

Acetylcholinesterase Histochemistry of Rectal Suction Biopsies in the Diagnosis of Hirschsprung's Disease

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Rectal suction biopsy with acetylcholinesterase (AChE) histochemistry has gained increased acceptance as the means of definitely diagnosing Hirschsprung's disease (HD) as well as of excluding this diagnosis when evaluating children with low intestinal obstruction or chronic constipation since the report of Meier-Ruge et al. in 1972. But this AChE histochemical study has not been reported yet in Korea.

During the 14-month period from April, 1991 through June, 1992, 37 children, aged 3 days to 17 years had rectal suction biopsies for the diagnosis or exclusion of HD. In this study, AChE histochemistry (N = 37) was compared with hematoxylin & eosin (H&E) staining of same suction biopsy specimens (N = 35) for diagnostic accuracy. The histochemical criterion used for the diagnosis of Hirschsprung's disease was that of Chow et al. (1977), i.e., the presence of many coarse discrete cholinergic fibers in the muscularis mucosae and in the immediately subjacent submucosa regardless of an infiltration of cholinergic fibers in the lamina propria.

Of 13 biopsies from the patients with Hirschsprung's disease (N = 13), there were 12 positive reactions, and one false negative reaction in a neonate with total colonic aganglionosis. All biopsies from 24 unaffected children demonstrated negative reactions with no false positive reaction. In comparison, of the 35 specimens examined by H&E staining, ganglion cells were present in the submucosal Meissner's plexus only in 15 of these 24 unaffected children. In conclusion, a 97% diagnostic accuracy was achieved with AChE histochemistry compared with a 74% accuracy with H&E staining (P < 0.01).

AChE histochemistry in demonstrating cholinergic fibers in rectal suction biopsies was found to be a reliable, safe, and rapid tool for the diagnosis of HD and a number of advantages of this AChE histochemistry over manometric, radiologic, suction biopsy with routine histology, and full thickness biopsy techniques, were discussed.

Key Words: Acetylcholinesterase, Rectal suction biopsy, Hirschsprung's diseases.

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INTRODUCTION

Traditionally, the diagnosis of HD has been made by means of a full thickness rectal biopsy and confirmation of the absence of ganglion cells in Auerbach's plexus, but this requires general anesthesia and is associated with some morbidity and technical difficulty. Since the development of rectal suction biopsy tech-

initiated by Noblett (1969) and the introduction of AChE histochemistry by Meier-Ruge et al. (1972), AChE histochemical staining of rectal suction biopsies has gradually replaced conventional full thickness biopsy at many institutions.

The fact that there is no general agreement on the criteria necessary for establishing the diagnosis of HD, disputes the validity of this method as the sole means of diagnosing HD (Hamoudi et al., 1982; Goto et al., 1983; Trigg et al., 1974).

The aim of this study is to evaluate the validity of AChE histochemistry for the diagnosis or exclusion of HD, using the criteria advocated by Chow et al. (1977) and de Brito & Maksoud (1987). Furthermore, we compare the diagnostic accuracy of AChE histochemistry with routine histology using H&E performed simultaneously.

MATERIALS & METHODS

Patients profile

From April 1991 to June 1992, 37 patients, aged from 3 days to 17 years, underwent rectal suction biopsies with AChE histochemistry (N=41) and with routine histology using H&E (N=35) performed simultaneously (Table 1).

The main clinical features are bowel obstruction in 18 patients, chronic constipation in 14 patients, and colo- or ileostomy state in 7 patients.

Thirteen (32%) of the 41 suction biopsies were taken from the neonates and 30(73%) of the 41 suction biopsies were taken from the infants including the neonates (Table 2).

Inadequate specimens showing only lamina propria

Table 1. Patients Profile

	No. of Pts	No. of Bx	Age Range
Hirschsprung D	13	16	3d-17y
Unaffected	24	25	3d-5y
Total	37	41	

* During 14 months from April 91 to June 1992

Table 2. Age Distribution at Biopsy

AGE	HD	Unaffected	Total
-1m	6	7	13
2-6m	5	7	12
7-12m	3	2	5
1-3y	1	3	4
4-6y	0	5	5
7y<	1	1	2

or stratified squamous epithelium were excluded in this study.

Suction biopsy technique

Suction biopsies were performed without preparation or general anesthesia at the bedside or in an outpatient setting using the Quinton multipurpose pediatric biopsy instrument (Model 4.7mm). The capsule with two apertures was inserted approximately 2 to 3cm above the dentate line, suction of 15-20 inch Hg was applied to the system and the capsule knife activated. The average size of the specimen was 3-4mm at its longest diameter. At least two specimens were taken. Half of the specimens were frozen for AChE histochemistry and the remainder were fixed in formalin for routine H&E staining.

