Embryogenesis of the enteric ganglia in normal mice and in mice that develop congenital aganglionic megacolon

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SUMMARY

Embryological studies on the mouse indicate that, contrary to the classical concept, all the enteric ganglia are from a single, vagal, neural crest source. The immature ganglion cells first enter the gut by way of the newly formed vagal outgrowth at 10 days gestation. The neuroblasts then migrate down the gut in a cranio-caudal direction, being replaced by more neuroblasts from the vagus. By 15 days 12 h the process is complete and ganglion cells are present throughout the gut. The process is continuous and there is no evidence of a lumbo-sacral origin for any of the intramural ganglia.

In mice which develop aganglionic megacolon the process is basically the same except that the neuroblasts appear to migrate at a slower rate than normal. The result is that the migration of the neuroblasts and the elongation of the gut are out of phase so the migrating neuroblasts cannot reach the end of the gut, despite the fact they migrate 6–7 days longer than normal.

INTRODUCTION

Aganglionic megacolon, or Hirschsprung’s disease, is a congenital disease of mammals occurring in man at an incidence of between 1 in 2000 and 1 in 10000 live births in England (Bodian & Carter, 1963). The disease is due primarily to an absence of enteric ganglion cells from the rectum and a varying but continuous length of gut above the rectum. The present paper compares the development of the enteric ganglia in normal mice with the development in a strain which is characterized by congenital aganglionic megacolon due to the recessive gene piebald-lethal ($s^l$).

ORIGIN AND GENETICS

The gene piebald-lethal, $s^l$, was first detected in the Jackson Laboratories U.S.A. in 1959. It occurred in the $F_2$ generation of a cross between C3H/HeJ and C57BL/6J strains (Lane, 1966). Piebald-lethal, $s^l$, segregates as a simple recessive gene (Lane, 1966; Webster, 1971), it is allelic to and nearly recessive

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to piebald, s, and completely recessive to wild-type, (+). All the homozygote recessives develop megacolon with a complete lack of ganglion cells in the distal gut.

MATERIALS AND METHODS

To study the development of the enteric ganglia in the normal and sl/sl mice; embryos from over 150 pregnant females were examined. Three main crosses were used: s'/s' × s'/s', s'/s' × s'/+ and +/+ × +/+ . The mice were mated overnight and the day a plug was recorded was considered day 1 of pregnancy. Instead of using the conventional but capricious silver impregnation techniques to identify the developing neuroblasts a histochemical method was used. In the adult mouse the mature enteric ganglia are characterized by a cholinesterase positive reaction. A similar but weaker reaction can be obtained from the immature ganglia in the embryo. Maximal staining was obtained by using a non-specific esterase technique; α-naphthyl acetate was used as the substrate (Gomori, 1950; Davis & Ornstein, 1959; see Bancroft, 1967). Embryos of known age were frozen in hexane cooled to −90°C by an acetone/solid CO₂ mixture then serially sectioned in a cryostat at 16 μm. The sections were melted on to slides, air-dried, then stained for 2–30 mins at 37°C in the incubating medium. The sections were counter-stained in 2% methyl green (chloroform extracted).

The distribution of ganglion cells in the adult mouse intestine was examined by use of frozen sections stained with haematoxylin and eosin.

RESULTS

1. Pathology

Throughout this study the term ‘colon’ is used to represent all the large intestine distal to the caecum as in the mouse the rectum is not clearly delimited. In normal mouse colon groups of myenteric ganglion cells are present approximately every 50 μm between the outer longitudinal and inner circular muscle layers as seen in 10 μm sections (Fig. 1). Ganglia normally extend as far distally as about 1 mm from the striated sphincter surrounding the anal canal. The submucous plexus, although present, is very sparse in the colon.

In mice with aganglionic megacolon there are no ganglion cells in either the myenteric or submucous plexuses in the distal 25 mm of colon, there is a gradual increase in the next 20 mm (transitional zone) and the proximal half of the colon is comparable to that of a normal mouse. In the aganglionic region large abnormal nerve fibres are frequently seen in the zone normally occupied by the ganglion cells between the longitudinal and the circular muscle layers (Fig. 2). These nerve trunks, which are considered to be part of the sacral parasympathetic outflow, will be discussed in a future paper.
Embryogenesis of enteric ganglia in mice

575

Fig. 1. Longitudinal section through the wall of colon of a normal adult mouse. Note groups of ganglion cells (arrows) between the outer longitudinal and inner circular muscle layers. × 60.

