boddy (1986): Associated developmental abnormalities of the anterior end of the neural crest: Hirschsprung's disease - Waardenburg's syndrome. J. Pediatr. Surg. 21:248-250.

Demos, T.C., J. Blonder, W.L. Schey, S.S. Braithwaite, and P.L. Goldstein (1983): Multiple endocrine neoplasia (MEN): syndrome. Type IIB: Gastrointestinal manifestations. Am. J. Radiol. 140:73-78.

Dennis, R.D., H. Antonicek, H. Wiegandt, and M. Schachner (1988): Detection of the L2/HNK-1 carbohydrate epitope on glycoproteins and acidic glycolipids of the insect calliphora vicina. J. Neurochem. 51:1490-1496.

Derrick, E.H., and B.M. St. George-Grambauer (1957): Megacolon in mice. J. Pathol. Bacteriol. 73:569-571.

De Villiers, D.R. (1966): Ischemia of the colon: an experimental study. Br. J. Surg. 53:497-03.

Dickson, G., H.J. Gower, C.H. Barton, H.M. Prentice, V.L. Elsom, S.E. Moore, R.D. Cox, C. Quinn, W. Putt, and F.S. Walsh (1987): Human muscle neural cell adhesion molecule (N-CAM) identification of a muscle-specific sequence in the extracellular domain. Cell 50:1119-1130.

DiLorenzo, M., S. Yasbeck, and P. Brochu (1985): Aganglionosis of the entire bowel: four new cases and review of the literature. Br. J. Surg. 72:657-658.

DiVirgilio, G., N. Lavenda, and J.L. Worden (1967): Sequence of events in neural tube closure and the formation of neural crest in the chick embryo. Acta Anat. 68:127-146.

Dobbins, W.O., and A.H. Bill (1965): Diagnosis of Hirschsprung's disease excluded by rectal suction biopsy. New Engl. J. Med. 272:990-993.

Dodd, J. and J.M. Jessel (1986): Cell surface glycoconjugates and carbohydrate-binding proteins: possible recognition signals in sensory neuron development. J. Exp. Biol. 124:225-238.

Dressler, G.R., and P. Gruss (1988): Do multigene families regulate vertebrate development? Trends Neurosci. 4:214-219.

Drickamer, K. (1987): Membrane receptors that mediate glycoprotein endocytosis: structure and biosynthesis. Kidney Internatl. 32:S167-S188.

Duband, J-L., and J.P. Thiery (1982): Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. Dev. Biol. 93:308-323.

Duband, J-L., S. Rocher, W-T. Chen, K.M. Yamada, and J.P. Thiery (1986): Cell adhesion and migration in the early vertebrate embryo: location and possible role of the putative fibronectin receptor complex receptor complex. J. Cell Biol. 102:160-178.

Duband, J-L., Tucker G.C., T.J. Poole, M. Vincent, H. Aoyama, and J.P. Thiery (1985): How do the migratory and adhesive properties of the neural crest govern ganglia formation in the avian peripheral nervous system? J. Cell. Biochem. 27:189-203.

Duband, J-L., and J.P. Thiery (1987): Distribution of laminin and collagens during avian neural crest development. Development 101:461-478.

Duband, J-L., S. Dufour, K. Hatta, M. Takeichi, G.M. Edelman, and J.P. Thiery (1987): Adhesion molecules during somitogenesis in the avian embryo. J. Cell Biol. 104:1361-1374.

Duboule, D., B. Galliot, A. Baron, and M.S. Featherstone (1989): Murine homeo-genes: some aspects of their organization and structure. In: *Cell to cell signal in mammalian development.* Eds. S. de Laat, J.G. Bluemink, and C.L. Mummary. NATO ASI series, Springer Verlag.

Dufour, S., J-L. Duband, M.J. Humphries, M. Obara, K.M. Yamada, and J.P. Thiery (1988a): Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. EMBO J. 7:2661-2671.

Dufour, S., J-L. Duband, A.R. Kornblihtt, and J.P. Thiery (1988b): The role of fibronectins in embryonic cell migrations. Trends Genet. 4:198-203.

Duffly, T., E.E. Erickson, G.L. Jordan, and H.D. Bennet (1962): Megacolon and bilateral pheochromocytoma. Am. J. Gastroenterol. 38:555-563.

Earlam, R.J. (1972): A vascular cause for aganglionic bowel - a new hypothesis. Dig. Dis. 17:255-261.

Earlam, R.J. (1985): A vascular cause for Hirschsprung's disease? Gastroenterology 88:1274-1276.

Edelman, G.M. (1984): Cell-adhesion molecules: a molecular basis for animal form. Sci. Amer. 250 (april):118-129.

Edelman, G.M. (1984): Cell adhesion and morphogenesis: the regulator hypothesis. Proc. Natl. Acad. Sci. USA 81:1460-1464.

Edelman, G.M. (1985): Cell adhesion and the molecular processes of morphogenesis. Ann. Rev. Biochem. 54:135-169.

Edelman, G.M., and J.P. Thiery (Eds) (1985): *The cell in contact.* Wiley, New York.

Edelman, G.M. (1988): Morphoregulatory molecules. Biochemistry 27:3533-3542.

Ehrenpreis, Th. (1947): *Hirschsprung's disease.* Year Book Medical Publisher, Chicago.

Ehrenpreis, Th. (1966): Pseudo-Hirschsprung's disease. Arch. Dis. Child. 41:143-144.

Emanuel, B., M.P. Padorr, and O. Swenson (1965): Familial absence of myenteric plexus (congenital megacolon). A study of 6 families. J. Pediatr. 67:381-386.

Enemar, A., B. Falck, and R. Hakanson (1965): Observations on the appearance of norepinephrine in the sympathetic nervous system of the chick embryo. Dev. Biol. 11:268-283.

Epstein, M.L., D. Sherman, and M.D. Gershon (1980): Development of serotonergic neurons in the chick duodenum. Dev. Biol. 77:22-40.

Erdohazi, M. (1974): Retarded development of the enteric nerve cells. Dev. Med. Child Neurol. 16:365-368.

Erickson, H.P., and J.L. Inglesias (1984): A six-armed oligomer isolated from cell surface fibronectin preparations. Nature (London) 311:267-269.

Fadda, B., W.A. Maier, W. Meier-Ruge, and R. Daum (1983): Neuronal intestinal dysplasia. A critical 10-year analysis of clinical and bioptic results. Z. Kinderchir. 38:305-311.

Feinstat, T., H. Testnik, and M.D. Schuffler (1984): Megacolon and neurofibromatosis: a neuronal intestinal dysplasia. Gastroenterology 86:1573-1579.

Fekété, C.N., C. Ricour, H. Martelli, S. Lortat-Jacob, and D. Pellerin (1986): Total colonic aganglionosis (with or without ileal involvement): a review of 27 cases. J. Pediatr. Surg. 21:251-254.

Felten, D.L., S.Y. Felten, D.L. Bellinger, S.L. Carlson, K.D. Ackerman, K.S. Madden, J.A. Olschowski, and S. Livnat (1988): Noradrenergic sympathetic neural interactions with the immune system: structure and function. Immunol. Rev. 100:225-260.

Ferri, G-L., L. Probert, D. Cocchia, F. Michetti, P.J. Marangos, and J.M. Polak (1982): Evidence for the presence of S-100 protein in the glial component of the human enteric nervous system. Nature (London 297:409-410.

Finne, J., U. Finne, H. Deagostini-Bazin, and C. Goridis (1983): Occurrence of a a 2-8 linked polysialosyl units in a neural cell adhesion molecule. Biochem. Biophys. Res. Comm. 112:482-487.

Fontaine-Perus, P., M. Chanconie, J.M. Polak, and N.M. Le Douarin (1981): Origin and development of VIP and substance P containing neurons in the embryonic avian gut. Histochem. 71:313-323.

Fraser, G.R. (1976): The causes of profound deafness in childhood. A study of 3535 individuals with severe hearing loss present at birth or of childhood onset. John Hopkins University Press, Baltimore. pp. 90-132.

Fried, K., and S. Beer (1980): Waardenburg' syndrome and Hirschsprung's disease in the same patient. Clin. Genet. 18:91-92.

Friedlander, D.R., S. Hoffman, and G.M. Edelman (1988): Functional mapping of cytotactin: proteolytic fragments active in cell-substrate adhesion. J. Cell Biol. 107:2329-2340.

Furness, J.B., and M. Costa (1980): Types of nerves in the enteric nervous system. Neuroscience 5:1-20.

Gaisie, G., O.H. Kook Sang, and L.W. Young (1979): Coexistent neuroblastoma and Hirschsprung's disease - Another example of the neurocristopathy. Paediatr. Radiol. 8:161-163.

Galjaard, H. (1980): *Genetic metabolic diseases: Early diagnosis and prenatal analysis.* Elsevier, Amsterdam.

Garret, J.R., E.R. Howard, and H.H. Nixon (1969): Histochemical diagnosis of Hirschsprung's disease. Lancet ii:436.

Gaunt, S.J., P.T. Sharpe, and D. Duboule (1989): Spatially restricted domains of homeo-gene transcripts in mouse embryos: relation to a segmental body plan. Development 104:169-179.

Gehring, W. (1987): Homeo boxes in the study of development. Science (Washington DC) 236:1245-1252.

Gershon, M.D., M.L. Epstein, and L. Hegstrand (1980): Colonization of the chick gut by progenitors of enteric serotonergic neurons: distribution, differentiation, and maturation within the gut. Dev. Biol. 77:41-51.

Gershon, M.D., and S.M. Erde (1981): The nervous system of the gut. Gastroenterology 80:1571-1594.

Gershon, M.D., P. Sherman, and A.R. Gintzler (1981): An ultrastructural analysis of the developing enteric nervous system of the guinea-pig small intestine. J. Neurocytol. 10:271-296.

Gershon, M.D., and T.P. Rothman (1984): Experimental and genetic approaches to the study of the development of the enteric nervous system. Trends Neurosci. may:150-155.

Gershon, M.D., R.F. Payette and T.P. Rothman (1984): Development of the enteric nervous system. Fed. Proc. 42:1620-1625.

Gherardi, G.J. (1960): Pathology of the ganglionic-aganglionic junction in congenital megacolon. Arch. Pathol. 69:520-523.

Gintzler, A.R., T.P. Rothman, and M.D. Gershon (1980): Ontogeny of opiate mechanisms in relation to the sequential development of neurons known to be components of the guinea pig's enteric nervous system. Brain res. 189:31-48.

Goldberg, R.B., and R.J. Sphrintzen (1981): Hirschsprung megacolon and cleft palate in two sibs. J. Craniofac. Genet. Dev. Biol. 1:185-189.

Goldberg, E. (1984): An epidemiological study of Hirschsprung's disease. Int. J. Epidemiology 13:479-485.

Gordon, H., M. Torrington, J.H. Louw, and S. Cywes (1966): Genetical study of Hirschsprung's disease. S. Afr. Med. J. 40:720-721.

Graivier, L., and W.K. Sieber (1966): Hirschsprung's disease and mongolism. Surgery 60:458-461.

Greve, J.M. and D.I. Gottlieb (1982): Monoclonal antibodies which alter the morphology of cultured chick myogenic cells. J. Cell. Biochem. 18:221-230.

Griffith, S.G., and G. Burnstock (1983): Serotoninergic neurons in human fetal intestine: an immunohistochemical study. Gastroenterology 85:929-937.

Grumet, M., and G.M. Edelman (1984): Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by specific cell adhesion molecule. J. Cell Biol. 98:1746-1756.

Grumet, M., S. Hoffman, K.L. Crossin, and G.M. Edelman (1985): Cytotactin, an extracellular matrix protein of neural and non-neural tissues that mediates glia-neuron interaction. Proc. Natl. Acad. Sci. USA 82:8075-8079.

Gulotta, F., and G. Straaten (1970): Hirschsprungsche Krankheit mit gleichzeitiger Aganglionose und sogenannter neuronaler Kolondysplasie. Z. Kinderchir. 20:42-49.

Haddad, G.G., N.M. Mazza, R. Defendini, W.A. Blanc, J.M. Driscoll, M.A.F. Epstein, R.A. Epstein, and R.B. Melllins (1978): Congenital failure of automatic control of ventilation, gastrointestinal motility and heart rate. Medicine 57:517-526.

Hall, J.G., E.K. Powers, R.T. McIlvaine, and V.H. Ean (1978): The frequency and burden of genetic disease in a pediatric hospital. Am. J. Med. Genet. 1:417-436.

Hall, C.L. and P.W. Lampert (1985): Immunocytochemistry as an aid in the diagnosis of Hirschsprung's disease. Am. J. Clin. Pathol. 83:177-181.

Hamilton, J., and J.N. Bodurtha (1989): Congenital central hypoventilation syndrome and Hirschsprung's disease in half sibs. J. Med. Genet. 26:272-279.

Hammond, W.S., and C.L. Yntema (1964): Depletions of pharyngeal arch cartilages following extirpation of cranial neural crest in chick embryos. Acta Anat. 56:21-34.

Hammond, W.S. and C. L. Yntema (1947): Depletions in the thoracolumbar sympathetic system following removal of neural crest in the chick. J. Comp. Neurol. 86:237-265.

Haney, P.J., J.L. Hill, and C-C.J. Sun (1982): Zonal colonic aganglionosis. Pediatr. Radiol. 12:258-261.

Harvey, R.P., and D.A. Melton (1988): Microinjection of synthetic Xhox-1a homeobox mRNA disrupts somite formation in developing Xenopus embryos. Cell 53:687-697.

Hatta, K., and M. Takeichi (1986): Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature (London): 320:447-449.

Hatta, K., S. Takagi, H. Fujisawa, and M. Takeichi (1987): Spatial and temporal expression pattern of B-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev. Biol. 120:215-227.

Hé, H.T., J. Barbet, J-C. Chaix, and C. Goridis (1986): Phosphatidylinositol is involved in the membrane attachment of N-CAM 120, the smallest component of the neural cell adhesion molecule. EMBO J. 5:2489-2494.

Hill, R.E., P.F. Jones, A.R. Rees, C.M. Sime, M.J. Justice, N.G. Copeland, N.A. Jenkins, E. Graham, and D.R. Davidson (1989): A new family of mouse homeo box-containing genes: molecular structure, chromosomal location, and developmental expression of Hox-7.1. Genes and Development 3:26-37.

Hirschsprung, H. (1887): Stuhlträgheit Neugeborener in Folge von Dilatation und Hypertrophie des Colons. Jahrb. Kinderh. 27:1-7.

Hirschsprung, H. (1890): Festschrift fur Henoch. 83:27.

Hirschsprung, H. (1904): Dilatation congenitale du colon. In: *Traité des maladies de l'enfance*. Grancher and Comby, Paris. Vol. 2.

His, W. (1868): *Untersuchungen über die erste Anlage des Werbeltierleibes. Die erste Entwicklung des Hünchens im Ei.* Vogel, Leipzig.

His, W. (1897): Über die Entwicklung des Bauchs sympaticus beim Hühnchen und Menschen. Arch. Anat. Physiol., Anat. Abt. (Suppl.) 137-170.

Hoffmann, S., and F. Rehbein (1966): Hirschsprungsche Krankheit im Neugeborenenalter. Z. Kinderchir. 3:182-194.

Hoffman, S. and G.M. Edelman (1983): Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. Proc. Natl. Acad. Sci. USA 80:5762-5766.

Hoffman, S., and G.M. Edelman (1987): A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. Proc. Natl. Acad. Sci. USA 84:2523-2527.

Hoffman, S., K.L. Crossin, and G.M. Edelman (1988): Molecular forms, binding functions, and developmental expression patterns of cytotactin and cytotactin-binding proteoglycan, an interactive pair of extracellular matrix molecules. J. Cell Biol. 106:519-532.

Holland, P.W.H., and B.L.M. Hogan (1988a): Expression of homeo box genes during mouse development: a review. Genes and Development 2:773-782.

Holland, P.W.H., and B.L.M. Hogan (1988b): Spatially restricted patterns of expression of the homeobox-containing gene Hox 2.1. during mouse embryogenesis. Development 102:159-174.

Holley, J.A., and R.K. Yu (1987): Localization of glycoconjugates recognized by the HNK-1 antibody in mouse and chick embryos during early neural development. Dev. Neurosci. 9:105-119.

Holmdahl, D.E. (1928): Die Entstehung und weitere Entwicklung der Neuralleiste (Ganglienleiste): bei Vögeln und Säugetieren. Z. Mikrosk.-Anat. Forsch. 14:99-298.

Holmes, L.B. (1976): Congenital malformations. New. Engl. J. Med. 295:204-207.

Holtfreter, J. (1944): Significance of the cell membrane in embryonic processes. Ann. NY Acad. Sci. 49:709-760.

Hope, J.W., P.F. Borns, and P.K. Berg (1965): Roentgenologic manifestations of Hirschsprung's disease in infancy. Am. J. Radiol. 95:217.

Howard, E.R., and J.R. Garret (1984): Hirschsprung's disease and other neuronal disorders of the hindgut. In: *Neonatal gastroenterology - contemporary issues.* Eds. M.S. Tanner, and R.J. Stocks. Intercept, New Castle upon Tyne. pp. 121-137.

Hukuhara, T., S. Kotani, and G. Sato (1961): Effects of destruction of intramural ganglion cells on colon motility. Possible genesis of congenital megacolon. Jap. J. Physiol. 11:635-640.

Hultgren, B.D. (1982): Ileocolonic aganglionosis in white progeny of overo spotted horses. J. Am. Vet. Med. Ass. 180:289-292.

Humphries, M.J., S.K. Akiyama, A. Komoriya, K. Olden, and K. Yamada (1988): Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell binding domain and the alternative spliced type III connecting segment. J. Cell Biol. 106:1289-1297.

Hüther, W. (1954): Die Hirschsprungsche Krankheit als Folge einer Entwicklungsstorung der intramural Ganglien. Beitr. Pathol Anat. Allg. Pathol. 114:11-191.

Hyafil, F., C. Babinet, C. Huet, and F. Jacob (1983): Uvomorulin and compaction. In: *Teratocarcinoma stem cells.* Eds. L.M. Silver, G.R. Martin, and S. Strickland. Cold Spring Harbor Laboratory, Cold Spring Harbor. pp. 197-207.

Hynes, R.O. (1987): Integrins, a family of cell surface receptors. Cell 48:549-554.

Ikeda, K., and S. Goto (1984): Diagnosis and treatment of Hirschsprung's disease in Japan. Ann. Surg. 199:400-405.

Ikeda, K., and S. Goto (1986): Additional anomalies in Hirschsprung's disease: an analysis based on the nationwide survey in Japan. Z. Kinderchir. 41:279-282.

Ilyas, A.A., R.H. Quarles, M.C. Dalakas, P.H. Fishman, and R.O. Brady (1984): IgM in a human neuropathy related to paraproteinemia binds to a carbohydrate determinant in the myelin-associated glycoprotein and to a ganglioside. Proc. Natl. Acad. Sci. USA 81:1225-1229.

Ito, Y., P.K. Donahoe, and W.H. hendren (1977): Differentiation of intramural ganglia in the dissociated rectosigmoid of the rat: an organ culture study. J. Pediatr. Surg. 12:969-974.

Jacob, L., M. Opper, B. Metzroth, B. Phannavong, and B.M. Mechler (1987): Structure of the l(2)gl gene of Drosophila and delimitation of its tumor suppressor domain. Cell 50:215-225.

Jacobs-Cohen, R.J., R.F. Payette, M.D. Gershon, and T.P. Rothman (1987): Inability of neural crest cells to colonize the presumptive aganglionic bowel of ls/ls mutant mice: requirement for a permissive microenvironment. J. Comp. Neurol. 255:425-438.

Jayle, F. (1909): La dilatation congenitale idiopatique du colon. Presse Med. 17:803.

Jessen, K.R., and R. Mirsky (1980): Glial cells in the enteric nervous system contain glial fibrillary acidic protein. Nature (London) 286:736-737.

Jones, K.L (1988): *Smith's recognizable patterns of human malformation.* Saunders, Philadelphia, USA.

Jones, D.S. (1942): The origin of the vagi and the parasympathetic ganglion cells of the viscera of the chick. Anat. Rec. 82:185-197.

Jones, F.S., M.P. Burgoon, S. Hoffman, K.L. Crossin, B.A. Cunningham, and G.M. Edelman (1988): A cDNA clone for cytotactin contains sequences similar to epidermal growth factor-like repeats and segments of fibronectin and fibrinogen. Proc. Natl. Acad. Sci. USA 85:2186-2190.

Kaiser, G., and M. Bettex (1982): Clinical generalities. In: *Hirschsprung's disease.* Ed. A.M. Holschneider. Hippokrates, Stuttgart.

Kamagata, S., and P.K. Donahoe (1985): The effect of fibronectin on cholinergic differentiation of the fetal colon. J. Pediatr. Surg. 20:307-314.

Karnovski, M.J. and L. Roots (1964): A direct-coloring thiocholine method for cholinesterases. J. Histochem. Cytochem. 12:219-221.

Kawana, T., O. Nada, and K. Ikeda (1988): An immunohistochemical study of glial fibrillary acidic (GFA)

protein and S-100 protein in the colon affected by Hirschsprung's disease. Acta Neuropathol. 76:159-165.

Keilhauer, G., A. Faissner, and M. Schachner (1985): Differential inhibition of neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2 and N-CAM antibodies. Nature (London): 316:728-730.

Keller, H.P. (1976): The development of the intramural nerve plexus of the gastrointestinal tract. Anat. Embryol. 150:1-6.

Keuning, F.J. (1944): The development of the intramural nerve elements of the digestive tract in tissue culture. Acta Neerlan. Morph. 5:237-247.

Keuning, F.J. (1948): Histogenesis and origin of the autonomic nerve plexus in the upper digestive tube of the chick. Acta Neerl. Morph. 6:8-42.

Kirby, M.L., T.F. Gale, and D.E. Stewart (1983): Neural crest cells contribute to aorticopulmonary septum. Science (Washington DC) 220:1059-1061.

Kissel, P., J.M. André, and A. Jacquier (1982): *The neurocristopathies.* Masson, New York.

Kleinhaus, S., S.J. Boley, M. Sheran, and W.K. Sieber (1979): Hirschsprung's disease. A survey of the members of the surgical section of American academy of pediatrics. J. Pediatr. Surg. 14:588-597.

Klos, I., W.A. Maier, R. Morger, and P. Schweizer (1978): Neuronal colon dysplasia. Z. Kinderchir. 23:53-54.

Klück, P., G.N.P. van Muijen, A.W.M. van der Kamp, D. Tibboel, W.A. van Hoorn, S.O. Warnaar, and J.C. Molenaar (1984): Hirschsprung's disease studied with monoclonal antineurofilament antibodies on tissue sections. Lancet i: 652-655.

Klück, P., D. Tibboel, K. Leendertse-Verloop, A.W.M. van der Kamp, F.JW. ten Kate, and J.C. Molenaar (1986): Diagnosis of congenital neurogenic abnormalities of the bowel with monoclonal anti-neurofilament antibodies. J. Pediatr. Surg. 21:132-135.

Koch, T.R., J.A. Carney, L. Go, and V.L.W. Go (1988): Idiopathic chronic constipation is associated with decreased colonic vasoactive intestinal peptide. Gastroenterology 94:300-310.

Krotoski, D.M., C. Domingo, and M. Bronner-Fraser (1986): Distribution of a putative cell surface receptor for fibronectin and laminin in the avian embryo. J. Cell Biol. 103:1061-1071.

Krumlauf, R., P.W.H. Holland, J.H. McVey, and B.L.M. Hogan (1987): Developmental and spatial patterns of expression of the mouse homeobox gene, Hox 2.1. Development 99:603-617.

Kruse, J., R. Mailhammer, H. Wernecke, A. Faissner, I. Sommer, C. Goridis, and M. Schachner (1984): Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. Nature (London): 311:153-155.

Kruse, K., G. Keilhauer, A. Faissner, R. Timpl, and M. Schachner (1985): The J1 glycoprotein - a novel nervous system cell adhesion molecule of the L2/HNK-1 family. Nature (London): 316:146-148.

Künemund, V., F.B. Jungalwala, G. Fischer, D.K.H. Chou, G. Keilhauer, and M, Schachner (1988): The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. J. Cell Biol. 106:213-223.

Kuntz, A. (1910): The development of the sympathetic nervous system in birds. J. Comp. Neurol. 20:283-308.

Kurcherer, A., A. Faissner, and Melitta Schachner (1987): The novel carbohydrate epitope L3 is shared by some neural cell adhesion molecules. J. Cell Biol. 104:1597-1602.

Lallier, T.E., and M. Bronner-Fraser (1988): A spatial and temporal analysis of dorsal root and sympathetic ganglion formation in the avian embryo. Dev. Biol. 127:99-112.

Lake, B.D., P. Puri, H.H. Nixon and A.E. Claireux (1978): Hirschsprung's disease. An appraisal of histochemically demonstrated acetylcholinesterase activity in suction rectal biopsy specimens as an aid to diagnosis. Arch. Pathol. Lab. Med. 102:244-247.

Lamont, M.A., M. Fitchett, and N.R. Dennis (1989): Interstitial deletion of distal 13q associated with

Hirschsprung's disease. J. Med. Genet. 26:100-104.

Lane, P.W. (1966): Association of megacolon with two recessive spotting genes in the mouse. J. Hered. 57:181-183.

Lane, P.W., and H.M. Liu (1984): Association of megacolon with a new dominant spotting gene (DOM): in the mouse. J. Hered. 75:435-439.

Langenauer, C., and V. Lemmon (1987): An L1-like molecule, the 8D9 antigen is a potent substrate for neurite extension. Proc. natl. Acad. Sci. USA 84:7753-7757.

Langley, J.N. (1921): *The autonomic nervous system.* Part I. Heffer and Sons, Cambridge.

Lassmann, G., and P. Wurnig (1973): Local hypertrophy of the ganglion cells in the submucosa of the oral end of the aganglionic segment in Hirschsprung's disease. Z. Kinderchir. 12:236-243.

Laurence, K.M., R. Prossner, I. Rocker, J.F. Pearson, and C. Richards (1975): Hirschsprung's disease associated with congenital heart malformation, broad big toes, and ulnar polydactyly in sibs: a case for fetoscopy. J. Med. Genet. 12:334-338.

Le Douarin, N.M., and C. Le Lièvre (1971): Sur l'origine des cellules a calcitonine du corps ultimobranchial de l'embryon d'oiseau. C. R. Assoc. Anat. 152:558-568.

Le Douarin, N.M., and M-A. Teillet (1973): The migration of neural crest cells to the wall of the digestive tract in avian embryo. J. Embryol. Exp. Morphol. 30:31-48.

Le Douarin, N.M., and M-A. Teillet (1974): Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives, using a biological cell marking technique. Dev. Biol. 41:162-184.

Le Douarin, N.M. (1982): *The Neural Crest.* Cambridge University Press, Cambridge, UK.

Le Douarin, N.M. (1986): Cell line segregation during peripheral nervous system ontogeny. Science (Washington DC) 231:1515-1521.

Le Lièvre, C. (1974): Role des cellules mesectodermiques issues des cretes neurales cephaliques dans la formation des arcs branchiaux et du squelette visceral. J. Embryol. Exp. Morphol. 31:453-477.

Le Lièvre, C., and N.M. Le Douarin (1973): Contribution du mesectoderme a la genese des arcs aortiques chez l'embryon d'oiseau. C. R. Acad. Sci. 276:383-386.

Le Lièvre, C., and N.M. Le Douarin (1974): Origine ectodermique du derme de la face et du cou, montree par des combinaisons interspecifiques chez l'embryo d'oiseau. C.R. Acad. Sci. 278:517-520.

Lipson, A.H. and J. Harvey (1987): Three-generation transmission of Hirschsprung's disease. Clin. Genet. 32:175-178.

Lister, J. (1966): Abnormal arteries in Hirschsprung's disease. Arch. Dis. Childh. 41:149.Lee, V.M. (1955): Megacolon, with particular reference to Hirschsprung's disease. Surgery 37:762-777.

Louw, J.H. (1952): Congenital intestinal atresia and severe stenosis in the newborn. South African Clin. Sci. 3:109-129.

Louw, J.H. (1964): Investigations into the etiology of congenital atresia of the colon. Dis. Col. Rectum 7:471-478.

Lowry, R.B. (1975): Hirschsprung's disease and congenital deafness. J. Med. Genet. 12:114-115.

Lütschelschwab, R., C. Kambt, R. Rossa, and O. Schmidt (1987): A protein product of the Drosophila recessive tumor gene, l(2) giant gl, potentially has cell adhesion properties. EMBO J. 6:1791-1797.

MacKinnon, A.E., and S.J. Cohen (1977): Total intestinal aganglionosis. An autosomal recessive condition? Arch. Dis. child. 52:898-899.

Mackey, H.M., R.F. Payette, and M.D. Gershon (1988): Tissue effects on the expression of serotonin, tyrosine hydroxylase and GABA in cultures of neurogenic cells from the neuraxis and branchial arches. Development 104:205-217.

Mackie, E.J., R.P. Tucker, W. Halfter, R. Chiquet-Ehrismann, and H.H. Epperlein (1988): The distribution of tenascin coincides with pathways of neural crest cell migration. Development 102:237-250.

Madsen, C.M. (1964): *Hirschsprung's disease*. Munksgaard, Copenhagen.

Mahakrishnan, A., and M.S. Srinivasan (1980): Piebaldness with Hirschsprung's disease. Arch. Dermatol. 116:1102.

Masson, P., and L. Berger (1923): Sur un nouveau mode de secretion interne: la neurocrine. C. R. Hebd. Seanc. Acad. Sci. Paris 176:1748-1750.

Mayer, E.A., Schuffler, M.D., J.I. Rotter, P. Hanna, and M.A. Mograd (1988): A familial visceral neuropathy with autosomal dominant transmission. Gastroenterology in press.

McGarry, R.C., S.L. Helfand, R.H. Quarles, and J.C. Roder (1985): Recognition of myelin-associated glycoprotein by the monoclonal antibody HNK-1. Nature (London) 306:376-378.

McKusick, V.A. (1973): Congenital deafness and Hirschsprung's disease (letter). New Engl. J. Med. 288:691.

Meier-Ruge, W. (1969): New aspects in the pathology of the hypoganglionic megacolon. Verh. Dtsch. Ges. Pathol. 53:237-239.

Meier-Ruge, W. (1971): Ueber ein Erkrankungsbild des Colon mit Hirschsprung-Symptomatik. Verh. Dtsch. Ges. Pathol. 55:506-510.

Meier-Ruge, W., P.M. Lütterbeck, B. Herzog, R. Morger, R. Moser, and A. Scharli (1972): Acetylcholinesterase activity in suction biopsies of the rectum in the diagnosis of Hirschsprung's disease. J. Pediatr. Surg. 7:11-17.

Meier-Ruge, W. (1982): Morphological diagnosis. In: *Hirschsprung's disease.* Ed. A.M. Holschneider. Hippokrates, Stuttgart.

Michna, B., N.B. McWilliams, T.M. Krummel, M.A. Hartenberg, and A.M. Salzberg (1988): Multifocal ganglioneuroblastoma coexistent with total colonic aganglionosis. J. Pediatr. Surg. 23:57-59.

Moscona, A.A. (1952): Cell suspensions from organ rudiments of chick embryos. Exp. Cell Res. 3:536-539.

Munakata, K., K. Morita, and H. Sueoka (1985): Clinical and histologic studies of neuronal intestinal dysplasia. J. Pediatr. Surg. 20:231-235.

Murphy, J.B., and P. Rous (1912): The behavior of chicken sarcoma implanted in the developing embryo. J. Exp. Med. 15:119-132.

Nagafuchi, A., Y. Shirayoshi, K. Okazaki, K. Yasuka, and M. Takeichi (1987): Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. Nature (London) 329:341-343.

Neff, N.T., C. Lowrey, C. Decker, A. Tovar, C. Damsky, C. Buck, and A.F. Horwitz (1982): A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. J. Cell Biol. 95:654-666.

Newgreen, D.F., I. Jahnke, I.J. Allan, and I.L. Gibbins (1980): Differentiation of sympathetic and enteric neurons of the fowl embryo in grafts to the chorioallantoic membrane. Cell Tissue Res. 208:1-19.

Newgreen, D.F., and J.P. Thiery (1980): Fibronectin in early avian embryos: synthesis and distribution along the migration pathways of neural crest cells. Cell Tissue Res. 224:145-160.

Newgreen, D.F., I.L. Gibbins, J. Sauter, B. Wallenfels, and R. Wutz (1982): Ultrastructural and tissue culture studies on the role of fibronectin, collagen and glycosaminoglycans in the migration of neural crest cells in the fowl embryo. Cell Tissue Res. 203:115-140.

Newgreen, D.F., and I. Gibbins (1982): Factors controlling the time of onset of the migration of neural crest cells in the fowl embryo. Cell Tissue Res. 224:145-160.

Nixon, H.H. (1955): Intestinal obstruction in the newborn. Arch. Dis. Child. 30:13-xx.

Noden, D. (1975): An analysis of the migratory behaviour of avian cephalic neural crest cells. Dev. Biol. 24:106-130.

Noden, D. (1978): The control of avian cephalic neural crest cytodifferentiation. I. Skeletal and connective

tissues. Dev. Biol. 67:296-312.

Noden, D.M. (1983): The embryonic origins of avian cephalic and cervical muscles and associated connective tissues. Am. J. Anat. 168:257-276.

Noden, D.M. (1984): Craniofacial development: new views on old problems. Anat. Rec. 208:1-13.

Nolf, P. (1934): Les nerfs extrinsiques de l'intestin chez l'oiseau. Le nerf de Remak. Arch. Inr. Physiol. 39:227-256.

Noronha, A.B., A. Ilyas, H. Antonicek, M. Schachner, and R.H. Quarles (1986): Molecular specificity of L2 monoclonal antibodies that bind to carbohydrate determinants of neural cell adhesion molecules and their resemblance to other monoclonal antibodies recognizing the myelin-associated glycoprotein. Brain Res. 385:237-244.

Nose, A., and M. Takeichi (1986): A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. J. Cell Biol. 103:2649-2658.

Nusse, R. (1988): The *int* genes in mammary tumorigenesis and in normal development. Trends Genet 4:291-295.

Obara, M., M.S. Kang, and K.M. Yamada (1988): Site-directed muatagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. Cell 53:649-657.

Ohama, C. (1982): Prenatal detection of conceptuses with chromosomal abnormalities. Cong. Anom. (Japan): 22:391.

Okamoto, E., and T. Ueda (1967): Embryogenesis of intramural ganglia of the gut and its relation to Hirschsprung's disease. J. Pediatr. Surg. 2:437-443.

Okamoto, E., T. Iwasaki, T. Kakutani, and T. Ueda (1967): Selective destruction of the myenteric plexus: its relation to Hirschsprung's disease, achalasia of the esophagus and hypertrophic pyloric stenosis. J. Pediatr. Surg. 2:444-454.

Okata, K.M., M. Oide, and S. Handa (1977): Effects of glycolipids on in vitro development of neuromuscular junctions. Nature (London) 266:269-371.

Omenn, G.S., and V. A. McKusick (1979): The association of Waardenburg syndrome and Hirschsprung megacolon. Am. J. Med. Genet. 3:217-223.

Orr, J.D., and W.G. Scobie (1983): Presentation and incidence of Hirschsprung's disease. Br. M. J. 287:1671.

Passarge, E. (1967): The genetics of Hirschsprung's disease. Evidence for heterogeneous etiology and a study of sixty-three families. New Engl. J. Med. 276:138-143.

Passarge, E. (1983): Hirschsprung disease and other developmental defects of the gastrointestinal tract. In: *Principles and practice of medical genetics.* Emery, A.E.H., and D.L. Rimoin Eds. Churchill Livingstone, Edingburgh.

Payette, R.F., G.S. Bennett, and M.D. Gershon (1984): Neurofilament expression in vagal neural crest-derived precursors of enteric neurons. Dev. Biol. 105:273-287.

Payette, R.F., V.M. Tennyson, T. Duc Pham, G.M. Mawe, H.D. Pomeranz, T.P. Rothman, and M.D. Gershon (1987): Origin and morphology of nerve fibers in the aganglionic colon of the lethal spotted (ls/ls): mutant mouse. J. Comp. Neurol. 257:237-252.

Payette, R.F., V.M. Tennyson, H.D. Pomeranz, T. Duc Pham, T.P. Rothman, and M.D. Gershon (1988): Accumulation of components of basal laminae: association with the failure of neural crest cells to colonize the presumptive aganglionic bowel of ls/ls mice. Dev. Biol. 125:341-360.