AChE histochemical staining

For the demonstration of AChE activity, the method of Karnovsky and Roots (1964), modified by Meier-Ruge et al. (1972), was used.

The specimens were stored immediately at -70°C in a deep freezer (Cryostar, Queue Systems). Six micron-thick cryostat sections were taken and checked with H&E staining to ensure that both mucosa and submucosa were present. Before use of the incubation medium part A&B (Table 3), they were mixed and prewarmed in a coplin jar to 37°C . Air-dried sections fixed in cold (4°C) 4% neutral formalin (5 min.) were thoroughly rinsed in distilled water, then incubated at 37°C for 60 min. in an incubator (Sanyo). Sections were rinsed again in distilled water, then slightly counterstained with hematoxylin. They were then dehydrated over absolute alcohol, cleared by xylene and covered with balsam.

Interpretation of AChE histochemistry

The histochemical criterion used for the diagnosis of HD was that of Chow, i.e., "the presence of many coarse discrete cholinergic fibers in the muscularis mucosae and in the immediately subjacent submucosa regardless of an infiltration of cholinergic fibers in the lamina propria."

Positive reaction in patients with HD was further classified into three patterns, advocated by de Brito and Maksoud (1987): Pattern I-many thick fibers exclusively in the muscularis mucosae and submucosa (Newborn pattern). Pattern II-many thin fibers in the muscularis mucosae and submucosa with a clear infiltration of cholinergic fibers in the lamina propria (Classical pattern). Pattern III-an intermediate pattern showing morphological characteristics of the two patterns with

predominance of one or the other.

Negative reaction was defined as an absence of

AChE positive fibers or their bare presence in the muscularis mucosae or immediately subjacent submucosa.

Table 3. Incubation media

Incubation Medium Part A:		
Acetylthiocholine iodide		0.24g
0.06M Sodium acetate 3H ₂ O	(820mg/100ml)	302.4ml
0.1M Acetic acid	(600mg/100ml)	9.6ml
0.1M Sodium citrate	(2.94g/100ml)	24ml
0.03M Copper sulfate 5H ₂ O	(750mg/100ml)	48ml
0.004M Tetraisopropyl phosphoramidate	(137mg/100ml)	9.6ml
Distilled water		37.2ml
Snap freeze and store in volumes of 9ml at -25°C		
Incubation Medium Part B:		
Potassium ferricyanide		0.165g
Distilled water		100ml
Store in volumes of 1ml at -25°C		

Table 4. AChE Histochemistry

	HD	Unaffected
Positive	12	0
Pattern I → (5)		
Pattern II → (4)		
Pattern III → (3)		
Negative	1	24

* Sensitivity 92%, Specificity 100%
 Positive predictive value 100%
 Negative predictive value 96%
 Accuracy 97%

Table 5. Analysis of Patients with Hirschsprung's Disease by Pattern

Case	Age	Sex	AChE Pattern	Final Diagnosis
1 CBB	3d	m	I	short segment HD
2 NBB	3d	m	I	short segment HD
3 PBB	13d	m	I	short segment HD
4 LHY	4d	m	I	long segment HD
	5m*		II	
5 KSH	12d	m	I	total colonic aganglionosis
	7m*		III	
6 KSM	21d	m	Neg.	total colonic aganglionosis
	7m*	m	III	
7 KYH	2m	m	III	long segment HD
8 KCS	3m	m	III	short segment HD
9 PMJ	3m	f	II	short segment HD
10 LCW	5m	m	II	short segment HD
				with rectal perforation
11 HBJ	6m	m	III	short segment HD
12 KMH	2y	m	II	short segment HD
13 LWB	17y	m	II	short segment HD

*: second biopsy

Statistical analysis

We used Fisher's exact test to compare the diagnostic accuracy of AChE staining with routine H&E staining.

RESULTS

Positive AChE reaction

Of 13 biopsies from the patients with HD (N=13), 12 demonstrated a positive reaction representing a sensitivity of 92% (Table 4). The patients with positive reactions were established to have HD at surgery. There was one false negative reaction in a 21-day old neonate (Case 6) with total colonic aganglionosis but a second biopsy taken at the age of 7 months

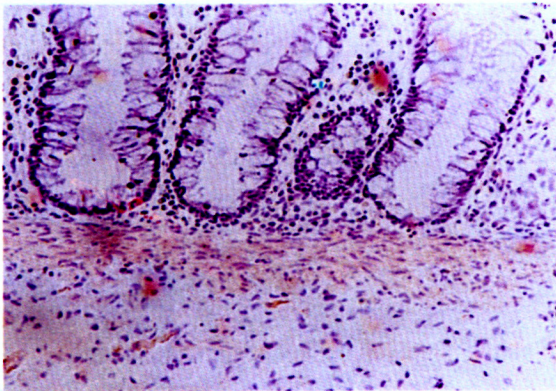


Fig. 1. A 2 week-old female neonate with a normal AChE activity. There is no evidence of cholinergic fibers in the mucosa and submucosa. AChE, counterstained with hematoxylin ($\times 100$).

