IMMUNOHISTOCHEMICAL DEMONSTRATION OF SEROTONIN (5HT)-CONTAINING CELLS IN THE HUMAN AND RAT INTESTINES

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5HT)-containing cells were successfully demonstrated by the indirect immunoperoxidase and immunofluorescence methods in both frozen and formalin-fixed paraffin sections of the human and rat intestines. Immunohistochemically, the 5HT-positive cells were distributed at the base of the crypts and intestinal villi with a long cytoplasmic process toward the luminal surface. Cells observed to fluoresce in the formaldehyde-induced fluorescence (FIF) method showed intense immunoreactivity to 5HT when treated successively by the indirect immunoperoxidase method. The applicability and advantages of the immunohistochemical demonstration of 5HT in the gut endocrine cells are discussed.

Serotonin (5-hydroxytryptamine, 5HT) is one of the biogenic amines distributed throughout the body and is synthesized from tryptophan by 5-hydroxylation followed by decarboxylation (22).

The biological actions of 5HT are currently being investigated especially in the gastrointestinal tract and central nervous system (CNS). In the gastrointestinal physiology, it is known that 5HT may be released from the duodenum by acidification as well as by instillation of hypertonic glucose in the duodenum under experimental conditions (10).

The cells containing 5HT are enterochromaffin (EC) cells dispersed in the gut epithelium. These cells have been described as typical members of the paraneuron family by Fujita, and their paraneuronal nature and functions have been well investigated (3, 4, 5). Recent investigations have supported the view that the EC cells contain not only 5HT but also one or more bioactive peptides. Substance P (15, 18), enkephalin (1) and motilin (8, 17) have been suggested to occur in the EC cells but the views of researchers are very variable concerning this point (11). The 5HT-containing neurons are distributed widely in the CNS of various species.

Lackner (12) and Poitras *et al.* (19) showed a monoaminergic neuron system in the cat CNS, and Hubbard *et al.* (9) demonstrated such neurons in the monkey, and Olson *et al.* (16) in the human fetal brain, using the formaldehyde-induced fluorescence (FIF) method.

Steinbusch *et al.* (21) showed detailed serotonergic neuron systems in the rat CNS with indirect immunofluorescence. However, to our knowledge, no immunohistochemical demonstration of 5HT-containing cells in the gut has been published.

The purpose of this paper is to describe the immunohistochemically detected 5HT-containing cells, to compare their localization with FIF-bearing cells, and to evaluate the procedures of tissue preparation for demonstration of 5HT in the tissue sections.

MATERIALS AND METHODS

Animal Materials

Five male albino Wistar rats weighing 170-250 g were used. Two were used for the FIF study and the others were used for the immunohistochemical study. For the immunohistochemis-

try, the animals were perfused through the left heart ventricle with 4% paraformaldehyde in phosphate buffer with or without saturated picric acid, under ether anesthesia. After the perfusion, the intestinal segments were postfixed with the same fixatives for 2–4 h at 4°C, and embedded in paraffin. In addition, some of them were frozen by dry ice-acetone after treatment with a sucrose solution.

Human Materials

The duodenal and colonic segments resected from the patients with gastric or colonic cancer were fixed immediately with 10% buffered formalin and 4% paraformaldehyde in phosphate buffer with or without saturated picric acid, and then embedded in paraffin.

Antisera

The anti-5HT antisera were produced in rabbits by injecting biweekly 1–5 mg of hapten (serotonin-creatinin sulfate; MERCK)-protein conjugates on the back. The hapten-protein conjugates were prepared according to Grotz's method (7), with a slight modification. The specificity of the antisera used was described elsewhere (14). The anti-rabbit IgG antisera were raised in goats. The hyperimmune goat IgG was isolated from the sera by ammonium sulfate fractionation and gelchromatography, and labelled with horseradish peroxidase (HRP; SIGMA type VI) by the method of Nakane and Kawaoi (13). For the indirect immunofluorescence method, fluorescein-conjugated swine anti-rabbit IgG antisera (DAKO-immunoglobulins, Copenhagen, Denmark) (FITC-labelled anti-RGG) were used.

Formaldehyde-Induced Fluorescence

The intestinal segments were freeze-dried and exposed to gaseous formaldehyde for 1 h at 80°C according to the method of Falck-Hillarp (2), and embedded in paraffin *in vacuo*. Five micrometer tissue sections were mounted in xylene and examined under a fluorescence microscope (Nikon Fluophot) with excitation filters V and a barrier filter 460. As a negative control, the tissue preparation was processed without exposure to gaseous formaldehyde.

Immunostaining

In order to demonstrate 5HT-containing cells in

the human and rat intestines, the indirect immunoperoxidase method was used. Five micrometer deparaffinized tissue sections were incubated with anti-5HT antisera (diluted 1: 5,000 to 1: 20,000) for 1 h at 37°C and washed three times in cold PBS. Then the tissue sections were incubated with HRP-labelled goat anti-rabbit IgG (anti-RGG) (diluted 1: 80 to 1: 160) or FITC-labelled swine anti-RGG (diluted 1: 20 to 1: 40) for 30 min at room temperature and washed three times in cold PBS. The tissue-bound HRP activity was visualized by the method of Graham and Karnovsky (6). For the immunofluorescence method, the tissue sections were mounted in glycerol and examined under a fluorescence microscope.

To examine the specificity of immunostaining, the first antibody (anti-5HT antibody) was replaced by 1) normal rabbit IgG (NRS) or 2) anti-5HT antibody previously absorbed with 5HT-BSA conjugates (1 µg to 10 µg/ml diluted antisera).

For comparison of the immunohistochemical localization with FIF localization, the tissue sections processed for FIF were photographed under a fluorescence microscope, and then were processed for immunostaining; thereafter the same areas were photographed again.