Pearse, A.G.E., J.M. Polak, F.W.D. Rost, J. Fontaine, C. LeLievre, and N. Le Douarin (1973): Demonstration of the neural crest origin of type I (APUD) cells in the avian carotid body, using a cytochemical marker system. Histochemistry 34:191-203.

Penzlin, H. (1985): Stomatogastric nervous system. In: *Comprehensive insect physiology, biochemistry, and pharmacology.* G.A. Kerkut, and L.I. Gilbert, Eds. Vol. 5, pp. 371-406. Pergamon, Oxford.

Perris, R., Y. von Boxberg, and J. Löfberg (1988): Local embryonic matrices determine region-specific phenotypes in neural crest cells. Science (Washington DC) 241:86-89.

Pesheva, P., A.F. Horwitz, and M. Schachner (1987): Integrin, the cell surface receptor for fibronectin and laminin, expresses the L2/HNK-1 and L3 carbohydrate structures shared by adhesion molecules. Neurosci. Lett. 83:303-306.

Petersen, T.E. (1983): Proc. Natl. Acad. Sci. USA 80:137-141.

Phat, V.N., A. Sezeur, M. Danne, D. Dupuis, G. de la Vaissiere, and J-P. Camilleri (1980): Primary myenteric plexus alterations as a cause of megacolon in von Recklinghausen's disease. Path. Biol. 28:585-588.

Pierschbacher, M.D., E. Ruoslahti, J. Sundelin, P. Lind, and P.A. Peterson (1982): The cell attachment domain of fibronectin. J. Biol. Chem. 257:9593-9597.

Pobert, L., J. de Mey, and J.M. Polak (1981): Distinct subpopulations of enteric p-type neurones contain substance P and vasoactive intestinal polypeptide. Nature (London): 294:470-471.

Pollerberg, G.E., M. Schachner, and J. Davoust (1986): Differentiation state-dependent surface mobilities of two forms of the neural cell adhesion molecule. Nature (London) 324:462-465.

Pollerberg, G.E., K. Burridge, K.E. Krebs, S.R. Goodman, and M. Schachner (1987): The 180-kD component of the neural cell adhesion molecule NCAM is involved in cell-cell contacts and cytoskeleton-membrane interactions. Cell Tissue Res. 250:227-236.

Polley, T.Z., and A.G. Coran (1986): Hirschsprung's disease in the newborn. An 11-year experience. Pediatr. Surg. Int. 1:80-83.

Poltorak, M., R. Sadoul, G. Keilhauer, C. Landa, T. Fahrig, and M. Schachner (1987): Myelin associated glycoprotein, a meber of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte andoligodendrocyte-oligodendrocyte interaction. J. Cell Biol. 105:1893-1899.

Pompino, H.J. (1969): Berichte über experimentelle Untersuchungen zur Frage des erworbenen Megacolon. Z. Kinderchir. 7:262-72.

Puri, P., B.D. Lake, H.H. Nixon, H. Mishalany, and A.E. Claireaux (1977): Neuronal colonic dysplasia: an unusual association of Hirschsprung's disease. J. Pediatr. Surg. 12:681-685.

Rathjen, F.G. and M. Schachner (1984): Immunological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. EMBO J. 3:1-10.

Rathjen, F.G., J.M. Wolff, R. Frank, F. Bonhoeffer, and U. Rutishauser (1987): Membrane glycoproteins involved in neurite fasciculation. J. Cell Biol. 104:342-353.

Ravitch, M. (1958): Pseudo-Hirschsprung's disease. Ann. Surg. 147:781-795.

Regan, L.J., J. Dodd, S.H. Barondes, and T.M. Jessel (1986): Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons. Proc. Natl. Acad. Sci. USA 83:2248-2252.

Revel, J.P.,and S.S. Brown (1975): Cell junctions in development, with particular reference to the neural tube. Cold Spring Harbor Symp. Quant. Biol. 40:443-455.

Reynolds, J.F., J.C. Barber, B.A. Alford, J.G. Chandler, and T.E. Kelly (1983): Familial Hirschsprung's disease and type D brachydactyly: a report of four affected males in two generations. Pediatrics 71:246-249.

Rickham P.P., J. Lister, and I.M. Irving (1978): *Neonatal surgery.* Butterworths, London.

Richardson, W.R., and I.H. Brown (1962): Hirschsprung's disease in infants and children. Am. J. Surg. 28:149.

Riopelle, R.J., R.C. McGarry, and J.C. Roder (1986): Adhesion properties of a neuronal epitope recognized by the monoclonal antibody HNK-1. Brain Res. 367:20-25.

Ringald, M., R. Schuh, D. Vestweber, H. Eistetter, F. Lottspeich, J. Engel, R. Dolz, F. Jahnig, J. Epplen, S. Mayer, and R. Kemler (1987): The structure of cell adhesion molecule uvomorulin. Insights into the molecular mechanism of Ca2+-dependent cell adhesion. EMBO J. 6:3647-3653.

Robert, B., D. Sassoon, B. Jacq, W. Gehring, and M. Buckingham (1989): Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. EMBO J. 8:91-100.

Roth, S.E.,E.J. Maguire, and S. Roseman (1971): Evidence for cell surface glycosyltransferases: their potential in cellular recognition. J. Cell Biol. 536-547.

Rothman, T.P., and M.D. Gershon (1982): Phenotypic expression in the developing murine enteric nervous system. J. Neurosci. 2:381-393.

Rothman, T.P., and M.D. Gershon (1984): Regionally defective colonization of the terminal bowel by precursors of enteric neurons in lethal spotted mutant mice. Neuroscience 12:1293-1311.

Rothman, T.P., V.M. Tennyson, and M.D. Gershon (1986): Colonization of the bowel by the precursors of enteric glia: studies of normal and congenitally aganglionic mutant mice. J. Comp. Neurol. 252:493-506.

Rothman, T.P., M.D. Gershon, J.C. Fontaine-Perus, M. Chanconie, and N.M. Le Douarin (1987): The effect of back-transplants of embryonic gut wall on growth of the neural tube. Dev. Biol. 124:331-346.

Rothman, T.P., V.M. Tennyson, and M.D. Gershon (1987): Loss of the capacity of intrinsic neural precursors to express a catecholaminergic phenotype is correlated with invasion of the developing chick gut by sympathetic nerves. Neurosci. Abstr. 13:925.

Roy, A.D., H. Barucha, N.C. Nevin, and G.W. Odling-Smee (1980): Idiopathic intestinal pseudo-obstruction: a familial visceral neuropathy. Clin. Genet. 18:291-297.

Rovasio, R.A., A. Delouvee, K.M. Yamada, R. Timpl, and J.P. Thiery (1983): Neural crest cell migration: requirements for exogenous fibronectin and high cell density. J. Cell Biol. 96:462-473.

Rudin, Ch., P.M. Jenny, C.P. Fliegel, H. Ohnacker, and Ph. U. Heitz (1986): Zuelzer-Wilson's syndrome and absence of the enteric nervous system. Two rare forms of anomalies of the enteric nervous system with identical clinical symptoms. Z. Kinderchir. 41:287-292.

Runyan, R.B., G.D. Maxwell, and B.D. Shur (1986): Evidence for a novel enzymatic mechanism of neural crest cell migration on extracellular glycoconjugate matrices. J. Cell Biol. 102:432-441.

Runyan, R.B., J. Versalovic, and B.D. Shur (1988): Functionally distinct laminin receptors mediate cell adhesion and spreading: the requirement for surface galactosyl-transferase in cell spreading. J. Cell Biol. 107:1863-1871.

Ruoslahti, E. (1988): Fibronectin and its receptors. Ann. Rev. Biochem. 57:375-413.

Rutishauser, U. (1986): Differential cell adhesion through spatial and temporal variations. Trends Neurosci. 9:374-378.

Rutishauser, U., and T.M. Jessel (1988): Cell adhesion molecules in vertebrate neural development. Physiol. Rev. 68:819-857.

Ruysch, F. (1691): *Observationum Anatomico-Chirurgicarum Centuria.* Amsterdam, H & T Boom.

Sacher, P., J. Briner, and U.G. Stauffer (19XX): Clinical aspects of neuronal intestinal dysplasia. Z. Kinderchir. XX:96-97.

Sadoul, R., M. Hirn, H. Deagostini-Bazin, G. Rougon, and C. Goridis (1983): Adult and embryonic mouse neural cell adhesion molecules have different binding properties. Nature (London) 304:347-349.

Saffrey, M.J., J.M. Polak, and G. Burnstock (1982): Distribution of vasoactive intestinal polypeptide-, substance P-, enkephalin-, and neurotensin-like immunoreactive nerves in the chicken gut during development. Neuroscience 7:279-293.

Salton, S.R., C. Richter-Landsberg, and L.A. Greene (1983): Nerve growth factor-inducible large external (NILE) glycoprotein: studies of a central and peripheral neuronal marker. J. Neurosci. 3:441-454.

Santos, H., J. Mateus, and M.J. Leal (1988): Hirschsprung's disease associated with polydactyly, unilateral renal agenesis, hypertelorism, and congenital deafness: a new autosomal recessive syndrome. J. Med. Genet. 25:204-208.

Saul, R.A., R.A. Sturber, and P.C. Burger (1982): Hyperplasia of the myenteric plexus. Its association with

early infantile megacolon and neurobromatosis. Am. J. Dis. Child. 136:852-854.

Saul, R.A., and R.E. Stevenson (1988): The nosology of megacolon. Am. J. Med. Genet. S.4:180.

Schärli, A.F., and W.Meier-Ruge (1981): Localized and disseminated forms of neuronal intestinal dysplasia mimicking Hirschsprung's disease. J. Pediatr. Surg. 16:164-170.

Schoeller, J. (1964): Recherches descriptives et experimentales sur la cephalogenese de Calliphra erythrocephala (Meigen) au cours des developpements embryonnaire et postembryonnaire. Arch. Zool. Exp. Gen. 103:1-216.

Schuffler, M.D., T.D. Bird, S.M. Sumi, and A. Cook (1978): A familial neuronal disease presenting as intestinal pseudoobstruction. Gastroenterology 75:889-898.

Schuller-Petrovic, S., W. Gebhart, H. Lassmann, H. Rumpold, and D. Kraft (1983): A shared antigenic determinant between natural killer cells and nervous tissue. Nature (London): 306:179-181.

Schwarting, G.A., F.B. Jungalwala, D.K.H. Chou, A.M. Boyer, and M. Yamamoto (1987): Sulfated glucuronic acid-containing glycoconjugates are temporally and spatially regulated antigens in the developing mammalian nervous system. Dev. Biol. 120:65-76.

Shah, K.N., J. Dalal, M.P. Desai, P.N. Sheth, N.C. Joshi, and L.M. Ambani (1981): White forelock, pigmentary disorder of the irides, and long segment Hirschsprung disease: possible variant of Waardenburg syndrome. J. Pediatr. 99:432-435.

Shocket, E., and H.A. Telok (1957): Aganglionic megacolon, pheochromocytoma, megaloureter, and neurofibroma. Am. J. Dis. Child. 94:185-195.

Shur, B.D. (1982): Evidence that galactosyltransferase is a surface receptor for poly(N)-acetyllactosamine glycoconjugates on embryonal carcinoma cells. J. Biol. Chem. 257:6871-6878.

Shur, B.D., and N.G. Hall (1982): A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. J. Cell Biol. 95:574-579.

Skinner, R., and D. Irvine (1973): Hirschsprung's disease and congenital deafness. J. Med. Genet. 10:337-339.

Slack, J.M.W. (1980): A series threshold theory of regeneration. J. Theor. Biol. 82:105-140.

Smith, B. (1968): Pre- and postnatal development of the ganglion cells of the rectum and its surgical implications. J. Pediatr. Surg. 3:386-391.

Smith, J., P. Cochard, and N.M. Le Douarin (1977): Development of choline acetyltransferase and cholinesterase activities in enteric ganglia derived from presumptive adrenergic and cholinergic levels of the neural crest. Cell Differ. 6:199-216.

Spouge, D.,and P.A. Baird (1985): Hirschsprung's disease in a large birth cohort. Teratology 32:171-177.

Staple, T.W., W.H. McAllister, and M.S. Anderson (1964): Plexiform neurofibromatosis of the colon simulating Hirschsprung's disease. Am. J. Roentgenol. 91:840-845.

Stallcup, W.B., and L. Beasly (1985): Involvement of the nerve growth factor-inducible large external glycoprotein (NILE) in neurite fasciculation in primary culture of rat brain. Proc. Natl. Acad. Sci. USA 82:1276-1280.

Stern, M., H.H. Hellwege, L. Gravinghoff, and W. Lambrecht (1981): Total aganglionosis of the colon (Hirschsprung's disease): and congenital failure of automatic control of ventilation (Ondine's curse): Acta Pediatr. Scan. 70:121-124.

Sundler, F., J. Alumets, J. Fahrenkrug, R. Hakanson, and O.B. Schaffalitzky de Muckadell (1979): Cellular localization and ontogeny of immunoreactive vasoactive intestinal polypeptide (VIP) in the chicken gut. Cell Tissue Res. 196:193-201.

Sunshine, J., K. Balak, U. Rutishauser, and M. Jacobson (1987): Changes in neural cell adhesion molecule (NCAM) structure during vertebrate neural development. Proc. Natl. Acad. Sci. USA 84:5986-5990.

Suzuki, H., T. Chiba, and M. Kasai (1978): Entire colon aganglionosis, and extensive aganglionosis: analysis of 94 cases in Japan. Jap. J. Surg. 8:119-122.

Svajger, A., B. Levek-Svajger, and N. Skreb (1986): Rat embryonic ectoderm as renal isograft. J. Embryol. Exp. Morphol. 94:1-27.

Swenson, O., and A.H. Bill (1948): Resection of rectum and rectosigmoid with preservation of sphincter for benign spastic lesions producing megacolon. Surgery 24:212-220.

Swenson, O., H.F. Rheinlander, and I. Diamond (1949): Hirschsprung's disease: a new concept of the etiology. Operative results in thirty-four patients. New Engl. J. Med. 241:551-556.

Swenson, O. (1959): Hirschsprung's disease (aganglionic megacolon): New Engl. J. Med. 260:972-976.

Swenson, O., J.O. Sherman, and J.H. Fisher (1973): Diagnosis of congenital megacolon: an analysis of 501 patients. J. Pediatr. Surg. 8:581-593.

Tagushi, T., K. Tanaka, K. Ikeda, and A. Hata (1983): Double zonal aganglionosis with a skipped oligoganglionic ascending colon. Z. Kinderchir. 38:312-315.

Taguchi, T., K. Tanaka, and K. Ikeda (1985): Fibromuscular dysplasia of arteries in Hirschsprung's disease. Gastroenterology 88:1099-1103.

Takeichi, M.(1987): The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development 102:639-655.

Talwalker, V.C. (1976): Aganglionosis of the entire bowel. J. Pediatr. Surg. 11:213-216.

Tam, P.K.H., and J. Lister (1986): Development profile of neuron-specific enolase in human gut and its implications in Hirschsprung's disease. Gastroenterology 90:1901-1906.

Tan, S-S., K.L. Crossin, S. Hoffman, and G.M. Edelman (1987): Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest cell distribution. Proc. Natl. Acad. Sci. USA 84:7977-7981.

Tanner, M.S., B. Smith, and J.K Lloyd (1976): Functional intestinal obstruction due to deficiency of argyrophilic neurones in the myenteric plexus. Familial syndrome presenting with short small bowel, malrotation, and pyloric hypertrophy. Arch. Dis. Child. 51:837-841.

Teillet, M-A. (1978): Evolution of the lumbo-sacral neural crest in the avian embryo: origin and differentiation of the ganglionated nerve of Remak studied in interspecific quail-chick chimerae. W. Roux's Arch. 184:251-268.

Teitelman, G., H. Baker, T.H. Joh, and D.J. Reis (1979): Appearance of catecholamine-synthesizing enzymes during development of rat sympathetic nervous system: possible role of tissue environment. Proc. Natl. Acad. Sci. USA 76:509-513.

Tello, J.F. (1924): La precocité embryonaire du plexus d'Auerbach et ses differences dans les intestine anterieur at posterior. Trab. Inst. Cajal Invest. Biol. 22:317-328. Quoted in Yntema and Hammond (1954), and Van Campenhout (1932)

Tello, J.F. (1925): Sur la formation des chaines primaire et secondaire du grand sympatique dans l'embryon de poulet. Trab. Lab. Invest. Biol. Univ. Madrid 23:1-28. Quoted in Van Campenhout (1932).

Tennyson, V.M., T. Duc Pham, T.P. Rothman, and M.D. Gershon (1986): Abnormalities of smooth muscle, basal laminae, and nerves in the aganglionic segments of the bowel of lethal spotted mutant mice. Anat. Record 215:267-281.

Thiery, J.P., J-L. Duband, and A. Delouvee (1982): Pathways and mechanisms of avian trunk neural crest cell migration. Dev. Biol. 93:324-343.

Thiery, J.P., J-L. Duband, U. Rutishauser, and G.M Edelman (1982): Cell adhesion molecules in early chicken embryogenesis. Proc. Natl. Acad. Sci. USA 79:6737-6741.

Thiery, J.P., A. Delouvee, W.J. Gallin, B.A. Cunningham, and G.M. Edelman (1984): Ontogenetic expression of cell adhesion molecules: L-CAM is found in epithelia derived from the three primary germ layers. Dev. Biol. 102:61-78.

Thiery, J.P., A. Delouvee, M. Grumet, and G.M. Edelman (1985): Initial appearance and regional distribution of the neuron-glia cell adhesion molecule in the chick embryo. J. Cell Biol. 100:442-456.

Thiery, J.P., J-L., Duband, and A. Delouvee (1985): The role of cell adhesion in morphogenetic movements during early embryogenesis. In: *The cell in Contact.* Eds. J.P. Thiery and G.M. Edelman. Riley, New York.

Thiery, J.P., and J-L. Duband (1986): Role of tissue environment and fibronectin in the patterning of neural crest derivatives. Trends Neurosci. nov/dec:565-570.

Thorpe, S.J., R. Bellairs R., and T. Feizi (1988): Developmental patterning of carbohydrate antigens during early embryogenesis of the chick: expression of antigens of the poly-N-acetyllactosamine series. Development 102:193-210.

Tibboel, D., J.H.C. Meijers, P. Klück, A.W.M van der Kamp, F.W.J. ten Kate, C.C.M. van Haperen-Heuts, J.C. and Molenaar (1987): Use of monoclonal antibodies for diagnosis and research in pathology of the enteric nervous system. A Review. Dev. Neurosci. 9:133-143.

Tiffin, M.E., L.R. Chandler, and H.K. Faber (1940): Localized absence of the ganglion cells of the myenteric plexus in congenital megacolon. Am. J. Dis. Childh. 59:1071-1982.

Toriello, H.V., K.Komar, C. Lawrence, J.V. Higgins, and D.F. Waterman (1988): Macrocephaly, Hirschsprung's disease, brachydactyly, vertebral defects and other minor anomalies. Dysmorphol. Clin. Genet. 1:155-157.

Tosney, K.W. (1978): The early migration of neural crest cells in the trunk region of the avian embryo: an electron microscopic study. Dev. Biol. 62:317-333.

Tosney, K.W. (1982): The segregation and early migration of cranial neural crest cells in the avian embryo. Dev. Biol. 89:13-24.

Tucker, G.C., H. Aoyama, M. Lipinski, T. Tursz, and J.P. Thiery (1984): Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Differ. 14:223-230.

Tucker, G.C., G. Ciment, and J.P. Thiery (1986): Pathways of avian neural crest cell migration in the developing gut. Dev. Biol. 116:439-450.

Tucker, G.C., M. Delarue, S. Zada, J-C. Boucaut, and J.P. Thiery (1988): Expression of the HNK-1/NC1 epitope in early vertebrate neurogenesis. Cell and Tissue Res. 251:457-465.

Tucker, G.C., J-L.Duband, S. Dufour, and J.P. Thiery (1988): Cell adhesion and substrate adhesion molecules: their instructive role in neural crest cell migration. Development 103:82-94.

Tucker, R.P. and C.A. Erickson (1984): Morphology and behavior of quail neural crest cells in artificial three-dimensional extracellular matrices. Dev. Biol. 104:390-405.

Van Campenhout, E. (1931): L'origine du systeme nerveux sympatique chez le poulet. Arch. Biol. Lib. Mem. Brachet 42:479-507.

Van Campenhout E. (1932): Further experiments on the origin of the enteric nervous system in the chick. Physiol. Zoology 5:333-353.

Vaos, G.C. and J. Lister (1987): Elastic fibers in musculature of rectosigmoid colon: normal findings in children and changes in Hirschsprung's disease -- a preliminary report. J. Pediatr. Surg. 22:252-256.

Vaos, G.C., and J. Lister (1988): Anatomic evidence for coexistence of cholinergic and adrenergic neurons in the developing human intestine: new aspects in the pathogenesis of developmental neuronal abnormalities. J. Pediatr. Surg. 23:231-236.

Vaughan, L., S. Huber, M. Chiquet, and K. H. Winterhalter (1987): A major, six-armed glycoprotein from embryonic cartilage. EMBO J. 6:349-353.

Vincent, M., J-L. Duband, and J.P. Thiery (1983): A cell surface determinant expressed early on migrating neural crest cells. Dev. Br. Res. 9:235-238.

Vincent, M., and J.P. Thiery (1984): A cell surface marker for neural crest and placodal cells: further evolution in peripheral and central nervous system. Dev. Biol. 103:468-481.

Vinores, S.A. and E. May (1985): Neuron-specific enolase as an immunohistochemical tool for the diagnosis of Hirschsprung's disease. Am. J. Surg. Pathol. 9:281-285.

Walker, A.W., R.L. Kempson and J.L. Ternberg (1966): Aganglionosis of the small intestine. Surgery 60:449-457.

Walther, F.G., and G. Kootstra (1981): Total small bowel and colon aganglionosis. Z. Kinderchir. 33:310-315.

Warkany, J. (1967): *Congenital malformations.* Year Book Medical Publishers Inc. Chicago. pp. 704-709.

Weatherall, D.J. (1985): *The new genetics and clinical practice.* Oxford University Press, Oxford.

Webster, W.S. (1973): Embryogenesis of the enteric ganglia in normal mice and in mice that develop congenital aganglionic megacolon. J. Embryol. Exp. Morphol. 30:573-585.

Weinberg, A.G., G. Currarino, and A. M. Besserman (1977): Hirschsprung's disease and congenital deafness. Familial association. Hum. Genet. 38:157-161.

Westerveld, A., A.P.M. Jongsma, P. Meera-Khan, H. van Someren, and D. Bootsma (1976): Assignment of the AK1:Np:ABO linkage group to human chromosome 9. Proc. Natl. Acad. Sci. USA 73:895-899.

Wheelock, M.J., C.A. Buck, K.N. Bechtol, and C.H. Damsky (1987): Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. J. Cell. Biochem. 34:187-202.

Whitehouse, F.R., and J.W. Kernohan (1948): Myenteric plexus in congenital megacolon. Study of eleven cases. Arch. Int. Med. 82:75-111.

Wilkinson, D.G., S. Bhatt, P. Chavrier, R. Bravo, and P. Charnay (1989): Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. Nature (London): 337:461-464.

Williams, A.F. (1987): A year in the life of the immunoglobulin superfamily. Immun. Today 8:298-303.

Wingate, D.L. (1986): Neurophysiology of the gastrointestinal tract. Gastroenterol. Ann. 3:257-283.

Wolff, J.M., F.G. Ratjen, R. Frank, and S. Roth (1987): Distribution of stage-specific neurite-associated proteins in the developing murine nervous system recognized by a monoclonal antibody. Eur. J. Biochem. 168:551-561.

Wolgemuth, D.J., R.R. Behringer, M.P. Mostoller, R.L. Brinster, and R.D Palmiter (1989): Transgenic mice overexpressing the mouse homeobox-containing gene Hox-1.4 exhibit abnormal gut development. Nature (London): 337:464-467.

Wood, J.D. (1979): Congenital megacolon (Hirschsprung's disease). In: *Spontaneous animal models of human disease.* Vol I. Eds. Andrews, E.J. et al. Academic Press, New York, USA.

Wright, T.C., R.W. Orkin, M. Destrempes, and D.M. Kurnit (1984): Increased adhesiveness of Down syndrome fetal fibroblasts in vitro. Proc. Natl. Acad. Sci. USA 81:2426-2430.

Yntema, C.L., and W.S. Hammond (1945): Depletions and abnormalities in the cervical sympathetic system of the chick following extirpation of neural crest. J. Exp. Zool. 100:237-263.

Yntema, C.L., and W.S. Hammond (1953): Experiments on the sacral parasympathetic nerves and ganglia of chick embryo. Anat. Rec. 115:382.

Yntema, C.L. and W.S. Hammond (1954): The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J. Comp. Neurol. 101:515-541

Yntema, C.L., and W.S. Hammond (1955): Experiments on the origin and development of the sacral autonomic nerves in the chick embryo. J. Exp. Zool. 129:375-414.

Yoshida-Noro, C.N., N. Suzuki, and M. Takeichi (1984): Molecular nature of the calcium-dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. Dev. Biol. 101:19-27.

Yunis, E.J., A.W. Dibbins, and F.E. Sherman (1976): Rectal suction biopsy in the diagnosis of Hirschsprung's disease. Z. Kinderchir. 34:36-42.

Yunis, E., W.K. Sieber, and D.R. Akers (1983): Does zonal aganglionosis really exist? Report of a rare variety of Hirschsprung's disease and review of the literature. Pediatr. Pathol. 1:33-49.

Ziegler, M.M., A.J. Ross, and H.C. Bishop (1987): Total intestinal aganglionosis: a new technique for prolonged survival. J. Pediatr. Surg. 22:82-83.

Zuelzer, W.W., and J.L. Wilson (1948): Functional intestinal obstruction on a congenital neurogenic basis in infancy. Am. J. Dis. Childh. 75:40-64.

CHAPTER 7.1

THE EFFECT OF ISCHEMIA ON THE DEVELOPING ENTERIC NERVOUS SYSTEM: AN EXPERIMENTAL STUDY IN THE CHICKEN EMBRYO AND A CLINICAL STUDY IN HUMANS

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SUMMARY. Local intestinal ischemia has been proposed as a cause of Hirschsprung's disease. To test this hypothesis we induced permanent and temporary local intestinal ischemia in chicken embryos. One of the main branches of the omphalomesenteric artery was either ligated or crushed. Other embryos were subjected to periods of general hypoxia. The effect of the interventions was studied at diverse stages of development. Enteric neurons or neural crest cells were visualized using monoclonal antibody HNK-1. Enteric neurons were absent or pyknotic if severe ischemic changes had also occurred in other parts of the bowel wall, irrespective of the type of ischemia induced. Permanent or temporary local intestinal ischemia at E16 led to intestinal atresia or stenosis. We also studied the enteric nervous system in resected bowel specimens of 24 neonates that underwent surgery for intestinal atresia (small bowel and colonic atresia) or meconium peritonitis. Enteric neurons were absent or pyknotic if severe ischemic changes had also occurred in other parts of the bowel wall. No differences were found between small bowel and colonic atresia. We conclude that local intestinal ischemia occurring at an early stage of development does not lead to selective disappearance of enteric neurons.

Abbreviations: E.. = day of embryonic development; HNK-1 = Human Natural Killer cell.

INTRODUCTION

Hirschsprung's disease (congenital aganglionosis) is characterized by the absence of enteric neurons in the hindgut and hypertrophic nerve trunks in the lamina propria, submucosa, and smooth muscle layers (1). The exact cause of aganglionosis is not known although several pathogenetic mechanisms have been proposed (2-4). Recently, attention has refocused on ischemia as the causative factor in aganglionosis when abnormal

intramural blood vessels were rediscovered in the transitional zone in patients with Hirschsprung's disease (5). The association of abnormal arteries with Hirschsprung's disease was first reported in the 1960s (6). At the time, the effect of ischemia on the enteric nervous system was studied experimentally by several investigators (7-12), based on the early studies of Cannon and Burket (13). All these studies were carried out in adult animals. Consequently, any conclusions about the pathogenesis of aganglionosis concerned acquired aganglionosis only. If ischemia plays a part in the pathogenesis of congenital aganglionosis, then the ischemic insult must occur before birth. It might well be that the response of fetal enteric neurons to ischemia differs from that of adult enteric neurons.

Previous experiments have demonstrated the merits of the chicken embryo as a laboratory animal for studying congenital anomalies of the digestive tract (14,15). It provides a suitable model for studying the effect of ischemia on the developing enteric nervous system in view of the detailed knowledge concerning its normal development in chickens (16). Both enteric neurons and enteric glial cells derive from the neural crest opposite somite 1-7, while there is controversy about a slight contribution by the sacral neural crest (16-19). The moment that neural crest cells reach the various segments of the gut has well been documented (17-19). Neural crest cell colonization of the gut is completed by the eighth day (E8) of development (16-20).

We studied the effect of either permanent or temporary local ischemia on the ENS in the postumbilical bowel by manipulating branches of the omphalomesenteric artery in the embryo. We chose E9 and E16 as the stages to induce intestinal ischemia, examples of newly formed and well established enteric ganglia respectively. We also investigated the effect of general hypoxia of the fetus on the developing enteric nervous system.

In order to extrapolate our experimental findings to human diseases, we investigated surgical specimen from patients with diseases with proven ischemic etiologies. Antenatal intestinal ischemia can lead to intestinal atresia (14,21). It is likely that the bowel segments adjacent to the obstruction also experience a period of moderate ischemia; they are thus most suited to investigate whether enteric neurons are selectively killed by moderate ischemia. To exclude regional differences in the susceptibility of enteric neurons for moderate ischemia, we investigated material from both small-bowel and colonic atresia. Ischemic changes of the bowel wall have also been reported for patients with meconium peritonitis (22) and premature infants with necrotizing enterocolitis (23) frequently leading to intestinal stenosis (24). Consequently, we also looked for anomalies of the enteric nervous system in surgical specimens of patients with meconium peritonitis, and necrotizing enterocolitis.

MATERIALS AND METHODS

EXPERIMENTAL STUDY

Fertilized eggs of white leghorn chickens (*Gallus Domesticus*) were incubated in a forced

draught incubator at a relative humidity of 70% at 38°C. The embryos were staged according the number of incubation days (E..). To study the normal enteric plexus at various stages of development, we removed the bowel of control embryos at E9, E16 and E21 and performed immunoperoxidase staining for neural crest cells or enteric neurons, using the HNK-1 antibody.

Induction of permanent local ischemia. Permanent local ischemia was induced by crushing a branch of the omphalomesenteric artery at E9 in 100 embryos and at E16 in 315 embryos. After opening the egg shell and shell membranes, the physiologic hernia was located following the umbilical artery over the yolk sac. The physiologic umbilical hernia was opened and a number of bowel loops, including the mesentery, were luxated and a branch of the omphalomesenteric artery supplying the postumbilical bowel was crushed. After the intervention, the bowel loops were put back into the physiologic hernia and the opening in the eggshell was sealed off with sellotape. The effect of this induced permanent ischemia was studied between 4 and 8, as well as 24 hours after the intervention and again at hatching. Two groups of, respectively, 100 and 169 embryos served as controls. These embryos underwent a sham operation at either E9 or E16, whereby the bowel loops were only lifted out of the umbilical hernia without crushing any blood vessels.

Induction of temporary local ischemia. To determine whether temporary local ischemia followed by reperfusion would cause a selective disappearance of enteric neurons, we ligated a main branch of the pedicle of the omphalomesenteric artery with monofilament ethilon 9/0 which was left in situ for a variable length of time. The ischemic period lasted 5 minutes in 25 embryos (group A); 25 minutes in 26 embryos (group B), and 60 minutes in 31 embryos (group C). A fourth group (D) of 23 embryos underwent a sham operation. The effect of reperfusion of the pertinent bowel segment was studied both macroscopically and microscopically. Eggs were re-incubated and at E21 (normal day of hatching) the surviving embryos sacrificed for investigation. Special attention was paid to signs of obstruction and secondary changes in the bowel proximal and distal to the site of induced ischemia.

Induction of general hypoxia. We also determined whether selective disappearance of enteric neurons might be caused by the diving reflex, which is a reduction in mesenteric blood flow caused by general hypoxia of the embryo (25,26). To this effect, E16 eggs were transferred to a glass pot within the incubator and the oxygen content of this pot lowered to variable degrees. One group of 20 embryos was exposed to an oxygen concentration of 12 vol% for 180 minutes and another group of 20 embryos to 4 vol% for 45 minutes. The technique has been described in detail (14). Twenty embryos with undisturbed incubation served as controls.

CLINICAL STUDY

Resected bowel of 24 neonates that underwent surgery for intestinal atresia, meconium peritonitis or necrotizing enterocolitis was investigated. There were 20 patients with intestinal atresia, in 6 of them located in the jejunum, in 7 in the ileum, and in 5 in the colon, while the other 2 patients had multiple atresias. In four cases the intestinal atresia

was associated with meconium peritonitis. Two other patients had meconium peritonitis only. The remaining two patients, premature born after 34 and 35 weeks gestation respectively, had necrotizing enterocolitis and they developed intestinal atresia 7 and 8 weeks postnatally. Cystic fibrosis was excluded in all cases by negative sweat tests, using pilocarpine iontophoresis.

Histology. Tissue specimen were fixed in 4% formaldehyde and embedded in paraffin. Sections were made at 7 μm. Routine staining methods were used (hematoxylin/eosin; von Gieson's elastica), as well as special staining methods to identify bile pigments (Fouchet), hemosiderin pigment or iron. The specimen used for transmission EM studies was fixed in glutaraldehyde and embedded in Araldite^{R}.

Immunocytochemistry. Endogenous peroxidase activity was blocked by incubating the tissue sections in methanol with 1% hydrogen-peroxide for 20 minutes. The HNK-1 antibody, directed against both migrating and nonmigratory neural crest cells, was applied for one hour in a humidified incubator at room temperature (27,28). The HNK-1 hybridoma cell line was purchased from the American Type Culture Collection (ATCC TIB 200). After rinsing, peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark) were applied for one hour under the same conditions. Subsequently, peroxidase was visualized with 3,3' diaminobenzidine 4HCl with 0.01% hydrogen peroxide. Sections were counterstained with hematoxylin for one minute. Phosphate-buffered saline with 1% Tween 20 was used for rinsing and diluting.

RESULTS

Aggregates of neural crest cells were visualized using HNK-1 immunoperoxidase staining. Neural crest cells had reached the cloacal end of the bowel by E8.

Permanent ischemia of the bowel at E9 and E16. Crushing of a branch of the omphalomesenteric artery at E9 immediately resulted in pallor in the distribution area of the branch. Twenty-four hours after the intervention the bowel had returned to normal. At hatching (E21), 82 out of 100 embryos so treated were alive, versus 92 out of 100 sham-operated embryos. Deaths were mainly due to severe blood losses. No gross anomalies were found in either group. Light microscopy showed the normal structure of the layers of the bowel wall and HNK-1 positive enteric neurons or neural crest cells in the myenteric and submucous plexuses throughout the bowel (Fig 1a). The size of enteric ganglia in the experimental group matched that found in the sham-operated group. Apparently the ischemic insult had not disturbed the formation of the ENS in the ischemic bowel segment. The results are summarized in table 1.

The intervention at E16 resulted in higher mortality compared to the intervention at E9; 100 out of 315 embryos survived in the experimental group, versus 100 out of 169 sham-operated embryos. This time we did find anomalies in the chickens that were alive at hatching: 49 of them presented with intestinal atresia, and 25 with stenosis. The remaining 26 did not show gross anomalies. Light microscopy in the latter revealed HNK-1 immunoreactive neurons throughout the entire bowel, normally arranged in

concentric plexuses. Light microscopy of the obstruction area in the 49 embryos with intestinal atresia revealed that the normal structure of the bowel wall had been replaced by connective tissue. HNK-1 immunoperoxidase staining did not reveal enteric neurons or neural crest cells. We did find HNK-1 positive enteric neurons in the myenteric and submucous plexuses in the bowel segments adjacent to the obstruction (Fig 1b). Light microscopy of the 25 cases with intestinal stenosis did not reveal HNK-1 positive neurons at the site of obstruction; however, they were found as soon as the normal structure of the layers of the bowel wall was present. The results are summarized in table 1.

Temporary local ischemia of the bowel at E16. As permanent intestinal ischemia resulted in major anomalies of the bowel (e.g. atresia and stenosis), we argued that selective disappearance of enteric neurons might be caused by a milder ischemic insult. Therefore, we induced temporary local ischemia to determine whether ischemia followed by reperfusion would cause a selective disappearance of enteric neurons. The gross abnormalities found at E21 are summarized in table 1.

