Histochemical diagnosis of Hirschsprung’s disease and a comparison of the histochemical and biochemical activity of acetylcholinesterase in rectal mucosal biopsies

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SUMMARY Three hundred and seventy-two rectal mucosal biopsies, taken from 150 children and young adults with chronic constipation, were subjected to histochemical and biochemical analysis of acetylcholinesterase to exclude Hirschsprung’s disease. The relative merits of the procedures were compared. The histochemical method was considered to be the most practical for laboratories handling small numbers of biopsies but the biochemical estimation of acetylcholinesterase activity was found to be a useful complementary procedure and an accurate quantitative assessment of enzyme activity.

The introduction of acetylcholinesterase histochemistry has resulted in a reliable means of excluding Hirschsprung’s disease in rectal mucosal biopsies.

Previously, the diagnosis of Hirschsprung’s disease depended on standard histological techniques, although some workers have used histochemical methods to confirm the diagnosis on resected specimens.

Acetylcholinesterase histochemistry is now used in preference to routine histological methods in many centres. Its diagnostic reliability in rectal mucosal biopsies has been emphasised by Meier-Ruge and his colleagues and confirmed by other workers and ourselves.

The present paper is an account of our experience over a two-year period of the use of acetylcholinesterase histochemistry as a screening procedure to exclude Hirschsprung’s disease in rectal mucosal biopsies and compares the histochemical findings with the biochemical activity of the enzyme.

Material and methods

Over a two-year period, which ended in January 1979, 372 rectal mucosal biopsies were performed on 150 children and young adults aged between 6 days and 28 years (Table 1) and the specimens were processed for acetylcholinesterase histochemistry.

Table 1 Age and sex incidence of patients biopsied: numbers of cases of Hirschsprung’s disease in parentheses

<table>
<thead>
<tr>
<th>Age group</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4 weeks</td>
<td>7 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1-12 months</td>
<td>11 (0)</td>
<td>7 (1)</td>
</tr>
<tr>
<td>1-4 years</td>
<td>30 (4)</td>
<td>18 (1)</td>
</tr>
<tr>
<td>5-12 years</td>
<td>52 (6)</td>
<td>18 (0)</td>
</tr>
<tr>
<td>13-20 years</td>
<td>3 (1)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>&gt; 21 years</td>
<td>1 (0)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>Totals</td>
<td>104 (15)</td>
<td>46 (2)</td>
</tr>
</tbody>
</table>

The biopsies were taken by means of laryngeal punch biopsy forceps at several levels from the anal margin (Table 2). The patients attended hospital as day cases on prearranged days of the week, and the procedure was carried out with sedation or under mild general anaesthesia. The biopsy specimens were transported to the laboratory on ice and orientated under a dissecting microscope; frozen sections were taken for histochemistry within an hour of removal.

Table 2 Levels of individual biopsies: number of biopsies from cases of Hirschsprung’s disease in parentheses

<table>
<thead>
<tr>
<th>Level (cm)</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td>180 (23)</td>
<td>50</td>
</tr>
<tr>
<td>6-10</td>
<td>35 (18)</td>
<td>10</td>
</tr>
<tr>
<td>11-15</td>
<td>9 (4)</td>
<td>3</td>
</tr>
<tr>
<td>&gt; 15</td>
<td>3 (3)</td>
<td>1</td>
</tr>
<tr>
<td>Not stated</td>
<td>130 (27)</td>
<td>36</td>
</tr>
<tr>
<td>Totals</td>
<td>357 (75)</td>
<td>100</td>
</tr>
</tbody>
</table>

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Fifteen specimens were subsequently excluded because they were either too badly traumatised or because they were of anal as opposed to rectal mucosa.

One hundred and sixteen of the original 372 biopsy specimens were divided longitudinally so that half of the specimen went for histochemical and the other half for biochemical analysis of acetylcholinesterase. The piece of tissue for biochemistry was wrapped in aluminium foil, snap frozen, and stored for up to one year in the vapour phase of liquid nitrogen before biochemical analysis.

