The Korean Journal of internal Medicine
Vol 6, No 1, January, 1991

Autoradiographic and Immunohistochemical Study on the Proliferative Kinetics of Intestinal Metaplasia

Hee Seung Bom, M.D., Chong Mann Yoon, M.D.
Jong Sun Rew, M.D. and Sung Kyu Choi, M.D.

Department of Internal Medicine, Chonnam University Medical School, Kwangju, Korea

Sang Woo Juhng, M.D.

Department of Pathology

In order to elucidate the proliferative behavior of the intestinal metaplasia around gastric cancer, the authors used both in vitro tritiated thymidine (^{3}H-thymidine) autoradiography and in vivo bromodeoxyuridine (BrdUrd) immunohistochemistry for labeling the proliferative cells of the normal pyloric glands and metaplastic gastric glands.

The results of the methods were comparable: The labeling pattern and the rate of labeling were very similar. In the normal pyloric mucosa, the labeled cells were confined to the isthmus region, indicating that pyloric glandular cells are normally renewed from the isthmus region. On the other hand, a zone of the labeled cells was found in the lower half of the intestinalized mucosa, indicating that cell proliferation took place deep in the mucosa, just like the case of normal intestinal glands. The labeling indices of the pyloric mucosa were 19.4% by autoradiography and 18.0% by immunohistochemistry, and that of the intestinalized gastric glands were 25.2% by autoradiography and 24.2% by immunohistochemistry.

In conclusion, both ^{3}H-thymidine autoradiography and BrdUrd immunohistochemistry showed that the proliferative kinetics of the intestinalized gastric glands was similar to that of the normal intestinal glands rather than the pyloric glands, i.e. a lower level of proliferative zone and higher labeling index were present.

Key Words: *Tritiated thymidine autoradiography, Bromodeoxyuridine immunohistochemistry, Intestinal metaplasia, Proliferative zone, Labeling index*

INTRODUCTION

The intestinal metaplasia of the stomach, which may be associated with a change in the direction of differentiation from the normal pyloric pattern to that of the intestine, is often accompanied by gastric cancer[1,2] and so considered to be a precancerous condition[3,4,5]. However, it also has been regarded as a normal repair mechanism[6] because it accompanied by peptic ulcer or chronic gastritis[1,2].

In order to understand the meaning of the metaplastic change of the gastric mucosa around the gastric cancer, it is necessary to elucidate the proliferative characteristics of the intestinal metaplasia. As to the site of cell proliferation in the

*A preliminary report for this paper was presented at the Eighth Asian-Pacific Congress of Gastroenterology, October 9-13, 1988.

*Address reprint requests to: Chong Mann Yoon, M. D., Department of Internal Medicine, Chonnam University Hospital, #8 Hak-Dong, Dong-Ku, Kwangju, 501-190, Korea

intestinalized gastric mucosa, there are two different sets of data. Winawer and Lipkin[7] report ^{3}H-thymidine incorporating cells concentrated at the middle level and even located at the surface of the mucosa. On the other hand, Hattori and Fujita[8] demonstrate a zone of labeled cells in the lower one-thrid of the mucosa.

Bromodeoxyuridine (BrdUrd), like ^{3}H-thymidine, is incorporated into the DNA of the proliferating cells. So an immunohistochemical method using monoclonal antibodies to BrdUrd can be used to study the proliferative kinetics of the gastric mucosa. So far, however, BrdUrd immunohistochemistry was mainly used to study the proliferative kinetics of human tumor[9,10].

In this study, the authors used both ^{3}H-thymidine autoradiography and BrdUrd immunohistochemistry to elucidate the proliferative behavior of the inestinal metaplasia found in the pyloric mucosa around the gastric cnacer.

MATERIALS AND METHODS

1. Autoradiographic Study

The materials used in this study were biopsy specimens taken from 9 patients suffering from advanced gastric cancer (Table 1). The specimens were carefully taken from the nondiseased portions of the pyloric mucosa, and 3 biopsy specimens were obtained from each individual. ^{3}H-thymidine autoradiography was performed using the in vitro method described by Hattori and Fujita[8]. Briefly, the specimens were cut into 1 mm pieces and washed in sterile saline solution. They were then immersed in MEM (modified Eagle's medium) supplemented with 10% fetal calf serum containing 20 μCi of ^{3}H-thymidine (methyl-^{3}H-thymidine, Amersham, England, specific activity, 25 μCi/mmol) per 1 ml and incubated for 60 min at 37°C. After fixation with 10% formalin, they were dehydrated in a graded alcohol series and embedded in paraffin. The embedded tissues were carefully oriented and sectioned serially and longitudinally to the long axis of the glandular tubule. Sectons of about 2~3 μm in thickness were mounted on glass slides treated with tissue adhesives, dipped in SAKURA NR-M_2 nuclear emulsion, and developed in FD-111 after 6 weeks' exposure. They were stained with hematoxylin and eosin.