Fig. 2. Longitudinal section through the wall of colon from a mouse with aganglionic megacolon. Note large nerve fibres (arrow) in place of ganglion cells seen in Fig. 1. × 60.

Fig. 3. Coat pigmentation of the different mouse genotypes. (a) Black, s'/+ or +/+; (b) white, s'/s' easily recognizable coat pigmentation of animals with aganglionic megacolon; (c) piebald, s'/s'; (d) black and white, Is/Is, Is is another gene causing aganglionic megacolon; see Discussion; (e) agouti, Is/+ or +/+.

Fig. 4. Colon and part of small intestine from five adult mice. (a) Normal mouse – note 4 or 5 faecal pellets in the colon; (b), (c) From well but affected s'/s' mice – pellet formation is masked by faecal accumulation in the distal half of the colon; (d), (e) From sick s'/s' mice; note grossly enlarged proximal segment including the caecum and the normal sized empty distal colon (aganglionic). × ½.

2. Clinical presentation

All mice homozygous for the gene piebald-lethal are easily recognizable as s', besides causing megacolon, also causes an almost complete absence of coat pigmentation (Fig. 3). Approximately 10% of these homozygotes died before postnatal day 3 and a further 25% before weaning. The surviving mice lived an average of 14 weeks.

Death in the preweaned mouse was not usually due to intestinal obstruction, but was characterized by diarrhoea and presumably enterocolitis. In older mice,
Embryogenesis of enteric ganglia in mice

however, the sick mouse invariably showed a grossly enlarged faeces-packed, proximal colon; followed by a normal sized empty distal colon (aganglionic) (Fig. 4).

3. Embryonic development

The development of the enteric plexus in the mouse was examined by serial frozen sections stained for esterase activity. The esterase activity was localized in the perikaryon of the early neuroblasts (Fig. 5). The developing liver, the gut mucosa and the dorsal root ganglia also show high activity.

(a) Control (+/+ or s¹/+)

At the 10th day of gestation the normal mouse embryo has 26–28 somites and is structurally comparable to a 96 h chick embryo (Rugh, 1968) or a 28-day human embryo (Otis & Brent, 1954). The gut is a simple tube with the lungs, liver and dorsal pancreas all present as small primordia. There are no ganglion cells in any portion of the alimentary tract at this stage. There are however a pair of vagal downgrowths from the post-otic vagal ganglia which make contact with the primitive oesophagus. Neuroblasts are present along the vagus presumably migrating from the vagal ganglia to the gut.

By 10½–11 days there are considerable numbers of neuroblasts present in the gut. The intestine has grown rapidly and because of its great elongation a loop has formed to the left. The stomach can be clearly distinguished by its expanded lumen. Neuroblasts are present along the oesophagus in conjunction with the vagus just outside the circular muscle layer (Fig. 6). These neuroblasts can be traced on serial sections throughout the stomach and into the intestine (Fig. 7). The distal part of the alimentary canal is completely aganglionic at this stage.