HISTOCHEMICAL METHOD

Reagents
(1) Substrate: Acetylthiocholine iodide (AThCh) 5 mg, dissolved in 0.1 M-acetate buffer at pH 5.5, with the addition of 0.5 ml 0.1 M-sodium citrate, 1.0 ml 30 mM-cupric sulphate, 1.0 ml distilled water, 1.0 ml 5 mM-potassium ferricyanide, and 0.2 ml 0.01% (w/v) tetraminoisopropylpyrophosphoramide (iso-OMPA).
(2) DAB/Hanker-Yates solution: 3.3-diaminobenzidine tetrahydrochloride (DAB) 5 mg, dissolved in 10 ml 0.1 M-sodium phosphate buffer at pH 6.8; subsequently Hanker-Yates reagent, 5 mg, dissolved in 10 ml 0.1 M-sodium phosphate buffer at pH 6.8.

Procedure
The histochemical method used was based on the modification by Karnovsky and Roots of that originally described by Koelle and Friedenwald using acetylthiocholine iodide as substrate.

Cryostat sections, cut at 10 μm, were fixed in standard formol calcium for 1 minute, washed in distilled water, and incubated with the substrate for 1 hour at 37°C in the presence of iso-OMPA as a selective irreversible inhibitor of non-specific cholinesterase activity. After incubation the sections were washed in distilled water, and the reaction was enhanced by treatment with DAB for 45 minutes and osmium tetroxide for 1 minute. The sections were counterstained with haematoxylin and mounted.

The histochemical procedure was carried out routinely on three sections; a fourth control section was incubated in the absence of substrate, and a fifth was stained with haematoxylin and eosin.

BIOCHEMICAL METHOD

Reagents
(1) Substrate (0.015 M AThCh): Acetylthiocholine iodide (AThCh), 21.67 mg, added to 4.95 ml distilled water followed by 0.05 ml 1 M-hydrochloric acid.
(2) Colour reagent (0.01 M DTNB): 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 39.5 mg, and sodium bicarbonate 15 mg, dissolved in 10 ml 0.1 M-phosphate buffer pH 7.0.
(3) Inhibitor (8.52 × 10⁻⁴ M Lysivane): 10-(2-diethylaminopropyl)phenothiazine hydrochloride (ethopropazine HCl, Lysivane), 27.1 mg, dissolved in 30 ml 2 M-hydrochloric acid and made up to 100 ml with 0.1 M-phosphate buffer pH 7.0.

Procedure
The biochemical method used was a modification by Dale and his colleagues of a photometric method originally described by Ellman et al.

The assays were carried out without prior knowledge of the histochemical findings as single determinations as there was usually insufficient material to do otherwise. The biopsies were trimmed to give a final specimen weighing less than 10 mg. The tissue was hand-homogenised in 0.1 M-phosphate buffer at pH 8.0 in a glass Potter-Elvehjem type homogeniser using 15 strokes. The volume of buffer was sufficient to give a final concentration of 10 mg tissue per ml. The homogenate was centrifuged at 12 000 g for 4 minutes to remove cellular debris. Each stage of the procedure was carried out at approximately 5°C.

Two enzyme determinations were made on each sample, corresponding to (1) total acetylcholinesterase activity and (2) true acetylcholinesterase activity. The total (AChE + ChE) activity was determined at 25°C in microcuvettes by adding 80 μl of tissue extract to 480 μl 0.1 M-phosphate buffer at pH 8.0 and 20 μl colour reagent (DTNB). After allowing the solutions to stabilize for 5 minutes, 20 μl of substrate (AThCh) was added. The rate of change of absorbance per minute was measured at 412 nm with a recording spectrophotometer. Following determinations of total activity, specific acetylcholinesterase (AChE) activity was measured by the addition of 10 μl of the non-specific cholinesterase (ChE) inhibitor, Lysivane.

Results
The product of the histochemical reaction appears as a dark brown precipitate at the site of nerve fibres and ganglia.