Group A seemed the most suitable for testing the selective disappearance of enteric neurons, since gross anomalies were absent in this group. In group A embryos, HNK-1 antibody staining revealed a normal enteric nervous system throughout the bowel. Light microscopy of embryos from groups B and C that showed gross anomalies revealed that the normal bowel had been replaced by connective tissue. Proximal to the obstruction, the bowel wall was stretched and the mucosa showed an undulating single-layered epithelium. HNK-1 positive enteric neurons were observed both proximal and distal to the obstruction, organized in concentric enteric ganglia. At the site of the obstruction, the bowel had changed into a necrotic remnant, consisting of connective tissue and pyknotic cells. Using the HNK-1 antibody, enteric neurons could not be identified in the amorphous structure (Figure 1b).

General hypoxia at E16. Mortality amounted to 50% for each group of embryos exposed to general hypoxia. At E21, the edematous bowel was pale at gross examination. Intestinal atresia was never observed. HNK-1 antibody staining revealed normal enteric neurons (see table 1).

CLINICAL STUDY

Ischemic changes were observed in the bowel wall of all 24 patients under investigation. These changes were restricted to the mucosa in a minority of cases (n=5), leaving the other layers of the bowel wall intact. The intestinal lumen was replaced by connective tissue and histiocytic giant cells, loaded with bile and iron pigment. Enteric ganglia, containing neurons, were present at the level of the obstruction.

In the majority of the patients with small-bowel atresia, ischemic changes were also found in the smooth muscle layers and consisted of nuclear pyknosis and accumulations of iron pigment. Ischemic changes of enteric neurons, such as pyknotic nuclei, were only observed together with ischemic changes of the circular smooth muscular layer and the mucosa (Figure 2a and 2b). Aganglionosis was never observed

in the ischemic segments adjacent to the atresia. Electron microscopy revealed normal nerve trunks and enteric neurons in the ischemically damaged bowel (Figure 3 and 4). In some cases, hyperplastic nervous tissue was observed in segments adjacent to the stenosis. There was an increase in nerve fibers and enteric neurons.

TABLE 1

THE EFFECT OF LOCAL INTESTINAL ISCHEMIA OR GENERAL HYPOXIA ON THE DEVELOPING BOWEL

	No of embryos			enteric	Abnormalities		
	E9	E16	E21	neurons	A	S	D
P.L.	100		82	+	0	0	0
SHAM	100		92	+	0	0	0
P.L.		315	100	+	49	25	0
SHAM		100	92	+	0	0	0
T.L. 5'		25	20	+	2	0	0
25'		26	20	+	11	2	1
60'		31	20	+	13	2	3
GH 1		20	10	+	0	0	0
2		20	10	+	0	0	0

PL = Permanent Local ischemia; TL = Temporary Local ischemia; GH = General Hypoxia; A = Atresia; S = Stenosis; D = Dilatation

In the patients with meconium peritonitis, we found arterial thrombosis in the mesentery with extensive calcification or recanalization of the thrombosed vessels. Enteric neurons were present in the severe ischemic segments of the bowel close to the site of the perforation.

Histological evaluation of our five patients with colonic atresia revealed the same abnormalities as seen in small-bowel atresia, consisting of acute and chronic ischemic changes of the bowel wall. In bowel segments that had undergone extensive transmural ischemic changes enteric neurons had pyknotic nuclei if they were present at all.

Extensive permanent ischemic changes were observed in the bowel wall of the two patients with stenosis or atresia secondary to necrotizing enterocolitis. These ischemic changes consisted of ulcerative lesions in the mucosa, absence of villi, collagen deposits in the submucosa, numerous macrophages containing iron and bile pigment, and necrosis of the smooth muscle layers. A small number of enteric neurons was observed.

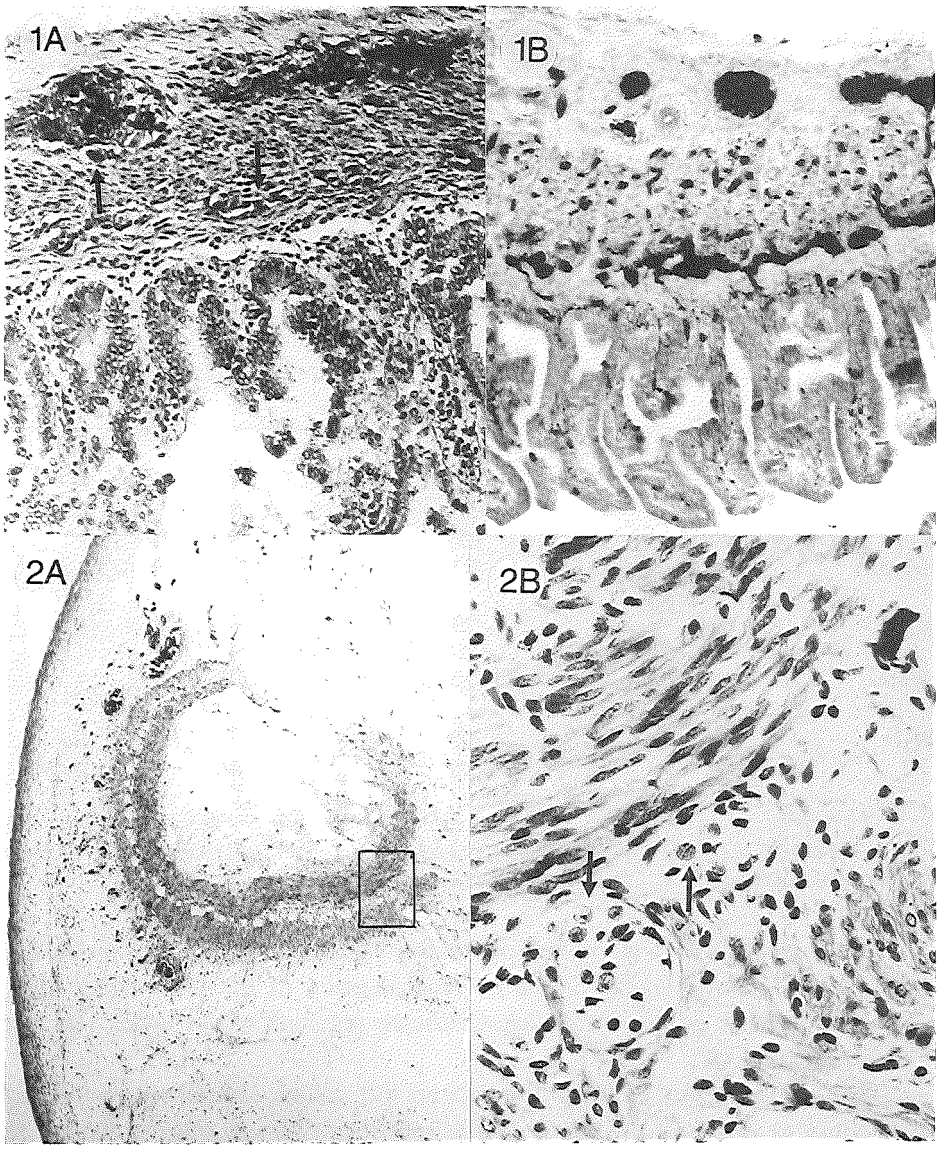

Figure 1 (A): HNK-1 immunoperoxidase staining of paraffin section of E21 bowel subjected to local ischemia at E16. Note extensive ischemic damage in the mucosa and the presence of enteric neurons. Magnification 25x. (B) HNK-1 immunoperoxidase staining of paraffin section of normal E21 bowel. Magnification 25x. Figure 2 (A) H/E stained paraffin section of bowel of a patient with intestinal atresia. Chronic ischemia has replaced the mucosa partly transforming muscle layers into connective tissue. Magnification 25x. (B) Detail of (A), showing ischemic destruction of the enteric nervous system and other layers of the bowel wall.

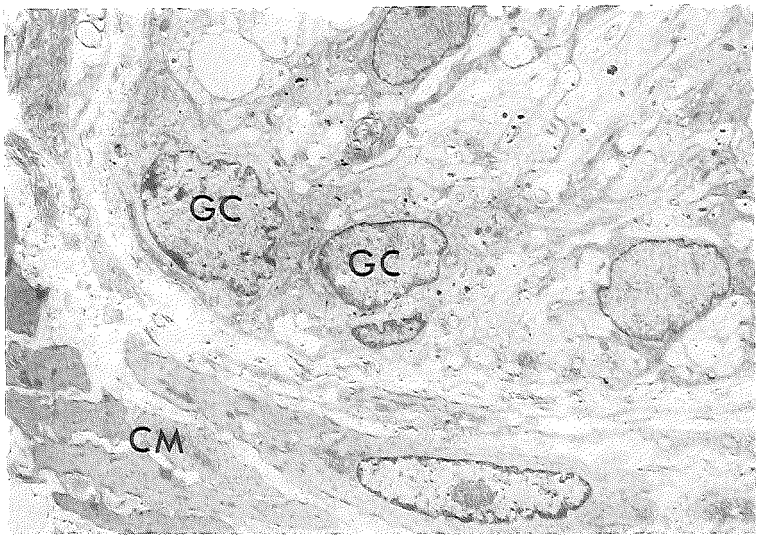

Figure 3: Electronmicrograph of the myenteric plexus immediately adjacent to an atretic bowel segment showing ganglion cells (GC) and unmyelinated axons in direct proximity to circular smooth muscle layer (CM). Magnification 4,800x.

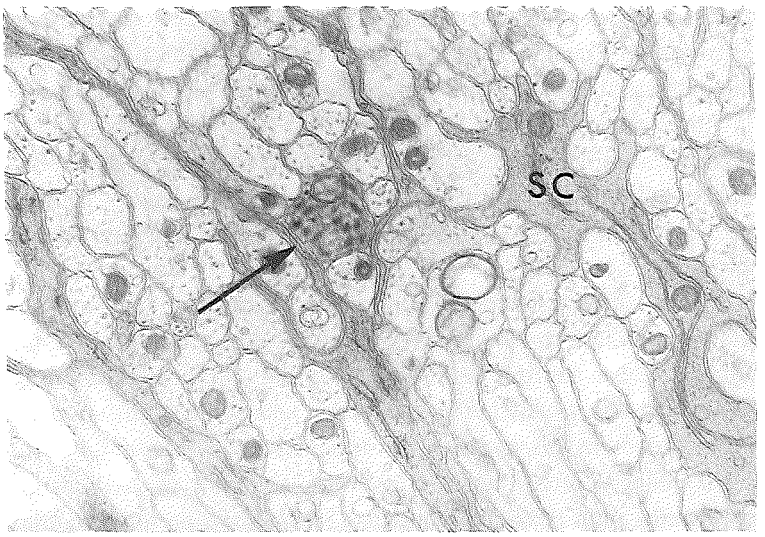

Figure 4: Electronmicrograph of atretic bowel segment showing normal axon bundles enveloped by Schwann cell cytoplasm (SC). Note single axon containing large dense core vesicles (arrow). Magnification 23,500x.

DISCUSSION

Local intestinal ischemia has been proposed as the causative factor for a selective disappearance of enteric neurons, resulting in aganglionosis. A number of investigators have tried to substantiate this premise experimentally by inducing ischemia of the enteric nervous system in adult animals (7-12). We investigated the effect of three different types of ischemic insults to the developing enteric nervous system in chicken embryos.

Permanent local ischemia, induced by damaging a branch of the omphalomesenteric artery at E9, did not result in permanent abnormalities of either bowel or enteric nervous system. The development of vascular anastomoses between segmental vessels or the regeneration of enteric ganglia due to neural crest cell proliferation (29) might account for this. In contrast, both permanent and temporary local ischemia of the bowel at E16, resulted in atresia or stenosis of the small bowel. No selective disappearance of enteric neurons was observed in normal bowel adjacent to the atretic segments. Temporary ischemia followed by reperfusion also resulted in atresia or stenosis of the bowel, but aganglionosis was never found. The induction of general hypoxia did not result in atresia, stenosis or aganglionosis. We conclude that neither permanent nor temporary local ischemia of the bowel nor general hypoxia of the fetus leads to the selective disappearance of enteric neurons in the developing bowel.

These results contradict Earlam's premise that the enteric nervous system is more sensitive to anoxia than other tissues (30). Our results do substantiate Earlam's finding that intestinal ischemia led to midgut atresia and stenosis in chicken embryos (31), as demonstrated by us in previous experiments (14). Analogous results were obtained in other animal models of intestinal atresia, such as lambs and dogs. No selective disappearance of enteric neurons was reported in any of these studies (32-34). Likewise, an animal model of necrotizing enterocolitis produced by inducing ischemia in neonatal rabbits and dogs, did not result in a selective disappearance of enteric neurons (35-37).

Based on the original work of Cannon and Burket (13), several methods have been employed to induce ischemia experimentally and thereby promote the disappearance of enteric neurons in the hindgut of adult animals. There is, however, no consensus about the effect of ischemia on enteric neurons in adult animals (7,8,10,11). Segmental differences in susceptibility to ischemia might account for the divergent findings. It has been reported that in mice the hindgut enteric nervous system is less sensitive to ischemia than proximal segments (38), supporting this hypothesis.

Our evaluation of patients with intestinal atresia revealed that there is no selective destruction of enteric neurons in bowel segments with proven antenatal or postnatal ischemia (intestinal atresia, meconium peritonitis and necrotizing enterocolitis with secondary stenosis). These results are in agreement with other pathological studies

of patients with atresia (32). It is important to note that enteric neurons were only absent in conjunction with extensive transmural ischemic changes of the bowel wall, regardless of the location of atresia (small bowel or colon). These observations allow for extrapolation of our experimental findings. If ischemia of the postumbilical bowel does not lead to aganglionosis, then ischemia of the hindgut will have the same effect.

Selective disappearance of enteric neurons due to ischemia of the gut has been proposed as the causative factor in Hirschsprung's disease (4). The observation of fibromuscular dysplasia of arteries in the transitional zone supports this theory (5,6). Tagushi speculated that bowel ischemia affects enteric neurons (neural crest cells) or the migratory pathways (5). Our experiments revealed that none of three different types of ischemic insult affected the enteric neurons selectively. A deleterious effect of ischemia on the migratory pathways also seems unlikely. Bowel segments grafted onto the chorioallantoic membrane experience an ischemic episode lasting one hour, before they are fixed in position. Furthermore, it requires at least 6 hours to establish vascular connections between the graft and the vessels in the chorioallantoic membrane. This ischemic period does not affect neural crest cell colonization (18).

Two other pathogenetic mechanisms have been proposed as cause of Hirschsprung's disease, viz. a) a temporal disparity between on the one hand neural crest cell migration on the other hand the longitudinal growth and differentiation of the bowel, and b) a defective microenvironment in the hindgut which would prevent neural crest cell colonization.

Webster proposed that in piebald lethal mice the hindgut was not colonized by neural crest cells because these had arrived late (39). However, we have recently demonstrated that an advanced stage of differentiation of the hindgut does not hamper the colonization of neural crest cells in chicken embryos (40). Therefore, aganglionosis cannot be caused by a purely temporal difference between neural crest cell migration and the differentiation of the distal gut.

Rothman and Gershon proposed that in the lethal spotted mouse model for Hirschsprung's disease the microenvironment of the hindgut was defective (41). The abnormal microenvironment would prevent either the colonization as such, or the survival of neural crest cells after colonization of the distal segment. There is experimental evidence for the former possibility (42). The developing bowel of this mutant mouse strain shows ultrastructural differences compared to control embryos. These consist of a thickening of mucosal basal laminae and exceptionally wide intercellular spaces, containing irregular fibrillar material, between smooth muscle myoblasts (42-44). The physiological role of the microenvironment in the homing of neural crest cells during normal development deserves further investigation.

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REFERENCES

1. Meier-Ruge W. Hirschsprung's disease: its etiology, pathogenesis and differential diagnosis. Curr Top Pathol 1974;59:131-79.

2. Okamoto E, Ueda T. Embryogenesis of intramural ganglia of the gut and its relation to Hirschsprung's disease. J Pediatr Surg 1967;2:437-43.

3. Rothman TP, Gershon MD. Regionally defective colonization of the terminal bowel by the precursors of enteric neurons in lethal spotted mutant mice. Neuroscience 1984;12:1293-311.

4. Earlam R. A vascular cause for Hirschsprung's disease. Gastroenterology 1985;88:1274-76.

5. Tagushi T, Tanaka K, Ikeda K. Fibromuscular dysplasia of arteries in Hirschsprung's disease. Gastroenterology 1985;88:1099-103.

6. Lister J. Abnormal arteries in Hirschsprung's disease. Arch Dis Child 1966;41:149.

7. Hukuhara T, Kotani S, Sato G. Effects of destruction of intramural ganglion cells on colon motility: possible genesis of congenital megacolon. Jap J Physiol 1961;11:635-40.

8. De Villiers DR. Ischemia of the colon: an experimental study. Br J Surg 1966;53:497-03.

9. Okamoto E, Iwasaki T, Kakutani T, Ueda T. Selective destruction of the myenteric plexus: its relation to Hirschsprung's disease, achalasia of the esophagus and hypertrophic pyloric stenosis. J Pediatr Surg 1967;2:444-54.

10. Pompino HJ. Berichte uber experimentelle Untersuchungen zur Frage des erworbenen Megacolon. Z Kinderchir 1969;7:262-72.

11. Kyi Kyi J, Daniel EE. The effects of ischemia on intestinal nerves and electrical slow waves. Am J Dig Diseases 1970;15:959-61.

12. Earlam RJ. Ganglion cell changes in experimental stenosis of the gut. Gut 1971;12393-98.

13. Cannon WB, Burket IR. The endurance of anaemia by nerve cells in the myenteric plexus. Am J Physiol 1913;32:347-57.

14. Tibboel D, van Nie CJ, Molenaar JC. The effects of temporary general ischemia and local ischemia on the development of the intestines: an experimental study. J Pediatr Surg 1980;15:57-61.

15. Tibboel D, van der Kamp AWM, Molenaar JC. The effect of experimentally induced intestinal perforation at an early developmental stage. J Pediatr Surg 1981;16:1017-21.

16. LeDouarin NM. The Neural crest. Cambridge, UK: Cambridge University Press, 1982.

17. Payette RF, Bennet GS, Gershon MD. Neurofilament expression in vagal neural crest derived precusors of enteric neurons. Dev Biol 1984;105:272-87

18. Allan IJ, Newgreen DF. The origin and differentiation of enteric neurons in the intestine of the fowl embryo. Am J Anat 1980;157:137-54.

19. LeDouarin NM, Teillet M-A. The migration of neural crest cells to the wall of the digestive tract in avian embryo. J Embryol exp Morphol 1973;30:31-48.

20. Tucker G, Ciment G, Thiery JP. Pathways of avian neural crest cell migration in the developing gut. Dev Biol 1986;116:439-50.

21. Louw JH, Barnard CN. Congenital intestinal atresia. Observations on its origin. Lancet 1955;ii:1065-67.

22. Tibboel D, Gaillard JLJ, Molenaar JC: The importance of mesenteric vascular insufficiency in meconium peritonitis. Hum Pathol 1986;17:411-16.

23. Brown EG, Sweet AY: Neonatal necrotizing enterocolitis. Pediatr Clin North Am 1982;29:1149-70.

24. Virjee JP, Gill GJ, Desa D, Somers S, Stevenson GW. Strictures and other late complications of neonatal necrotizing enterocolitis. Clin Radiol 1979;30:25-31.

25. Edelstone DI, Holzman IR. Regulation of perinatal intestinal oxygenation. Seminars in Perinatology 1984;8:226-33.

26. Scholander PF. The master switch of life. Sci Am 1963;209:92-106.

27. Abo T, Balch CM. A Differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J Immunol 1981;127:1024-29.

28. Tucker GC, Aoyama H, Lipinski M, et al.: Identical reactivity of monoclonal antibodies HNK-1 and NC1: conservation in vertebrates on cell derived from the neural primordium and on some leukocytes. Cell Differen 1984;14:223-30.

29. Meijers JHC, Tibboel D, van der Kamp AWM, et al. Cell division in migratory and aggregated neural crest cells in the developing gut. An experimental approach of innervation-related motility disorders of the gut. J Pediatr Surg 1987;22:243-45.

30. Earlam RJ. A Vascular cause for aganglionic bowel. A new hypothesis. Am J Dig Dis 1972;17:255-61.

31. Earlam, RJ. A study of the aetiology of congenital stenosis of the gut. Ann R Coll Surg Engl 1972;51:126-30.

32. Hamdy MH, Man DWK, Bain D, et al. Histochemical changes in intestinal atresia and its implications on surgical management: a preliminary report. J Pediatr Surg 1986; 21:17-21.

33. Tepas JJ, Wyllie RG, Shermeta HE et al. Comparison of histochemical studies of intestinal atresia in the human newborn and fetal lamb. J Pediatr Surg 1979;14:376-80.

34. Pickard LR, Santoro S, Wyllie RG, et al. Histochemical studies of experimental fetal intestinal obstruction. J Pediatr Surg 1981;16:256-60.

35. Touloukian RJ, Posch JN, Spencer R. The pathogenesis of ischemic gastroenterocolitis of the neonate: selective gut mucosal ischemia in asphyxiated neonatal piglets. J Pediatr Surg 1972;7:195-05.

36. Barlow B, Santulli TV. Importance of multiple episodes of hypoxia or cold stress on the development of enterocolitis in an animal model. Surgery 1975;77:687-90.

37. Harrison MW, Connell RS, Campbell JR, Webb MC. Microcirculatory changes in the gastrointestinal tract of the hypoxic puppy: an electron microscope study. J Pediatr Surg 1975;5:599-07.

38. Wood JD, Brann LR, Daugherty CK. Regional effect of hypoxia and hypothermia on rebound excitation in large intestine of piebald mouse model for Hirschsprung's disease. Dig Dis Sci 1986;31:859-64.

39. Webster W. Embryogenesis of the enteric ganglia in normal mice and in mice that develop congenital aganglionic megacolon. J Embryol Exp Morphol 1973;30:573-85.

40. Meijers JHC, Tibboel D, van der Kamp AWM et al. The influence of the stage of differentiation of the gut on the migration of neural cells. An experimental approach to Hirschsprung's disease. Pediatr Res 1987;21:466-70.

41. Rothman TP, Gershon MD. Regionally defective colonization of the terminal bowel by the precursors of enteric neurons in lethal spotted mutant mice. Neuroscience 1984;12:1293-311.

42. Jacobs-Cohen RJ, Payette RF, Gershon MD, Rothman TP. Inability of neural crest cells to colonize the presumptive aganglionic bowel of ls/ls mutant mice: requirement for a permissive microenvironment. J Comp Neurol 1987;255:425-38.

43. Tennyson VM, Duc Pham T, Rothman TP, Gershon MD. Abnormalities of smooth muscle, basal laminae, and nerves in the aganglionic segments of the bowel of lethal spotted mutant mice. Anat Rec 1986;215:267-81.

44. Payette RF, Tennyson VM, Pomeranz HD, et al: Accumulation of basal laminae: association with the failure of neural crest cell to colonize the presumptive aganglionic bowel of ls/ls mutant mice. Dev Biol 1988; 125:341-60.

CHAPTER 7.2

THE INFLUENCE OF THE STAGE OF DIFFERENTIATION OF THE GUT ON THE MIGRATION OF NEURAL CELLS: AN EXPERIMENTAL STUDY OF HIRSCHSPRUNG'S DISEASE.

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ABSTRACT. Based on experimental studies in mutant mouse strains, an imbalance between the rate of migration of neural crest cells and the rate of differentiation of the mesenchyme of the distal gut has been proposed as an etiological factor in Hirschsprung's disease. We studied the influence of the stage of differentiation of embryonal chick gut on the migration of neural crest cells in an in vivo culture system: the chorioallantoic membrane (CAM). Neural crest cells in cultured gut, were demonstrated with antibodies directed against the HNK-1 epitope. Enteric neurons were demonstrated with neurofilament immunoreactivity. By culturing isolated gut segments of E4 embryos, we obtained aneuronal (neurofilament-negative) embryonal chick gut up to 25 days of development. In co-cultures of aneuronal gut and the neural anlage (NA = neural tube and neural crest) neural crest cell colonization was observed, even in advanced stages of differentiation. The significance of the results is discussed in terms of the etiology of Hirschsprung's disease.

ABBREVIATIONS: E = embryonal day; Ex + y = embryonal day x + y days culture; CAM = chorioallantoic membrane; HNK-1 = human natural killer cell; NA = neural anlage; NF = neurofilament; NC = neural crest.

INTRODUCTION

ETIOLOGY OF HIRSCHSPRUNG'S DISEASE

Nearly one hundred years after the first clinical description of Hirschsprung's disease (1) there is still little understanding concerning the etiology and pathogenesis. The basic defect is variously attributed to (a) intrinsic abnormalities of migrating neural crest (NC) cells (2), (b) muscular dysplasia of mesenteric vessels (3,4); or (c) antenatal viral destruction of enteric neurons (5). In an embryologic study of the piebald lethal mouse

strain with aganglionosis of the distal colon, resembling Hirschsprung's disease, an apparent slowing of the proximo-distal sequence of the non-specific esterase staining of NC cells was noted (6). It was surmised that aganglionosis had been caused by NC cell migration being out of phase with the longitudinal growth and differentiation of the developing gut. Studies with the lethal spotted mouse strain provided experimental evidence for the localization of the effect of the ls gene in the distal gut (7). We investigated whether mere temporal factors in the distal gut, hamper the NC cell colonization of the gut. An advanced stage of differentiation of the gut mesenchyme might inhibit the normal invasion of NC cells.

NEURAL CREST CELL MIGRATION TO THE DEVELOPING GUT

Little is known about NC cell migration in mammalian embryos, but it has been studied extensively in the chicken embryo (8, 9, 10). When the vagal NC is deleted, the distal gut becomes aganglionic (11). Studies with chick-quail chimeras demonstrated that most enteric neurons derive from the NC opposite somite 1-7 (8). These NC cells use a pathway between the ectoderm and the somite to reach the region of the third branchial arch. They enter the developing gut at the level of the pharynx (12).

At the axial level of early embryos, the normal process of NC cell migration can be affected by temporal factors (13, 14).We studied the influence of temporal factors on the migration of NC cells at the peripheral level, in the embryonal postumbilical gut.

IN VIVO CULTURES OF EMBRYONAL GUT

We used the nutritive properties of the chorioallantoic membrane (CAM) of the chicken embryo to culture segments of embryonal gut. In comparison with in vitro culture systems, CAM-cultures have the advantage, that grafted embryonal tissues follow an almost normal course of differentiation (15). CAM vessels anastomose with the developing vessels in the graft, resulting in normal vascularisation. The grafting technique is reviewed in detail by Coulombre (16).

The postumbilical gut of E4 embryos contains no NC cells (17). We obtained aneuronal gut of different stages of development by culturing postumbilical gut of E4 embryos for different periods of time. When an isolated segment of embryonal gut is cultured on the CAM in combination with the neural anlage (NA = neural tube + NC) of an E2 embryo, the segment is colonized and innervated in a physiological fashion (18).

Monoclonal antibodies have recently been introduced that stain (migrating) NC cells or enteric neurons selectively. Most NC cells express an epitope, which is recognized by antibodies produced by the HNK-1 hybridoma cell line (19). At the vagal level of the NC, a subpopulation of premigratory NC cells can be stained selectively with antibodies directed against neurofilament (NF) epitopes (20, 21). We used HNK-1

epitopes for the identification of NC cells, while NF epitopes served as a marker for the neuronal subpopulation of NC cells in sections of cultured segments of embryonal gut.

MATERIALS AND METHODS

Animals and stages of development. Eggs of white leghorn chickens (*Gallus Domesticus*) were incubated at 38°C in a forced draft incubator at a relative humidity of 80%. For young embryos, the stage of development was determined by the number of paired somites, while for older embryos we used the table of Hamburger and Hamilton (22) or the number of days of incubation.

Removal of organs and tissues for transplantation. The hindgut (the region between the cecal primordia and the cloaca) was dissected from embryos before its colonization by NC cells, i.e. at day 4 and 5 of incubation. The ganglion of Remak, wich is formed at stage 24-25 (23) and is located in the dorsal mesentery, was removed from the explants (E5 embryos) by dissection using tungsten needles.

Segments of the vagal neural tube (somite 1-7) together with the NC, were isolated from 12-21 somite embryos (E2) by using microdissection with tungsten needles after 10 minutes of trypsinization (porcine trypsin 0.25% diluted in phosphate buffered saline (PBS) at room temperature.

Transplantation techniques. Segments of aneuronal gut were grafted alone or in combination with vagal NA onto the CAM of 7 day old chick embryos after abrasion of the superficial CAM layer, at the bifurcation of two great vessels. The grafts were fixed in position with a piece of cellophane (Clingo wrap sterilized in 70% ethanol and dried in air). The egg was sealed with scotch tape and incubated for one week.

Immunocytochemical techniques. Grafts were embedded in Tissue Tek II O.C.T. Compound (Miles) and snap-frozen in liquid nitrogen cooled isopentane. Frozen sections (10 μm) were mounted on slides coated with chrome alum, fixated in acetone for 5 minutes, and then dried in air. Other grafts were fixated in 4% formaldehyde in PBS, dehydrated and embedded in paraffin. Sections (7 μm) were deparaffinized, hydrated and used for immunocytochemical investigation or stained with hematoxylin-eosin (HE).

Frozen sections were stained with three monoclonal antibodies raised against NF-triplet proteins. The 3G6 Ig-M antibody is specific for the 160kD and 200kD NF-protein (24), the 2F11 antibody (Sanbio, Holland) for the 70kD (24) and C90 for the 200kD. The antibody raised against the HNK-1 (Becton & Dickinson) antigen was used on both paraffin and frozen sections. Rabbit-antimouse peroxidase conjugated immunoglobulins (Dako, Denmark) were used in a dilution of 1:100. In order to reduce background staining, we added 2% chick serum to the conjugate. Peroxidase was visualized by 0.1% 3,3' diamino benzidine.4HCl (Serva) and 0.02% hydrogen peroxide.

All rinsing and diluting was done in PBS (pH 7.4) with 0.1% Tween 20.

RESULTS

Localization of HNK-1 and NF epitopes in embryonal gut. Post-umbilical gut of E4 embryos does not show HNK-1 or NF immunoreactivity. The expression of the HNK-1 antigen was first detected in the post-umbilical gut of E6 embryos. No differentiated cell types were recognized at this stage in the mesenchyme of the gut. The stratified mucosal epithelium is surrounded by a mesenchyme that shows no regional specializations. The mesenchyme itself is enclosed within a simple cuboidal serosal epithelium. No nervous tissue was recognized histologically with HE staining.

3G6 immunorectivity was first detected in postumbilical gut of E6 embryos. In the postumbilical segment of the gut of E7 embryos, the neurons of the myenteric and submucous plexus were identified with 3G6 anti-NF antibodies. Thus both the HNK-1 and the 3G6 anti-NF antibodies enabled detection of neuronal (precursor) cells at very early stages of development. Staining of the C90 anti-NF antibody first appeared in postumbilical gut of E8 embryos, while the 2F11 anti-NF antibody only revealed nervous tissue in embryonal gut of advanced stages of differentiation, i.e. in E18 embryos.

Culture of differentiated embryonal aneuronal gut. We grafted postumbilical segments of embryonal gut of E4 embryos onto the CAM of E7 embryos. After one week CAM culture, the segment showed a stratified epithelium. The mesenchyme had differentiated into a circular, smooth muscle layer. The lumen of the gut was circular. No NF or HNK-1 immunoreactivity was revealed, demonstrating the absence of enteric neurons and NC cells. Likewise, the post-cecal part of E5 embryos did not show immunoreactivity with HNK-1 or anti-NF antibodies after 7 days culture (Fig. 1). In the gut segment between the umbilicus and the ceca of E5 embryos, however, NC cells were visualized with HNK-1 immunoreactivity, while enteric neurons stained with the 3G6 NF-antibody (Figs. 2, 3 and 5). In a series of experiments in which subsequent levels of the postumbilical gut were cultured on the CAM, we did not observe any staining in the distal parts of the gut prior to staining in the proximal parts.

In order to obtain aneuronal gut of advanced stages of development, the CAM-cultured aneuronal gut was freed from the surrounding membranes and grafted anew to the CAM of an E7 embryo. By subsequent subculturing, we obtained aneuronal gut of 11 and 18 and 25 days of development (Fig. 6). In comparison with normal in situ development, the histological picture of these cultured gut segments corresponded to age. In cultured segments of E4+14, the lumen had lost its circular appearance. The stratification of the epithelium had changed into a single layered cuboidal epithelium. The mesenchyme had differentiated into longitudinal and smooth muscle layers. Apparently, the grafting procedure had only minor effects on the normal course of

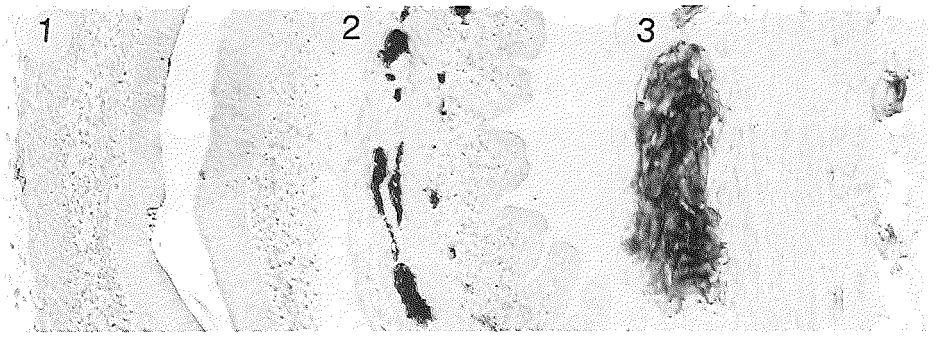


Fig. 1: Paraffin section of CAM-cultured postcecal gut of an E5 embryo. The circular smooth muscle layer and the epithelium have differentiated. There is no HNK-1 immunoreactivity, indicating the absence of NC cells. Magnification: 25x. Comparable age: E12. Fig. 2: Paraffin section of CAM-cultured prececal gut of an E5 embryo. Note the presence of HNK-1 immunoreactivity at the sites of the myenteric and submucous plexus. Magnification: 25x. Comparable age E12. Fig. 3. Detail of the myenteric and submucous plexus of Fig. 2, showing HNK-1 positive ganglioncells. Magnification 100x.