By 11½ days gestation further growth of the intestine has led to the formation

<table>
<thead>
<tr>
<th>Figures 5-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 5. Section of 16 μm from the intestine of a normal 11½-day embryo showing esterase stained neuroblasts. Note the nucleus is not stained. × 1000.</td>
</tr>
<tr>
<td>Fig. 6. Transverse section through a normal 10½-day embryo. The vagal downgrowths (arrows) have made contact with the upper oesophagus and neuroblasts are present around the oesophagus. Stained for esterase activity. × 50.</td>
</tr>
<tr>
<td>Fig. 7. Transverse section through a normal 11-day embryo showing esterase stained neuroblasts (arrows) in the stomach and intestine. × 50.</td>
</tr>
<tr>
<td>Fig. 8. Transverse section through a normal 11-day embryo at the level of the tracheal bifurcation. Neuroblasts are seen around the oesophagus outside the circular muscle layer. × 100.</td>
</tr>
<tr>
<td>Fig. 9. Transverse section through a normal 11-day embryo showing esterase stained neuroblasts in the stomach and duodenum × 100.</td>
</tr>
<tr>
<td>Fig. 10. Longitudinal section of a normal 11½-day embryo showing neuroblasts as far distally as the caecum. The distal gut is completely aganglionic at this stage. The arrows show distal limit reached by neuroblasts. Section stained for esterase activity. Inset shows section of whole embryo. × 35.</td>
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</table>
of a slight umbilical hernia. The trachea has now separated from the oesophagus and the cloacal cavity has now divided into urogenital and rectal portions. Neuroblasts are now present in the oesophagus (Fig. 8), stomach and duodenum (Fig. 9), and small intestine as far distally as the caecum (Fig. 10).

By 12\(\frac{3}{2}\) days further elongation of the gut has caused a sizeable umbilical hernia. Intramural neuroblasts are now present throughout most of the intestinal tract; only the distal three-quarters of the colon is still aganglionic (Fig. 11). By day 13\(\frac{1}{2}\) this is reduced to the distal quarter of the colon, about 1-5 mm. However, because of the continuous rapid growth of the large intestine it is a further 2 days, to 15\(\frac{1}{2}\) days gestation, before the neuroblasts reach the end of the gut (Fig. 15). When the longitudinal muscle differentiates these neuroblasts form the myenteric plexus. The submucous or Meissner’s plexus becomes apparent later.

The time taken for the complete migratory process is about 5\(\frac{1}{2}\) days; despite the fact that three-quarters of the alimentary tract has ganglia after only 24 h. This emphasizes the great elongation of the gut, which increases from approximately 1 mm at 10 days to 50 mm by 15\(\frac{1}{2}\) days gestation.

(b) Piebald-lethal \((s^l/s^l)\)

The embryology of the \(s^l/s^l\) mice was examined by comparing sections of embryos from \(s^l/s^l \times s^l/s^l\) crosses with normal embryos. Also complete litters from \(s^l/s^l \times s^l/+\) crosses were examined by serial sections of each embryo. The distance the ganglia had migrated along the gut was determined for each embryo and if possible the embryos from each litter were divided into two groups \(s^l/s^l\) and \(s^l/+\). Using this method no difference could be demonstrated between ++ or ++/++ and \(s^l/s^l\) genotypes in 10\(\frac{1}{2}\)- or 11\(\frac{1}{2}\)-day embryos. By 12\(\frac{1}{2}\) days gestation, however, a definite and constant difference could be demonstrated.

In the normal 12\(\frac{1}{2}\)-day embryo, ganglia are present as far distally as one-quarter of the way down the colon, which is 4 mm long (Fig. 11). In \(s^l/s^l\) embryos of the same age neuroblasts only extend as far distally as the caecum (Fig. 12). There is no evidence of any difference in size of the embryo or length.

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**Figures 11, 12**

*Fig. 11. Longitudinal section through normal 12\(\frac{1}{2}\)-day embryo showing the proximal half of the colon and a section of the small intestine. Neuroblasts are present throughout the small intestine and the proximal quarter of the colon. The arrows mark the distal limit reached by the neuroblasts. \(\times 40\).*

*Fig. 12. Longitudinal section through 12\(\frac{1}{2}\)-day \(s^l/s^l\) embryo showing the proximal half of the colon and a section of the small intestine. Neuroblasts are present throughout the small intestine but have only just started to enter the colon. The arrow marks the distal limit reached by neuroblasts. \(\times 40\).*
Embryogenesis of enteric ganglia in mice

By 13½ days gestation the difference has increased, in the normal embryo only one quarter of the colon is still aganglionic while in the $s^I/s^I$ embryos it is the distal three-quarters. In the 14½-day normal embryo only 0-7 mm is still aganglionic compared with 3-5 mm in the $s^I/s^I$ embryos (Figs. 13, 14). When the process is normally complete at 15½ days only 50% of the colon is ganglionic in $s^I/s^I$ embryos (Figs. 15 and 16 show 16½-day embryos). The process does not stop here, however; the ganglia continue to migrate for a further 6–7 days, until 1–2 days after birth, so in the newborn only the distal 25% of the colon remains aganglionic; the colon at this stage measures 25 mm compared with 4 mm at 12½ days gestation.

