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Some observations on the intrinsic nervous mechanism in Hirschsprung's disease

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SUMMARY Both at rest and during transmural stimulation acetylcholine output from isolated longitudinal and circular muscle strips is significantly higher in the spastic segment than in the proximal dilated bowel. No difference has been found in the tissue concentration of acetylcholine between ganglionic and aganglionic specimens.

The pattern of response to transmural stimulation is also similar in the spastic and dilated bowel. However, after cholinergic and adrenergic blockade transmural stimulation fails to induce relaxation in aganglionic specimens, as it does in normal colon.

The hypotheses are advanced that the increase in acetylcholine output may be partly dependent on a failure of the intrinsic modulating mechanisms and that an alteration of the non-adrenergic inhibitory neurons may be involved in the motor disturbances of the aganglionic tract.

Several authors have investigated the structural changes in the intramural plexuses in the aganglionic segment of Hirschsprung's disease (for review see Smith, 1970; Ehrenpreis, 1971). Most recent investigations have been carried out on the distribution of adrenergic nerves in the smooth muscle layers and on the qualitative and quantitative modifications of cholinergic bundles of nerve fibres (Smith, 1967; Bennett, Garrett, and Howard, 1968; Gannon, Noblet, and Burnstock, 1969; Howard and Garrett, 1970a; Weinberg, 1970). Since the earliest histochemical observations on Hirschsprung's disease it has been recognized that the most striking feature in the aganglionic segment is the increase in number and size of intramural cholinergic fibres (Bodian, Carter, and Ward, 1951; Niemi, Kouvalainen, and Hjelt, 1961; Howard and Nixon, 1968; Garrett and Howard, 1969; Howard and Garrett, 1970b). However, little information exists about the activity of intramural nerves in Hirschsprung's disease with regard both to the relationships between the nervous elements and the functioning of the neuroeffector junction (Ehrenpreis, 1970).

The only available data concerning the intramural cholinergic system are often conflicting. For example, the sensitivity of the smooth muscle in the aganglionic portion to cholinergic stimulation has been found to be decreased (Kamijo, Hiatt, and Koelie, 1953; Wright and Shepherd, 1965) or normal (Adams, Marples, and Trounce, 1960; Trounce and Nightingale, 1961). By administering mecholyl, Davidson, Sleisenger, Steinberg, and Almy (1955) have shown that stimulation of muscarinic receptors fails to produce inhibition in the aganglionic segment as it does in the normal descending colon. Similar results, using nicotine, have been obtained by Howard and Nixon (1968).

Few investigations have been carried out on the storage, release, and metabolism of neurotransmitter substances in Hirschsprung's disease. Kamijo *et al* (1953) and Niemi *et al* (1961) found that the content of acetylcholinesterase was increased in the spastic segment but no increase was detected by Adams *et al* (1960). Locally released substances, which are believed not to be putative neurotransmitters, could also be responsible for the hypertonus of the smooth muscle in the spastic segment. Substance P, however, is not likely to be involved because it is present in the aganglionic segment at a lower concentration than in normal bowel (Ehrenpreis and Pernow, 1953).

In order to understand the functional changes in the motor innervation of the smooth muscle in the spastic segment, it is necessary to obtain more detailed information of the disposition of the neurotransmitter substances. The present paper deals with the acetylcholine content and release in the aganglionic part as well as with the pattern of muscular responses during stimulation of the intramural nerve

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fibres. We believe that such observations may lead to a better understanding of the relationships between morphological and functional changes in Hirschsprung's disease.

Material and Methods

Sixty preparations were taken from the large intestine of seven children, aged 4 to 8 years, with fully developed megacolon. Typical Hirschsprung's disease was selected from clinical signs such as severe intestinal obstruction and chronic constipation. Experiments were also carried out in 17 surgical specimens of rectosigmoid colon taken from five patients undergoing extensive resection of the large intestine for carcinoma. Care was taken not to include damaged tissue in the specimens and histological examinations were performed to check their integrity. After mucosal and submucosal layers had been removed and longitudinal muscle thickenings clearly recognized on the serosal surface, strips 25-30 mm long and 4 mm wide were cut from each taenia. Circular strips of similar size were cut transversally from muscular tissue of the intertaenial space. To avoid damage of the intramural plexuses, no attempt has been made to separate the muscular layers so that both the taenial and the intertaenial preparations actually contained longitudinal and circular muscle fibres. However, it is assumed that in the intertaenial preparations circular muscle fibres were predominant.