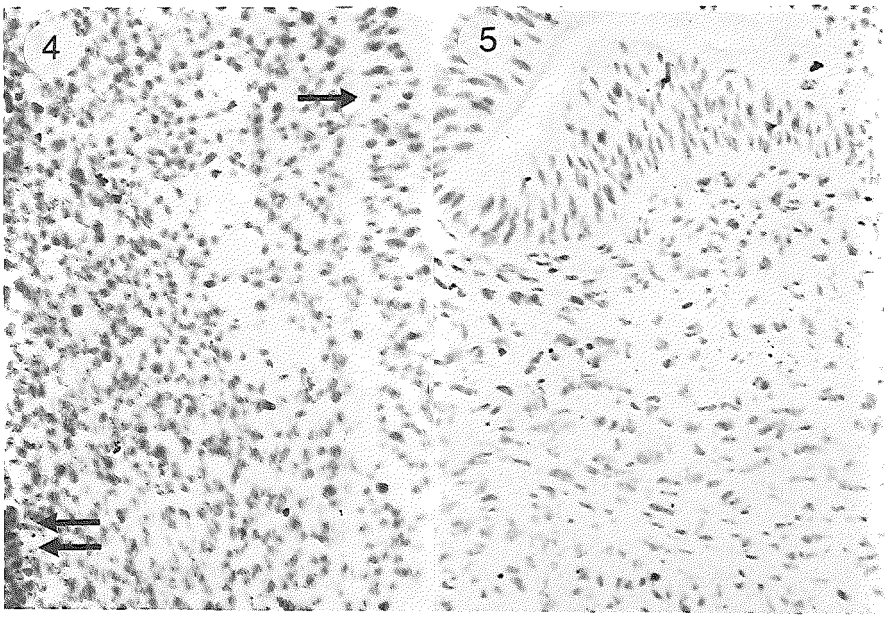
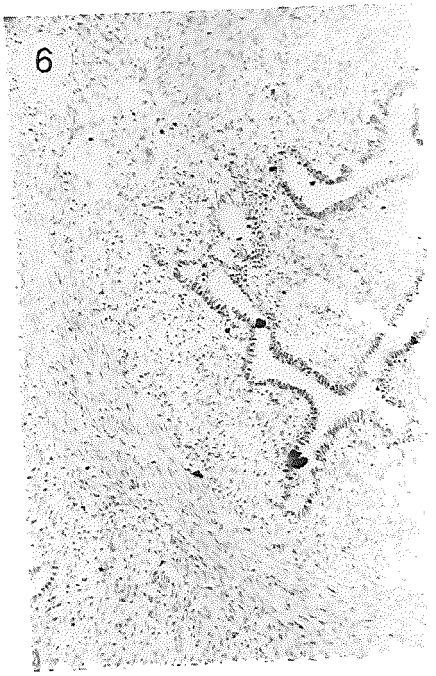


Fig. 4. Frozen section of post-umbilical gut of an E4 embryo, stained with C90 anti-NF antibody. No NF immunoreactivity is observed. The mesenchyme has no regional specializations. Arrow indicates the lumen. The double arrow indicates the serosal side. Magnification: 40x. Fig. 5. Frozen section of postumbilical gut of an E4 embryo (cultured for 7 days on CAM) stained with anti-NF antibody C90. There is no NF-immunoreactivity, indicating the absence of enteric neurons. Magnification 40x. Comparable age E11. (Compare with the paraffin section of the same segment of the gut, figure 1).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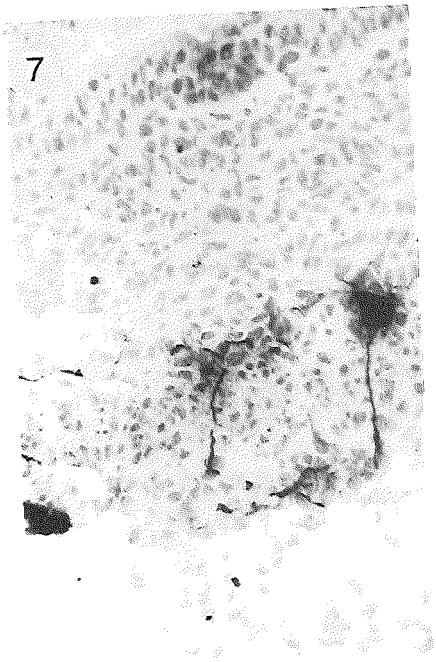
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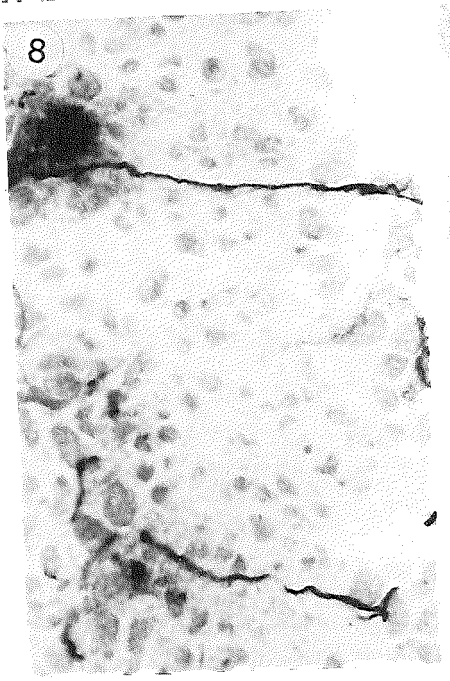
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differentiation (Fig. 6).

The longitudinal growth of the graft decreased with the repetition of grafting. At removal at E4, the postumbilical segment of gut measured 1 mm in length. After 7 days CAM-culture, the 1 mm aneuronal gut had elongated to 3-4 mm. For the second transplantation, this segment was divided into parts of 1 mm each, which were subsequently grafted and cultured. After 7 days culture, these segments had elongated to 2-3 mm. These grafts were isolated and again divided into 1 mm segments and grafted to the CAM. At the time of removal, after 21 days of culturing, these grafts measured approximately 1.5 mm.

Coculture of vagal neural anlage and "old" aneuronal gut. To determine whether cultured aneuronal gut of advanced stages of differentiation would still accept NC cells, we cocultured the aneuronal gut with the vagal NA. Sections of the CAM of E7 and E16 did not stain with anti-NF antibodies. NF-immunoreactivity did not appear in sections of postumbilical gut of E4 (see Fig. 4) and the cultures of E4 gut. NF immunoreactivity only appeared in cocultures of aneuronal gut of E4 with NA (Fig. 7, 8 and 9). Aneuronal gut of E4+7, E5+7, E4+14, and E5+14 was also invaded by NC cells (table 1 and Fig. 7, 8 and 9). The observed NF-immunoreactivity was localized at the sites of the myenteric and submucous plexus. With relevance to the size of the ganglia, no major divergence was observed comparing cocultures of NA with aneuronal gut of E4 and E4+7. Cocultures of NA with aneuronal gut of E4+14 showed less NF immunoreactivity. Compared to sections of explanted gut of corresponding developmental stages the ganglia appeared to be smaller in all the cocultures.

Fig. 6. Frozen section of aneuronal postumbilical gut of an E4 embryo, cultured for 21 days on CAM, stained with C90 anti-NF antibody. There is no NF immunoreactivity. Note the single layered cuboidal epithelium and the differentiated smooth muscle layer. Magnification: 16x. Comparable age: 3 days after hatching. Fig. 7. Frozen section of a coculture (7 days) of postumbilical gut (E4 embryo) and NA (E2 embryo) stained with C90 anti-NF antibody. Note the NF immunoreactivity at the sites of the myenteric and submucous plexus. Magnification 40x. Compare with figure 5. Fig. 8. Detail of Fig. 7 showing NF immunoreactivity in cells and neurites in the submucous plexus. Magnification: 100x. Fig. 9. Frozen section of a coculture (7 days) of aneuronal gut of 18 developmental days (E4 + 14) and the NA (E2 embryo). Note C90 NF immunoreactivity at the sites of the myenteric and submucous ganglia. Magnification 16x. Comparable age: 3 days after hatching. Compare with Fig. 6.

TABLE 1

NUMBER OF ANEURONAL BOWEL EXPLANTS SHOWING NEUROFILAMENT POSITIVE NERVOUS TISSUE AFTER A 7 DAYS COCULTURE WITH NEURAL ANLAGE E2 ON CAM

Neurofilament staining	Day of development (aneuronal gut)			
	4	5	11*	18¶
	(n=8)	(n=8)	(n=12)	(n=10)
3G6	7	7	10	8
C90	7	7	9	8
Controls (cultures without neural anlage)				
	(n=13)	(n=4)	(n=3)	(n=26)
3G6	1	0	0	3
C90	1	0	0	3

* *11 = 4 days in situ + 7 days CAM culture;* ¶ *18 = 4 days in situ + 14 days CAM culture*

DISCUSSION

Neural crest cell invasion of "old" target organ. In the present study, we cultured aneuronal gut up to the age of E4+21 days. We observed NC cell invasion in both aneuronal gut of E4 and E4+14 in cocultures with NA. In other words, NC invasion occurred despite a developmental difference of 14 days at the peripheral target organ. It is interesting to note that temporal factors do not hamper NC migration in a peripheral organ while they do at the axial level. Weston and Butler transplanted radioactively labelled, neural tubes into progressively older recipients at the axial level (13). They found that migration of the implanted, labelled cells was restricted when the recipients were 9-12 hours older with the NC cell migration already in progress. However, their conclusion was undermined by the damage inflicted during grafting, particularly in the case of older embryos. Bronner-Fraser & Cohen circumvented this problem by injecting cultured NC cells directly into somites. They, likewise, observed that the migration was limited depending on the developmental age (14). The difference between the axial and

peripheral levels can be explained by the assumption that at the axial levels the fibronectin and laminin rich migratory pathways are destroyed in a period of 9-12 hours and that the migratory pathways are preserved in the postumbilical segment of the gut. It might also well be that in the distal gut, NC cells use other glycoproteins as substrates for migration than at the axial levels (12).

In cocultures of NA and aneuronal gut at different stages of development, the myenteric and submucous ganglia are of a comparable size. The ganglia in the cocultures, however, are smaller than those in the gut of corresponding ages. We presume that this is due a loss of NC cells on theCAM, or to the absence of factors stimulating NC cell proliferation in the CAM-cultured isolated segments of embryonal gut. In our the coculture experiments, we combined aneuronal gut and NA, two tissues that normally do not confront one another. It remains to be studied whether NC cells from the region of the third branchial arch and innervated gut, will also colonize "old" aneuronal gut in CAM- cultures. The NA of E2 embryos might contain a subpopulation of NC cells capable of migration on diverse migratory substrates. NC cells from older NC cell suppliers might be more selective as to the migratory substrate.

Etiological factors in Hirschsprung's disease. It has been demonstrated that, at least at the axial level, there are no major differences between the migratory pathways in chicken or mouse embryos, related to either the fibronectin or laminin lining (25). Furthermore, it is propable that NC cell migration in chicken embryos occurs in the same fashion as in mouse embryos, despite the fact that they use slighly different cell-adhesion molecules. For example, the HNK-1 epitope is not found on NC cells in mice (19). Therefore, we feel the results of our experiments with chicken embryos apply to the hypothesis of the development of aganglionosis in mice (6). As comparative research of the migratory pathways in man has not been reported to date, the relevance of our results to NC cell migration and the etiology of Hirschsprung's disease in man is entirely hypothetical.

The inaccessibility of differentiated distal parts of the gut for slowly migrating NC cells, has been proposed as a causative factor of aganglionosis in the piebald lethal mouse (6) and for the aganglionosis in Hirschsprung's disease and (26). The results of our experiments, indicate that the gut, at advanced stages of development, can still be colonized by NC cells. Consequently, it is not feasible that the migration of NC cells being out of phase with the longitudinal growth and differentiation of the gut is an etiological factor in Hirschsprung's disease in mice or in man.

Other factors, like intrinsic abnormalities of NC cells, and structural defects within the embryonal gut remain possible. Further research in those directions, has to elucidate which of these etiologies has validity for Hirschsprung's disease.

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REFERENCES

1. Hirschsprung H, 1887. Stuhlträgheit Neugeborener infolge von Dilatation und Hypertrophie des Colons. Jahrbuch Kinderheilkunde 27:1-7.

2. Bolande RP, 1974. The neurocristopathies; A unifying concept of disease arising in neural crest maldevelopment. Hum Pathol 5:409-429.

3. Taguchi T, Tanaka K, Ikeda K, 1985. Fibromuscular dysplasia of arteries in Hirschsprung's disease. Gastroenterology 88:1099-1103.

4. Earlam R, 1985. A vascular cause for Hirschsprung's disease? Gastroenterology 88:1274-1279.

5. Hershlag A, Ariel I, Nissan S, Rosenmann E, 1984. Cytomegalic inclusion virus and Hirschsprung's disease. Z Kinderchir 39:252-254.

6. Webster W, 1973. Embryogenesis of the enteric ganglia in normal mice and in mice that develop congenital aganglionic megacolon. J Embryol Exp Morphol 30:573-585.

7. Rothman TP, Gershon MD, 1984. Regionally defective colonization of the terminal bowel by the precursors of enteric neurons in lethal spotted mutant mice. Neuroscience 12:1293-1311.

8. LeDouarin NM, 1982. The neural crest. Cambridge University Press, Cambridge.

9. Duband JL, Thiery J-P, 1982.Distribution in the early phase of avian cephalic neural crest migration. Dev Biol 93:308-323.

10. LeDouarin NM, 1984. Cell migrations in embryos. Cell 38:353-360.

11. Yntema CL, Hammond WS, 1954. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J Comp Neurol 101:515-541.

12. Weston JA, Butler SL, 1966. Temporal factors affecting localization of neural crest cells in the chicken embryo. Dev Biol 14:246-266.

13. Tucker GC, Ciment G, Thiery JP, 1986. Pathways of avian neural crest cell migration in the developing gut. Dev Biol 116:439-450.

14. Bronner-Fraser M, Cohen AM, 1980. Analysis of the neural crest ventral pathway using injected tracer cells. Dev Biol 77: 130-141.

15. Murray PDF, 1928. Chorio-allantoic grafts of fragments of the two-day chick, with special reference to the development of the limbs, intestine and skin. Austr J Exp Biol Med Sci 5:237-256.

16. Coulombre AJ, 1967. Grafting of embryonic rudiments. In: Wilt FH, Wessels NK (eds) Methods in Developmental Biology. TY Crowell Co, New York, pp 457-469.

17. Allan IJ, Newgreen DF, 1980. The origin and differentiation of enteric neurons of the intestine of the fowl embryo. Am J Anat 157:137-154.

18. Smith J, Cochard P, LeDouarin NM, 1977. Development of choline acetyltransferase and cholinesterase activities in enteric ganglia derived from presumptive adrenergic and cholinergic levels of the neural crest. Cell Diff 6:199-216.

19. Tucker GC, Aoyama H, Lipinski M, Tursz T, Thiery JP, 1984. Identical reactivity of monoclonal antibodies HNK-1 and NC1:conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Diff 14:223-230.

20. Tapscott SJ, Bennett GS, Holtzer H, 1981. Neuronal precursor cells in the chick neural tube express neurofilament proteins. Nature 292:836-838.

21. Payette RF, Bennett GS, Gershon MD, 1984. Neurofilament expression in vagal neural crest derived

precursors of enteric neurons. Dev Biol 105:272- 287.

22. Hamburger V, Hamilton HL, 1951. A series of normal stages in the development of the chick embryo. J Morphol 188:49-92.

23. Teillet MA, 1978. Evolution of the lumbo-sacral neural crest in the avian embryo: origin and differentiation of the ganglionated nerve of Remak studied in interspecific quail-chick chimerae. W Roux Arch 184:251-268.

24. Klück P, van Muijen GNP, van der Kamp AWM, Tibboel D, van Hoorn WA, Warnaar SO, Molenaar JC, 1984. Hirschsprung's disease studied with monoclonal antineurofilament antibodies on tissue sections. Lancet i:652-655.

25. Sternberg J, Kimber SJ, 1986. Distribution of fibronectin, laminin and entactin in the environment of migrating neural crest cells in early mouse embryos. J Embryol Exp Morphol 91:267-282.

26. Okamoto E, Ueda T, 1967. Embryogenesis of intramural ganglia of the gut and its relation to Hirschsprung's disease. J Pediatr Surg 2:437-443.

CHAPTER 7.3

NEURAL CREST CELL COLONIZATION OF EMBRYONIC MURINE BOWEL

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Submitted

Summary. The development of the enteric nervous system in mice was studied to determine when neural crest cell colonization of distal bowel segments occurs. Explants of murine embryonic bowel were grown for ten days in the renal subcapsular space of adult mice, after which visualization of enteric neurons by means of acetylcholinesterase histochemistry and neurofilament immunocytochemistry served to determine the presence of neural crest cells in the explants. The success rate for bowel segments explanted at E11 to E15 and grown for ten days in the renal subcapsular space amounted to over 82 per cent, whereas the succes rate of explants from E9 and E10 embryos amounted to 44 per cent. Cultured whole bowel explanted at embryonic day 9 (E9) contained enteric neurons, indicating that neural crest cells colonization of the bowel begins on or before E9. Acetylcholinesterase and neurofilament positive enteric neurons were consistently absent in the cultures of distal 1-2 mm of hindgut explanted at E10-E12, indicating that neural crest cells do not colonize the entire bowel at E9. It appeared that between E10 and E12, the aneuronal of the distal bowel had a constant length of 1 to 2 mm. Cultures of the distal 2 mm segments explanted at E13 did contain AChE and NF positive enteric neurons. We surmise that neural crest cell colonization of embryonic murine bowel occurs in distinctive phases. Phase I: migrating neural crest cells colonize the embryonic foregut and a major part of the midgut (E7.5-E9). Phase II: longitudinal growth of the bowel and neural crest cell proliferation increase the length of the innervated bowel segments (E10-E12). Phase III: neural crest cells colonize the distal 1-2 mm of hindgut by active migration (E13-E14).

List of abbreviations: AChE = AcetylCholineEsterase; CA = catecholaminergic; CNS = central nervous system; E..= Embryonic day ..; ENS = enteric nervous system; GFAP = Glial Fibrillary Acidic Protein; NF = neurofilament; H..= Hindgut segment ..; TH = Tyrosine Hydroxylase.

INTRODUCTION

The enteric nervous system (ENS) is a division of the autonomic nervous system, but resembles the central nervous system (CNS) in ultrastructure and in the content of putative and established neurotransmitters (Furness and Costa, '80). Like the CNS, the ENS contains glial cells but no internal collagen (Gabella, '71, '72; Cook and Burnstock, '76; Jessen et al., '84). A distinct basal lamina surrounds the ganglia and separates them from collagenous connective tissue and blood vessels (Gabella, '79, '81). Specialized blood vessels just outside the myenteric plexus give rise to a blood-plexus barrier analogous to the blood-thymic and blood-brain barriers (Gershon and Bursztajn, 1978). Enteric ganglia are connected to the CNS by extrinsic nerve fibers, but they are capable of functioning autonomously. Despite the resemblance to the CNS, the cells of the ENS, such as enteric glial cells and enteric neurons, derive from the same neural crest source as the neurons and supportive cells of the peripheral nervous system.

The neural crest is a transient ridge on the dorsal surface of the neural tube. Vagal neural crest cell migrate along defined migration pathways to the developing bowel (Le Douarin, '82; Tucker et al., '86). After their arrival in embryonic murine bowel, neural crest cells express a sequence of differentiation antigens and acquire the mature phenotype of enteric neurons. Nonspecific esterase activity is one of the earliest differentiation antigens expressed by neural crest cells after arrival in the bowel. This enzyme activity was first observed in the foregut at E9.5 and in the distal colon at E14.5 (Webster, '73 - embryonic staging adjusted). The expression of cytoskeleton proteins, such as neurofilaments (NF), was first noticed in the foregut at E12 and in the hindgut at E14 (Cochard and Paulin, '84). The expression of NF coincides with the expression of acetylcholinesterase (AChE) and the uptake of ^{3}H 5-HT (Rothman and Gershon, '82). Neuroblasts were first observed at E12 in the foregut and at E14 in the distal colon, by means of electron microscopy (Rothman and Gershon, '82).

Various techniques, such as toluidine-blue staining, enzyme-histochemistry, or the demonstration of the uptake of radioactive precursors for neurotransmitters, have been employed to visualize enteric neurons in murine embryos. These techniques reveal relatively late differentiation antigens of enteric neurons only. More sensitive immunofluorescence staining techniques have become available, which require monoclonal or polyclonal antibodies that recognize migrating neural crest cells. Such monoclonal antibodies are available for avian but not for mammalian neural crest cells (Tucker et al., '84). Consequently, no staining method will serve to visualize migrating neural crest cells in mice. The difficulty of visualizing migrating neural crest cells in murine embryonic bowel may be circumvented by cultivating bowel explants in vivo or in vitro. During cultivation, the neural crest cells acquire mature neuronal phenotypes that can be visualized with staining techniques for late phenotypic neuronal markers.

However, organotypic tissue culture does not provide an optimal environment for the differentiation of all layers of the bowel wall. The renal subcapsular space is a suitable environment for the growth and differentiation of organ rudiments from murine embryos (Svajger et al., '86). The kidney capsule consists of an outer layer of fibrous connective tissue covering vascularized loose connective tissue adjacent to the renal parenchyma. There are no presumptive sources of AChE positive cells close to the subcapsular space. We wondered whether the renal subcapsular space would provide a suitable environment for the development of embryonic murine bowel and the determination of the presence of neural crest cells in bowel explants. Our objective was to determine the developmental stages at which bowel segments are colonized by neural crest cells. To this effect, we cultivated successive bowel segments, explanted at different developmental stages, in the renal subcapsular space of synergic adult mice.

MATERIALS AND METHODS

Animals. Embryos of C57BL/BL1 mice, which were bred in the Laboratory Animal Center at Erasmus University Medical school, were obtained by overnight controlled mating. The morning on which a vaginal plug was observed marked the start of day E0. Pregnant mice were killed by cervical dislocation and the embryos, enveloped by the uterine horns, were removed and placed in tissue culture medium F10+ (Flow Labs). Uterus and extraembryonic membranes were then removed. Embryos were staged according to the table of Theiler ('72) and to number of the gestational days.

Dissection of embryonic bowel. The bowel was dissected under sterile conditions using fine tungsten needles and a dissecting microscope. At E9, stage 14-15, the entire bowel measured 1.5 mm. We grafted distal bowel segments of 1 mm or less from eleven embryos, and the entire bowel from seven embryos. Bowel explanted at later stages was divided into successive segments (Fig. 1). At E10, stage 16-17, the bowel, measuring 2.5 mm, was divided into three separate segments, consisting of foregut (0.7 mm), midgut (0.8 mm), and hindgut (1 mm). At E11, stage 18-19, the explanted bowel was divided into four separate segments. Proximal to the cecal bulge the bowel, consisting of 1 mm foregut and 1.5 mm midgut, was divided in two segments, and distally the bowel was divided in two 1 mm segments of hindgut. The bowel explanted at subsequent stages (E12-E15) was likewise divided into segments of 1 mm, which were numbered in a caudocranial sequence with H1 referring to the most distal segment. At E12, stage 20, the hindgut was divided into three 1 mm segments (H1-H3). From E13 to E15 we only used segments H1-H3 for grafting. Remaining hindgut was processed for enzyme-histochemistry. Distal colon explanted at E17-E20, stage 25-27, was divided into five successive 1 mm segments, which were not used for grafting, but studied histologically.

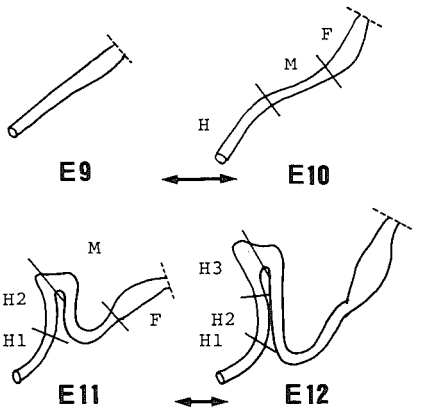

Fig. 1. Embryonic bowel at various developmental stages. The bowel was divided into successive segments as indicated. F = Foregut; M = Midgut; H = Hindgut.

Renal subcapsular grafting. Adult male mice were anesthetized by intraperitoneal injection of O.3 ml of 2.5% avertine (tribromoethyl alcohol diluted in tertiary amyl alcohol) per 20 g body weight. A 1 cm longitudinal paravertebral incision was made after disinfection with 70% ethanol. The left kidney was pulled out by its fat pad and a small cut was made in the renal capsule. The renal capsule was picked up and bowel explants were inserted in the subcapsular space using a 20 gauge mandrin needle. After closing of the skin, recipient mice were placed in a heated box for recovery from anesthesia. After ten days the recipients were sacrificed, and the kidneys, containing the graft, were immediately removed and put in phosphate buffered saline (PBS) (4°C).

Histology. The grafts were embedded in O.C.T. Compound (Tissue Tek 2, Miles) and frozen in liquid nitrogen cooled isopentane. Cryosections (10 μm) were mounted on slides coated with chrome alum and gelatin and stored at -20°C. To determine the degree of differentiation, the grafted bowel sections were routinely stained using hematoxylin and eosin. Sections were examined using a Zeiss microscope.

The presence of neural crest cells in explants was determined using AChE and NF staining in perikarya located on the serosal side of the circular muscle layer. Acetylcholinesterase activity served to visualize enteric neurons. The staining technique was used according to a modification of the method of Karnovski and Roots (Karnovski & Roots, '64; Hedreen et al, '85). The cryosections were fixed for 5 min in buffered 4% formalin, and then rinsed with 0.1M sodium acetate (pH=6.0). Nonspecific-esterase activity was inhibited by tetra-isopropyl-pyrophosphoramide (iso-OMPA) for 20 min. Subsequently, sections were incubated in a medium containing acetylthiocholine iodide for 45 min at room temperature, treated with 1% ammonium sulfide solution for 1 min

and exposed to 0.1% silver nitrate for 1 min. After counterstaining with hematoxylin, sections were dehydrated and coverslipped.

Neurofilament immunoperoxidase staining was also used to visualize enteric neurons. Rabbit polyclonal antibodies raised against 68, 160 and 200 kDa purified neurofilament (NF) triplet proteins (Nakazato, '84) were kindly provided by Dr. Y. Nakazato (Gunma University, Gunma, Japan). Cryosections were fixed in acetone for 5 min, and then dried in air. Peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako, Denmark) were used as second step antibodies in a dilution of 1:80. Peroxidase was visualized by 0.1% 3,3'diaminobenzidine.4HCl (Serva) and 0.02% hydrogen peroxide. All rinsing and diluting was done in PBS (pH 7.4) with 0.1% Tween-20. A graft was considered to contain neural crest cells when positive staining was observed in at least two serial sections. If no AChE activity or NF immunoperoxidase staining was observed in at least 6 serial sections it was concluded that the bowel segment did not contain neural crest cells at the time of explantation.

Statistical analysis. Statistical analysis was performed according to the X^2 test.

RESULTS

Development of embryonic bowel in the renal subcapsular space. Table 1 gives the number of bowel segments grafted for each embryonic stage and the fate of these grafts. Altogether 148 bowel explants were grafted in the same number of recipient mice. Fifteen mice died during the culture period. From the 133 surviving recipient mice 110 kidneys were retrieved that contained the grafted bowel segments. Of these, 95 grafts showed normal differentiation, whereas 15 grafts did not. The latter were not included in the assessment of enteric neurons or their precursor cells. Cystic dilatation of the graft occurred in 25 cases. In 11 out of 25 cases with cystic dilatation, the mucosa had not differentiated into villi or crypts, and the circular muscle layer was underdeveloped. In addition to the enteric cysts, four grafts showed poor development of the epithelium and the circular muscle layer. The success rate of the grafting procedure amounted to 71% (histological investigation/ surviving recipients, 95/133).

The first stage at which embryonic bowel could be explanted was E9. Hematoxylin eosin staining of E9 bowel revealed a tube of mesenchymal cells lined with stratified endodermal cells. Only 2 bowel segments out of a series of 6 showed growth and differentiation after the culture period. Bowel explants smaller than 1 mm did not grow in the subcapsular space (n=10; data not shown). Hematoxylin eosin staining of the cultured bowel explants (E10) revealed a single layered cuboidal epithelium with villi or crypt formation, the muscularis mucosa, the submucosal layer, and differentiated smooth muscle cells in the circular muscle layer. The bowel graft was covered by a

fibrous serosal layer which was continuous to the renal capsule. Compared with the bowel of E20 embryos (thus the same developmental age) the cultured bowel explants showed differentiation according to the developmental stage (Fig. 2). This pattern of differentiation of the bowel explant was observed in the majority of cases.

The success rate of the growth and differentiation of bowel segments explanted at E10 was 44%. The use of larger bowel explants (>1.5mm) slightly increased the success rate (data not shown). Table 1 shows that growth and differentiation of bowel explants of older embryos (E11-E15) was much better than that of younger embryos (over 82% for the older explants versus 33 and 44 percent for explants from E9 and E11 embryos). The differences in growth and differentiation of grafts explanted before or after E11 are statistically significant ($p<0.01$ X^2 test).

Distribution of AChE activity in adult kidney. AChE histochemistry of kidneys of adult mice showed that the enzyme was only present in nerve fibers accompanying arteries in the renal hilus. The renal parenchyma, the subcapsular space and the fibrous renal capsule were devoid of AChE activity (Fig. 3).

Presence of neural crest cells in embryonic bowel explants. The AChE and NF staining techniques matched each other, except for three cases in which NF positive enteric neurons did not show AChE activity. At E12 to E15, neurofilament immunoperoxidase staining is more apparent than AChE activity.

At E9, no AChE activity was found in the bowel *in situ*. Both successful grafts of bowel segments explanted at this stage showed AChE activity in neuronal cells (7 bowel explants were grafted). Table 2 summarizes the AChE and NF staining results of cultured bowel segments, explanted at various developmental stages. Regarding E10 bowel explants, all 4 foregut grafts showed AChE activity and NF staining (Figs. 4 and 5). Only 1 out of 6 explanted segments of midgut showed AChE activity and NF staining. None of 5 explanted segments of hindgut contained AChE and NF positive enteric neurons.

At E12, AChE activity was noticed in the foregut *in situ*, but not in the hindgut. Cultures of the H3 segment of E12 embryos showed AChE and NF positive enteric neurons. Cultures of the H2 segment of E12 embryos contained AChE and NF positive enteric neurons in 2 out of 5 cases, whereas cultures of H1 segments did not contain enteric neurons at all. We conclude that the H1 segment remains consistently aneuronal until E13 (stage 21). Cultured H1 segments of E13 embryos contained AChE and NF positive enteric neurons in 2 out of 4 cases, while the adjacent H2 segment contained enteric neurons in 4 out of 5 cases. At E14, AChE activity was demonstrated in the hindgut in situ. All segments explanted at E14 and E15 and grown in the subcapsular space contained AChE and NF positive enteric neurons. All bowel segments that were explanted and processed for histology at E17 and E20 contained AChE and NF positive neurons.

TABLE 1.

SURVIVAL, GROWTH AND DIFFERENTIATION OF EXPLANTS OF EMBRYONIC BOWEL CULTURED IN THE RENAL SUBCAPSULAR SPACE.

	EMBRYONIC STAGE AT EXPLANTATION							
	E9	E10	E11	E12	E13	E14	E15	TOTAL
Number of grafts	7	36	39	15	16	15	20	148
Surviving recipients	6	34	39	15	13	12	14	133
Growth failure	4	17	2	0	0	0	0	23
Growth	2	17	37	15	13	12	14	110
Normal development	2	15	32	14	11	11	10	95
Success rate (%)	33	44	82	93	85	92	71	71

TABLE 2.

NUMBER OF CULTURED BOWEL SEGMENTS CONTAINING AChE AND NF POSITIVE NEURONS AFTER 10 DAYS' CULTURE IN THE RENAL SUBCAPSULAR SPACE

	E9	E10	E11	E12	E13	E14	E15
Whole gut	2/2	nd	nd	nd	nd	nd	nd
Foregut	*	4/4	5/5	nd	nd	nd	nd
Midgut	*	1/6	8/9	nd	nd	nd	nd
H(indgut)-3	*	*	*	4/4	2/2	4/4	2/2
H(indgut)-2	*	*	3/10	2/5	4/5	4/4	4/4
H(indgut)-1	*	0/5	0/8	0/5	2/4	3/3	4/4

*nd = not done; * not possible to dissect at these stages.*

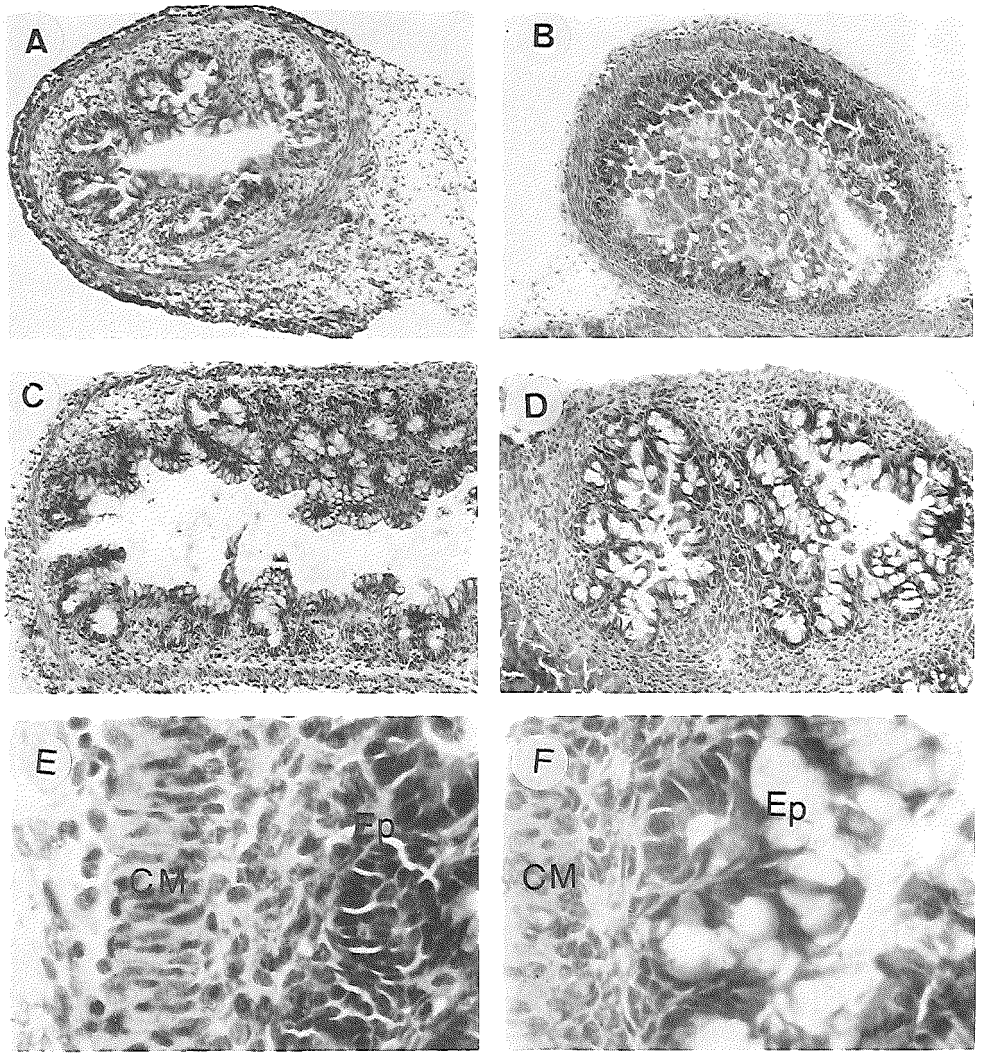

Fig. 2. Comparison of cultured bowel explants and embryonic bowel of corresponding age. A = H/E stained cryosection of E20 ileum; B = H/E stained cryosection of E20 colon; C = H/E stained cryosection of explant of E10 midgut grown for 10 days in the renal subcapsular space; D = H/E stained cryosection of E10 hindgut explant grown for 10 days in the renal subcapsular space; E = detail of B; F = detail of D. Ep = epithelium; CM = circular smooth muscle layer. A, B, C, D magnification 16x; E + F magnification 40x.

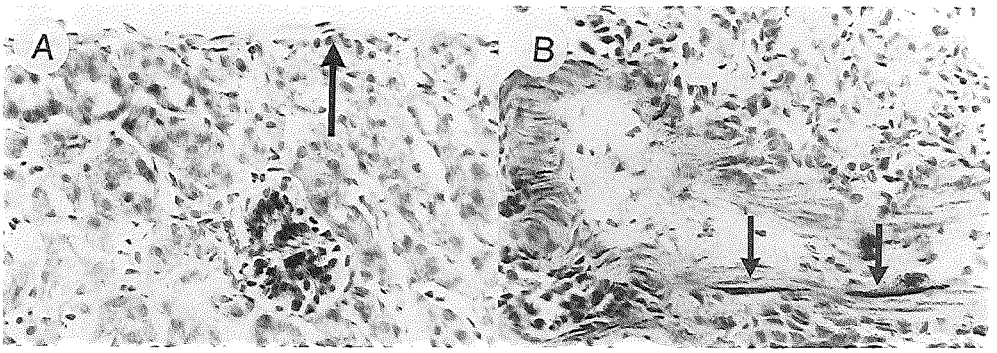

Fig. 3. AChE staining of adult murine kidney. A = renal cortex with renal capsule (arrow) Magnification 25x. B = renal hilus with AChE-positive nerve fibers (arrow) alongside the renal artery (RA). Magnification 25x.

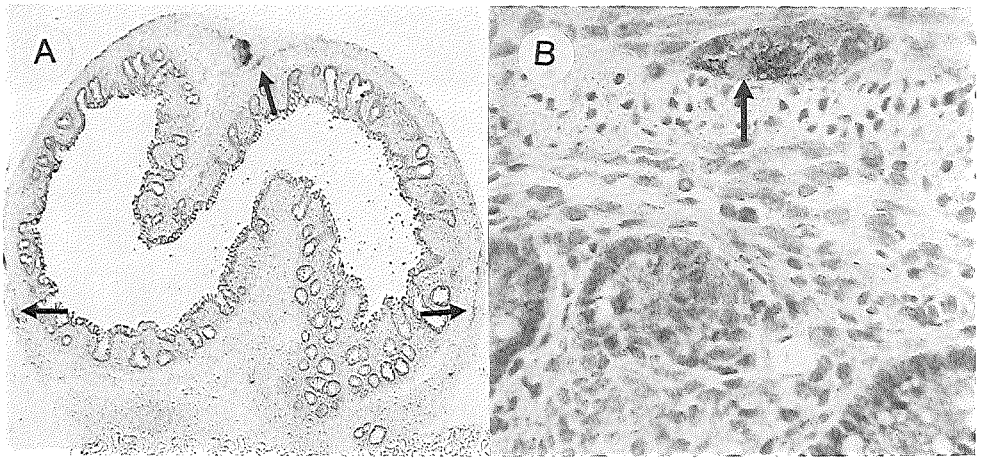

Fig. 4. A = AChE staining of E10 bowel explant grown for 10 days in the renal subcapsular space. Note groups of AChE positive enteric neurons (arrows). K = kidney parenchyma. Magnification 25x. B = detail of A showing AChE positive neurons grouped into a myenteric plexus. Note the differentiated circular smooth muscle layer (CM). Magnification 63x.