**DISCUSSION**

The use of animal mutations to study human congenital disease has often been disappointing as superficial similarities have been shown to have quite different pathogenesis. However, in the case of the piebald-lethal mutation in mice the similarity to Hirschsprung's disease in man is extensive. Histologically the condition is almost identical; a detailed comparison will be published elsewhere.

The present study shows that the intramural ganglionic plexus of the mouse intestine is formed by neuroblasts which migrate along the alimentary tract in a cranio-caudal direction. The process starts on the 10th day of gestation and is completed by 15½ days gestation. All the neuroblasts are of vagal origin.

This system of development is in agreement with the study made by Okamoto & Ueda (1967) on the embryogenesis of the myenteric plexus in man; and the results from Yntema & Hammond's (1954) ablation experiments on the chick embryo. However, although it is generally agreed that the enteric ganglia of the upper intestine are of vagal origin, a number of authors have reported that the ganglia of the terminal gut have a separate lumbosacral origin (Abel, 1909,

**Figures 13–16**

an = region of anus

Fig. 13. Longitudinal section of a normal 14½-day embryo showing the distal colon (rectum). Neuroblasts are present almost to the end of the gut (arrows). Section stained for esterase activity. × 50.

Fig. 14. Longitudinal section of a 14½-day $s^I/s^I$ embryo showing almost the whole colon. Neuroblasts are present only in the proximal quarter of the colon. The arrows mark the distal limit reached by the neuroblasts. × 40.

Fig. 15. Longitudinal section of a 16½-day normal embryo showing distal colon. Ganglion cells are now present throughout the whole intestine. Section stained for esterase activity. × 50.

Fig. 16. Longitudinal section of a 16½-day $s^I/s^I$ embryo showing the distal colon. There are no ganglion cells present in this part of the gut. Section stained for esterase activity. × 50.
1912; Kuntz, 1910a, b, 1920, 1922, 1953; Uchida, 1927; van Campenhout, 1930, 1931, 1932; Jones, 1942; Dereymaeker, 1943; Huther, 1954; Andrew, 1963, 1964, 1969, 1970; Cantino, 1970). Recently Andrew (1971) reviewed the experimental evidence and concluded that the only work carried out early enough in development to be of value was that of Yntema & Hammond (1954) and her own work (Andrew, 1963, 1964, 1969, 1970). The work of Yntema & Hammond supports the hypothesis of a single vagal origin for all the enteric ganglia. Andrew’s work has primarily consisted of culturing as chorio-allantoic grafts pieces of chick blastoderm. She has demonstrated that both vagal and more caudal trunk levels of neural crest can give rise to ‘enteric ganglia’ in these grafts. However, as Andrew (1971) points out, although trunk neural crest gives rise to enteric ganglia in grafts it is quite possible that this ability may not be realized in the intact embryo. The morphological investigations have been carried out almost exclusively by silver impregnation techniques despite the fact that identification of neuroblasts is difficult at early stages by this method. However, Cantino (1970) used a cholinesterase staining method on rat and rabbit embryos and still concluded that neuroblasts travel along the pelvic nerves to the hind gut.

Although the distal intestine in the mouse grows in close proximity to the pelvic plexus there is no evidence in the present study that ganglion cells pass from the plexus into the intestine. It is possible that in mammals at least, investigators have interpreted the adjacent pelvic plexus with its many ganglion cells as part of the distal gut. Certainly before the longitudinal muscle differentiates the boundary of the distal gut is difficult to determine.

The strongest evidence against a dual origin, at least in the mouse, comes from the embryology of mice that will develop megacolon. It has been shown in this study that the absence of ganglia in the distal gut is not caused by a deficient pelvic supply. Instead the neuroblasts of vagal origin migrate down the gut at a slower rate than normal. This results in the migrating neuroblasts being out of phase with the lengthening of the gut, so despite the fact they migrate 6–7 days longer than normal they cannot reach the end of the gut.