In normal mucosal biopsies, a few clearly defined slender nerve fibres are present in the connective tissue of the lamina propria and between muscle bundles in the muscularis mucosae (Fig. 1). The nerve fibres in the submucosa occur in bundles, and ganglion cells are usually prominent, the reaction appearing as fine particulate deposit in the cytoplasm. The supportive Schwann cells are also con-
The range of AChE and ChE activity in nine biopsies showing the histochemical features of Hirschsprung's disease was 5.3-30.2 units (mean 15.2, SD 9.0) and 2.1-7.9 units (mean 3.8, SD 2.0) respectively (Figs 4 and 5). In six biopsies which were histochemically equivocal, that is to say, biopsies showing some increase in the number of nerve fibres in the muscularis mucosae and, to a lesser extent, the lamina propria, AChE activity (Fig. 4) was within the normal range 1.7-5.7 units (mean 3.7, SD 1.7).

AChE activity expressed as a percentage of total activity in the 101 normal biopsies (Fig. 6) was in the range 27.79% (mean 54.6, SD 10.5), and, for the nine biopsies showing the histochemical features of Hirschsprung's disease, in the range 70.84% (mean 78.9, SD 6.8).

Discussion

Over 75% of the patients investigated in our series were aged between 1 and 12 years (Table 1). A higher proportion of the cases presenting in infancy were found to have Hirschsprung's disease. Males outnumbered females both in the number of cases presenting with a history of chronic constipation and in the number who subsequently were shown to have Hirschsprung's disease. This remarkable preponderance of males with Hirschsprung's disease has been noted by others,28-30 and our male to female ratio is close to the 8:1 ratio reported in negro infants by Leenders and his colleagues.29

The histochemical criteria for the diagnosis of Hirschsprung's disease as stated above are those generally accepted by other workers.13-17,19 The increase in the number and size of nerve fibres in the muscularis mucosae and lamina propria is a particularly striking feature, and a diagnosis can be made confidently without reference to the ganglia in the submucosa,13,19 a view that conflicts with that of at least two other groups who consider that the presence of submucosa is essential to accurate diagnosis.10,16 The absence of abnormal nerve fibres in the normal hypoganglionic zone immediately above the pectinate line described by Aldridge and Campbell31 would exclude the disease.

In the histochemical assessment of the sections, care must be taken to be certain that structures resembling ganglia in the submucosa do actually contain ganglion cells and are not just a cluster of supportive cells. As Naik and Cauna32 pointed out, both types of cell show moderate AChE activity. Difficulty may also be encountered if cellular detail in the lamina propria is obscured by haemorrhage.17,19

Accurate definition of the aganglionic segment in Hirschsprung's disease would require biopsies to be

spicuous, and the reaction usually outlines smooth muscle elements.

The biopsies in cases of Hirschsprung's disease show a marked increase in the number and size of positively staining nerve fibres in the muscularis mucosae and lamina propria (Figs 2 and 3), an increase in the size of the nerves in the submucosa, and an absence of submucosal ganglia. The abnormal nerve plexuses in the muscularis mucosae and lamina propria are not readily seen in haematoxylin and eosin stained sections.

The biochemical activity of acetylcholinesterase was expressed according to Dale and his colleagues26 as units of specific (AChE) and non-specific (ChE) activity. The specific AChE activity was also expressed as a percentage of total (AChE + ChE) activity.

In 101 biopsies showing a normal histochemical reaction, AChE activity (Fig. 4) was in the range 0.3-7.9 units (mean 2.7, SD 1.4) and ChE activity (Fig. 5) in the range 0.3-5.8 units (mean 2.6, SD 1.0).
Fig. 2  Rectal mucosa in Hirschsprung's disease with an increase in the number and size of AChE positive nerve fibres (single arrows) in the connective tissue of the lamina propria (lp) and muscularis mucosae (mm), and aggregates of Schwann cells in the submucosa (sm) that could be mistaken for ganglia (short arrow). × 38.