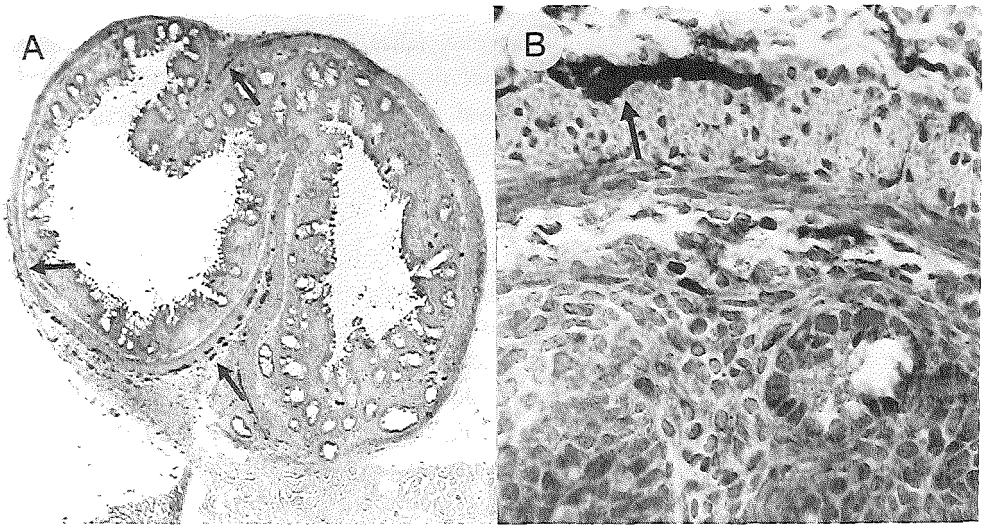

Fig. 5. A = NF immunostaining of enteric neurons (arrows) in same explant as in Fig. 4. Magnification 25x. B = detail of A. Magnification 63x.

DISCUSSION

CULTURE OF EMBRYONIC BOWEL

Our present study has shown that segments of embryonic murine bowel (explanted at E11 to E15) develop optimally in the renal subcapsular space. The differentiation of the layers of the bowel wall in the grafts matches the differentiation of the bowel segments which develop *in situ*. The lower succes rate for the development for the bowel explants from younger embryos (E9 and E10) possibly reflects a critical period in the development of the bowel. An influence of the environment in the renal subcapsular space, which is not embryonic, cannot be excluded entirely. On the other hand, nonspecific constituents of the substratum and the culture medium may elicit the expression of unnatural phenotypes in organotypic tissue culture. The striking difference in succes rate between bowel explants of E9-10 embryos and explants of older embryos has also been observed in organotypic tissue culture (Rothman and Gershon, '82; Rothman and Gershon, '84). Since explants from the distal bowel of older embryos (E11-E15) develop normally in the renal subcapsular space and consistently lack enteric neurons, and, that explants from the proximal bowel contain enteric neurons, we deduce that the environment in the renal subcapsular space does allow for normal differentiation of neural crest cells.

Neural crest cell derivatives that are located close to the kidney (e.g. adrenal medulla) do not colonize aneuronal bowel explants that are inserted into the subcapsular space. Therefore, the renal subcapsular space provides a suitable environment for a) growth and differentiation of embryonic bowel, b) the differentiation of enteric neural crest cells, and c) determination of the presence of neural crest cells in embryonic bowel explants.

NEURAL CREST CELL COLONIZATION OF MURINE EMBRYONIC BOWEL

The cultures of bowel segments explanted at E9 contained enteric neurons, indicating that neural crest cell colonization of the bowel begins at or before E9. The consistent absence of AChE and NF staining in the distal hindgut segment explanted at E10 to E12 indicates that the distal hindgut is devoid of neural crest cells up till E13. This implies that neural crest cells do not colonize the entire bowel at E9. The presence of AChE and NF positive enteric neurons in cultivated bowel segments explanted at E9 must be due to the presence of neural crest cells in the distal foregut at the time of explantation. These findings are in agreement with the observations of Rothman and Gershon ('82), who detected "neuronal" elements in bowel explants (E9 and E10) that were grown in organotypic tissue culture for two weeks. They concluded that the entire bowel was colonized as early as E9. Our findings do not support this conclusion, as segments of distal hindgut explanted at more advanced stages (stages 16 to 20 = E10 to E12) remained aneuronal. The supposition that the entire bowel is colonized at E9 is also contradicted by experiments carried out by the group of Jacobs-Cohen and co-workers (' 87) who reported that cultivated explants of embryonic bowel will remain aneuronal if explantation occurs prior to stage 16 (E10). The fact that we obtained aneuronal bowel from segments explanted at later stages might be related to the size of the explants or to the type of the culture system.

From E10-E12, tyrosine hydroxylase (TH) is expressed transiently by a small number of (neural crest) cells in the bowel. These cells are predominantly localized in the distal esophagus, the stomach, and the proximal small bowel. By E13.5 most of the TH-positive cells have disappeared. Tyrosine hydroxylase expression coincides with the appearance of catecholamine (CA) positive cells in the bowel (Gershon et al., '79; Gershon et al., '81b). After a pulse label of ^{3}H thymidine at E10-E12, some of the CA-positive cells are radioactively labeled, which is an indication that they proliferate (Teitelman et al., '81). A subpopulation of enteric neural crest cells differentiates into the glial phenotype. Immunoreactivity of the marker for enteric glial cells, glial fibrillary acidic protein (GFAP), was first observed at E16 (Rothman et al., '86). Consequently, GFAP immunoreactivity is a relatively late marker for enteric glia and lags behind the development of morphologically recognizable neurons.

Neural crest cell colonization of murine bowel has been summarized in figure 6. We conjecture that, in mice, neural crest cells colonize the foregut and at least part of the midgut at an early stage (E9) (phase I). The elongation of the innervated segment

is due to a combination of longitudinal bowel growth and neural crest cell proliferation (phase II). There is no active craniocaudal migration of neural crest cells through the bowel at these stages. At E13, however, the distal 1-2 mm of hindgut is colonized by actively migrating neural crest cells (phase III). Whether these neural crest cells derive from the proximal innervated segments of the bowel or from the sacral neural crest remains to be investigated.

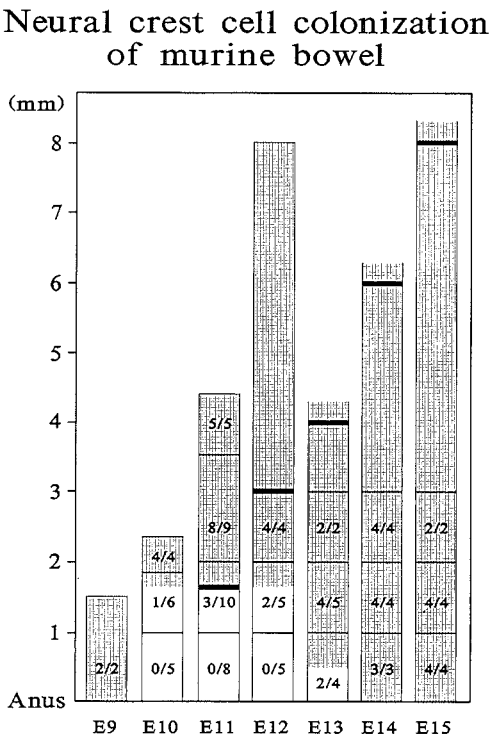

Fig. 6. Graph illustrating neural crest cell colonization of murine embryonic bowel. The bars are divided in accordance with the division of the bowel into segments for grafting. The figures represent the number of grafts that contained AChE and NF positive enteric neurons, indicating the presence of neural crest cells in the explants. Bowel segments containing enteric neurons are shaded. The transition of innervated bowel to aneuronal bowel is located in segment H2 (E10-E12). To locate the presumptive front of migrating neural crest cells we chose the anal end of the bowel as the fixed point of reference. In only a few cases, neural crest cells have passed line A at E10. This indicates that the putative front of migrating neural crest cells is located just proximal to line A. Analogously, the putative front of migrating neural crest cells is located close to line A at E11 and E12. Neural crest cell colonization of this aneuronal segment starts at E13. The putative front of neural crest cells appears as a straight line. In conclusion: neural crest cells did not advance to the anal end of the bowel during E10 to E12, and there is a distal aneuronal bowel segment with a constant length (between 1.5 and 2 mm). The bottom line represents the anal end of the bowel. Ileocecal junction is depicted as a bold line.

In conclusion: a) the renal subcapsular space provides a suitable microenvironment for the grow and development of bowel explants of murine embryos. b) neural crest cells that are present in bowel segments at the time of explantation will

differentiate into enteric neurons; c) neural crest cell colonization of the distal 1-2 mm of the bowel does not occur before E13; d) there is no active neural crest cell migration through the bowel from E10 to E12; e) neural crest cell colonization of the bowel occurs in at least three, distinct phases.

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LITERATURE CITED

Andrew, A. (1971) The origin of intramural ganglia. IV. The origin of enteric ganglia: a critical review and discussion of the present state of the problem. J. Anat. 108:169-184.

Cochard, P., M. Goldstein, and I.B. Black (1978) Ontogenetic appearance and disappearance of tyrosine hydroxylase and catecholamines in the rat embryo. Proc. Natl. Acad. Sci. USA. 75:2986-2990.

Cochard, P., and D. Paulin (1984) Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. J. Neurosci. 4:2080-2094.

Cook, R.D., and G. Burnstock (1976) The ultrastructure of Auerbach's plexus in the guinea-pig. II Non-neuronal elements. J. Neurocytol. 5:195-206.

Furness, J.B., and M. Costa (1980) Types of nerves in the enteric nervous system. Neuroscience 5:1-20.

Gabella, G. (1971) Glial cells in the myenteric plexus. Zeit. Naturforsch. 26B:244-245.

Gabella, G. (1972) Fine structure of the myenteric plexus in the guinea-pig ileum. J. Anat. Lond. 111:69-97.

Gabella, G. (1979) Innervation of the gastrointestinal tract. Int. Rev. Cytol. 59:129-193.

Gabella, G. (1981) *Structure of muscles and nerves in the gastrointestinal tract. In L.R. Johnson (ed): Physiology of the gastrointestinal tract.* New York: Raven Press, p.p. 197-241.

Gershon, M.D., and S. Bursztajn (1978) Properties of the enteric nervous system: limitation of access of intravascular macromolecules to the myenteric plexus and muscularis externa. J. Comp. Neurol. 180:467-488.

Gershon, M.D., G. Teitelman, T.P. Rothman, and D.J. Reis (1979) Proliferation and lack of species and organ specificity in the transient catecholaminergic cells of developing mammals. Neurosci Abstr. 5:334

Gershon, M.D., P. Sherman, and A.R. Gintzler (1981a) An ultrastructural analysis of the developing enteric nervous system of the guinea-pig small intestine. J. Neurocytol. 10:271-296.

Gershon, M.D., G. Teitelman, and T.P. Rothman (1981b) Development of enteric neurons from non-recognizable precursor cells. In: *Development of the autonomic nervous system* (Ciba Foundation Symposium 83). London: Pitman Medical, p.p.51-69.

Hedreen, J.C., S.J. Bacon, and D.L. Price (1985) A modified histochemical technique to visualize acetylcholinesterase-containing axons. J. Histochem. Cytochem. 33:134-140.

Jacobs-Cohen, R.J., R.F. Payette, M.D. Gershon, and T.P. Rothman (1987) Inability of neural crest cells to colonize the presumptive aganglionic bowel of ls/ls mutant mice: requirement for a permissive microenvironment. J. Comp. Neurol. 255:425-438.

Jessen, K.R., R. Thorpe, and R. Mirsky (1984) Molecular identity, distribution and heterogeneity of glial fibrillary acidic protein: An immunoblotting and immunohistochemical study of Schwann cells, satellite cells, enteric glia and astrocytes. J. Neurocytol. 13:187-200.

Karnovsky, M.J., and L. Roots (1964) A "direct-coloring" thiocholine method for cholinesterase. J. Histochem. Cytochem. 12:219-221.

Le Douarin, N.M. (1982) *The neural crest.* Cambridge: Cambridge University Press.

Meijers, J.H.C., D. Tibboel, A.W.M. van der Kamp, C.C.M. van Haperen-Heuts, and J.C. Molenaar (1987) Cell division in migratory and aggregated neural crest cells in the developing gut: an experimental approach to innervation-related motility disorders of the gut. J. Pediatr. Surg. 22:243-245.

Nakazato, Y. (1984) Immunohistochemical localization of neurofilament protein in neuronal degenerations. Acta Neuropathol. 64:30-36.

Rothman, T.P., and M.D. Gershon (1982) Phenotypic expression in the developing murine enteric nervous system. J. Neurosci. 2:381-393.

Rothman, T.P., and M.D. Gershon (1984) Regionally defective colonization of the terminal bowel by the precursors of enteric neurons in lethal spotted mutant mice. Neuroscience 12:1293-1311.

Rothman, T.P., G. Nilaver, and M.D. Gershon (1984) Colonization of the developing murine enteric nervous system and subsequent phenotypic expression by the precursors of peptidergic neurons. J. Comp. Neurol. 225:13-23.

Rothman, T.P., V.M. Tennyson, and M.D. Gershon (1986) Colonization of the bowel by the precursors of enteric glia: Studies of normal and congenital aganglionic mutant mice. J. Comp. Neurol. 252:493-506.

Rothman, T.P., H.M. Mackey, and M.D. Gershon (1987) Loss of the capacity of intrinsic neural precursors to express a catecholaminergic phenotype is correlated with invasion of the developing chick gut by sympathetic nerves. Neurosci. Abstr. 13:925.

Svajger, A., B. Levek-Svajger, and N. Skreb (1986) Rat embryonic ectoderm as renal isograft. J. Embryol. Exp. Morphol.94:1-27.

Teitelman, G., M.D. Gershon, T.P. Rothman, T.H. Joh, and D.J. Reis (1981) Proliferation and distribution of cells that transiently express a catecholaminergic phenotype during development in mice and rats. Dev. Biol. 86:348-355.

Theiler, K. (1972) *The house mouse.* New York: Springer Verlag.

Thiery J.P., and J-L. Duband (1986) Role of tissue environment and fibronectin in the patterning of neural crest derivatives. Trends Neuroscience 565-570.

Tucker, G.C. et al. (1984) Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Diff. 14:223-230.

Tucker, G.C., G. Ciment, and J.P. Thiery (1986) Pathways of avian neural crest cell migration in the developing gut. Dev. Biol. 116:439-450.

Webster, W. (1973) Embryogenesis of the enteric ganglia in normal mice and in mice that develop congenital aganglionic megacolon. J. Embryol. Exp. Morphol. 30:573-585.

CHAPTER 7.4

DEVELOPMENT OF INTESTINAL AGANGLIONOSIS IN LETHAL SPOTTED MICE

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Submitted

ABSTRACT. To gain more insight in the pathogenesis of congenital malformations of the enteric nervous system, we compared the development of the enteric nervous system in lethal spotted and control mice. To this end, we grew dissected bowel segments of lethal spotted embryos in the renal subcapsular space of adult recipients and visualized neurons using acetylcholinesterase and neurofilament staining. We found that both in lethal spotted and control mice neural crest cells are present in explants of foregut at E10 and that the vanguard of migrating neural crest cells does not advance until E13. Neural crest cell colonization of the distal hindgut started at E13. In lethal spotted mice the vanguard of migrating neural crest cells was located in the corresponding bowel segment until E13 but the neural crest cell colonization of the 1-1.5 mm hindgut segment did not occur. We conclude that ls/ls gene only affects neural crest cell colonization in the distal 2 mm of the bowel and this effect is first noticed at E13.

Abbreviations used: ls/ls = lethal spotted; NF = Neurofilament; AChE = Acetylcholinesterase; ENS = Enteric Nervous System; E .. = Embryonic day of development; H.. = Hindgut segment.

INTRODUCTION

The enteric nervous system (ENS) plays an important role in intestinal motility, absorption, and immunity (1-3). Congenital ENS malformations result in disturbances in intestinal motility leading to life-threatening situations in newborns and infants. The pathology underlying these disorders varies from aganglionosis to hyperganglionosis (4,5). Hirschsprung's disease (or congenital aganglionosis) is the most frequent ENS anomaly with an incidence of one in 5,000 newborns. The occurrence of intestinal aganglionosis in such diverse disorders as trisomy 21, Waardenburg syndrome and other forms of deafness, in addition to classical Hirschsprung's disease supports the contention that the development of aganglionosis is etiologically heterogeneous. Familial occurrence in 3 to 12 percent of non-syndromic cases is thought to be due to sex-modified multifactorial inheritance (6,7).

The formation of the ENS has been extensively studied in avian embryos. There is general agreement that enteric neurons derive from the neural crest (8-12). Vagal neural crest cells migrate to and through the developing bowel along defined migration pathways, lined with extracellular matrix molecules (13,14). Neural crest cells interact with these extracellular matrix molecules using integral membrane glycoproteins (substrate adhesion molecules), such as integrin (15,16). After homing in the target organ, neural crest cells form clusters and adhere to each other. These processes are mediated by cell adhesion molecules, such as the neural cell adhesion molecule (N-CAM) and the neuron-glia cell adhesion molecule (Ng-CAM) (17,18).

Intestinal aganglionosis also occurs in mammals, e.g. in mice and rats, frequently on a genetic basis (19,20). At least three different genes cause aganglionosis in mice, viz. the lethal spotted (ls), piebald lethal, and the dominant spotting gene (DOM) (21,22). The ls/ls mouse is most suited for embryologic studies because homozygous litters are easy to obtain.

The renal subcapsular space is an established environemnt to culture rudiments of embryonic organs (23). Previously we demonstrated that segments of embryonic bowel will grow and differentiate in the renal subcapsular space of adult mice (submitted for publication). Furthermore, neural crest cells, if present in the bowel segments at the time of explantation, could be visualized after neuronal differentiation. In that study, we found that the vanguard of migrating neural crest cells is located 1.5 mm cranial to the anus but does not advance to the anal end of the bowel until E12. Neural crest cells start to colonize the distal 1 to 1.5 mm of hindgut at E13.

The present study was undertaken to compare neural crest cell colonization of the embryonic bowel of ls/ls and control mice. To this end, we grew dissected bowel segments of ls/ls mouse embryos in the renal subcapsular space of adult recipient mice and visualized neurons using AChE and NF staining.

MATERIALS AND METHODS

Animals. Experiments were carried out according to the Dutch law on the use of laboratory animals. C57BL/BL1 and ls/ls mice were bred in the Laboratory Animal Center at Erasmus University Medical School. To obtain exclusively homozygous ls/ls embryos for the embryological experiments, homozygous ls/ls mice were mated. Mouse embryos of gestational ages E10 to E15 were obtained after overnight matings. The morning on which a vaginal plug was observed was called day E0. Mice were killed by cervical dislocation and the uterine horns or the bowel were removed. Embryos were removed from the uterus and extraembryonic membranes in tissue culture medium F10+ (Flow Labs). Embryos were staged according to the table of Theiler (24) and the number of gestational days.

Dissection of embryonic bowel. The bowel was dissected under sterile conditions using fine tungsten needles and a dissection microscope (Nikon). The levels at which the bowel was divided after explantation are shown in Fig. 1. At E10, stage 16-17, the bowel

was divided into three successive segments, representing 0.7 mm of foregut, 0.8 mm of midgut, and 1 mm of hindgut. At E11, stage 18-19, the explanted bowel was divided into four successive segments. Proximal to the cecal bulging the bowel was divided in two segments, representing 1 mm foregut and 1.5 mm midgut; distal to the cecal bulging the bowel was divided in two 1 mm segments. H1 refers to the distal 1 mm of the hindgut and successive segments are numbered in a caudocranial sequence.

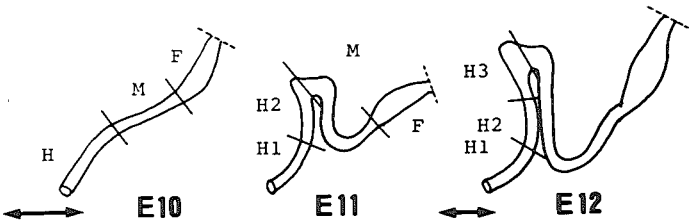

Fig. 1. Embryonic bowel at various developmental stages. The bowel was divided into successive segments as indicated. F = foregut; M = midgut; H = hindgut.

Renal subcapsular grafting. Dissected bowel segments were transplanted to heterozygous (ls/+) adult male mice which were anesthetized by intraperitoneal injection of O.3 ml of 2.5% avertine (tri-bromo-ethyl alcohol diluted in tertiary amyl alcohol) per 20g body weight. A 1 cm longitudinal paravertebral skin incision was made and the left kidney was exposed. Bowel explants were inserted in the subcapsular space using a 20G mandrin needle. After closing of the skin, recipients recovered from anesthesia in a heated box, and they were sacrificed after ten days.

Histology. The kidneys containing the graft were embedded in O.C.T. Compound (Tissue Tek 2, Miles) and frozen in liquid nitrogen cooled isopentane. Cryostat sections (10 μm) were mounted on slides coated with chrome alum and stored at -20°C. To determine the degree of differentiation of the grafted bowel, sections were routinely stained using hematoxylin and eosin, and observed with bright field optics (Zeiss).

Acetylcholinesterase activity was used to visualize enteric neurons, and was demonstrated according to a modification of the method of Karnovsky and Roots (25,26). The cryostat sections were fixed for 5 min in buffered 4% formalin, and then rinsed with 0.1M sodium acetate (pH=6.0). Nonspecific-esterase activity was inhibited by tetra-isopropyl-pyrophosphoramide (iso-OMPA) for 20 min. Then the sections were incubated for 45 min at room temperature using acetylthiocholine iodide as substrate, treated with 1% ammonium sulfide solution for 1 min and exposed to 0.1% silver nitrate for 1 min. Sections were counterstained with hematoxylin.

Neurofilament immunoperoxidase staining was also used to visualize enteric neurons. Polyclonal rabbit antibodies raised against 70, 160 and 200 kDa purified

neurofilament (NF) proteins were kindly provided by Dr. Y. Nakazato (Gunma University, Gunma, Japan) (27). The immunostaining procedure is described elsewhere (28). The presence of neural crest cells in explants was determined by demonstrating AChE activity and NF immunoperoxidase staining in perikarya located on the serosal side of the circular muscle layer. Both AChE and NF staining had to be present in at least two serial sections. If AChE activity or NF immunoperoxidase staining was absent in at least 6 serial sections it was concluded that the bowel segment did not contain neural crest cells at the time of explantation.

RESULTS

Adult bowel. The colon of adult ls/ls and control mice was investigated macroscopically and microscopically. In control mice the distal 5 mm of hindgut contained AChE and NF positive enteric neurons in the myenteric plexus. By contrast, the distal 2-3 mm hindgut of ls/ls mice did not contain AChE and NF positive neurons. We found nerve fibers at the site of the myenteric and submucous plexus in the aganglionic segment of ls/ls mice. We did not find ectopic neurons at the serosal surface of the bowel. The differentiation of mucosa and smooth muscle layers was equivalent in both strains.

Presence of neural crest cells in the bowel of control and lethal spotted embryos. Table 1 summarizes the AChE and NF staining results of bowel segments removed from E10-E15 embryos and grown for 10 days in the renal subcapsular space. We never observed clusters of AChE and NF positive neurons at ectopic sites. In control E10 embryos, AChE and NF positive enteric neurons were found in all cultures of explanted foregut, whereas only one of the midgut cultures, and none of the hindgut cultures contained enteric neurons. Almost identical results were found in ls/ls embryos (Figure 2). In control E11 embryos, all cultured bowel explants, except for those of the distal 1-2 mm contained enteric neurons. Cultures of bowel segments of E11 ls/ls embryos all contained enteric neurons except for those of the H1 segment. In control E12 embryos, AChE activity and NF immunoperoxidase staining was found in all bowel segments cranial to the H2 segment. In ls/ls E12 embryos, AChE activity and NF immunoperoxidase staining was found in all cultures cranial to the H2 segment.

The H1 and H2 segments of control embryos contained enteric neurons when explanted at E13. At E14, all cultures of the distal hindgut segments contained enteric neurons. By contrast, cultures of H1 and H2 segments of ls/ls embryos never contained enteric neurons when explanted at E13-E15 (figure 3). The bowel segments proximal to the H2 segment usually contained enteric neurons.

TABLE 1.

RATIO OF CULTURED BOWEL SEGMENTS CONTAINING AChE AND NF POSITIVE NEURONS AFTER 10 DAYS' CULTURE IN THE RENAL SUBCAPSULAR SPACE

	C57BL/BL-1 (Control mice)					
	E10	E11	E12	E13	E14	E15
Foregut	4/4	5/5	nd	nd	nd	nd
Midgut	1/6	8/9	nd	nd	nd	nd
H-3	*	*	4/4	2/2	4/4	2/2
H-2	*	3/10	2/5	4/5	4/4	4/4
H-1	0/5	0/8	0/5	2/4	3/3	4/4
	ls/ls mice					
	E10	E11	E12	E13	E14	E15
Foregut	3/3	6/6	2/2	nd	nd	nd
Midgut	0/5	6/6	7/7	nd	nd	nd
H-3	*	*	*	5/5	4/5	2/3
H-2	*	*	0/6	0/4	0/4	0/4
H-1	0/2	0/5	0/5	0/3	0/2	0/4

** not present*
nd not determined

DISCUSSION

The present study was undertaken to compare the developmental biology of the ENS in ls/ls and control mice. We demonstrated that both in ls/ls and control mice neural crest cells are present in explants of foregut at E10. The distal 1-2 mm does not contain neural crest cells at this stage, both in ls/ls and control mice. These hindgut segments (H1 and H2) remain devoid of neural crest cells until E13. In control mice, neural crest cells start to colonize the distal 1-2 mm bowel segment at E13. By contrast, there is no neural crest cell colonization of the distal 2 mm hindgut in ls/ls mice. During subsequent

development, this aneuronal hindgut segment shows little longitudinal growth, measuring 2-3 mm at E20 and 4-5mm in adult mice.

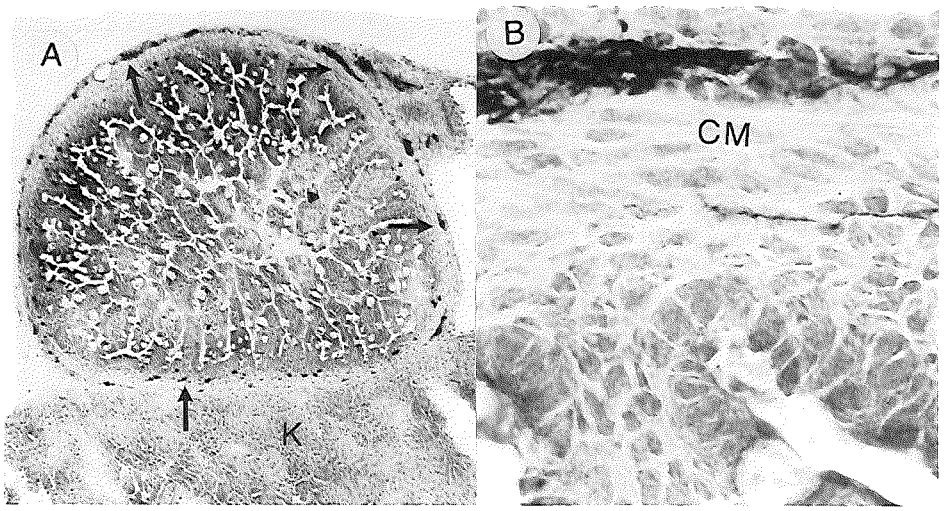

Fig. 2. A = NF immunostaining of E13 bowel explant from control mice cultured for 10 days in the renal subcapsular space. Note groups of NF positive enteric neurons (arrows). K = kidney parenchyma. Magnification 25x. B = detail of A showing NF positive neurons grouped into a myenteric plexus. Note the differentiated circular smooth muscle layer (CM). Magnification 63x.

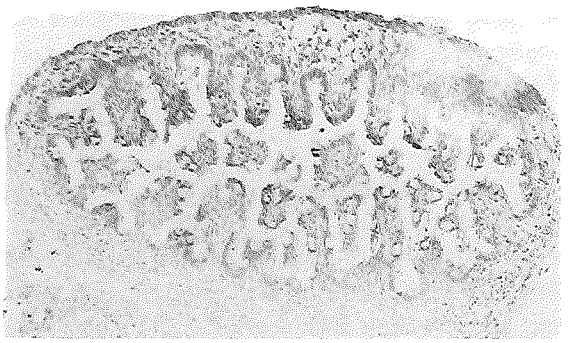

Fig. 3. NF immunostaining of E13 bowel explant from ls/ls mice grown for 10 days in the renal subcapsular space. Enteric neurons are abent. Magnification 25x.

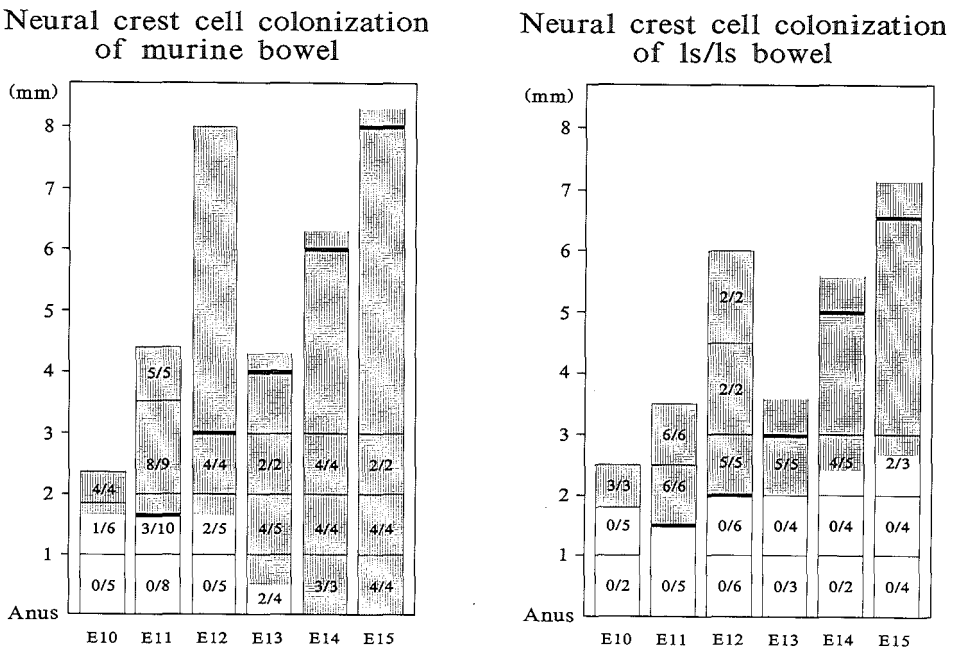

Fig. 4. Neural crest cell colonization of the bowel of control and ls/ls mice. The bars are divided in accordance with the division of the bowel into segments for grafting. The figures represent the number of grafts that contained AChE and NF positive enteric neurons, corresponding with the presence of neural crest cells in the explants. Bowel segments containing enteric neurons are shaded. The vanguard of neural crest cells appears as a straight line in both mouse strains. The difference between control mice and ls/ls mice is evident at E13: the third phase of colonization of the bowel does not occur. The bottom line represents the anal end of the bowel. Ileocecal junction is depicted as a bold line.

To prevent the error that elongation of the innervated bowel is misunderstood. as neural crest cell migration, which occurs when the umbilicus is used as reference point, we took the anal end of the bowel as reference point to determine the position of the vanguard of migrating enteric neural crest cells. The vanguard of migrating neural crest cells does not advance until E13, leaving a distal aneuronal segment with a constant length (approximately 1.5-2 mm). Neural crest cell colonization of the distal hindgut starts at E13. In ls/ls mice the vanguard of migrating neural crest cells is located at the same site as in control mice until E13. The difference in colonization pattern between ls/ls and control mice is evident: the final phase of colonization of the 1-1.5 mm hindgut segment, which starts at E13 in control mice, does not occur in ls/ls mice.

It has been suggested that a slowing of the craniocaudal migration of enteric

neural crest cells results in aganglionosis in mice and men (29,30). The present results show that neural crest cell colonization of the bowel proceeds similarly in ls/ls mice and control mice until E13; thus there is no slowing down of the migration speed of enteric neural crest cells in ls/ls mice.

The receptivity of presumptive aganglionic ls/ls bowel to colonization by neural crest cells has been analyzed using in vitro culture systems in which aneuronal hindgut was combined with a variety of sources of migrating neural crest cells (31). The presumptive aganglionic ls/ls hindgut (E11) was not colonized by neural crest cells from various sources, whereas neural crest cells colonized recipient bowel of chicken and quail embryos. Rothman and Gershon surmised that the presumptive aganglionic segment was intrinsically abnormal at an very early embryonic stage and does not elongate during development (32). Compared to normal mice, several differences in constituents of extracellular matrices (laminin and collagen type IV) of the basal lamina of the mucosa, and an accumulation of proteoglycans in the enteric mesenchyme have been reported in embryonic (starting at E11) ls/ls bowel (33). These differences were observed, however in other caudal mesenchymes of the embryos as well. Therefore, these observations do not explain why neural crest cell colonization of the distal bowel segment fails to occur, since other neural crest derivatives in the caudal part of the embryo presented no abnormalities and since enteric neural crest cells do not migrate along the basal lamina of the mucosa.

We propose that neural crest cell colonization of the bowel proceeds in distinct phases. This proposition is supported by our observations that the vanguard of migrating neural crest cells does not advance to the anus from E10 to E12, and that the hindgut is colonized at E13. It may well be that the colonization of distal 1-2 mm of hindgut at E13 requires a particular subset of cell-substratum adhesion molecules. Recently Wolgemuth and coworkers described megacolon in transgenic mice carrying the homeobox gene Hox-1.4 (34). Using *in situ* hybridization, they found that the Hox-1.4 gene was overexpressed in the bowel of E12.5 embryo. It might well be that during normal development the Hox-1.4 gene product activates the expression of substrate adhesion molecules that are required for the migration in various bowel segments. Overexpression of the Hox-1.4 gene product could result in accumulation of substrate adhesion molecules, that result in the cessation of enteric migration. The ls/ls gene however is located on chromosome 2, wheras the Hox-1.4 gene is located on chromosome 5. There are no Hox-containing genes known, that are close to the ls/ls locus on chromosome 2.

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REFERENCES

1. Gershon MD, Erde SM. The nervous system of the gut. Gastroenterology 1981;80:1571-94.

2. Cooke HJ. Neurobiology of the intestinal mucosa. Gastroenterology 1986;90:1057-81.

3. Felten DL, Felten SY, Bellinger DL, Carlson SL, Ackerman KD, Madden KS, Olschowski, Livnat S. Noradrenergic sympathetic neural interactions with the immune system: structure and function. Immunological Reviews 1987;100:225-260.

4. Schärli A. Pathophysiology of Hirschsprung's disease. In: Hirschsprung's disease. Ed. Holschneider AM. Hippokrates Verlag, Stuttgart, FRG. 1982. pp.23-40.

5. Tibboel D, Meijers JHC, Klück P, van der Kamp AWM, ten Kate FWJ, van Haperen-Heuts ICCM, Molenaar JC. Monoclonal antibodies for diagnosis and research in enteric nervous system pathology. A review. Dev Neurosci 1987;9:133-43.

6. Passarge E. The genetics of Hirschsprung's disease. Evidence for heterogeneous etiology and a study of sixty-three families. N Engl J Med 1967;276:138-43.

7. Passarge E. Hirschsprung disease and other developmental defects of the gastrointestinal tract. In: *Principles and practice of medical genetics.* Eds. Emery AEH, Rimoin DL. London, Churchill Livingstone, 1983 pp 886-889.

8. Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J Comp Neurol 1954;101:515-541.

9. Le Douarin NM, Teillet MA. The migration of neural crest cells to the wall of the digestive tract in avian embryo. J Embryol Exp Morphol 1973;31-48.