Preparations were taken from both the narrow segment and, for comparison, from the proximal dilated bowel 5-10 cm above the upper border of the spastic segment. Care was taken not to cut the specimens from the cone-shaped transitional zone. Although structural changes of the intramural plexus from the narrow to the dilated segment are usually abrupt (Ehrenpreis, 1970), nevertheless a microscopic examination was always performed to check the aganglionosis in the former and the presence of normal-sized nerve fibres and ganglion cells in the latter.

The muscle strips were mounted in a 5 ml organ bath containing Krebs solution at 36°C, gassed with a 95% O_2 – 5% CO_2 mixture, and placed under a tension of 2·5 to 3·0 g. After the appearance of spontaneous motility, the ability of the specimens to contract in response to electrical stimulation was checked by applying trains of rectangular pulses lasting 30 sec, of supramaximal strength and 0·5 msec duration at 20 Hz; stimuli were delivered by a modified high impedance Grass S5 stimulator, suitable for transmural stimulation, through a pair of silver sheets (30 mm × 4 mm × 0·3 mm) mounted in parallel 5 mm apart and facing the strip.

The amount of acetylcholine released was collected and assayed in a manner similar to that described for human colonic preparations by Del Tacca, Soldani, Selli, and Crema (1970). Acetylcholine output was measured both at rest and during transmural stimulation. For this purpose, rectangular pulses of 2 msec duration at supramaximal voltage, with a frequency of 10 Hz, were applied throughout a period of 15 min. The total tissue content of acetylcholine was extracted and estimated by the method of Beani and Bianchi (1963) after incubation in Tyrode solution.

The following drugs were used: eserine sulphate, bretylium tosylate, atropine sulphate, dibenamine hydrochloride, propranolol hydrochloride, acetylcholine chloride, (—)-nor-adrenaline bitartrate, (—)-isoprenaline bitartrate, and crystalline tetrodotoxin (Sankyo). The concentrations refer to the salts.

Results

Due to the individual variation in the acetylcholine output from human colonic preparations as well as the difficulty in obtaining samples of normal descending or rectosigmoid colon from healthy children, a comparison has had to be made between samples of the narrow and dilated segments. In order to check the validity of the comparison, the pattern of resting and evoked release of acetylcholine has also been studied in normal specimens taken from adult subjects at the level of the rectosigmoid colon where the spasm more frequently occurs in Hirschsprung's disease. In such preparations, the mean acetylcholine output (± SE) during a 15-min resting period was 12·14 ± 2·75ng/g (five experiments) in the longitudinal specimens and 9·86 ± 1·42 ng/g (five experiments) in the circular ones, while the release associated with transmural stimulation was 113·76 ± 29·97 and 105·46 ± 17·97 ng/g respectively.

The results obtained in Hirschsprung's disease are shown in Figure 1. It appears that both during rest and during transmural stimulation acetylcholine output from the aganglionic specimens is significantly higher than that from the proximal dilated bowel. Analysis of the results obtained in longitudinal and circular strips indicates that the difference between pathological and normal tissue is highly significant in both kinds of preparation, although it is greater in the longitudinal one. The mean percentage difference between acetylcholine output from longitudinal aganglionic and ganglionic specimens (six experiments) was 146·89 ± 30·06 at rest and 169·72 ± 37·57 during stimulation, while in the circular ones (six experiments) it was 71·20 ± 13·18 and 63·58 ± 11·14 respectively.