RESULTS

When the FIF and indirect immunoperoxidase methods were compared, all of the FIF-bearing cells showed a positive immunoreactivity for 5 HT, and both FIF substances and 5HT-like immunoreactivity were localized in the same cells (Figs. 1 and 2).

In the rat intestine, 5HT-containing cells were dispersed in the epithelium of the intestinal crypts, especially in their bases, and in the intestinal villi. Both FIF and immunoreactivity were concentrated in the basal cytoplasm, and were present less intensely in the long cytoplasmic process toward the luminal surface (Figs. 2 and 3). In the FIF study, fine granular or beaded fluorescence, probably indicating the nerve fibers, was observed near the basement membrane of the crypts and around the vascular channels (Figs. 1 and 2a). However, no neural constituents could not be detected by the immunostaining for 5HT (Fig. 2b). On the other hand, these 5HT-containing cells were also well demonstrated on the cryostat sections of the rat intestine by the indirect immunofluorescence method (Fig. 4).

In the human material fixed with formalin and

Fig. 1 Fluorescence photomicrograph of the rat small intestine. Intense fluorescence can be seen in the epithelium of the intestinal crypts and in some neural constituents (arrows), Falck-Hillarp's method. $\times 170$
 Fig. 2 Medium power view of Fig. 1. a: Six FIF-bearing cells in the intestinal crypts and fluoresced nerve fibers (arrows) around the cryptal basement membrane, Falck-Hillarp's method. b: The same field stained with anti-5HT antiserum. The 5HT-like immunoreactivity is seen in the same cells as Fig. 2a. The nerve fibers are not stained, indirect immunoperoxidase method. Numbers indicate the corresponding cells. $\times 310$

embedded in paraffin, 5HT-containing cells were satisfactorily demonstrated by the indirect immunoperoxidase and immunofluorescence methods. Immunohistochemically, 5HT-containing cells were detected occasionally in groups and distributed in the bases of the intestinal crypts (Figs. 5 and 6). They showed a cytoplasmic

elongation toward the luminal surface as observed in the rats. Hematoxylin-eosin staining of the adjacent sections revealed that the 5HT-positive cells were flask-like and had slightly eosinophilic cytoplasm.

No positive staining was observed when the sections were incubated with either NRS or

Figs. 3-5

Fig. 6 Consecutive paraffin sections of the normal human colon. a: 5HT-immunoreactive cells in the crypts. Indirect immunoperoxidase method. b: Arrows indicate 5HT-containing cells. Hematoxylineosin stain. $\times 350$

anti-5HT antisera previously absorbed with an appropriate amount of 5HT-BSA conjugates.

Fig. 3 5HT-containing cells in the villi of the rat intestine. One of them shows a long cytoplasmic process to the luminal surface. The section was first observed by the Falck-Hillarp's method (a) and then treated with the indirect immunoperoxidase method (b). $\times 730$

Fig. 4 Fluorescence photomicrograph of 5HT-immunoreactive cells in the rat small intestine. Frozen section. Indirect immunofluorescence method. $\times 340$

Fig. 5 Fluorescence photomicrograph of 5HT-immunoreactive cells in the human colon. Paraffin section. Intense immunofluorescence can be seen in the cryptal cells. Indirect immunofluorescence method. $\times 320$

DISCUSSION

Today's knowledge of the gastro-entero-pancreatic (GEP) endocrine system and its pathophysiology owes much to the advances in the immunohistochemistry and ultrastructural morphology. More than 18 types of endocrine cells in the GEP system have been identified (20). The heterogeneity of EC cells has also pointed out in terms of the variety of their granule morphology as well as of their peptide hormone immunoreactivity, such as for motilin, substance P or enkephalin (1, 8, 15, 17, 18).

Hitherto, 5HT could be demonstrated only by FIF method. Although this method is very useful, there are some disadvantages, such as the complicated tissue preparation, rapid decline of fluorescence and inapplicability to the electron microscopic examination. Moreover, the FIF method cannot discriminate 5HT from catecholamines, *i.e.*, dopamine, noradrenalin and adrenalin.

In this study, the FIF method showed noradrenalin-containing neural constituents as well as 5HT-positive cells. However, the immunoreactivity for 5HT was confined within the epithelial cells in the intestinal crypts and villi, and no nerve fibers could be demonstrated on the same sections (Fig. 3).

Although the immunohistochemical localization of 5HT in the CNS has already been reported by Steinbusch and his coworkers (21), we could not find any reports regarding the gut.

By employing both FIF method and immunostaining on the same tissue sections, we confirmed that the FIF reactive cells possess 5HT immunoreactivity.

The staining activity of the antisera against 5HT used in this study was inhibited completely by preincubation with serotonin-BSA conjugates and incompletely by serotonin creatinin sulfate, but not by BSA alone. The details of cross-reactivity of the antisera with tryptophan derivatives will be described elsewhere. The fact that these antisera could be absorbed by free serotonin creatinin sulfate suggests their reactivity with free 5HT as well as formaldehyde-induced condensed form of 5HT (6-hydroxy-3, 4-dihydro- β -carbolin) in the tissue sections. However, the amount of the free 5HT remaining in routine tissue sections is unknown.

In conclusion, this paper confirms the usefulness of immunohistochemical demonstration of 5HT-containing cells in the gut, especially in routine histological specimens. It will be a powerful tool for the study of the GEP endocrine cells and their pathological, especially neoplastic, changes.

We thank Misses M. Hanawa and M. Kaneko for their technical assistance and help with the manuscript.

This study was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture, Japan (No. 56770207), and from the Foundation of Nihon University.

Received for publication 19 January 1982; and in revised form 19 February 1982

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