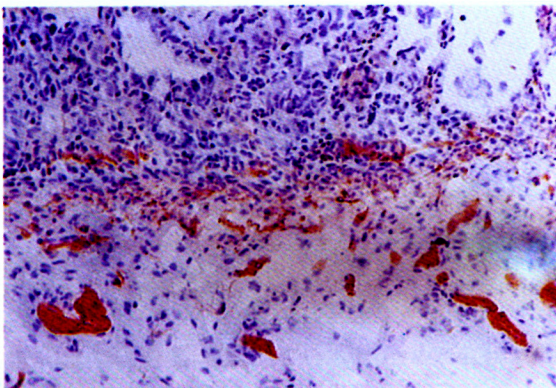


Fig. 2. Case 5 with pattern I (newborn pattern). Thick AChE positive cholinergic fibers distribute diffusely in the muscularis mucosae and submucosa, but the lamina propria is clear. AChE, counterstained with hematoxylin ($\times 100$).

demonstrated pattern III reaction (Table 5). Pattern I was exclusively observed in 5 out of 6 neonatal patients while pattern II and III were seen in 5 children each beyond the neonatal period at an average age of 58 months and 5 months, respectively (Table 4&5, and Figure 2, 3&4).

Negative AChE reaction

All biopsies from 24 unaffected children demonstrated a negative reaction (Table 4 and Figure 1). All these children except one thrived normally and their problems were managed successfully without surgical procedure. A 4 year-old girl with chronic constipation and hydrocephalus was wrongly diagnosed to have short segment HD by a typical cone shaped tran-

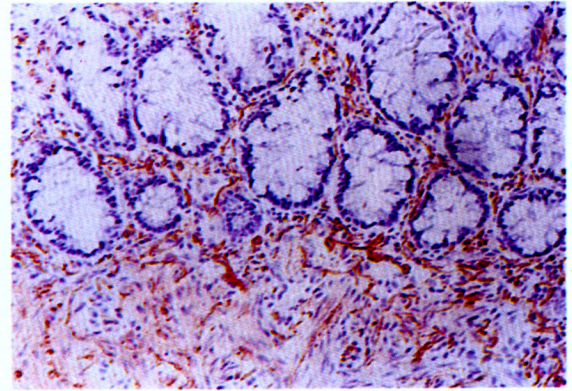


Fig. 3. Case 10 with pattern II (classical pattern). Thin AChE positive fibers distribute diffusely in the muscularis mucosae, submucosa, and in the lamina propria. AChE, counterstained with hematoxylin ($\times 100$).

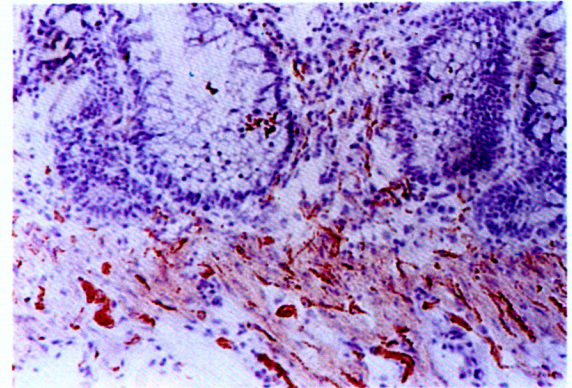


Fig. 4. Case 7 with pattern III (intermediate pattern). Thick and thin AChE positive cholinergic fibers distribute in the muscularis mucosae and submucosa. Also noted is the presence of this AChE activity in the lamina propria. AChE, counterstained with hematoxylin ($\times 100$).

sitional zone on barium enema but HD was ruled out by negative reaction on AChE histochemistry and positive ganglion cells on H&E staining.

The overall diagnostic accuracy with AChE histochemistry was 97% (36/37) (Table 4).