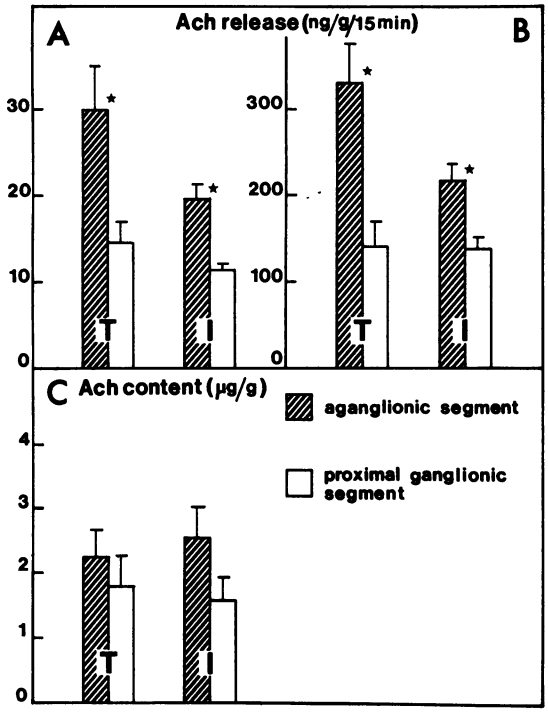

Fig. 1 *Isolated large intestine taken from patients with Hirschsprung's disease. Acetylcholine content and release from longitudinal (T) and circular (I) muscle strips cut in the taenial and intertaenial space of spastic or dilated bowel.*

A: *spontaneous release.* B: *release during transmural stimulation at a frequency of 10 Hz, at twice threshold voltage and 2 msec duration. Each value is the mean of six experiments.*

The asterisk indicates significant differences ($P < 0.05$) *between ganglionic and aganglionic specimens.*

The extent of the increase in release associated with transmural stimulation and the changes of acetylcholine output after blockade of nerve impulses could provide some information about the origin of the acetylcholine released into the bath. In this respect the behaviour of the different groups of preparations seemed to be quite similar. From the normal rectosigmoid preparations (10 experiments) the average ratio between stimulated and spontaneous release was $10{\cdot}24 \pm 1{\cdot}15$. From Hirschsprung's disease preparations the ratio was $11{\cdot}89 \pm 1{\cdot}09$ in the aganglionic segment and $11{\cdot}27 \pm 1{\cdot}21$ in the proximal one, each value being calculated from 12 experiments.

In some experiments acetylcholine output was also measured after tetrodotoxin had been added to the perfusing fluid in a final concentration of 1×10^{-6} g/ml. Tetrodotoxin was able to reduce both spontaneous and evoked release, as it was previously observed in specimens taken from human normal descending colon (Del Tacca *et al*, 1970). The effect was greater on the evoked than on spontaneous release, but no significant difference could be found among the three preparations. The mean percentage reduction of acetylcholine output (each from six experiments) after tetrodotoxin was $55{\cdot}09 \pm 5{\cdot}28$ at rest and $88{\cdot}83 \pm 3{\cdot}95$ during stimulation in normal rectosigmoid preparations, $59{\cdot}83 \pm 4{\cdot}04$ and $92{\cdot}18 \pm 2{\cdot}90$ in Hirschsprung's proximal segment, and $67{\cdot}34 \pm 4{\cdot}68$ and $95{\cdot}36 \pm 2{\cdot}08$ in the aganglionic preparations. The increased release associated with transmural stimulation was almost completely inhibited by tetrodotoxin and the average ratio of evoked to spontaneous release (six experiments) fell to $1{\cdot}96 \pm 0{\cdot}45$ in the normal rectosigmoid colon, $1{\cdot}71 \pm 0{\cdot}28$ in the dilated segment, and $1{\cdot}39 \pm 0{\cdot}30$ in the aganglionic specimens.

The total tissue amount of acetylcholine has been measured in 24 Hirschsprung's specimens and the results obtained are shown in Figure 1C. No significant difference could be found between the specimens taken from the aganglionic and the ganglionic tract and between the longitudinal and the circular preparations of the same segment.

Functional experiments have been carried out in each type of preparation. Spontaneous and stimulated motor activities have been studied in seven longitudinal and circular strips cut from normal rectosigmoid colon and in 12 specimens taken from the narrow and dilated segment of Hirschsprung's disease. There was no difference between the patterns of spontaneous motility; this was fully developed within one hour of incubation in an organ bath and consisted of regular pendular movements with a frequency ranging from 6 to 12 per min. No attempt has been made to test the smooth muscle sensitivity to cholinergic stimulation, eg, by constructing the dose-response curves for acetylcholine. However, no difference could be observed with respect to the threshold concentration of acetylcholine required to elicit a contraction or the relationship between the contractile response and the frequency of transmural stimulation. In each type of preparation, the effect of transmural stimulation was enhanced by pretreatment with eserine at concentration ranging from 5×10^{-7} and 1×10^{-6} g/ml. Contraction elicited by transmural stimulation could always be abolished by tetrodotoxin and by atropine, both at concentrations ranging from 1×10^{-7} and 1×10^{-6} g/ml.