10. Andrew A. The origin of intramural ganglia. IV. The origin of enteric ganglia: a critical review and discussion of the present stage of the problem. J Anat 1971;108:169-84.

11. Allan IJ, Newgreen DF. The origin and differentiation of enteric neurons of the fowl embryo. Am J Anat 1980;157:137-54.

12. Le Douarin NM. *The neural crest.* Cambridge University Press, Cambridge, 1982.

13. Duband JL, Thiery JP. Appearance and distribution of fibronectin during chick embryo gastrulation. Dev Biol 1982;93:308-23.

14. Tucker GC, Ciment G, Thiery JP. Pathways of avian neural crest cell migration in the developing gut. Dev Biol 1986;116:439-450.

15. Boucaut JC, Darribiere T, Poole TJ, Aoyama H, Yamada KM, Thiery JP. Biologically active synthetic peptides as probes of embryonic development: a competitive inhibitor of fibronectin function inhibits gastrulations in amphibian and neural crest migration in avian embryos. J Cell Biol 1984;99:1822-1830.

16. Bronner-Fraser M. Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. J cell Biol 1985;101:610-17.

17. Thiery JP, Duband JL, Rutishauser U, Edeman GM. Cell adhesion molecules in early chicken embryogenesis. Proc Natl Acad Sci USA 1982;79:6737-6741.

18. Thiery JP, Delouvee A, Grumet M, Edelman GM. Initial appearance and regional distribution of the neuron-glia cell adhesion molecule in the chick embryo. J Cell Biol 1985;100:442-56.

19. Derrick EH, St George-Grambauer B. Megacolon in mice. J Path Bact 1957;73:569-571.

20. Ikadai H, Fujita H, Agematsu Y, Imamichi T. Observation of congenital aganglionosis rat (Hirschsprung's disease rat) and its genetical analysis. Cong Anom 1979;19:31-36.

21. Lane PW. Association of megacolon with two recessive spotting genes in the mouse. J. Heredity 1966;57:181-83.

22. Lane PW, Liu HM. Association of megacolon with a new dominant spotting gene (Dom) in the mouse. J Heredity 1984;75:435-39.

23. Svajger A, Levek-Svajger B, Skreb N. Rat embryonic ectoderm as renal isograft. J Embryol exp Morphol 1986;94:1-27.

24. Theiler K. *The house mouse.* New York: Springer Verlag, 1972.

25. Karnovsky MJ, Roots L. A "direct-coloring" thiocholine method for cholinesterase. J Histochem Cytochem 1964;12:219-21.

26. Hedreen JC, Bacon SJ, Price DL. A modified histochemical technique to visualize acetylcholinesterase-containing axons. J Histochem and Cytochem 1985;33:134-40.

27. Nakazato Y. Immunohistochemical localization of neurofilament protein in neuronal degenerations. Acta Neuropathol. 1984;64:30-36.

28. Meijers JHC, Tibboel D, van der Kamp AWM, van Haperen-Heuts CCM, Klück P, Molenaar JC. The influence of the stage of differentiation on the migration of neural cells: an experimental study of Hirschsprung's disease. Pediatr Res 1987;21:466-70.

29. Webster WS. Embryological study of the development of aganglionic megacolon in piebald-lethal and lethal-spotting mice. PhD Thesis, University of London, 1971.

30. Webster W. Embryogenesis of the enteric ganglia in normal mice and in mice that develop congenital aganglionic megacolon. J Embr Exp Morphol 1973;30:573-85.

31. Jacobs-Cohen RJ, Payette RF, Gershon MD, Rothman TP. Inability of neural crest cells to colonize the presumptive aganglionic bowel of ls/ls mutant mice: requirement for a permissive microenvironment. J Comp Neurol 1987;255:425-38.

32. Rothman TP, Gershon MD. Regionally defective colonization of the terminal bowel by the precursors of enteric neurons in lethal spotted mutant mice. Neuroscience 1984;12:1293-311.

33. Payette RF, Tennyson VM, Pomeranz HD, Pham TD, Rothman TP, Gershon MD. Accumulation of components of basal laminae: Association with the failure of neural crest cells to colonize the presumptive aganglionic bowel of ls/ls mutant mice. Dev Biol 1988;125:341-60.

34. Wolgemuth DJ, Behringer RR, Mostoller MP, Brinster RL, Palmiter RD. Transgenic mice overexpressing the mouse homeobox-containing gene Hox-1.4 exhibit abnormal gut development. Nature 1989;337:464-467.

CHAPTER 7.5

THE ORIGIN OF ENTERIC NEURONS IN CHICKEN HINDGUT

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Submitted

Summary. For a better understanding of the etiology and pathogenesis of congenital anomalies of the enteric nervous system, we studied the origin of enteric neurons in chicken hindgut. It is generally agreed that enteric neurons derive from the vagal neural crest, i.e. the neural crest opposite somite pairs 1 to 7. There is still controversy about the involvement of the sacral neural crest, opposite somite pairs 28 and subsequent somites. To determine the contribution of the sacral neural crest to the development of hindgut ganglia, we prevented craniocaudal migration of vagal neural crest cells by transecting the postumbilical bowel in front of migrating neural crest cells. The transected bowel was left *in situ* till embryonic day 12 (E12) and was then examined for enteric neurons. We found that the hindgut was left aganglionic when the bowel was transected at E4, and observed an unexpected, consistent pattern of HNK-1 immunoreactivity in the hindgut: a neuron-free myenteric plexus and a diffuse layer of HNK-1 immunoreactive cells at the site of the submucous plexus. Electronmicroscopy revealed that the myenetric plexus was filled with unmyelinated axons. Occasionally some supportive cells were observed at the periphery of the plexus, engolfing axons. The pattern of HNK-1 immunoreactivity was present from the site of transection down to the colorectum, and was also found in 67% of cases in which the bowel was transected at E5. A normal pattern of HNK-1 immunoreactivity was found in the remaining 33%. Transection of embryonic bowel at E6 and E7 had no effect on the innervation of the hindgut either distal or proximal to the site of transection. In view of the absence of enteric neurons in the hindgut after transection at E4 and E5 we conclude that the sacral neural crest does not provide precursor cells for enteric neurons in the hindgut.

Abbreviations: ENS = Enteric Nervous System; E..= Embryonic day

INTRODUCTION

Congenital anomalies of the enteric nervous system (ENS), give rise to considerable morbidity and mortality in the neonatal period (Nixon, 1982). Despite detailed

knowledge of the underlying pathological defects and the availability of some animal models, the etiology and pathogenesis of these anomalies remain unclear. Investigation of normal ENS development, involving the intrinsic and extrinsic innervation of the bowel, might clarify pathogenetic mechanisms of these anomalies.

In chickens, enteric neurons and enteric glial cells derive from the neural crest, a transient ridge on the dorsal surface of the neural tube. The vagal neural crest, opposite somite pairs 1 to 7, provides precursor cells for enteric neurons in the entire bowel, as was shown by ablation studies (Yntema & Hammond, 1954) and in quail-chick chimeras (Le Douarin & Teillet, 1973). The vagal neural crest cells migrate from their site of origin to the region of the third and fourth branchial arches (Duband & Thiery, 1982), where they enter the developing foregut (Ciment & Weston, 1983; Tucker et al, 1986) and migrate in a craniocaudal direction to the colorectum (Allan & Newgreen, 1980). There is controversy concerning the extent to which the sacral neural crest, opposite somite pair 28 and subsequent somite pairs, provides precursor cells for enteric neurons in the postumbilical bowel. In quail-chick chimeras, sacral neural crest cells migrate to Remak's ganglia and a small number of quail neural crest cells colonize postumbilical bowel up to the umbilicus (Le Douarin & Teillet, 1973). Reports of dual gradients in neurofilament-, (Payette et al, 1984), vaso intestinal peptide- and substance P-immunoreactivity (Saffrey et al, 1982) also suggest that the sacral neural crest contributes to enteric neurons in the hindgut.

On the other hand, when successive segments of embryonic bowel, isolated at different stages of development, were grown on the chorioallantoic membrane, only a craniocaudal direction of enteric neurons was observed (Allan & Newgreen, 1980). In these experiments, hindgut explants taken at an early developmental stage had been cut off from any source of neural crest cells, either vagal or sacral.

To determine whether the sacral neural crest contributed to the hindgut ganglia, we prevented craniocaudal migration of vagal neural crest cells by transecting the postumbilical bowel *in ovo* in front of migrating neural crest cells. This would prohibit colonization of the hindgut by vagal neural crest cells, but would not impede colonization by sacral neural crest cells. The transected bowel was left *in situ* for a week and was then examined for enteric neurons. If enteric neurons were present these must have derived from the sacral neural crest. We also investigated whether the extrinsic innervation had developed normally in the absence of vagal neural crest cells. The proximal midgut was studied to establish whether an excess of vagal neural crest cells had resulted in an increase in the number of enteric neurons.

MATERIALS AND METHODS

Animals. Fertilized eggs from the white leghorn (*Gallus Domesticus*) were incubated in a forced draught incubator at 37°C, at a relative humidity of 75-80%. Embryos were staged according to the number of developmental days or according to the table of

Hamburger & Hamilton (1951).

Normal ENS development in the hindgut. Bowel segments were isolated from E4-E15 embryos and processed for HNK-1 immunoperoxidase staining.

Transection of embryonic bowel. We transected postumbilical bowel at various stages of development. This involved 130 embryos, 15 at E4, 88 at E5, 19 at E6 and 8 at E7. We removed approximately 1.5 cm^2 of both shell and shell membrane and repositioned the embryo using watchmaker's forceps to enable the introduction of microscissors into the umbilical stalk. The bowel was then transected just distal to the omphalomesenteric duct (E5-E7) or just distal to the cecal bulges (E4)(Fig. 1). After transection, the eggs were sealed with Scotch tape and put back in the incubator. To investigate the effect of manipulation of the umbilical stalk on ENS formation, a sham operation was performed in 26 control embryos.

Immunohistology. The embryos that underwent bowel transection were sacrificed at E12, whereupon the bowel was dissected, fixed in 4% formaldehyde in phosphate-buffered saline, dehydrated and subsequently embedded in paraffin. Alternatively, bowel segments were embedded in Tissue Tek II embedding compound (Miles) and snap-frozen in liquid nitrogen cooled isopentane. Sections were made at 10 μm. For immunohistology, sections were incubated with the primary antibody in a moist incubation chamber at room temperature for one hour. The HNK-1 hybridoma cell line was purchased from the American Type Culture Collection TIB 200 (Abo & Balch, 1981; Tucker et al., 1984). Two monoclonal antibodies, raised against purified human neurofilament triplet proteins but cross reacting to chicken neurofilaments, were used to identify enteric neurons or extrinsic nerve fibers. Monoclonal antibody 3G6 is specific for the 160kD and 200kD neurofilament protein (Klück et al, 1984) and C90 for the 200kD. Hybridoma E/C8 is directed against NAPA-73, a neurofilament associated glycoprotein; the supernatant of the culture was used undiluted (Ciment & Weston, 1982; Ciment et al, 1986). Peroxidase-conjugated, rabbit-anti-mouse immunoglobulins (Dako, Denmark) were used as second step antibodies (dilution 1:100). Peroxidase was visualized with 0.1% 3,3'diaminobenzidine.HCl (Serva) with 0,01% hydrogen peroxide. Sections were counterstained with hematoxylin for one minute. Phosphate-buffered saline with 0.1 % Tween 20 was used for all rinsing.

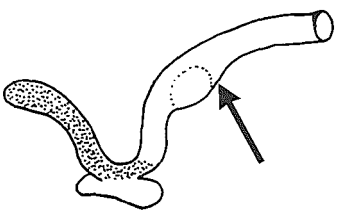


Fig. 1: Diagram of embryonic chicken bowel at stage 26 showing the anlage for ceca. The dotted area represents bowel that is colonized by neural crest cells. The arrow indicates the usual level of transection.

Electronmicroscopy. Dissected bowel was fixed in a mixture of 1% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3) for 24 hrs. After rinsing in 0.1M cacodylate buffer (pH 7.3) postfixation was performed, overnight, in 1% OsO_4 in the cacodylate buffer. After rinsing with the cacodylate buffer the specimens were incubated with tannic acid (Simionescu & Simionescu, 1976), dehydrated, and embedded in epon. Ultrathin sections were prepared with a Reichert OMU2-microtome and stained with a saturated solution of uranyl-acetate in water and leadcitrate (Reynolds, 1963).

RESULTS

Gross examination of the sham-operated embryos at E12 revealed no abnormalities of the bowel. Of 130 embryos that underwent bowel transection, 111 survived this procedure. All deaths were perioperative and due to excessive bleeding. We never observed adherence of bowel segments at the time of fixation (E12). HNK-1 immunocytochemistry revealed a normal ENS in the sham-operated embryos and in the segment proximal to the transection irrespective of the day of intervention (Figs. 2-5) (we will refer to the normal HNK-1 staining pattern as pattern B). There were no signs of an increase in the number of enteric neurons in the proximal bowel segments.

Table 1 summarizes the results of the transection experiments at the various stages of development. Gross examination of the 13 embryos which survived bowel transection at E4 showed complete separation of the bowel immediately distal to the ceca at E12. Histological investigation of both proximal and distal segments revealed a normal differentiation of the layers of the bowel wall. HNK-1 staining consistently revealed what we will call pattern A in the distal segment: an HNK-1 positive neuron-free, myenteric plexus and a diffuse layer of HNK-1 positive cells at the site of the submucous plexus (Figs. 6). The antibodies E/C8, 3G6 and C90 visualized nerve fibers but no enteric neurons or neural crest cells in cryosections.In ultrathin sections, we observed the presence of a large number of axons in the myenteric plexus and confirmed that enteric neurons were absent (Fig. 7). Occasionally supportive cells were observed both at the periphery of the plexus and in the centre (Figs 8-10). The number of neuron-free, myenteric plexuses was smaller than that of myenteric ganglia in control bowel at E12. The diffuse layer of HNK-1 positive cells in the submucosa was not present in control bowel. HNK-1 staining pattern A was present from the site of transection down to the colorectum, except for the most distal 2 mm. There were two two exceptions: a small number of enteric neurons was found in the distal hindgut of one and unidentifiable HNK-1 positive cells in another one.

Gross examination of the 80 surviving embryos that were operated at E5 showed complete separation of the bowel. HNK-1 staining of the distal segment revealed three patterns of innervation. In 31 embryos we found HNK-1 immunoreactivity pattern A, the same picture observed after transection at E4. In 26 embryos HNK-1 revealed normal innervation with enteric neurons and extrinsic nerve fibers (pattern B). In the remaining

23 embryos, the HNK-1 antibody revealed a combination of patterns A and B. Pattern A was found in the distal hindgut. Pattern B was found adjacent to the site of transection.

Two separate segments were consistently found in the surviving embryos operated at E6 and E7. HNK-1 immunoperoxidase staining revealed the normal pattern B of innervation in all the embryos. The antibodies E/C8, 3G6, and C90 confirmed that neural crest cells or enteric neurons were present at the normal sites both proximal and distal to the transection.

TABLE 1

HNK-1 IMMUNOREACTIVITY IN HINDGUT AT E12 AFTER BOWEL TRANSECTION AT DIFFERENT DAYS OF EMBRYONIC DEVELOPMENT

CONTROLS	DAY OF TRANSECTION				
	E4	E5	E6	E7	E4-E7
Pattern A	11	31	0	0	0
Pattern B	1	26	11	7	26
Pattern A + B	1	23	0	0	0
Total number of embryos	13	80	11	7	26

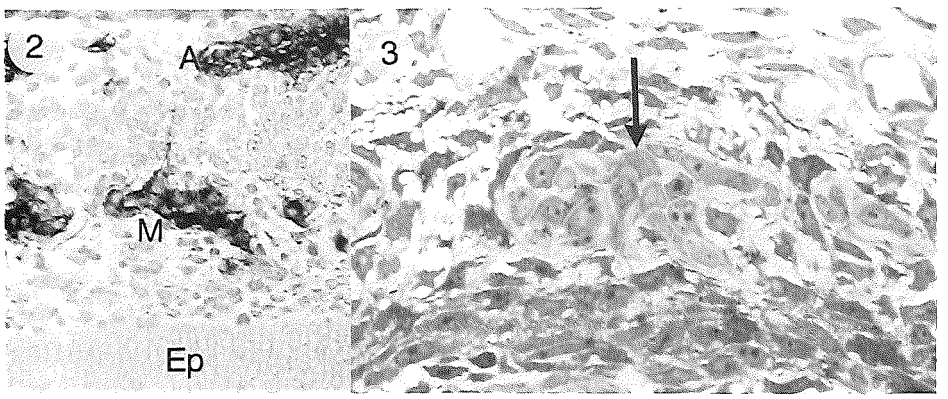


Fig. 2. Paraffin section of hindgut of E12 embryo stained with HNK-1. Note the presence of the myenteric and submucous ganglia containing HNK-1 enteric neurons (Pattern B). Magnification 25x. Fig. 3. Semithin section showing enteric neurons in the myenteric plexus. Magnification 63x.

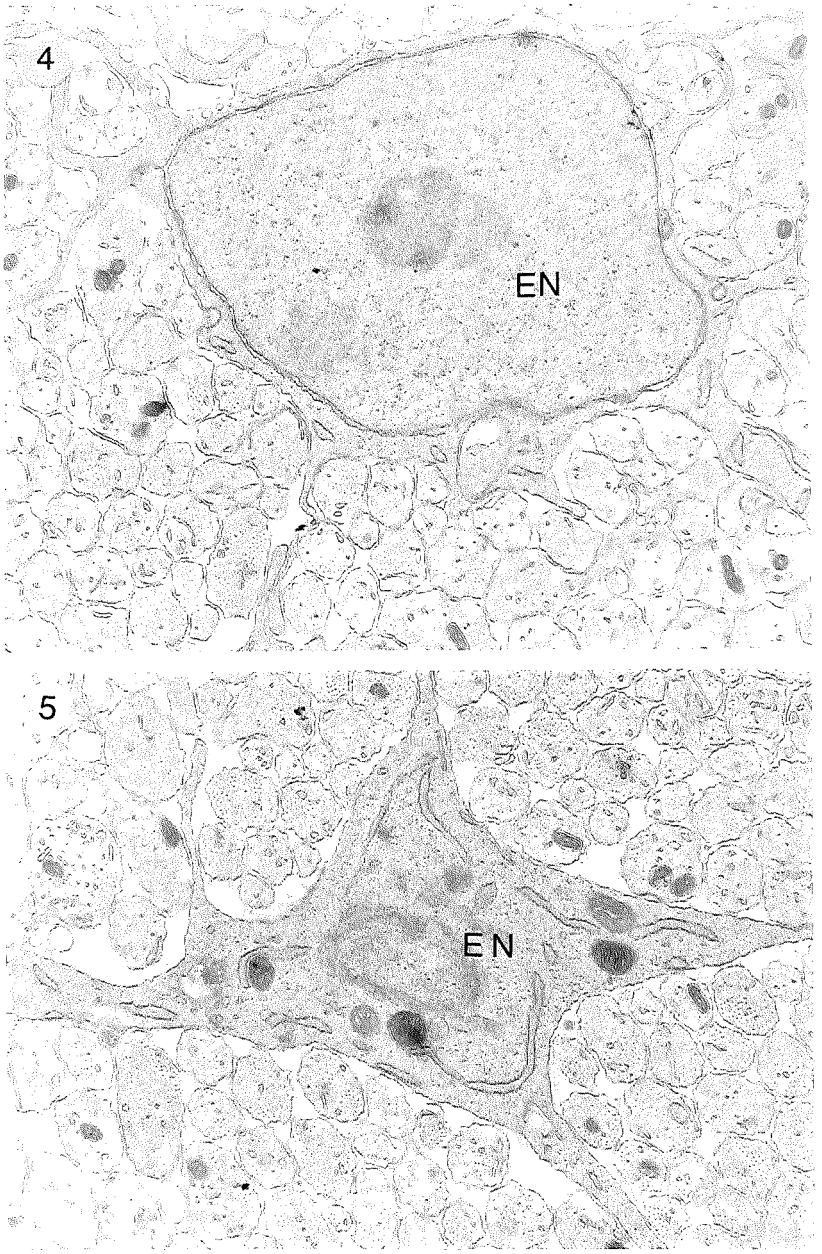
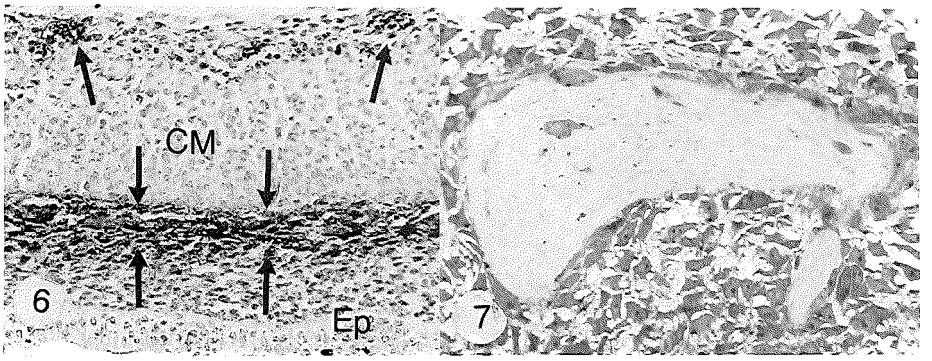


Fig. 4. Electronmicrograph of a normal myenteric plexus in proximal bowel showing several axons and an enteric neuron (EN).
Fig. 5. Electronmicrograph showing the cytoplasm of an enteric neuron (EN) in a normal myenteric plexus.



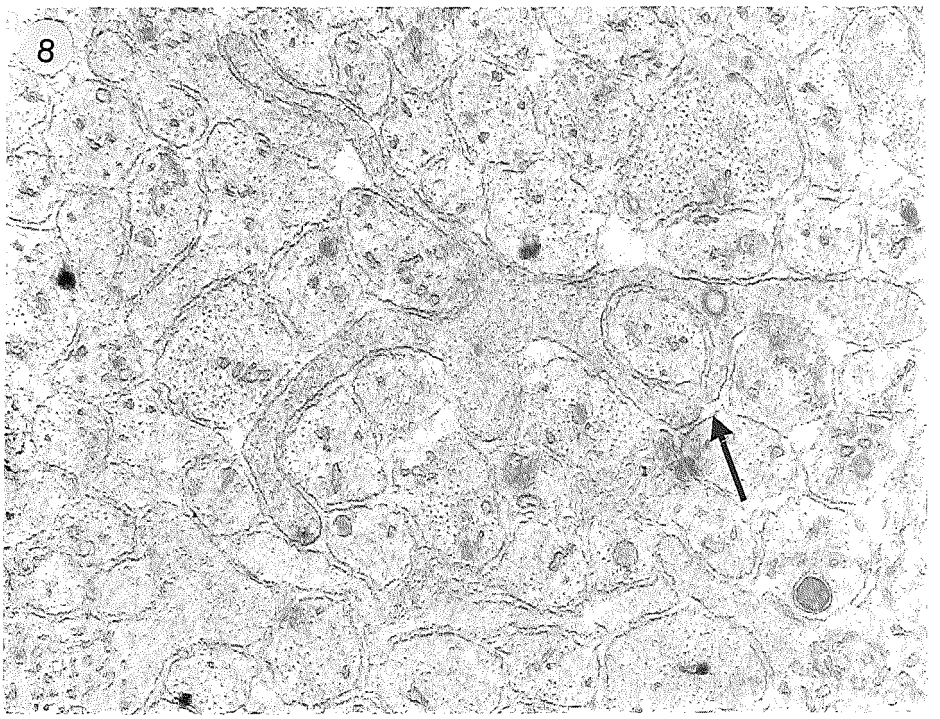


Fig. 6. Paraffin section of hindgut isolated at E12 from an embryo operated upon at E4. Note the small HNK-1 positive neuron-free, myenteric plexuses. Note also the diffuse layer of HNK-1 positive cells where the submucous plexus would have been (pattern A).
Fig. 7. Semithin section showing neuron-free myenteric plexus. Magnification 63x.
Fig. 8. Electronmicrograph of central of a neuron-free, myenteric plexus, showing processes of a supportive cell around axons (arrows). Magnification 37,800x.

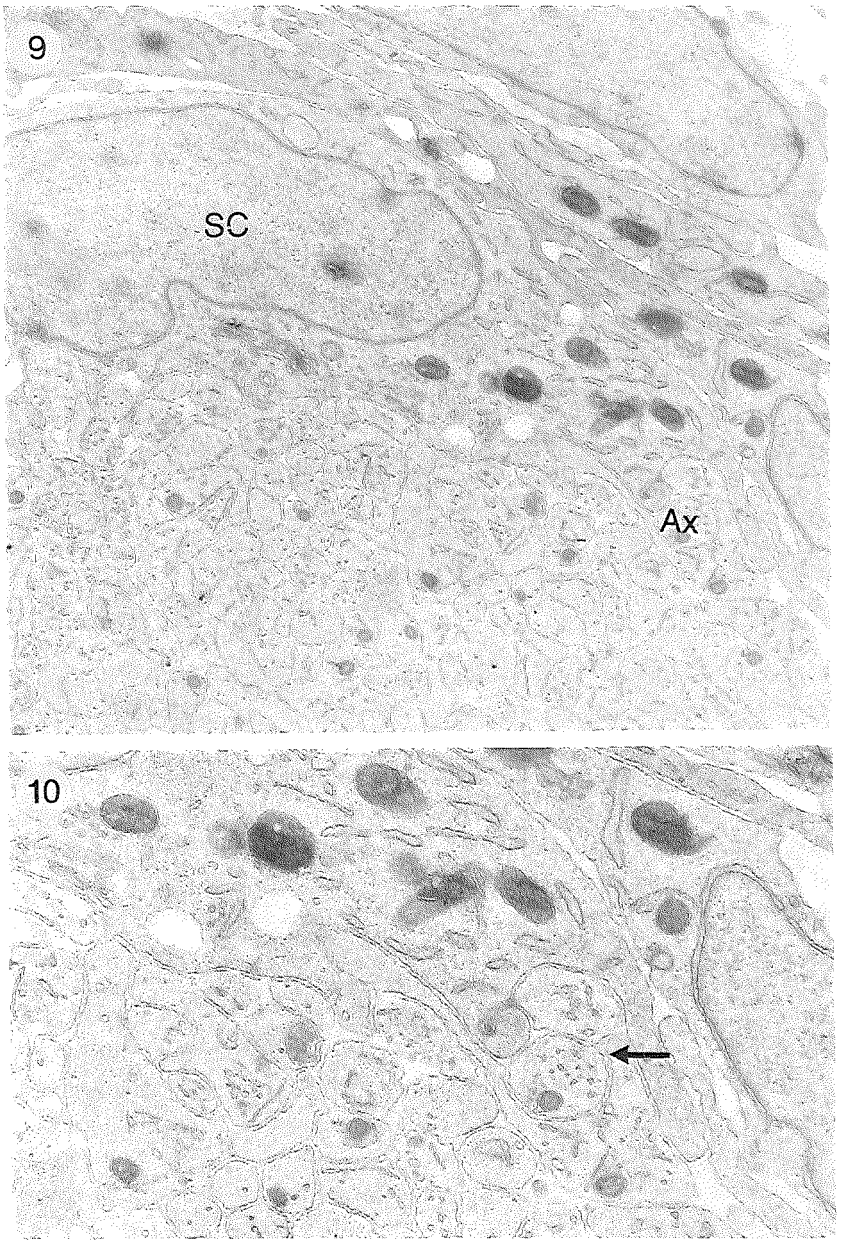


Fig. 9. Electronmicrograph of a neuron-free, myenteric plexus in the distal bowel showing a supportive cell (SC) at the periphery of the plexus and mulptiple axons. Magnification 16,800x. Fig. 10. Detail of Fig. 9 showing that the cytoplasm of the supportive cell surrounds several axons (arrow). Magnification 28,000x.

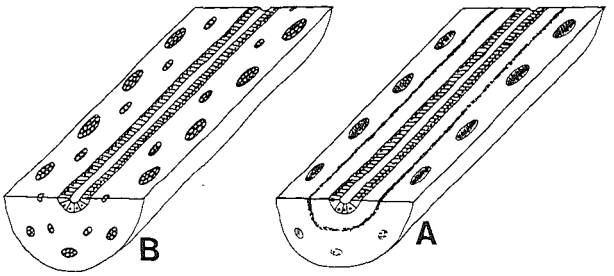


Figure 11. Distribution of HNK-1 immunoreactivity in hindgut of normal embryos (left) and in hindgut of embryos that underwent bowel transection at E4. HNK-1 immunoreactivity is located in the enteric ganglia in normal bowel (pattern B), but in a layer of cells in the submucosa and in neuron-free, myenteric plexus after bowel transection at E4 (pattern A).

DISCUSSION

To study the contribution of the sacral neural crest to enteric ganglia in the distal bowel, we prevented the craniocaudal migration of vagal neural crest cells trough the developing bowel. Embryonic bowel was transected distal to the omphalomesenteric duct at different stages of development. When transection took place in an aneuronal segment of the bowel (E4 and E5), HNK-1 positive neuron-free, myenteric plexuses developed in the hindgut (pattern A). The three HNK-1 staining patterns (A, B, A+B) found at E12 in different embryos operated upon at E5, might be due to a) variation in the site of transection or b) variation in the location of the vanguard of migrating neural crest cells. Normal ganglionic hindgut (pattern B) was observed when transection had taken place in already colonized bowel. Transection through the vanguard of migrating neural crest cells resulted in a mixed pattern, consisting of pattern B close to the transection and a much longer pattern A in the remaining part of the hindgut. Apparently, the number of neural crest cells present in the distal segment was not sufficient for complete ENS development in the hindgut. In view of the absence of enteric neurons in the hindgut after transection at E4 and E5 (pattern A), we conclude that sacral neural crest cells on their own, are not able to form enteric ganglia in the distal bowel.

A single, vagal origin of enteric neurons is in agreement with the results of Yntema and Hammond (1954), who ablated the vagal neural crest and subsequently obtained aganglionosis in the hindgut. The length of the aganglionic segment was determined by the extent of the neural crest ablation. A single vagal origin for enteric neurons was, likewise, demonstrated by Allan and Newgreen (1980). They reported that enteric neurons appeared in a craniocaudal direction in cultured explants of successive

segments of embryonic bowel, explanted at different stages of development. Using the same culture method and the more sensitive HNK-1 immunoperoxidase staining for the visualization of neural crest cells we have confirmed the craniocaudal direction of migrating neural crest cells.

Contradictory findings, suggestive for a dual vagal/sacral neural crest origin of enteric neurons in the hindgut have been reported by other investigators. In quail-chick chimeras, a small number of sacral quail neural crest cells was observed in the chick hindgut (Le Douarin & Teillet, 1973). We suggest the following explanations for the contradictory conclusions:

1) it may well be that the neuron-free, myenteric plexuses and the diffuse layer of HNK-1 immunoreactive cells in the submucosa (pattern A) resulted from colonization by sacral neural crest cells that did not acquire a neuronal phenotype.

2) it may well be that the presence of vagal neural crest cells in the hindgut is a prerequisite for its colonization by sacral neural crest cells.

3) the small number of quail neural crest cells observed in the hindgut of quail-chick chimeras might be inherent to the chimera technique. Quail neural crest cells are more invasive than chick neural crest cells, as is indicated by our finding that quail neural crest cells are present in chicken bowel after insertion of the quail neural primordium into the chick coelomic cavity (data not shown).

4) it may well be that in quail-chick chimeras whereby vagal quail neural primordium was grafted to the vagal segment of the neural tube in the chicken host, the chicken neural crest cells found in de hindgut derived from the sacral neural crest, but a vagal origin resulting from incomplete extirpation of the vagal neural primordium cannot be excluded.

Some (immuno)cytochemical and autoradiographical studies also suggest a dual vagal/sacral neural crest source for enteric neurons. Both caudocranial and craniocaudal expression sequences have been reported for acetylcholinesterase activty (Keller, 1976), uptake of radioactively labelled serotonin (Gershon et al, 1980), and for vasoactive intestinal peptide- (Saffrey, 1982), and neurofilament immunoreactivity (Payette et al, 1984). In contrast, single craniocaudal expression sequences have been reported for vaso intestinal peptide, substance P, Met-enkephalin, choline acetyl transferase (Fontaine-Perus et al, 1981; Saffrey et al, 1982; Smith et al, 1977). The expresion sequences of neurofilaments, neurotransmitter substances, and neuron specific enzymes reflect the differentiation of enteric neurons rather than the migration of neural crest cells. These sequences do not reveal the origin of the cells that express these differentiation characteristics.

Enteric ganglia developed normally in the hindgut (HNK-1 stainining pattern B) when innervated bowel was transected (E6 and E7). A normal ENS was also found in all sham-operated animals and in all bowel segments proximal to the site of transection. Despite the fact that neural crest cells continue to proliferate during migration (E4-E8) (Meijers et al, 1987), we observed no increase in the number of neural crest cells in the ganglia proximal to the site of transection.

There are few data about the development of the extrinsic component of the

ENS. The extrinsic adrenergic innervation of the bowel is characterized either chemically (Enemar et al, 1965) or through the transmitter uptake mechanism specific for adrenergic neurons from E12 (Gershon et al., 1980). Our results show that the absence of enteric neurons does not prevent the development of extrinsic nerve fibers in the bowel.

The immunohistological picture of the hindgut, obtained after the transection of embryonic bowel, resembles the one found in patients with aganglionosis. Therefore transection of the postumbilical bowel at E4 and E5 might provide a chicken model for aganglionosis.

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REFERENCES

Abo, T., and Balch, C.M. (1981). A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J. Immunol. 127, 1024-1029.

Allan, I.J., and Newgreen, D.F. (1980). The origin and differentiation of enteric neurons of the fowl embryo. Am. J. Anat. 157, 137-154.

Andrew, A. (1971). The origin of intramural ganglia. IV. The origin of enteric ganglia: a critical review and discussion of the present stage of the problem. J. Anat. 108, 169-184.

Ciment, G., and Weston, J.A. (1982). Early appearance in neural crest and crest-derived cells of an antigenic determinant present in avian neurons. Dev. Biol. 93, 355-367.

Ciment, G., and Weston, J.A. (1983) Enteric neurogenesis by neural crest-derived branchial arch mesenchymal cells. Nature (London) 305, 424-427.

Ciment, G., Ressler, A., Letourneau, P.C., and Weston, J.A. (1986). Identification of an interfilament associated protein, NAPA-73, binding to different filament types at different stages during neurogenesis. J. Cell Biol. 102, 246-251.

Duband, J.L., and Thiery, J.P. (1982). Appearance and distribution of fibronectin during chick embryo gastrulation and neurulation. Dev. Biol. 93, 308-323.

Enemar, A., Falck B., and R. Hankanson (1965). Observations on the appearance of norepinephrine in the sympathetic nervous system of the chick embryo. Dev. Biol. 11, 268-320.

Fontaine-Perus, J., Chanconie, M., Polak, J.M., and Le Douarin N.M. (1981). Origin and development of VIP and substance P containing neurons in the embryonic avian gut. Histochem. 71, 313-323.

Gershon, M.D., Epstein, M.L., and Hegstrand, L. (1980). Colonization of the chick gut by progenitors of enteric serotonergic neurons: distribution, differentiation, and maturation within the gut. Dev. Biol. 77, 41-51.

Hamburger, V., and Hamilton H.L. (1951). A series of normal stages in the development of the chick embryo. J. Morph. 88, 49-92.

Keller, H. (1976). The development of the intramural nerve plexus of the gastrointestinal tract. Anat. Embryol. 150, 1-6.

Klück, P., van Muijen, G.N.P., van der Kamp, A.W.M., Tibboel, D., van Hoorn, W.A., Warnaar, S.O., and Molenaar, J.C. (1984). Hirschsprung's disease studied with monoclonal antineurofilament antibodies on tissue sections. Lancet i, 652-654.

Le Douarin, N.M., and Teillet, M.A. (1973). The migration of neural crest cells to the wall of the digestive tract in avian embryo. J. Embryol. Exp. Morphol. 30, 31-48.

Le Douarin, N.M. (1982). *The neural crest.* Cambridge University Press, Cambridge.

Meijers, J.H.C., Tibboel, D., van der Kamp, A.W.M., van Haperen-Heuts, C.C.M., and Molenaar, J.C. (1987). Cell division in migratory and aggregated neural crest cells in the developing gut. An experimental approach of innervation-related motility disorders of the gut. J. Pediatr. Surg. 22, 243-245.

Nixon, H.H. (1982). Hirschsprung's disease in the newborn. In "*Hirschsprung's disease*" (A.M. Holschneider, ed.), pp. 103-113. Hippokrates Verlag, Stuttgart.