Fig. 3  Superficial rectal mucosa in Hirschsprung's disease with a marked increase in the number and size of AChE positive nerve fibres (single arrow) in the lamina propria: L = lumen. × 47.
Fig. 4 AChE activity in biopsy specimens showing a normal histochemical reaction (A), and in those with the histochemical features of Hirschsprung's disease (B). The specimens that were histochemically equivocal are represented by interrupted lines.

Fig. 5 ChE activity in biopsy specimens showing a normal histochemical reaction (A), and in those with the histochemical features of Hirschsprung's disease (B). The specimens that were histochemically equivocal are represented by interrupted lines.
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Fig. 6. AChE activity expressed as a percentage of total activity in biopsy specimens showing a normal histochemical reaction (top) and in those with the histochemical features of Hirschsprung's disease (bottom). The specimens that were histochemically equivocal are represented by interrupted lines.

taken at several levels from the anal margin. We aimed to make these levels below 5 cm, between 5 and 10 cm, and above 10 cm to correspond with our definition of ultrashort, short, and long segment Hirschsprung's disease respectively.83 The levels were proportionately less in younger children. In practice, eight of the 17 cases of Hirschsprung's disease in our series required repeat biopsies to define the aganglionic segment, 80% of all our cases had biopsies taken from only one or two levels, and 52% of the biopsies were from levels less than 5 cm from the anal margin. For 36% of the biopsies the level was not stated.

Biopsies were obtained routinely from more than one level in three of the previously reported series. Chow and his colleagues16 obtained a minimum of three biopsies 3, 4, and 5 cm from the anal verge in younger children and 5, 6, and 7 cm from the anal verge in older children; Martinez-Almoyna and his colleagues18 had biopsies taken at 3, 5, and 10 cm from the pectinate line; and in the series of Lake and his colleagues19 the biopsy specimens were taken at 2, 4, and 5 cm from the mucocutaneous junction in most cases. The levels chosen by Chow and his colleagues16 in younger children and by Martinez-Almoyna and his colleagues18 in older children are in line with our recommendations and diagnostic approach to children with chronic constipation20 and would be more likely to define accurately the length of any aganglionic segment. In our opinion, this would directly influence the surgical management and obvitate the need for costly radiographic and monometric investigations.84

Up to 150 sections may have to be examined to exclude Hirschsprung's disease using standard histological techniques,5–7 whereas with the histochemical method a maximum of five sections is all that is required for accurate diagnosis. In 96% of our cases the diagnosis was unequivocal, and in no instance did we have a false-positive result. This compares favourably with histochemical results reported by other authors and is as good as the results obtained using routine histological methods.7,15,30

Established techniques were used in this study for both the histochemical and biochemical methods. The essential difference between the two methods was in the inhibitors of non-specific cholinesterase (ChE) activity. The inhibitors used (iso-OMPA and Lysivane, respectively) were not the same for each method, but both have been shown to be selective inhibitors of ChE activity10,38 and therefore would not be expected in any way to complicate the results.

The results we obtained biochemically are in almost all instances directly comparable with the histochemical findings, and we agree with Dale and his colleagues21 that the biochemical estimation of AChE activity is a useful complementary procedure, particularly if the histochemical diagnosis is equivocal. Overall the levels of activity are lower in our series than in Dale's—something that may have been due to the fact that the tissue was stored before the analyses were carried out. The figures for AChE activity expressed as a percentage of total activity suggest that the increased activity in Hirschsprung's disease is a true increase in specific (AChE) activity.

Hirschsprung's disease was confirmed in 16 of our 17 cases (Table 1) by histological and histochemical examination of specimens obtained at myectomy or after resection. The remaining case was a microcephalic infant who died before definitive treatment could be undertaken. Of the 16 confirmed cases, six had long segment disease, eight had short segment
disease, and two had ultrashort segment disease by our definition. Four of the six histochemically equivocal biopsies with biochemical AChE activity within the normal range were from this single unconfirmed case of Hirschsprung’s disease in the series.

For most laboratories handling small numbers of biopsies the histochemical method is probably the more practical. Both methods are straightforward and inexpensive, but the biochemical assay has the additional advantage of offering an accurate quantitative assessment of enzyme activity.

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