In the ganglionic preparations, both from normal rectosigmoid colon and from Hirschsprung's proximal segment, after pretreatment with atropine, transmural stimulation was able to elicit an inhibi-

tory response, which could not be abolished by combined pretreatment with dibenamine ($2{\cdot}5 \times 10^{-6}$ g/ml), propranolol (1×10^{-6} g/ml), and bretylium (1×10^{-5} g/ml), but which disappeared after tetrodotoxin (5×10^{-7} g/ml) (Fig. 2, upper tracing). It is remarkable that the simultaneous pretreatment with these drugs was able to prevent the effect of acetylcholine, noradrenaline, and isoprenaline added to the bath at the concentration of 1×10^{-6} g/ml. In our preparations, nerve-mediated, non-adrenergic inhibition was a constant and reproducible finding in the rectosigmoid colon, even after repeated stimulation; this has also been observed in other segments of human colon (Crema, Del Tacca, Frigo, and Lecchini, 1968). But in the presence of atropine, dibenamine, propranolol, and bretylium, transmural stimulation failed to elicit relaxation in both the longitudinal and circular aganglionic specimens (Fig. 2, lower tracing). It is noteworthy that after atropine alone, in four out of six aganglionic preparations transmural stimulation elicited a transient relaxation, which could always be abolished by adding dibenamine, propranolol, and bretylium.

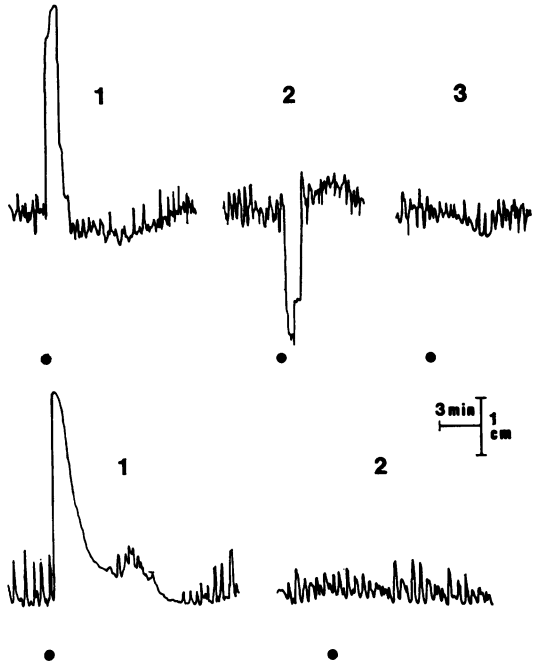

Fig. 2 *Human isolated large intestine from Hirschsprung's disease. Effect of transmural stimulation on the motor activity of two circular specimens taken from the dilated (upper tracing) and from the narrow (lower tracing) segments.*

At the mark (●) transmural stimulation lasting 30 sec, with 0·5 msec duration, 10 Hz frequency and supramaximal strength was applied.

Between 1 and 2 atropine, dibenamine, propranolol, and bretylium were added to the bath at the final concentrations of 1×10^{-6}, $2{\cdot}5 \times 10^{-6}$, 1×10^{-6}, and 1×10^{-5} g/ml respectively. Between 2 and 3 tetrodotoxin was added at the concentration of 5×10^{-7} g/ml.

Note that after cholinergic and adrenergic blockade, transmural stimulation relaxes the dilated bowel but fails to induce relaxation in the aganglionic segment.

Discussion

If one excludes an alteration of smooth muscle contractility, the hypertonus of the aganglionic segment could be accounted for by an hyperactivity of the excitatory nervous pathways or by a deficiency of the inhibitory ones.

The occurrence of an hypersensitivity of the smooth muscle to cholinergic stimulation has been ruled out by Kamijo *et al* (1953) and Wright and Shepherd (1965), who observed a decreased sensitivity of the aganglionic specimens to acetylcholine. It is possible that the reduced sensitivity to acetylcholine of the spastic segment is the consequence of the unusually rich cholinergic innervation. A similar decrease in sensitivity of the intestinal musculature has been observed by Kuzmicheva, Orlova, Rodionov *et al* (1969) for noradrenaline after an experimentally induced increased innervation by adrenergic fibres.