Routine histology with H&E

Of the 35 specimens examined by H&E staining, 24 were from the unaffected children and 11 from the patients with HD. However ganglion cells were present in the submucosal Meissner's plexus only in 15 of these 24 unaffected children representing a specificity of 63%. As expected, ganglion cells were not seen in any of the 11 children with HD. Overall diagnostic accuracy with H&E staining was 74% (26/35).

From this study, AChE histochemistry was more reliable than routine histology with H&E. (Accuracy; 97% versus 74%, $P < 0.01$)

DISCUSSION

Full thickness rectal biopsy was formerly established as the mainstay of the diagnosis of HD. With this technique, Swenson et al. (1959) reported a 100% diagnostic accuracy, however, several disadvantages became evident, including the need for general anesthesia, rare rectal bleeding, and scarring which renders the subsequent operative procedure difficult.

Since the introduction of rectal suction biopsy technique by Noblett in 1969 and the advent of AChE histochemistry on rectal suction biopsy by Meier-Ruge et al. in 1972, AChE histochemistry has gained increasing acceptance and has gradually replaced classical full thickness rectal biopsy, because it is a simple, safe, reliable, bedside procedure not requiring general anesthesia.

The histochemical diagnosis of HD is based on the fact that the cholinergic nerve fibers of the aganglionic segment are prominent and that these fibers contain an increased amount of AChE (Meier-Ruge et al. 1972) The AChE can be demonstrated by a simple technique whereby acetylthiocholine iodide is hydrolyzed by AChE to thiocholine, which reduces potassium ferricyanide to ferrocyanide to yield, after contact with copper sulfate, the copper ferrocyanide that shows a brown color (Karnovsky & Roots, 1964).

The classical histochemical criterion for the diagnosis of HD consists of the presence of many cholinergic fibers in the mucosa, ie., both in the muscularis mucosae and the lamina propria. Meier-Ruge et al. (1972) stressed the importance of cholinergic fibers in the lamina propria as diagnostic of HD. The expecta-

tion that positive AChE activity will be found in the lamina propria in all cases of HD, has caused controversy and called into question the validity of the method for the diagnosis of HD, showing many false negative reactions.

Using the classical histochemical criteria of Meier-Ruge et al. (1972), Lake et al. (1978), Ikawa et al. (1986), and Athow et al. (1990) described 100%, 99%, and 90% diagnostic accuracy respectively, but Hamoudi reported 16(29%) out of 56 cases classified as false negative reactions. Goto et al. (1983) stated that the diagnosis of HD using rectal suction biopsy for AChE histochemistry is only safe after 3 weeks of age. Accepting the strict criteria of Meier-Ruge et al. (1972), there have been difficulties with this method in the diagnosis of HD, particularly in the neonates. (Athow et al., 1990; Chow et al., 1977; de Brito & Maksoud, 1987; Goto et al., 1983; Huntly et al., 1982).

Chow et al. (1977) were the first to call attention to the fact that there could be more than one pattern for AChE activity in HD. This observation was also confirmed by Huntly et al. (1982), and de Brito & Maksoud (1987). Moreover, de Brito & Maksoud demonstrated evolution with age of the AChE activity in rectal suction biopsy in HD. In our three cases (Case 4, 5 & 6), evolution of the AChE activity was observed. De Brito & Maksoud concluded that pattern I was predominant in newborn to 3 months of age and pattern II in children older than 1 year. Five out of 6 neonatal patients with HD showed pattern I, correlating with the report of de Brito & Maksoud (1987), and Chow et al. (1977). According to the classical criteria by Meier-Ruge et al. (1972), pattern 1 reaction could be regarded as a false negative reaction.

Chow et al. (1977) emphasizes that the diagnostic feature does not depend so much on the number of cholinergic fibers but more on the localization and quality of these fibers, which are best appreciated under medium power, with the condenser lowered. The latter procedure brings out sharp staining of the fibers in distinct contrast to the diffuse staining of the background. They defined a positive reaction as many coarse discrete cholinergic fibers in the muscularis mucosae and in the immediately subjacent submucosa regardless of a clear infiltration of cholinergic fibers in the lamina propria since a clear infiltration of cholinergic fibers in the lamina propria was seen only in some cases of HD, and the presence of many coarse discrete cholinergic fibers in the muscularis mucosae and the immediately subjacent submucosa, was seen in all cases with HD. They warned that some fine discrete fibers were not uncommonly seen under high power, particularly in older children with

chronic constipation and that this should not be regarded as HD.