Payette, R.F., Bennet, G.S., and Gershon, M.D. (1984). Neurofilament expression in vagal neural crest derived precursors of enteric neurons. Dev. Biol. 105, 273-287.

Saffrey, M.J., Polak, J.M., and Burnstock, G, (1982). Distribution of vasoactive intestinal peptide-, substance P-, enkephalin- and neurotensin-like immunoreactive nerves in the chicken gut during development. Neuroscience 7, 279-293.

Smith, J., Cochard, P., and Le Douarin N.M. (1977). Development of choline acetyl transferase and cholinesterase activities in enteric ganglia derived from presumptive adrenergic and cholinergic levels of the neural crest. Cell Diff. 6, 199-216.

Tucker, G.C., Aoyama, H., Lipinski, M., Tursz, T., and Thiery, J.P. (1984). Identical reactivity of monoclonal antibodies HNK-1 and NC1: conservation in vertebrates on cell derived from the neural primordium and on some leukocytes. Cell. Diff. 14, 223-230.

Tucker, G.C., Ciment, G., and Thiery, J.P. (1986). Pathways of avian neural crest cell migration in the developing gut. Dev. Biol. 116, 439-450.

Yntema, C.L., and Hammond, W.S. (1954). The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J. Comp. Neurol. 101, 515-541.

CHAPTER 7.6

HNK-1 IMMUNOREACTIVE MESENCHYMAL CELLS ARE INVOLVED IN NEURAL CREST CELL HOMING IN THE BOWEL

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Submitted

Summary. During embryonic development, cells of the neural crest migrate throughout the embryo. After migration, these cells differentiate into a variety of phenotypes, depending on the microenvironment of their target organs. In the present study we provide evidence that the local embryonic microenvironment also determines the position of the neural crest cells in the target organs. The integration (or 'homing') of neural crest cells in a target organ was studied in the bowel of chicken embryos. Aneuronal bowel was isolated and cultured on the chorioallantoic membrane, either on its own, or in combination with the neural primordium. When aneuronal bowel was cultured on its own, HNK-1 immunocytochemistry revealed a layer of HNK-1 immunoreactive mesenchymal cells at the site where the submucous plexus would have been. Neural crest cell colonization, which was induced by coculturing with the neural primordium, changed the pattern of HNK-1 immunoreactivity completely; HNK-1 immunoreactive neural crest cells and enteric neurons were observed, whereas the mesenchymal cells had lost the HNK-1 epitope. We surmise that HNK-1 immunoreactive mesenchymal cells constitute the target for migrating neural crest cells, and that HNK-1 antigen(s) present on the neural crest cells interact with the HNK-1 antigen(s) of the subpopulation of mesenchymal cells.

INTRODUCTION

The neural crest is a transient embryonic structure in vertebrate embryos that arises from the dorsal ridges of the neural epithelium. The cells of the neural crest migrate to diverse sites in the embryo and give rise to the neurons and supportive cells of the peripheral and enteric nervous systems (Le Douarin, 1982; Le Douarin, 1986), to melanocytes, and to different tissues in the craniofacial and the cardiac region (Noden, 1984; Kirby, 1987). The initiation of neural crest cell migration coincides with a reduction of the expression of cell adhesion molecules, such as N-CAM and N-Cadherin (Thiery et al, 1982; Duband et al, 1987). Neural crest cells migrate along defined pathways lined with specific substrate adhesion molecules (Duband and Thiery, 1982).

The cessation of neural crest cell migration is also thought to be mediated by modulation of the expression of cell adhesion molecules, but the data in support of this hypothesis only concern the aggregation of neural crest cells into sympathetic ganglia (Duband et al, 1985). The mechanisms of the homing of neural crest cells in other regions of the embryo are largely unknown.

In the chicken embryo, precursor cells of the enteric nervous system originate at the neural crest opposite somites 1 to 7, the so-called vagal neural crest (Yntema and Hammond, 1954; Le Douarin and Teillet, 1973; Allan and Newgreen, 1980). There is no consensus about the contribution of sacral neural crest cells to enteric plexus formation. We found earlier that hindgut did not contain enteric neurons after bowel transection at embryonic day 4 (E4) (manuscript submitted). To determine whether neural crest cells were present in this hindgut we used monoclonal antibody HNK-1, which recognizes a carbohydrate moiety present on migrating neural crest cells (Vincent et al, 1983; Vincent and Thiery, 1984; Tucker et al, 1984; Abo and Balch, 1981). Instead of neural crest cells, HNK-1 immunoperoxidase staining of the distal hindgut at E12 revealed cell-free, ganglionic structures at the site of the myenteric plexus, and a layer of cells in the submucosa. Neither the layer of HNK-1 immunoreactive cells, nor the cell-free, ganglionic structures were found in normally innervated bowel. In the present study we investigated the role of the layer of HNK-1 immunoreactive cells.

MATERIALS AND METHODS

White leghorn chicken (*Gallus Domesticus*) embryos were staged following the table of Hamburger and Hamilton (1951) or according to the number of paired somites or the number of incubational days (E4-E21). Aneuronal E4 bowel was grafted alone, or in combination with neural primordium, onto E7 chorioallantoic membrane and cultured for one week. Neural primordium opposite somite 1-7 was dissected from 12 somite embryos. Grafts were fixed in 4% formaldehyde in phosphate buffered saline, dehydrated, embedded in paraffin and sectioned at 5-7 μm. Cryostatsections (10 μm) were fixated in acetone and immunostained by an indirect immunoperoxidase method. HNK-1 immunoperoxidase staining was performed using undiluted supernatant (American Type Culture Collection No TIB 200). Two monoclonal antibodies raised against purified human neurofilament proteins, but cross reacting to chicken neurofilaments, were used to identify enteric neurons (Klück et al, 1984). The supernatant containing monoclonal antibody E/C8 was not diluted (Ciment and Weston, 1982). Monoclonal antibody 15G8, directed against chicken N-CAM was used in a dilution of 1:20. A polyclonal rabbit antiserum raised against chicken N-CAM was used in a dilution of 1:80. Rabbit-anti-mouse peroxidase conjugated immunoglobulins (Dako, Denmark) and swine-anti-rabbit peroxidase conjugated immunoglobulins (Dako, Denmark) were used as second step antibodies (diluted 1:100 and 1:80 respectively).

RESULTS AND DISCUSSION

Successive segments of distal, postcecal bowel were dissected from E4, E5 and E6 embryos and grafted onto the chorioallantoic membrane of E7 embryos. After seven days' culture, HNK-1 visualized the layer of mesenchymal cells in the submucosa inside the circular smooth muscle layer, but did not visualize any structure at the site of the myenteric plexus (Fig. 1). This pattern of immunoreactivity will be referred to as HNK-1 mode I, in contrast to another pattern, to be referred to as HNK-1 mode II, found in grafts of prececal bowel dissected at E5 or E6, and in whole bowel dissected at later stages. Rather than a layer of mesenchymal cells, HNK-1 mode II visualized neurons and glial cells in the myenteric and submucous plexus (Figs. 2 and 3). The cells visualized in HNK-1 mode I did not express neurofilaments, or the neurofilament-associated glycoprotein NAPA-73, which is recognized by the monoclonal antibody E/C8 (Fig. 4a). The HNK-1 immunoreactive cells are present in the bowel before neural crest cell colonization. Therefore, these HNK-1 immunoreactive cells are probably mesenchymal cells and not neural crest cells or enteric neurons presenting at an unusual location. In contrast, the cells visualized in HNK-1 mode II did show neurofilament and E/C8 immunoreactivity (Fig. 4b) indicating that these HNK-1 immunoreactive cells are neural crest cells or enteric neurons.

To determine whether neural crest cells trigger the shift in pattern of HNK-1 immunoreactivity, we induced neural crest cell colonization of aneuronal E4 bowel by means of coculture with vagal neural primordium of E2 embryos. HNK-1 immunoperoxidase staining revealed HNK-1 mode II in these cocultures (Fig. 5). Immunostaining of cryosections of these cocultures showed neurofilament and NAPA-73 immunoreactivity in enteric ganglia. These findings indicate that neural crest cell colonization of aneuronal bowel mediates the shift in pattern of HNK-1 immunoreactivity. The results of immunoperoxidase staining of cultivated embryonic hindgut dissected at E4 and E5 are summarized in Table 1.

HNK-1 immunoperoxidase staining of cryosections of normal bowel revealed a combination of mode I and mode II in post-umbilical bowel from E6 to E15. After E15, HNK-1 mode I disappeared. We conclude that, during normal development, mesenchymal cells in the hindgut transiently express an HNK-1 antigen. The duration of expression of this HNK-1 antigen is extended when neural crest cell colonization is prevented. In this case, the initial diffuse distribution of the HNK-1 antigen within the mesenchyme is concentrated in a layer of HNK-1 immunoreactive mesenchymal cells.

We studied the tissue distribution of N-CAM in relation to the two modes of HNK-1 immunoreactivity. Table 1 shows that HNK-1 immunoreactive cells in cultured aneuronal bowel do not express N-CAM. In cultured aneuronal bowel, N-CAM immunoreactivity was only found in the lamina propria, but not in the submucosa adjacent to the smooth muscle layer (Fig. 6a). In cocultures of aneuronal E4 bowel and the neural primordium, or single cultures of neuronal bowel we found N-CAM immunoreactivity at the site of the enteric ganglia and on the HNK-1 immunoreactive cells in the submucosa (Fig. 6b).

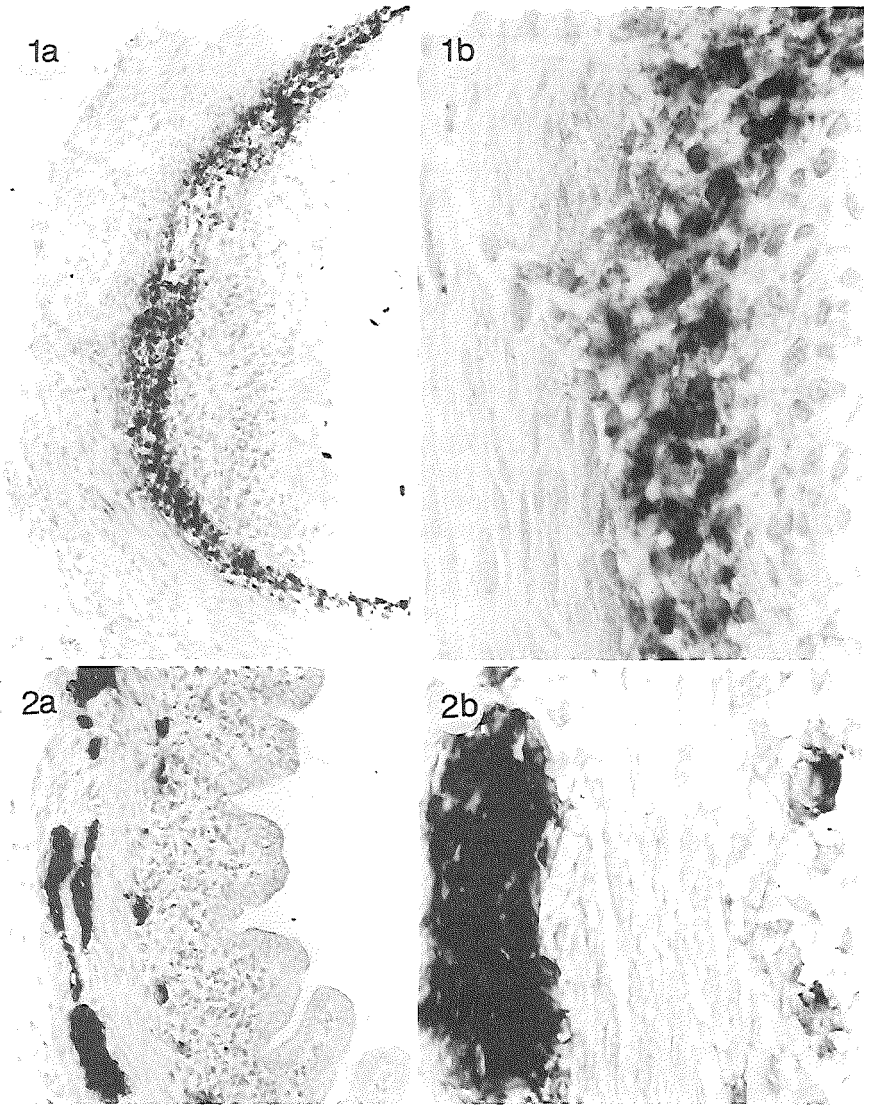


Fig. 1 (a) A layer of HNK-1 immunoreactive mesenchymal cells inside circular smooth muscle layer (HNK-1 mode I) in a paraffin section of cultured postcecal E5 bowel. x25. (b) Detail of (a) showing HNK-1 immunoreactivity both at cell membrane and in extracellular matrix. x63.

Fig. 2 (a) HNK-1 immunoreactive neural crest cells or enteric neurons in enteric ganglia (HNK-1 mode II) in paraffin section of cultured prececal E5 bowel (proximal to bowel segment of Fig. 1). Note the absence of the layer of HNK-1 immunoreactive mesenchymal cells. x25. (b) Detail of (a) showing HNK-1 immunoreactivity at the cell membranes of enteric neurons. x63.

TABLE 1

IMMUNOPEROXIDASE STAINING OF EMBRYONIC HINDGUT CULTURED ALONE OR IN COMBINATION WITH NEURAL PRIMORDIUM ON THE CHORIOALLANTOIC MEMBRANE

	HNK-1 mode	NF	E/C8	N-CAM
E4	I	-	-	-[a]
E4+NA	II	+	+	+
E5	II	+	+	+

I = HNK-1 mode I: a diffuse band of HNK-1 immunoperoxidase staining of mesenchymal cells in the submucosa inside the circular smooth muscle layer. II = HNK-1 mode II: HNK-1 immunoperoxidase staining of neural crest cells in enteric ganglia. [a] *N-CAM immunoreactivity was only found in the lamina propria but not at the site of HNK-1 mode I.*

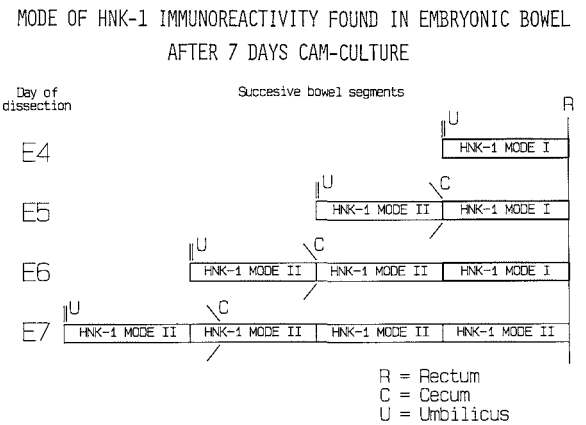
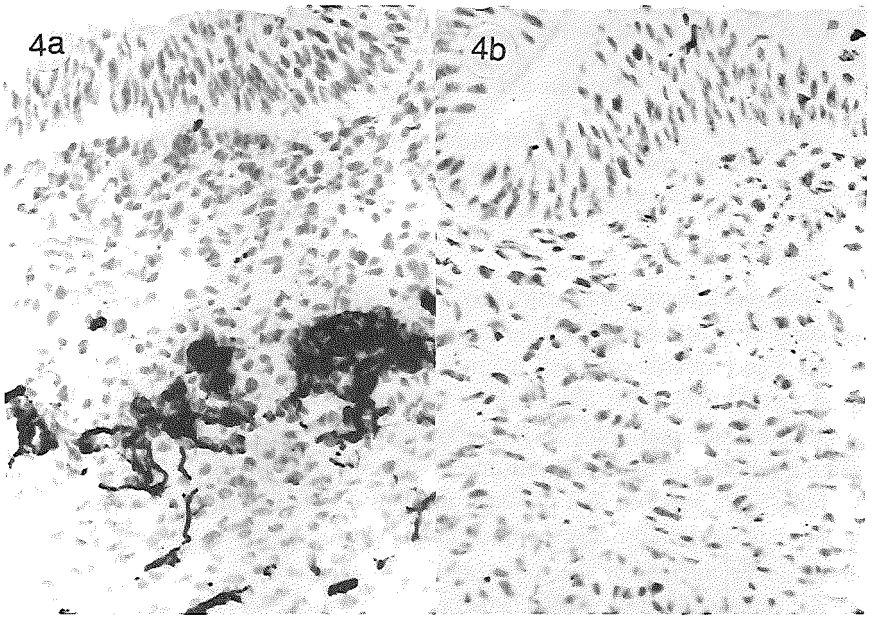
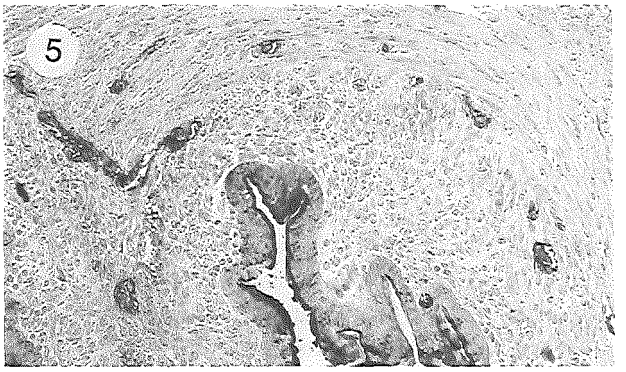


Fig. 3 Schematic presentation of two patterns of HNK-1 immunoreactivity in bowel segments isolated at diverse stages and grown on E7 CAM. The entire postumbilical E4 bowel was grafted, whereas E5, E6 and E7 bowel was divided into 2, 3 and 4 successive segments, respectively. R = rectum; C = cecum; U = umbilicus.


4a
4b


5

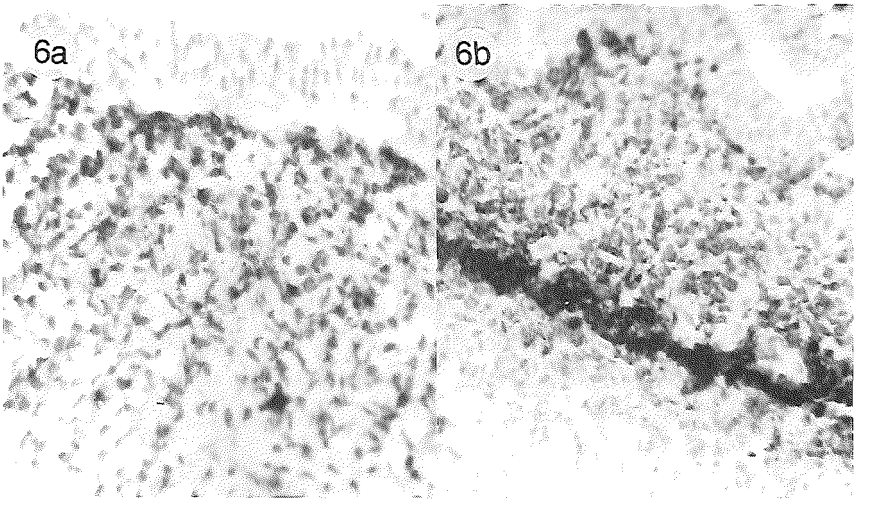

6a
6b

Fig. 4 (a) E/C8 immunoreactive enteric neurons in a cryosection of cocultured aneuronal E4 bowel and vagal neural primordium. x25. (b) E/C8 immunoreactive cells are notably absent in a cryosection of cultured aneuronal E4 bowel. x25.
Fig. 5 HNK-1 mode II immunoreactivity in paraffin section of cocultured aneuronal E4 bowel and E2 NA. x25.
Fig. 6 (a) N-CAM immunoperoxidase staining of cultured aneuronal E4 bowel. Compare with Fig. 1a and 4b. (b) N-CAM immunoperoxidase staining of cocultured E4 bowel and vagal neural primordium. x25.

Substrate adhesion molecules (e.g. fibronectin, tenascin) are present in the bowel at the stage of neural crest cell migration, but their distribution does not coincide with the areas where neural crest cell migration is supposed to occur (Tucker et al, 1986; Duband and Thiery, 1987; Mackie et al, 1988). The widespread distribution of these substrate adhesion molecules in the embryonic mesenchyme cannot account for the guided migration of neural crest cells to the sites of the myenteric and submucous plexus in the bowel. We suppose that the layer of HNK-1 immunoreactive mesenchymal cells in the submucosa does provide the directional cues for migrating neural crest cells. This premise is substantiated by the following findings:
1) HNK-1 immunoreactive mesenchymal cells are transiently present in the submucosa during normal ontogeny; 2) HNK-1 immunoreactive mesenchymal cells are present in the hindgut before the arrival of neural crest cells; 3) The arrival of neural crest cells generates the disappearance of HNK-1 immunoreactive mesenchymal cells; 4) HNK-1 immunoreactive mesenchymal cells persist when neural crest cell colonization is prevented.

The HNK-1 epitope itself is involved as a ligand in cell-cell and cell-substrate interactions (Keilhauer et al, 1985; Riopelle et al, 1986; Künemund et al, 1988). The HNK-1 carbohydrate moiety is present on several cell adhesion molecules such as N-CAM, Ng-CAM, and MAG (Kruse et al, 1984; Schachner et al, 1985; Edelman, 1985; Kruse et al, 1985; Poltorak et al, 1987), substrate adhesion molecules such as integrin (Pesheva et al, 1987) and extracellular matrix molecules such as cytotactin and cytotactin binding proteoglycan (Hoffman and Edelman, 1987; Tan et al, 1987). Furthermore, soluble diffusible HNK-1 bearing glycoproteins play a part in the cessation of neuronal migration, and in the promotion of neurite outgrowth (McGarry et al, 1985). Injection of HNK-1 into the migratory pathways disrupts cranial neural crest cell migration (Bronner-Fraser, 1987). Interaction between two different HNK-1 bearing glycoproteins, e.g. cytotactin and cytotactin binding proteoglycan (CBP), has been reported (Hoffman and Edelman, 1987; Tan et al, 1987). We suppose that HNK-1 immunoreactive migrating neural crest cells adhere to the HNK-1 immunoreactive mesenchymal cells, initiating neural crest cell aggregation at the site of the submucous plexus. The HNK-1 antigen(s) present on migrating neural crest cells remain to be identified, although the cell surface receptor for fibronectin and laminin, integrin, is a likely candidate (Bronner-Fraser, 1986; Pesheva et al, 1987). The HNK-1 antigen that is transiently expressed by the mesenchymal target cells is subject of present investigations.

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REFERENCES

Abo, T., and Balch, C.M. (1981). A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1) J. Immunol. 127, 1024-1029.

Allan, I.J., and Newgreen, D.F. (1980). The origin and differentiation of enteric neurons of the intestine of the fowl embryo. Am. J. Anat. 157, 137-154.

Bronner-Fraser, M. (1986). An antibody to a receptor for fibronectin and laminin perturbs cranial cranial neural crest development in vivo. Dev. Biol. 117, 528-536.

Bronner-Fraser, M. (1987). Perturbations of cranial neural crest migration by the HNK-1 antibody. Dev. Biol. 123, 321-331.

Ciment, G., and Weston, J.A. (1982). Early appearance in neural crest and crest-derived cells of an antigenic determinant present in avian neurons. Dev. Biol. 93, 355-367.

Duband, J-L., and Thiery, J.P. (1982). Distribution of fibronectin in the early phase of avian cephalic neural crest migration. Dev. Biol. 93, 308-323.

Duband, J-L., and Thiery, J.P. (1987). Distribution of laminin and collagens during avian neural crest development. Development 101, 461-478.

Duband, J-L. et al. (1985). How do the migratory and adhesive properties of the neural crest govern ganglia formation in the avian peripheral nervous system. J. Cell. Biochem. 27, 189-203.

Duband J-L. et al. (1987). Distribution of laminin and collagens during avian neural crest development. J. Cell Biol. 104, 1361-1374.

Edelman G.M. (1985). Cell adhesion and the molecular processes of morphogenesis. Annu. Rev. Biochem. 54, 135-169.

Hamburger, V., and Hamilton, H.L. (1951). A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92.

Hoffman, S., and Edelman, G.M. (1987). A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. Proc. Natl. Acad. Sci. U.S.A. 84, 2523-2527.

Keilhauer, G. et al. (1985). Differential inhibition of neurone-neurone, neurone-astrocyt and astrocyt-astrocyt adhesion by L1, L2 and N-CAM antibodies.Nature (London) 316, 728-730.

Kirby, M.L. (1987). Cardiac morphogenesis - Recent research advances. Pediatr. Res. 21, 219-224.

Klück, P. et al. (1984). Hirschsprung's disease diagnosed with monoclonal antineurofilament antibodies on tissue sections. Lancet i, 652-653.

Kruse, J. et al. (1984). Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. Nature (London) 311, 153-155.

Kruse, J. et al. (1985). The J1 glycoprotein - a novel nervous system cell adhesion molecule of the L2/HNK-1 family. Nature (London) 316, 146-148.

Künemund, V.J. et al. (1988). The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. J. Cell Biol. 106, 213-223.

Le Douarin, N.M. (1982). "*The Neural Crest.*" Cambridge University Press, Cambridge, Great Britain.

Le Douarin, N.M. (1986). Cell line segregation during peripheral nervous system ontogeny. Science 231, 1515-1522.

Le Douarin, N.M., and Teillet, M.A. (1973). J. Embryol. Exp. Morphol. 30, 31-48.

Mackie, E.J. et al. (1988). The distribution of tenascin coincides with pathways of neural crest cell migration. Development 102, 237-250.

McGarry, R.C. et al. (1985). Accelerated regenerative formation by a neuronal cell surface epitope reactive with the monoclonal antibody, Leu 7. Neurosci. Lett. 56, 95-100.

Noden, D.M. (1984). Craniofacial development: new views on old problems. Anat. Rec. 208, 1-13.

Pesheva, P. et al. (1987). Integrin, the cell surface receptor for fibronectin and laminin, expresses the L2/HNK-1 and L3 carbohydrate structures shared by adhesion molecules. Neurosci. Lett. 83, 303-306.

Poltorak, M. et al. (1987). The myelin-associated glycoprotein (MAG), a member of the L2/HNK-1 family of neural cell adhesion molecules, is involved in neuron-oligodendrocyte and oligodendro-oligodendrocyte adhesion. J. Cell Biol. 105, 1893-1899.

Riopelle, R.J. et al. (1986). Adhesion properties of a neuronal epitope recognized by the monoclonal antibody HNK-1. Brain Research 367, 20-25.

Schachner, M. et al. (1985). Functional and structural aspects of the cell surface in mammalian nervous development. In "*The Cell in Contact*" (G.M. Edelman and J.P. Thiery, eds.), pp. 257-275. Wiley & Sons, New York.

Tan, S-S. et al. (1987). Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correleated with neural crest cell distribution. Proc. Natl. Acad. Sci. U.S.A. 84, 7977-7981.

Thiery, J.P. et al. (1982). Cell adhesion molecules in early chicken embryogenesis.Proc. Natl. Acad. Sci. U.S.A. 79, 6737-6741.

Tucker, G.C. et al. (1984). Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Differ. 14, 223-230.

Tucker, G.C. et al. (1986). Pathways of avian neural crest cell migration in the developing gut. Dev. Biol. 116, 439-450.

Vincent, M., and Thiery, J.P. (1984). A cell surface marker for neural crest and placodal cells: further evolution in peripheral and central nervous system. Dev. Biol. 103, 468-481.

Vincent, M. et al. (1983). A cell surface determinant expressed early on migrating avian neural crest cells.Dev. Brain Res. 9, 235-238.

Yntema, C.L., and Hammond W.S. (1954). The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J. Comp. Neurol. 101, 515-541.

CHAPER 7.7

A CHICKEN MODEL FOR INTESTINAL AGANGLIONOSIS

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ABSTRACT. We investigated the ability of neural crest cells to colonize hindgut that had remained aneuronal due to bowel transection in ovo at an early stage. The fact that the bowel remained aneuronal proved that sacral neural crest cells do not give rise to enteric neurons in the hindgut. In the hindgut we did find cell-free ganglionic structures at the site of the myenteric plexus and mesenchyme in the submucosa. We cocultured this particular type of aneuronal bowel together with the vagal neural primordium of either quail or chicken embryos on the chorioallantoic membrane. After one week coculture, we found that neural crest cell colonization of the hindgut had taken place. Quail neural crest cells had given rise to submucous plexuses and a limited number of small myenteric plexuses. Chicken neural crest cells had only colonized the submucous region. These findings indicate that the cell-free, ganglionic structures hamper neural crest cell colonization in the myenteric region. Our findings also point at differences between quail and chicken neural crest cells with regard to their colonizing properties.

INTRODUCTION

In Hirschsprung's disease, or intestinal aganglionosis, there are no enteric neurons in the hindgut. This knowledge was the basis for the development of animal models to study the etiology, pathogenesis, and pathophysiology of this congenital anomaly of the enteric nervous system (ENS). Spontaneous aganglionosis was observed in mice (1,2), rats (3) and several methods were developed to induce aganglionosis in various animals (4,5).

Previous reports emphasized the suitability of the chicken embryo as a laboratory animal for the study of congenital anomalies of the digestive tract (6-8). Chicken embryos are easily accessible, while ENS development in the chicken is well documented (9). Unfortunately, there are no chicken strains with spontaneous aganglionosis. Investigating the origin of enteric ganglia, Yntema and Hammond induced aganglionosis in chicken hindgut by ablating the vagal neural crest. They found that the length of the aganglionic bowel segment was related to the size of the lesion to the neural crest (10).

The origin of enteric neurons has also been investigated in quail-chick chimera experiments. When quail neural primordium was grafted to either the vagal or the sacral region of the chicken embryo, quail enteric neurons were found in the bowel, although the number of enteric neurons deriving from the sacral neural crest was considerably smaller than that of neurons deriving from the vagal neural crest. These results indicated that enteric neurons might derive from both vagal *and* sacral neural crest (11). A dual origin of enteric neurons was negated by the findings of Allan and Newgreen (12). They isolated bowel segments from embryos at various stages of development and cultured these segments on the chorioallantoic membrane. Enteric neurons appeared in a craniocaudal sequence indicating a vagal source. In a previous experiment, we transected the bowel in ovo at an early stage of development, before the passage of neural crest cells had occurred, thus preventing craniocaudal migration of vagal neural crest cells. The fact that the hindgut remained aganglionic showed that sacral neural crest cells did not give rise to enteric neurons (submitted for publication). Immunocytochemical investigation of the aganglionic hindgut, using the monoclonal antibody HNK-1, which recognizes a sulphated carbohydrate moiety that is present on several cell adhesion molecules (13), revealed cell-free, ganglionic structures at the site of the myenteric plexus and a band of HNK-1 positive mesenchymal cells immediately inside the circular smooth muscle layer.

The present study was undertaken to determine whether neural crest cells would colonize aneuronal bowel that contained the HNK-1 positive cell-free, ganglionic structures and the submucous layer of HNK-1 positive mesenchymal cells. We therefore cocultured the neural primordium and aneuronal bowel segments that were obtained after bowel transection and checked for neural crest cell colonization.

MATERIALS AND METHODS

Embryos from the white leghorn (*Gallus Domesticus*) and the Japanese quail (*Coturnix coturnix Japonica*) were used as laboratory animals. Embryonic development was staged according to the number of paired somites in young embryos, or the number of incubational days (E..) for older embryos. The eggs were incubated at 38°C in a forced draft incubator at a relative humidity of 80%.

The postumbilical bowel was transected at E4, involving 15 embryos (Fig. 1). Approximately four square cm of both shell and shell membrane was removed and the embryo repositioned using Watchmaker's forceps to enable the introduction of microscissors into the umbilical stalk. The bowel was then transected close to the omphalomesenteric duct. After transection, the eggs were sealed with Scotch tape and put back in the incubator. To investigate the effect of manipulation of the umbilical stalk on ENS formation, a sham operation was performed in 26 control embryos. The embryos were sacrificed at E12 and the hindguts were removed. Postcecal segments were grafted onto the chorioallantoic membrane of other chick embryos (E7) together

with the neural anlage of either quail or chicken E2 embryos as described previously (14). The remainder of the dissected hindgut was investigated using the HNK-1 antibody to ascertain the presence of cell-free, ganglionic structures at the myenteric site and mesenchymal cells in the submucosa.

To prepare paraffin sections, grafts were fixed in 4% formaldehyde in phosphate-buffered saline, dehydrated, embedded in paraffin and cut at 5-7 μm. Quail neural crest cells in chick host gut were identified using Feulgen's DNA staining technique (15).

We used HNK-1 immunocytochemistry to visualize avian neural crest cells (16). The HNK-1 hybridoma cell line was purchased from the American Tissue Culture Collection (17). HNK-1 immunoperoxidase staining was performed using undiluted supernatant. Rabbit-antimouse peroxidase-conjugated immunoglobulins (Dako, Denmark) were used in a dilution of 1:100. In order to reduce background staining, 2% chick serum was added to the conjugate. Peroxidase was visualized by 0.1% 3,3'diaminobenzidine.4HCl (Serva) and 0.02% hydrogenperoxide. All rinsing and diluting was done in phosphate-buffered saline (pH 7.4) with 0.1% Tween 20.

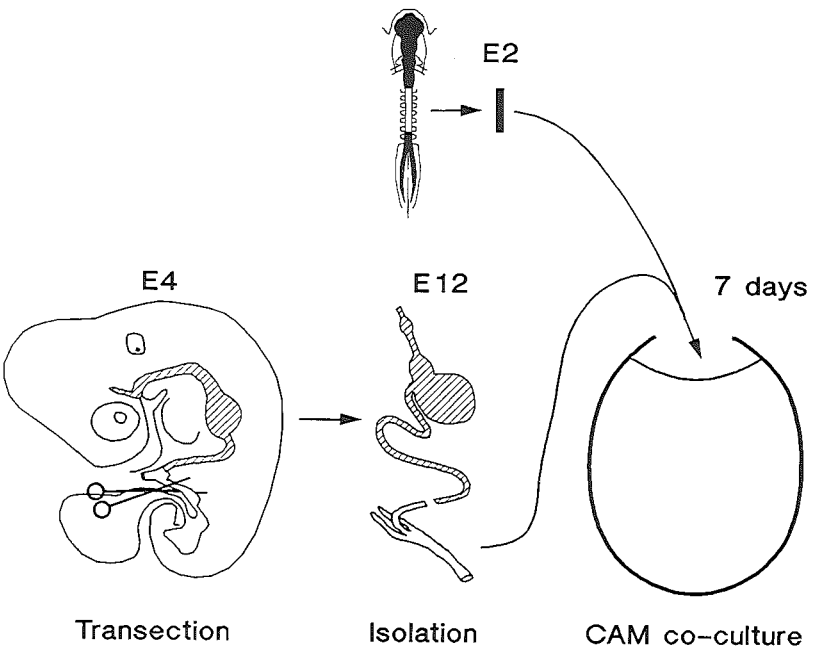


Fig. 1. Diagram showing the transection and coculture procedures.

RESULTS

Gross examination of the sham-operated embryos at E12 revealed no abnormalities of the bowel. HNK-1 immunocytochemistry revealed a normal ENS. In embryos that underwent bowel transection at E4, gross examination at E11 showed complete separation of the bowel immediately distal to the ceca. Histological investigation of both proximal and distal segments revealed a normal differentiation of the layers of the bowel wall. HNK-1 staining consistently revealed the cell-free, ganglionic structures at the site where the myenteric plexus would normally be and the layer of HNK-1 immunoreactive cells at the site of the submucous plexus (Fig. 2). The segment proximal to the transection showed normal innervation. There were no signs of an increase in the number of enteric neurons.

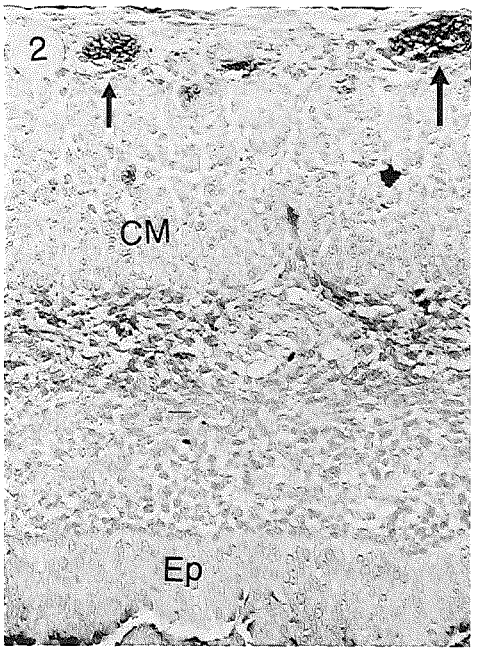
To determine whether aneuronal bowel that was obtained after bowel transection could be colonized by neural crest cells, the distal bowel was removed from the embryos at E11 and cocultured with the neural primordium on the chorioallantoic membrane. Table 1 summarizes the results of the coculture experiments. During the coculture of chicken neural primordium and chicken hindgut, three out of seven bowels were colonized by neural crest cells. Of these three two contained a normal submucous plexus and a rudimentary myenteric plexus. In one, also cell-free, ganglionic structures were observed. The remaining four did not contain neural crest cells at the site of the myenteric and submucous plexuses, nor cell-free, ganglionic structures and the layer of HNK-1 immunoreactive cells. In two of the cocultures the neural primordium was not present in the sections, which could explain the absence of neural crest cells in the enteric ganglia.

