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>
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<tr>
<th>Table 1</th>
<th>Age and sex incidence of patients biopsied: numbers of cases of Hirschsprung’s disease in parentheses</th>
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<tr>
<td>Age group</td>
<td>Males</td>
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<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td>0-4 weeks</td>
<td>7 (4)</td>
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<tr>
<td>1-12 months</td>
<td>11 (0)</td>
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<tr>
<td>1-4 years</td>
<td>30 (4)</td>
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<tr>
<td>5-12 years</td>
<td>52 (6)</td>
</tr>
<tr>
<td>13-20 years</td>
<td>3 (1)</td>
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<tr>
<td>&gt; 21 years</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Totals</td>
<td>104 (15)</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>
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<tr>
<th>Table 2</th>
<th>Levels of individual biopsies: number of biopsies from cases of Hirschsprung’s disease in parentheses</th>
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<tr>
<td>Level (cm)</td>
<td>Number</td>
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<td>----------</td>
<td>--------</td>
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<tr>
<td>1-5</td>
<td>180 (23)</td>
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<tr>
<td>6-10</td>
<td>35 (18)</td>
</tr>
<tr>
<td>11-15</td>
<td>9 (4)</td>
</tr>
<tr>
<td>&gt; 15</td>
<td>3 (3)</td>
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<tr>
<td>Not stated</td>
<td>130 (27)</td>
</tr>
<tr>
<td>Totals</td>
<td>357 (75)</td>
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</tbody>
</table>

Received for publication 8 October 1979
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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) tetramoisopropylpyrophosphoramide (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 6.8.22

Procedure
The histochemical method used was a modification by Dale and his colleagues26 of a photometric method originally described by Ellman et al.27

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 stabilise 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.

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-diethylamino-propyl)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 colleagues26 of a photometric method originally described by Ellman et al.27

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.

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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,18-30 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.18-17 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.15 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 Cauda32 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

Fig. 1 Normal rectal mucosa with slender AChE positive nerve fibres (single arrows) in the connective tissue of the lamina propria (lp) and muscularis mucosae (mm), and prominent ganglia (double arrow) in the submucosa (sm). × 25.

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).
Histochemical diagnosis of Hirschsprung's disease

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).  $\times$ 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.  $\times$ 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.
Histochemical diagnosis of Hirschsprung's disease

Histochemical diagnosis features of Hirschsprung's histochemical activity

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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.3 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-OPMA and

Lysivane, respectively) were not the same for each

method, but both have been shown to be selective

inhibitors of ChE activity 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 histo-

chemical findings, and we agree with Dale and his

colleagues21 that the biochemical estimation of AChE

activity is a useful complementary procedure, par-

ticularly 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 micro-

cephalic infant who died before definitive treatment
could be undertaken. Of the 16 confirmed cases, six

had long segment disease, eight had short segment

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 obviate the need for costly radiographic and

monometric investigations.34

Up to 150 sections may have to be examined to

exclude Hirschsprung's disease using standard

histological techniques,5-7 whereas with the histo-

chemical 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

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after resection. The remaining case was a micro-

cephalic infant who died before definitive treatment
could be undertaken. Of the 16 confirmed cases, six

had long segment disease, eight had short segment

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.33 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

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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 obviate the need for costly radiographic and

monometric investigations.34

Fig. 6 AChE activity expressed as a percentage of total

activity in biopsy specimens showing a normal histo-

chemical 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.

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<tr>
<th>% AChE ACTIVITY</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>2</td>
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<td>4</td>
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<td>6</td>
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<td>8</td>
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<table>
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<tr>
<th>% AChE ACTIVITY</th>
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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.

We thank Mr A R Cobb and Mr T McLaren for technical assistance; Mr F Coleman for help with the preparation of the illustrations; and Mrs Rhona Anderson for typing the manuscript. We are also grateful to Dr G Dale for supplying us with a sample of Lysivane.

References

26 Dale G, Bonham JR, Riley Katherine WA, Wagjet J. An improved method for the determination of acetylcholinesterase activity in rectal biopsy tissue
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Scobie WG. Personal communication, 1979.


Requests for reprints to: Dr WJA Patrick Department of Paediatric Pathology, Royal Hospital for Sick Children, Glasgow, UK.
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doi: 10.1136/jcp.33.4.336

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