Theories on the origin of aganglionic megacolon in man have been suggested by a number of authors. Huther (1954) and Svenson (1950, 1955), in accordance with the classical theory of a dual origin of enteric ganglion cells, claimed the ‘pelvic source’ of neuroblasts was defective. Additional evidence for this came from the occasionally reported case of a region of aganglionic colon followed both proximally and distally by normal ganglionic gut. However, the existence of such ‘skip segments’ has now largely been discredited or at least if such a lesion occurs it is very rare (Nixon, 1964).

Other suggestions include: some sort of vascular disturbance (Ehrenpreis, 1966) or temporary ischaemia (Earlam 1972) to the developing gut destroying ganglion cells. As pointed out by Gray & Skandalakis (1972), such vascular obstruction would presumably result in many cases of ‘skip segments’; again it is doubtful if such a condition exists.
A more plausible explanation comes from Okamoto & Ueda (1967); they propose that the neuroblast migration from the vagal neural crest source stops at an early stage leaving a continuous, distal, aganglionic region. None of these theories seem applicable to the situation seen in the piebald-lethal mouse. In this case, the formation and subsequent length of the aganglionic region is dependent upon the speed of migration of the progenitor ganglion cells. Whether this is the mechanism causing Hirschsprung’s disease in man is unknown.

There is considerable evidence that the primary effect of the \( s^l \), piebald-lethal gene is on the neural crest. Lane (1966) first showed that the myenteric ganglion cells of the distal colon were absent; the enteric ganglia have been shown by a number of authors to be of neural crest origin (Yntema & Hammond, 1954; review Andrew, 1971). Piebald-lethal mice also show extensive white spotting; as the mice have pigmented retinas there is nothing wrong with their genetic capacity to produce melanin so the melanocytes are presumed to be absent from the white areas (Billingham & Silvers, 1960). Melanocytes were first shown to be of neural crest origin by the elegant experiments of Rawles (1947, 1953, 1955). A third neural crest lesion was shown by Deol (1967) in the inner ear of the piebald-lethal mouse. The neural epithelium of the inner ear was invariably affected, although the degree and extent of the abnormality varied. Deol reasoned that the abnormalities of the neural epithelium are reflexions of a deficiency in the acoustic ganglion.

The way in which the mutant gene may act on the neural crest is unknown, but a considerable amount of experimental work has been done on the pigmentation aspect of the defect. Mayer & Maltby (1964), and Mayer (1965, 1967a, b) have investigated spotting in lethal-spotting \((ls)\) mice and in piebald \((s)\) mice. The lethal-spotting mice develop aganglionic megacolon in the same manner as do piebald-lethal mice (Webster, 1971). Mayer concluded that propigment cells migrate to all skin regions of the body, but the skin is a mosaic of specific areas which differ in the level of a factor promoting melanoblast differentiation. The \( s \) and \( ls \) genes make the melanoblasts more sensitive than normal to the effect of varying skin areas. Mayer (1967b) claimed the presence, in piebald skin, of a factor that prevented melanoblast expression only between day 11 and day 12 of gestation.

An alternative to this system has been proposed by Schaible (1969). From a series of breeding experiments in which pigment patterns were selected Schaible proposed that the complete pigmentation of most mammals and birds develops by expansion and merger of clones of pigment cells, from the same centres that appear as pigmented spots against a white background in piebald spotted mutants. Schaible’s conclusion on the way in which white areas are formed includes the idea introduced by Mayer that the neuroblasts must reach the skin at a certain stage and if they arrive late there is inadequate proliferation or no proliferation at all resulting in white areas. As Schaible was unable to demon-
strate any reduction in number of primordial melanoblasts he proposed that piebald, s, and other spotting genes have their effect by retarding melanoblast migration.

In man there have been greatly conflicting reports of other neural crest lesions associated with Hirschsprung’s disease.

REFERENCES

Embryogenesis of enteric ganglia in mice


(Received 14 March 1973, revised 14 June 1973)