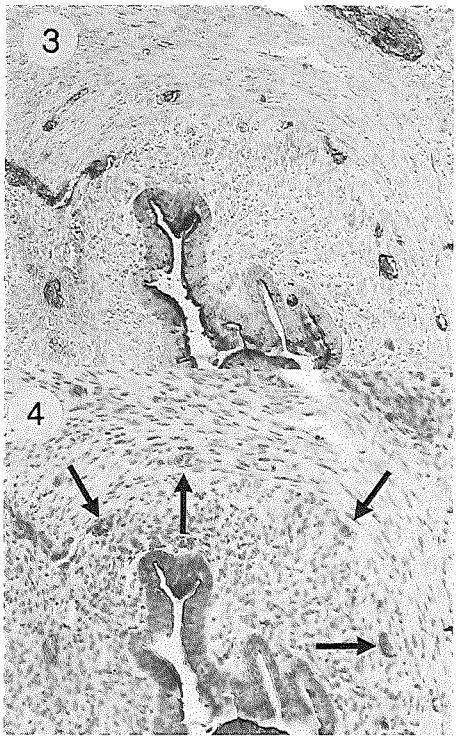
TABLE 1

NEURAL CREST CELL COLONIZATION OF EMBRYONIC BOWEL OF 11 DEVELOPMENTAL DAYS

NEURAL PRIMORDIUM	TYPE OF (ANEURONAL) BOWEL	COLONIZATION	
		M	S
Quail	Transection at E4	4/8*	8/8
Quail	Explantation at E4 + CAM	7/8	7/8
Quail	Explantation at E11	0/5	0/5
Chick	Transection at E4	0/7	3/7
Chick	Explantation at E4 + CAM	10/10	10/10

* *Few quail neural crest cells in myenteric plexus; CAM = Cultured on the chorioallantoic membrane for 7 days. M = Myenteric plexus; S = Submucous plexus.*





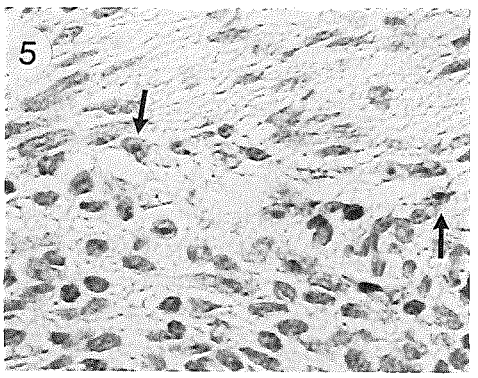


Fig. 2. HNK-1 immunostained paraffin section of the bowel of an E11 embryo operated in ovo at E4. Note the HNK-1 positive cell-free, ganglionic structures at the site of the myenteric plexus.
Fig. 3. HNK-1 immunoreactivity in a section of cocultured chicken bowel and quail neural primordium. 25x.
Fig. 4. Paraffin section stained according to Feulgen's technique visualizing DNA. Note the presence of quail cells (arrow) at the site of the submucous plexus. 25x.
Fig. 5. Detail of figure 4, showing quail cells (arrow) in a submucous plexus.

To facilitate the visualization of enteric neurons in the myenteric plexus, which is difficult in chicken-chicken cocultures, we cocultured aneuronal chicken bowel with the neural primordium of quail embryos. After one week coculture, quail neural crest cells were always present in submucous ganglia (6/6) and in only half of the cases in the myenteric ganglia (3/6) (Figs 3-5). The submucous ganglia were considerably larger than the rudimentary myenteric ganglia. We did not observe quail neural crest cells in enteric ganglia after coculture of quail neural primordium and neuronal bowel of E11 control embryos (6/6). We never found the layer of HNK-1 positive cells or cell-free ganglionic structures in such sections.

DISCUSSION

Transection of embryonic bowel at an early developmental stage resulted in aganglionic segments in the distal bowel. Thus embryonic bowel transection provides an experimental model for aganglionosis in the chicken embryo. In this experimental model the operated embryos survive longer (at least until E17) than in the other experimental model for intestinal aganglionosis, vagal neural crest ablation, which causes additional anomalies that lead to early death of the embryo. These experimental models for aganglionosis enable detailed investigations of ENS development that are relevant to pathogenesis and etiology of congenital ENS anomalies. This knowledge is essential for the prevention and the improvement of the diagnosis and treatments of such anomalies.

In the present coculture experiments neural crest cell colonization was hampered in the myenteric region of the bowel, but did occur in the region of the submucous plexus. One could argue that the impediment of neural crest cell colonization in the myenteric region is due to the stage of differentiation of the smooth muscle layers. We reported earlier, however, that chicken neural crest cells colonize aneuronal bowel at advanced stages of differentiation (14). In that study we cocultured the neural primordium of chicken embryos with aneuronal bowel of 11 developmental days, which was obtained by subculturing aneuronal E4 bowel. In the present study we cocultured the neural primordium with another type of aneuronal bowel of 11 developmental days, viz. that obtained from an E11 embryo that had undergone bowel transection at E4. This type of aneuronal bowel contains cell-free, ganglionic structures at the site of the myenteric plexus. Such structures develop between the smooth muscle layers after bowel transection at an early developmental stage, and we conclude that they inhibit the colonization by chicken neural crest cells, whereas they hamper the colonization by quail neural crest cells. Preliminary data in our laboratory indicate that the cell-free, ganglionic structures consist of hypertrophic axon bundles. If these findings are substantiated, the bowel transection experiment might serve as a model for classical Hirschsprung's disease. Presently, we are characterizing the cell-free, ganglionic structures and investigating their significance to neural crest cell colonization. With

regard to the pathogenesis of Hirschsprung's disease, it is important to determine when hypertrophic axon bundles appear in the aganglionic bowel segment.

The observed difference in colonizing properties of chicken and quail neural crest cells complicates the extrapolation of quail-chick chimera experiments to normal "in vivo" development. The difference between migration properties of quail neural crest cells and their chicken counterparts is taken into account in "in vitro" culture systems, in which quail neural crest are exclusively used. The differences between chicken and quail neural crest cells might also explain the presence of sacral neural crest cells in postumbilical bowel in quail-chicken chimeras (11). These chimeras provide the most important experimental data in support of a dual vagal/sacral neural crest origin of enteric neurons in the postumbilical bowel. By contrast, our transection experiments, the neural crest ablation experiments of Yntema and Hammond (10), and the chorio-allantoic membrane cultures of Allan and Newgreen (12), prove that enteric neurons in the postumbilacl bowel derive from a single vagal neural crest source.

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REFERENCES

1. Lane PW: Association of megacolon with two recessive spotting genes in the mouse. J Heredity 57:181-183, 1966.

2. Lane PW, Liu HM: Association of megacolon with a new dominant spotting gene (Dom) in the mouse. J Heredity 75:435-439, 1984.

3. Ikadai H, Fujita H, Agematsu Y, Imamichi T: Observation of congenital aganglionosis rat (Hirschsprung's disease rat) and its genetical analysis. Cong Anom 19:31-36, 1979.

4. Hukuhara T, Kotani S, Sato G: Effects of destruction of intramural ganglion cells on colon motility: possible genesis of congenital megacolon. Jap J Physiol 11:635-640, 1969.

5. Okamoto E, Iwasaki T, Kakutani T, Ueda T: Selective destruction of the myenteric plexus: its relation to Hirschsprung's disease, achalasia of the esophagus and hypertrophic pyloric stenosis. J Pediatr Surg 2:444-454, 1967.

6. Tibboel D, Molenaar JC, van Nie CJ: New perspectives in fetal surgery: the chicken embryo. J Pediatr Surg 14:438-440, 1979.

7. Tibboel D, van Nie CJ, Molenaar JC: The effects of temporary general hypoxia and local ischemia on the development of the intestines: an experimental study. J Pediatr Surg 15:57-62, 1980.

8. Klück P, Tibboel D, van der Kamp AWM, et al:The effect of fetal urine on the development of the bowel in gastroschisis. J Pediatr Surg 18:47-50, 1983.

9. Le Douarin NM: *The Neural Crest.* Cambridge, Cambridge University Press, 1982.

10. Yntema CL, Hammond WS: The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chicken embryo. J Comp Neurol 101: 515-541, 1954.

11. Le Douarin NM, Teillet MA: The migration of the neural crest cells to the wall of the digestive tract in the avian embryo. J Embryol Exp Morphol 30: 31-48, 1973.

12. Allan IJ, Newgreen DF: The origin and differentiation of enteric neurons of the intestine of the fowl embryo. Am J Anat 157: 137-154.

13. Kruse J, Mailhammer R, Wernecke H, et al: Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. Nature 311: 153-155, 1984.

14. Meijers JHC, Tibboel D, van der Kamp AWM, et al: The influence of the stage of differentiation

on the migration of neural cells: an experimental study of Hirschsprung's disease. Pediatr Res 21:466-470, 1987.

15. Le Douarin NM: Cell recognition based on natural morphological nuclear markers. Med Biol 52:281-319, 1974.

16. Tucker GC, Aoyama H, Lipinski M, et al: Identical reactivity of monoclonal antibodies HNK-1 and NC-1: Conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Differ 14: 223-230, 1984.

17. Abo T, Balch CM: A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1) J Immunol 127: 1024-1029, 1981.

CHAPTER 7.8

CELL DIVISION IN MIGRATORY AND AGGREGATED NEURAL CREST CELLS IN THE DEVELOPING GUT: AN EXPERIMENTAL APPROACH TO INNERVATION-RELATED MOTILITY DISORDERS OF THE GUT

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Summary. Extensive studies in the chicken embryo have recently supplied more insights into the development of the enteric nervous system, which mainly derives from the vagal neural crest (e.g. the neural crest opposite somite 1-7). Crest cells migrate from this region to and via the developing gut. By means of a double labelling technique of both neural crest cells and cells in the S-phase of the cell cycle, we found that these migrating crest cells still proliferate in the gut. Some cells even go through cell division after the formation of a nerve plexus. Some implications for the pathogenesis of congenital innervation abnormalities such as hyperganglionosis and aganglionosis in Hirschsprung's disease are discussed.

abbreviations: E = Embryonal day; HNK-1 = Human Natural Killer cells; ENS = enteric nervous system.

INTRODUCTION

Knowledge about the normal development of the enteric nervous system might supply some clues to the etiology of neuronal abnormalities of the bowel wall, such as Hirschsprung's disease. The absence of ganglion cells in the distal part of the gut in Hirschsprung's disease has been attributed to a disturbance in neural crest cell division[1] or in neural crest cell migration[2], and to local factors in the distal part of the gut inhibiting the normal aggregation of crest cells or killing aggregated cells[3]. The fact that chicken embryos are easily obtained while extensive knowledge is available aboutthe neurogenesis, renders this the most suitable animal model for experimental studies of the development of the enteric nervous system[4].

In birds, enteric neurons derive from the vagal neural crest[4-6], although a minor contribution to the enteric nervous system by the sacral neural crest cannot be excluded using current experimental methods[6]. The existence of migratory pathways at the axial level was emphasized in earlier studies [7]. Neural crest cells can be stained selectively by a set of antibodies directed against epitopes of cytoskeleton or cell membrane proteins (e.g. neurofilament and HNK-1 epitopes)[8,9]. It is known that neural crest cells

divide at the level of the crest and express differentiation characteristics at the same time (e.g. acetylcholinesterase activity and neurofilament proteins)[10,11]. It is not known whether the dividing capacity of neural crest cells is restricted to the neural crest area. The results of one study showed proliferative activity in peripheral ganglia (e.g.dorsal root ganglia) at 4.5 days of incubation[12]. There are some speculations about a dividing front of migratory crest cells in the gut[13] but no valid data support this theory. Therefore we studied the dividing properties of neural crest cells at the level of the developing gut.

From earlier studies we have learned that the formation of the enteric nervous system takes place from the fourth to eighth day of development[14,15]. We applied ^{3}H thymidine onto the chorio-allantoic membrane of the chicken embryo. ^{3}H thymidine is a nucleotide that is incorporated in cellular DNA during DNA replication which takes place in the S-phase of the cell cycle. By this method all cells passing through S-phase will incorporate the isotope and can be visualized by radioautography. We used HNK-1 antibodies as marker for neural crest cells during migration and at their final location.

MATERIALS AND METHODS

All eggs (fertilized White Leghorn - Gallus Domesticus) were incubated at 38°C in a forced draught incubator with a relative humidity of 75%. The experiments were carried out in two embryos per developmental stage from four to eight days of incubation. We applied 100 μL saline containing 100 μCi ^{3}H thymidine (specific activity 52 Ci/mmol) (Amersham) to the chorio-allantoic membrane. After one hour of incubation, the embryos were isolated and fixed for 24 hours in 4% formaldehyde in phosphate-buffered saline (pH 7.4). Subsequently, the specimens were dehydrated and embedded in paraffin. We used 5 μm sections for both immunohistology and radioautography. Sections were dewaxed, hydrated and incubated for one hour with the HNK-1 antibody (Becton & Dickinson). After rinsing in PBS, the second step antibody, peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Denmark), was applied in a dilution of 1:100, while 2% chick serum was added to the conjugate to reduce background staining. Peroxidase was visualized by 0.1% 3,3'diamino benzidine and 0.02 % hydrogen peroxide. Sections were counterstained with hematoxylin for one minute. After drying in air, the sections were dipped in Ilford K2 dipping fluid and exposed for one week. The sections were developed in KODAK D 19. We analyzed the neural crest cell marker and the silver grains appearing in one single section.

RESULTS

Histologic investigation of paraffin sections from E4-E8 embryos, stained with HNK-1 antibodies, revealed the epitope in the central nervous system, dorsal root ganglia, spinal

nerves, sympathetic chain as well as the enteric nervous system. Table 1 summarizes the staining results. The ^{3}H thymidine label was found over many cells of the developing embryo. Some HNK-1 positive cells also showed the ^{3}H thymidine label. This was confined to the early stages of the central nervous system, dorsal root ganglia and the sympathetic chain . The uptake of ^{3}H thymidine diminished in a central-peripheral direction.

TABLE 1

HNK-1 STAINING OF NEURAL TISSUES AT VARIOUS DEVELOPMENTAL STAGES (+) AND ^{3}H THYMIDINE UPTAKE ([1])

	E4	E5	E6	E7	E8
Central nervous system	+[1]	+[1]	+[1]	+	+
Dorsal root ganglia	+[1]	+[1]	+[1]	+	+
Spinal nerves	+[1]	+[1]	+[1]	+	+
Sympathetic chain	+[1]	+[1]	+[1]	+	+
Enteric nervous system					
Proximal gut	-	+[1]	+[1]	+	+
Esophagus	+[1]	+[1]	+	+	+
Stomach	-	+[1]	+[1]	+[1]	+
Ileum	-	-	-	+[1]	+[1]
Colon	-	-	-	+[1]	+[1]
Remak ganglion	-	-	+[1]	+[1]	+[1]

The HNK-1 staining of the enteric nervous system progressed in a proximo-distal manner. Starting diffusely in aneuronal areas, the HNK-1 epitope visualized migrating neural crest cells and eventually concentrated in the myenteric and submucous plexus. We observed the uptake of ^{3}H thymidine in both the migrating neural crest cells and the aggregated crest cells in the enteric ganglia (see fig. 1) . Frequently more than one single cell per ganglion had taken up the radioactive label. The uptake of ^{3}H thymidine within the ganglia ceased in a proximo-distal manner. The sympathetic ganglion of Remak showed the same proximo-distal wave of DNA-replication.

Some expression of the HNK-1 epitope was also found in the liver, the wing bud, mesonephros and the heart.

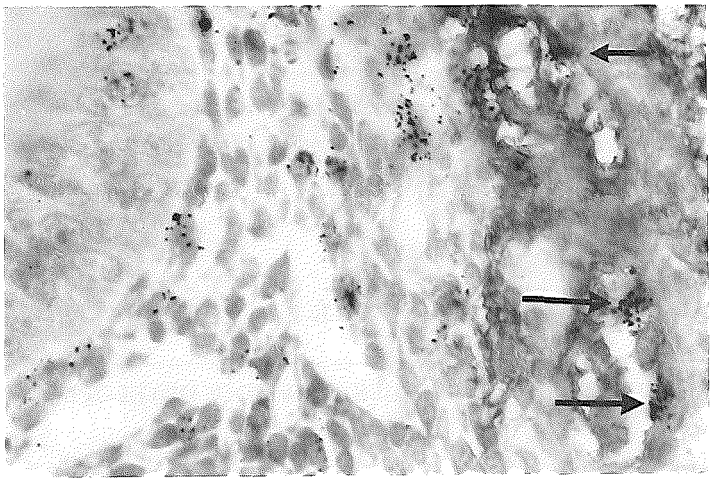

Fig. 1. Detail of myenteric plexus showing cells with HNK-1 antibody stain and ^{3}H thymidine label.

DISCUSSION

The dividing capacity of migrating neural crest cells was investigated in paraffin sections of whole embryos. Most of the neural crest derivatives (dorsal root ganglia, autonomic ganglia, enteric ganglia and melanocytes) stained with the HNK-1 antibody. The epitope appeared earlier in sites close to the neural tube (dorsal root ganglia) than at the more distal locations (enteric ganglia). This supports the theory of neural crest cell migration. The neural crest derivatives close to the central nervous system were the first cells to cease division. Some HNK-1 expression was also noted in the developing heart and liver. This can be explained by the fact that the neural crest plays a part in the septation of the heart[16].

Miki and associates[10] observed that neural crest cells, stained histochemically for the presence of acetylcholinesterase, could also be labeled with ^{3}H thymidine, indicating that at the neural crest level a certain proportion of crest cells is capable of proliferation. The same was observed by Payette and associates, by means of double staining of neurofilament proteins and metaphases[11]. These experiments indicate cell division at the axial level. We also observed the uptake of radioactive thymidine in migrating and aggregated crest cells, indicating that the proliferative capacity is not restricted to the axial levels only.

We surmise that the formation of enteric ganglia proceeds in the following way.

At the neural crest level opposite somite 1-7 some crest cells differentiate towards a neuronal precursor population. This population is still capable of division. In the course of development, the crest cells move in a lateral direction towards the developing gut, between the ectoderm and the somite. From there they move in a distal direction to reach the cloacal end of the gut. Some of the cells aggregate and divide to form the myenteric and submucous plexus. Others merely divide and continue migration until they have arrived at the distal end of the digestive tract.

Our observation of proliferating crest cells in the enteric ganglia as well as in the migratory crest cell population, provides new insights in the congenital, innervation-related motility disorders of the gut. We surmise that in hyperganglionosis the target organ does not provide the appropriate signals for the arrest of neural crest cell division. In case of Hirschsprung's disease and hypoganglionosis, factors may be present that hamper cell division in the distal part of the bowel. It still needs to be excluded that an insufficient number of cells leave the neural crest, which results in very few migratory cells reaching the distal locations.

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REFERENCES

1. Bolande RP. The neurocristopathies; A unifying concept of disease arising in neural crest maldevelopment. Hum Pathol 5:409-429, 1973.

2. Okamoto E, Ueda T. Embryogenesis of intramural ganglia of the gut and its relation to Hirschsprung's disease. J Pediatr Surg 2:437-443, 1967.

3. Earlam R. A vascular cause of Hirschsprung's disease? Gastroenterology 88:1274-1279, 1985.

4. LeDouarin NM. The neural crest. Cambridge University Press, Cambridge, 1982.

5. Yntema CL, Hammond WS. The origin of intrinsic ganglia of trunk viscera from vagal neural crest in the chick embryo. J Comp Neurol 101:515-541, 1954.

6. Le Douarin NM, Teillet M-A. The migration of neural crest cells to the wall of the digestive tract in avian embryo. J Embryol Exp Morphol 30:31-48, 1973.

7. Thiery JP, Duband JL, Delouvee A. Pathways and mechanisms of avian trunk neural crest migration and localisation. Dev Biol 93:324-343, 1982.

8. Tapscott SJ, Bennet GS, Holtzer H. Neuronal precursor cells in the chick neural tube express neurofilament proteins. Nature 292:836-838, 1981.

9. Tucker GC, Aoyama H, Lipinski M, Tursz, Thiery JP.Identical reactivity of monoclonal antibodies HNK-1 and NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leukocytes. Cell Differ 14:223-230, 1984.

10. Miki A, Fujimoto E, Mizoguti H. Acetylcholinesterase activity in neural crest cells of the early chick embryo. Histochemistry 78:81-93, 1983.

11. Payette RF, Bennett GS, Gershon MD. Neurofilament expression in vagal neural crest-derived precursors of enteric neurons. Dev Biol 105:273-287, 1984.

12. Carr VM, Simpson SB jr. Proliferative and degenerative events in the early development of chick dorsal root ganglia. J Comp Neurol 182:727-755, 1978.

13. Duband JL, Tucker GC, Poole TJ, Vincent M, Aoyama H, Thiery JP. How do the migratory and adhesive properties of the neural crest govern ganglia formation in the avian peripheral nervous system? J Cell Biochem 27:189-203, 1985.

14. Allan IJ, Newgreen DF. The origin and differentiation of enteric neurons of the intestine of the fowl embryo. Am J Anat 157:137-154, 1980.

15. Meijers JHC, Tibboel D, Van der Kamp AWM, CCM Van Haperen-Heuts, Klück P, Molenaar JC. The influence of the stage of differentiation of the gut on the migration of neural cells. An experimental study of Hirschsprung's disease. Pediatr Res 21:132-135.

16. Kirby ML, Bockman DE. Neural crest and normal development: a new perspective. Anat Rec 209:1-6, 1984.

SUMMARY

Chapter 1 A significant number of newborns suffer from congenital malformations related to the neural crest. The neural crest is a transient structure in vertebrate embryogenesis, that develops from the fusing ridges of the neural folds. Neural crest cells migrate throughout the entire embryo and give rise to craniofacial structures, the aorticopulmonary septum, the thymus, the enteric nervous system, the neurons and supportive cells of the peripheral nervous system, and melanocytes. Neural crest cells are particularly vulnerable during development and lesions to the neural crest have been related to a considerable number of congenital malformations, the neurocristopathies. The aim of this thesis is to clarify the pathogenetic mechanisms of one subgroup of neurocristopathies: congenital malformations of the enteric nervous system. To this end, the formation of the enteric nervous system was studied experimentally in chicken and murine embryos, and the literature concerning the pathology of congenital malformations was reviewed.

Chapter 2 Following an historical survey, the congenital malformations of the human enteric nervous system are classified according to developmental biological and genetic criteria. Two general types of congenital disorders are distinguished: developmental and degenerative disorders. The developmental disorders are subdivided into: 1) migration disturbances of neural crest cells; 2) proliferation disturbances of neural crest cells; 3) disorders of the enteric mesenchyme; and 4) differentiation disturbances of enteric neurons. The neuropathology of various congenital malformations and the occurrence of associated anomalies is described and related to developmental biological phenomena. Finally, the occurrence of congenital malformations of the enteric nervous system in other species is reviewed briefly.

Chapter 3 Knowledge of embryology facilitates the understanding of the pathogenesis of congenital malformations. During embryonic development cell-cell and cell-substrate interactions play an important part in organ development and pattern formation. These processes are mediated by morphoregulatory molecules. A particular carbohydrate moiety, a sulphated glucuronic acid, is recognized by monoclonal antibody HNK-1. The HNK-1 carbohydrate moiety is present on several morphoregulatory molecules and the epitope itself is involved in cell-cell and cell-substrate interactions. Three types of morphoregulatory molecules of the HNK-1 family involved in neural crest development are reviewed: a) cell adhesion molecules, which are involved in the adhesion of one cell to one another; b) extracellular matrix molecules, which are required for the migration of neural crest cells; c) substrate adhesion molecules, which are involved in the attachment of cells to extracellular matrix molecules.

Chapter 4 contains a review of the literature concerning the origin of enteric neurons,

the role of morphoregulatory molecules during neural crest development, the characteristics of vagal neural crest cells, their migration through the embryonic bowel and the homing at the site of the enteric ganglia. The vagal neural crest provides the precursor cells for the neurons in the entire bowel; there is no consensus of opinion about the contribution of the sacral neural crest. The vagal neural crest also contributes to craniofacial structures, the aorticopulmonary septum, the parathyroids, the thymus, and possibly the forelimb. The formation of the avian and murine enteric nervous system is compared with that of other species. Special attention is given to the development of the enteric nervous system in insects, viz. the moth, since there are no neural crest cells in invertebrates.

Chapter 5 contains a description of the experiments performed to unravel the formation of the enteric nervous system in chicken and murine embryos. For a summary of the experimental work the reader is referred to the abstracts of the appendix papers. In the experiments, embryonic bowel was cultured on the chorioallantoic membrane or in the renal subcapsular space. The characteristics of the two culture systems are described briefly.

Chapter 6 relates the experimental findings to clinicopathological data from patients. With regard to enteric neural crest cell migration it is speculated that these cells have specific receptors for the cell motility site of fibronectin. A possible role for the HNK-1 carbohydrate moiety in cell recognition is proposed. The knowledge about the formation of the enteric nervous system provided a framework for hypotheses about the pathogenesis of congenital malformations. Pathogenetic mechanisms are suggested for some congenital malformations of the enteric nervous system. In a final remark two fields of interest are indicated that require further research: on the one hand detailed neuropathological and clinical genetical studies, on the other hand developmental genetics studies concerning genes that are involved in segmental migration of neural crest cells.

SAMENVATTING

Hoofdstuk 1 Een aanzienlijk aantal pasgeborenen vertoont aangeboren afwijkingen, die in verband staan met de neurale lijst. De neurale lijst is een struktuur die tijdelijk aanwezig is tijdens de embryogenese van gewervelden, en ontstaat uit de fuserende neurale plooien. Neurale lijst cellen migreren door het gehele embryo en dragen bij aan de vorming van craniofaciale strukturen, het aorticopulmonale septum, de thymus, de darminnervatie, de neuronen en steuncellen van het perifere zenuwstelsel, en melanocyten. Neurale lijst cellen zijn bijzonder kwetsbaar gedurende de embryonale ontwikkeling; stoornissen in de neurale lijst ontwikkeling zijn in verband gebracht met aangeboren afwijkingen: de neurocristopathieën. Het doel van dit proefschrift is het verhelderen van pathogenetische mechanismen van een bepaalde groep neurocristopathieën: aangeboren misvormingen van de darminnervatie. Hiertoe werd de vorming van de darminnervatie experimenteel onderzocht bij kippe- en muize-embryo's; tevens werd een literatuurstudie verricht naar de pathologie van aangeboren misvormingen in de darminnervatie.

Hoofdstuk 2 Na een historisch overzicht worden de aangeboren misvormingen van de darminnervatie geclassificeerd op grond van ontwikkelingsbiologische en genetische criteria. Twee algemene typen van aangeboren stoornissen worden onderscheiden: ontwikkelingsstoornissen en degeneratieve stoornissen. De ontwikkelingsstoornissen worden onderverdeeld in: 1) migratiestoornissen van neurale lijst cellen; 2) proliferatiestoornissen van neurale lijst cellen; 3) afwijkingen in het mesenchym van de darm; 4) differentiatiestoornissen van ganglioncellen. De neuropathologie van de verschillende innervatiestoornissen wordt beschreven, alsmede het voorkomen van geassocieerde aangeboren afwijkingen. Deze laatste worden gerelateerd aan ontwikkelingsbiologische fenomenen. Tenslotte wordt het spontaan voorkomen van aangeboren afwijkingen in de darminnervatie bij andere soorten beschreven.

Hoofdstuk 3 Kennis van de embryologie is noodzakelijk voor een beter begrip van de pathogenese van aangeboren afwijkingen. Tijdens de embryonale ontwikkeling spelen cel-cel en cel-substraat interacties een belangrijke rol bij de organogenese en patroonvorming in die organen. Op moleculair niveau spelen vormregulerende moleculen ('morphoregulatory molecules') een sleutelrol bij deze processen. Een bijzondere koolhydraatgroep, een gesulfateerd glucuronzuur, wordt herkend door het monoclonale antilichaam HNK-1. Het HNK-1 koolhydraat is aanwezig op verscheidene vormregulerende moleculen en speelt zelf een rol in cel-cel en cel-substraat interacties. Drie typen vormregulerende moleculen van de HNK-1 familie, die betrokken zijn bij de ontwikkeling van de neurale lijst, worden besproken: a) cel-adhesie moleculen, die betrokken zijn bij de adhesie van de ene cel aan de andere; b) extracellulaire-matrix moleculen, die noodzakelijk zijn voor de migratie van neurale lijst cellen; c) substraat-adhesie moleculen, die betrokken zijn bij de hechting van cellen aan extracellulaire-

matrix moleculen.

Hoofdstuk 4 bevat een literatuuroverzicht over de oorsprong van darmwandneuronen, de rol van vormregulerende moleculen bij de neurale lijst ontwikkeling, de eigenschappen van vagale neurale lijst cellen, hun migratie door de embryonale darm, en de nesteling op de plaats van de intramurale ganglia. De vagale neurale lijst levert de precursor cellen voor neuronen in de gehele darm; er is verschil van mening over de bijdrage van de sacrale neurale lijst aan de ganglia in de einddarm. De vagale neurale lijst draagt eveneens bij tot de vorming van craniofaciale strukturen, het aorticopulmonale septum, de bijschildklieren, de thymus, en mogelijk tot de vorming van de voorste ledemaat. De ontwikkeling van de darminnervatie bij kippen en muizen wordt vergeleken met die bij andere soorten. Speciale aandacht wordt besteed aan de vorming van de darminnervatie bij insecten, met name de mot, daar neurale lijst cellen niet voorkomen bij ongewervelden.

Hoofdstuk 5 bevat een beschrijving van het experimentele werk dat werd verricht om de vorming van de darminnervatie te bestuderen bij kippen en muizen. Tijdens de experimenten werden embryonale darmen gekweekt op het chorioallantois membraan van kippe-embryos of in de ruimte onder het nierkapsel van muizen. De eigenschappen van beide kweekomstandigheden worden kort besproken.

Hoofdstuk 6 brengt de gegevens verkregen uit de experimenten in verband met clinicopathologische gegevens van patienten. Met betrekking tot de migratie van neurale lijst cellen door de darm wordt gespeculeerd dat enterale neurale lijst cellen over specifieke receptoren beschikken voor het cel motiliteits domein van fibronectine. Een mechanisme aangaande de rol van het HNK-1 koolhydraat bij cel-cel herkenning tijdens de embryonale ontwikkeling wordt besproken. De kennis van de vorming van de darminnervatie bood het raamwerk voor het opstellen van hypothesen omtrent de pathogenese van aangeboren misvormingen van de darminnervatie. Verschillende pathogenetische mechanismen die kunnen leiden tot aangeboren afwijkingen in de darminnervatie worden aangegeven. In een laatste opmerking worden aandachtsvelden aangegeven die nader onderzoek vereisen: enerzijds neuropathologische en klinische studies van aangeboren afwijkingen in de darminnervatie, en anderzijds ontwikkelings genetische studies met betrekking tot de segmentale migratie van neurale lijst cellen.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 28 juli 1960 geboren te Leerdam. Na het eindexamen gymnasium B (juni 1978) aan de Gereformeerde Scholengemeenschap te Rotterdam, studeerde hij Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens het keuzepraktikum onderzocht hij de differentiatie van teratocarcinoma cellen bij Dr. A.W.M. van der Kamp, afdeling Celbiologie en Genetica, Erasmus Universiteit, Rotterdam. Het onderzoek zette hij voort in een student-assistentschap (1981-1983). In dezelfde periode was hij ook student-assistent bij de contactcommissie tussen de medische faculteit en de centrale interfaculteit. Na het artsexamen (februari 1985, cum laude) startte hij het in dit proefschrift beschreven onderzoek als wetenschappelijk onderzoeker bij de afdeling Kinderheelkunde, Erasmus Universiteit te Rotterdam. In 1986 werd het onderzoek bekroond met de "Best paper prize" van de "12th International Meeting of the Greek Association of Paediatric Surgeons" te Rhodos, Griekenland. In 1987 werd het onderzoek bekroond met de prijs van de Heinz Karger Memorial Foundation; in 1988 met de onderzoeksprijs van de Erasmus Universiteit, Rotterdam.

LIST OF PUBLICATIONS J.H.C. MEIJERS

1. Meijers, J.H.C., D. Tibboel, A.W.M. van der Kamp, C.C.M. van Haperen-Heuts, and J.C. Molenaar (1987): Cell division in migratory and aggregated neural crest cells in the developing gut. An experimental approach of innervation-related motility disorders of the gut. J. Pediatr. Surg. 22:243-245.

2. Meijers, J.H.C., D. Tibboel, A.W.M. van der Kamp, C.C.M. van Haperen-Heuts, P. Klück, and J.C. Molenaar (1987): The influence of the stage of differentiation of the gut on the migration of neural cells. An experimental approach of Hirschsprung's disease. Pediatr. Res. 21:132-135.

3. Molenaar, J.C., D. Tibboel, A.W.M. van der Kamp, P. Klück, J.H.C. Meijers, C.C.M. van Haperen-Heuts, and F.W.J. ten Kate (1987): Etiology and diagnosis of innervation-related motility disorders of the gut. J. Jap. Soc. Pediatr. Surg. 23:7-17.

4. Tibboel, D., J.H.C. Meijers, P. Klück, A.W.M. van der Kamp, F.W.J. ten Kate, C.C.M. van Haperen-Heuts, and J.C. Molenaar JC (1987): Use of monoclonal antibodies for diagnosis and research in pathology of the enteric nervous system. A Review. Dev. Neurosci. 9:133-143.

5. Molenaar, J.C., D. Tibboel, A.W.M. van der Kamp, and J.H.C. Meijers (1989): Diagnosis of innervation- related motility disorders of the gut and basic aspects of enteric nervous system development. Progress in Pediatr. Surg. in press.

6. Meijers, J.H.C., D. Tibboel, A.W.M. van der Kamp, C.C.M. van Haperen-Heuts, and J.C. Molenaar (1989): Patterns of neural crest cell colonization of aneuronal bowel in a chicken model for intestinal aganglionosis. J. Pediatr. Surg. In press.

7. Molenaar, J.C., D. Tibboel, and J.H.C. Meijers (1989): The chick embryo model of fetal malformation. In: *The unborn patient.* Harrison MR, Golbus, MS, and RA Filly. (eds.) In press.

8. Meijers, J.H.C., D. Tibboel, A.W.M. van der Kamp, J.M. van Dongen, C.C.M. van Haperen-Heuts, and J.C. Molenaar (1989): The origin of enteric neurons in chicken hindgut. Submitted.

9. Meijers, J.H.C., D. Tibboel, A.W.M. van der Kamp, C.C.M. van Haperen-Heuts, and J.C. Molenaar (1989): HNK-1 immunoreactive mesenchymal cells are involved in neural crest cell homing. Submitted.

10. Meijers, J.H.C., D. Tibboel, J.I.J. Gaillard, N. Heaton, A.W.M. van der Kamp, and J.C. Molenaar (1989): The effect of ischemia on the developing enteric nervous system. An experimental study in the chicken embryo and a pathological study in humans. Submitted.

11. Nishijima, E., J.H.C Meijers, D. Tibboel, C.C.M. van Haperen-Heuts, A.W.M. van der Kamp, and J.C. Molenaar (1989): Neural crest cell colonization of embryonic murine bowel. Submitted.

12. Meijers, J.H.C., E. Nishijima, C.C.M. van Haperen-Heuts, D. Tibboel, A.W.M. van der Kamp, and J.C. Molenaar (1989): Development of intestinal aganglionosis in lethal spotted mice. Submitted.

13. Meijers J.H.C., D. Tibboel, A.W.M. van der Kamp, C.C.M. van Haperen-Heuts. and J.C. Molenaar (1989): Application of monoclonal antibodies directed against early neuronal markers in the study of the development of the enteric nervous system. Implications for pathogenetic studies of congenital anomalies of the enteric nervous system. Submitted.