The great increase in acetylcholine output both at rest and during transmural stimulation suggests that a hyperactivity of the intrinsic cholinergic mechanisms occurs in the aganglionic preparations. However, the question should be raised as to the origin of the acetylcholine in these preparations. At present, we do not know of a suitable method of distinguishing the neuronal from the non-neuronal sources of acetylcholine in these types of intestinal preparation. Since tetrodotoxin is known to prevent conduction in nerves, it may be concluded that at least 90% of the increased release of acetylcholine associated with transmural stimulation and 60% of the resting or spontaneous release is nervous in origin. Furthermore, there is no evidence that the remaining 40% tetrodotoxin-resistant release during rest does not also originate from neuronal sources (Ogura, Mori, and Watanabe, 1966; Goldenberg, 1971). In support of the conclusion that in our preparations the main source of acetylcholine was the intrinsic nerves is the finding that the ratio of the evoked to spontaneous release was so high. We cannot completely exclude damage of the tissue by surgical procedures (including the effect of anoxia and of anaesthetic substances). This is an inevitable disadvantage of all the experiments carried out on

human intestinal preparations. However it is remarkable that in our experiments the ratio of evoked to spontaneous release was remarkably similar to that found in animal colonic specimens where surgical damage is negligible (Beani, Bianchi, and Crema, 1969).

In our experiments, no significant increase in the acetylcholine content has been observed in the narrow segment compared with the dilated portion. With respect to possible alterations of disposition of acetylcholine in the aganglionic part, our results are not completely conclusive because they refer only to the total amount in the tissue. Even if the total content were unchanged, the mechanisms of storage of acetylcholine may be altered. At present, there is not sufficient information about the mechanism of storage and release in intramural plexuses in health and disease (Hoar, Paton, and Smith, 1971). Moreover, at present, although some data exist about the cholinesterase activity of the spastic segment, information about cholinacetylase activity is lacking.

Nevertheless, because we have found that the change of content does not parallel the increase of release, a defect in the intrinsic modulation of the acetylcholine release may be involved. Moreover, if the total number of cholinergic fibres is actually increased despite the lack of intramural ganglia, then, since the total content is unchanged, the cholinergic fibres of the aganglionic part would each contain a smaller amount of acetylcholine than those of the normal intestine.

As far as the inhibitory system is concerned, the ability of sympathetic nerve stimulation and that of sympathomimetic amines to modulate acetylcholine release from intramural plexuses has been demonstrated both in animals (Paton and Vizi, 1969; Beani *et al*, 1969; Kosterlitz, Lydon, and Watt, 1970) and in the human intestine (Del Tacca *et al*, 1970). Since it is known that adrenoceptors are located both on ganglia and on nerve endings, adrenergic modulation could also operate in the absence of ganglia by means of a direct action on the nerve fibres. In this connexion it is interesting to note that Bennett *et al* (1968) and Gannon *et al* (1969) have observed that more adrenergic fibres are present in the aganglionic segment than in the normal bowel. Therefore, since both the sympathetic modulating mechanism and the sites on which it could impinge are present, any failure of adrenergic modulation should be ascribed only to a functional defect for which there is no evidence at the morphological level.

If the muscular contraction of the narrow segment is due, at least in part, to a deficiency of an inhibitory nervous mechanism (Howard and Nixon, 1968; Shepherd and Wright, 1968; Gannon *et al*, 1969; Howard and Garrett, 1970a), obviously the adrenergic or non-adrenergic inhibitory structures could be involved (Burnstock, Campbell, Satchell, and Smythe, 1970). The presence of a non-adrenergic inhibitory system in the human colon has been postulated by Crema *et al* (1968), who observed that a nerve-mediated inhibition could be elicited by transmural stimulation after adrenergic blockade. However, if a functional deficiency of the adrenergic system alone occurred in the aganglionic segment and thus contribute to the contraction, it would still not be enough to account for a failure of propulsion. In fact, from animal experiments it appears that sympathetic denervation alone does not abolish peristaltic activity in the colon, rather the latter can be prevented by an impairment of the non-adrenergic inhibitory pathway involved in the peristaltic reflex (Crema, Frigo, and Lecchini, 1970).

In our experiments the failure of transmural stimulation to elicit a nerve-mediated, non-adrenergic relaxation in the aganglionic specimens supports the hypothesis that, in Hirschsprung's disease, the non-adrenergic inhibitory structures are in some way affected. We suggest that both a hyperactivity of the intramural cholinergic nerves as well as a deficiency of non-adrenergic inhibitory pathways may be involved in the disturbance of the balance between excitatory and inhibitory innervation in the spastic segment.

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