Even with Chow's histochemical criterion, false negative (Almoyana et al., 1978; Athow et al., 1990; Kurer et al., 1986; Staak, 1981) or false positive reactions (Ariel et al., 1983; Athow et al., 1990; Huntly et al., 1982; Staak, 1981) have been sporadically reported. Many authors have described false negative reactions, especially in patients with total colonic aganglionosis (Athow et al., 1990; Barr et al., 1985; Kurer et al., 1986; Staak, 1981). In this study, the 21 day-old infant (Case 6) with a false negative reaction, i.e., an absence of AChE positive fibers in the mucosa and submucosa, was proved to have total colonic aganglionosis at surgery but a second biopsy at 7 months of age demonstrated pattern III reaction. An equivocal or negative reaction when contradicting clinical features, seems to be an indication for a repeat biopsy or for the trial of other appropriate diagnostic modalities. Barr et al. (1985) described some situations in which the false negative reaction may occur: (1) Age; in some neonates possibly due to immaturity of the enzyme system (Almoyana et al., 1978; Elema et al., 1973; Goto et al., 1983; Staak, 1981). The majority of these false negative reactions can be avoided by the application of Chow's new criterion. (2) Some patients with total colonic aganglionosis (Athow et al., 1990; Barr et al., 1985; Kurer et al., 1986; Staak, 1981). An explanation for this false negative reaction has been offered on embryological grounds by Okamoto & Ueda (1967). (3) Height of the biopsy harvesting, especially in patients with ultrashort segment HD. (4) Technical faults such as a delay in freezing or insufficient incubation solution.

In regard to false positive reactions, Huntly et al. (1982) reported 3 occasions of false positive reaction in patients with megacystis microcolon, congenital heart disease with failure to thrive, and habitual constipation, respectively. The reason for this is unclear. False positive reactions have been reported in patients with neuronal intestinal dysplasia (Athow et al., 1990; Staak, 1981). But this false positive reaction can be avoided by demonstration of giant ganglia in the mucosa (Schärli & Meier-Ruge, 1981).

A number of diagnostic tests have been developed through the years for the investigation of HD. Barium enema has been helpful but some neonates with HD often do not develop typical cone shaped transition until a number of weeks after birth (Swenson et al., 1973). Smith & Cass (1991) stated that barium enema had a 24% false negative rate in the diagnosis of infantile HD.

Anorectal manometry is useful in the diagnosis of

HD as absence of the reflex relaxation of the internal sphincter with stretch stimulation of the rectum. There may be difficulties in gaining the child's cooperation and in interpretation of the results. Aarronson & Nixon (1972), and Park & Choi (1989) stated 91% and 93% accuracy in excluding HD and 74% and 88% accuracy in the diagnosis of HD, respectively. The restricted applicability and limited accuracy of anorectal manometry in the diagnosis of HD do not make this technique the sole means of diagnosis.

Routine histology with H&E staining on rectal suction biopsies is less reliable than AChE histochemistry. Ikawa et al. (1986) have demonstrated more reliable diagnostic accuracy with AChE staining versus standard H&E staining (99% versus 61%). It is also confirmed in our study that AChE histochemistry is more reliable than standard H&E staining (97% versus 74%, $P < 0.01$). For the detection of ganglion cells on rectal suction biopsies, some authors (Robey et al., 1988; Park et al., 1989) have suggested that immunohistochemistry for neuron specific enolase and S-100 protein is more reliable than routine H&E staining. Robey et al. (1988) suggested that the AChE stain is more specific than S-100 staining for identifying the mucosal nerve proliferations of HD.

By use of AChE histochemistry, the problem of inadequate specimen harvesting in rectal suction biopsy can be virtually eliminated because only the mucosa and immediately subjacent submucosa is quite enough to make a diagnosis or exclusion of HD. The presence of enough submucosa and the detection of the ganglion cells are not necessarily essential. Furthermore, it seems possible to make a diagnosis of HD even though the specimens are taken from the physiologic hypoganglionic zone within 2cm proximal to the dentate line, because abnormal distribution of the cholinergic nerve fibers may be expected at this level as well.

In the light of our experience with AChE histochemistry, we confirm that the criteria of Chow et al. (1977) and de Brito and Maksoud (1987) are highly reliable in newborns as well as in older children as long as it is kept in mind that there can be rare pitfalls with AChE histochemistry such as false negative reactions in some patients with total colonic aganglionosis and false positive reactions in patients with neuronal intestinal dysplasia. We strongly favor rectal suction biopsy with AChE histochemistry as the procedure of choice for the diagnosis or exclusion of HD when evaluating the child, especially the infant with bowel obstruction or chronic constipation since there are many aforementioned advantages such as high reliability, avoidance of many inadequate specimens, rapid

diagnosis within 2 hours, safety, and an ambulatory basis without general anesthesia. Therefore we encourage its more widespread use in Korea.

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