The in vitro labeling of the tissues with ^{3}H-thymidine did not provide constant results. Frequently, only the margins of the specimens were labeled with ^{3}H-thymidine. Sometimes, however, we were successful in labeling a whole layer of the specimens in which a zone of the labeled epithelial cells was found. In this study, we used these specimens in order to interpret cell proliferation kinetics in the pyloric and intestinalized mucosa.

2. Immunohistochemical Study

The materials used in the BrdUrd immunohistochemical study were resected stomachs from 4 patients suffering from advanced gastric cancer

Table 1. Profiles of Subjects Patients

No.	Age	Sex	Method	Diagnosis
1.	66	F	In vitro autoradiography	AGC*, III**
2.	68	M	In vitro autoradiography	AGC, IV
3.	55	F	In vitro autoradiography	AGC, II
4.	62	M	In vitro autoradiography	AGC, III
5.	68	M	In vitro autoradiography	AGC, III
6.	62	M	In vitro autoradiography	AGC, IV
7.	64	M	In vitro autoradiography	AGC, IV
8.	46	F	In vitro autoradiography	AGC, II
9.	56	M	In vitro autoradiography	AGC, III
10.	50	M	In vivo immunohistochemistry	AGC, II
11.	55	F	In vivo immunohistochemistry	AGC, III
12.	46	M	In vivo immunohistochemistry	AGC, IV
13.	68	M	In vivo immunohistochemistry	AGC, III

* AGC : advanced gastric cancer, ** Borrmann type

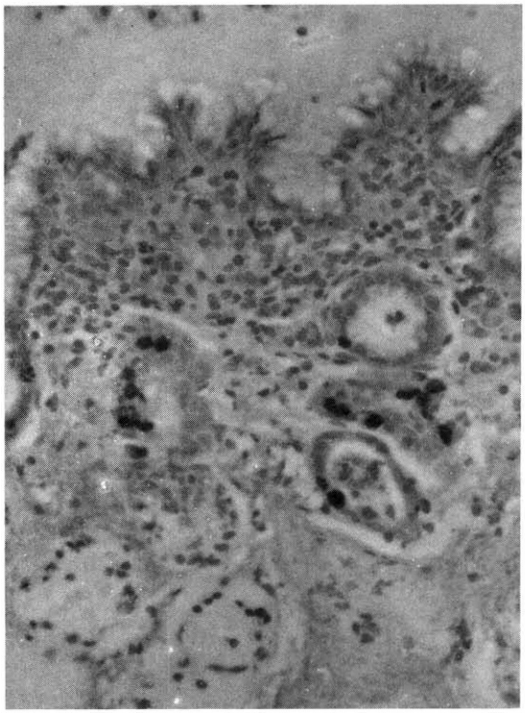

Fig. 1. *Representative presentation of labeled cells by in vitro ^{3}H-thymidine autoradiography. Black-silver grains indicate the incorporation of ^{3}H-thymidine into the DNA. H.E. X 200.*

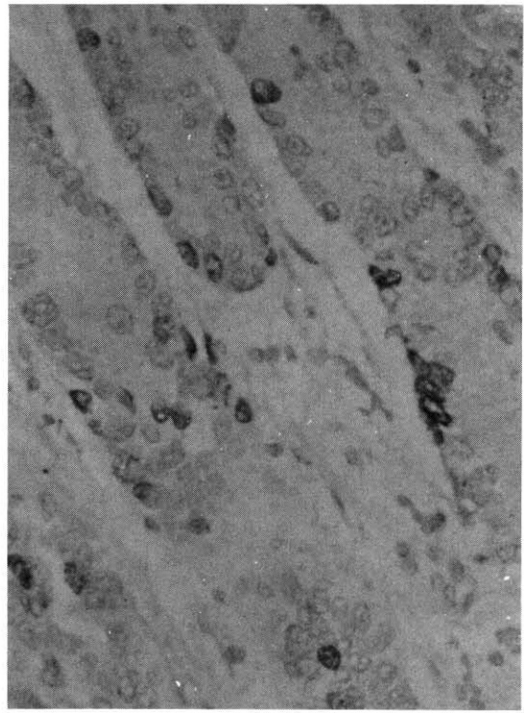

Fig. 2. *Representative presentation of labeled cells by in vivo bromodeoxyuridine immunohistochemistry. Dark brown colored nuclei indicate the incorporation of bromodeoxyuridine into the DNA. Immunoperoxidase X 400.*

Table 2. Comparison of Labeling Sites and Labeling Indices (LIs) of Pyloric and Intestinalized Glands Studied by in Vitro ^{3}H—thymidine Autoradiography and in Vivo Bromodeoxyuridine (BrdUrd) Immunohistochemistry

		Autoradiography	Immunohistochemistry
Pyloric gland			
Labeled cells	Counted No.	836	757
	Site*+	19 – 72	13 – 84
Nonlabeled cells	Counted No.	3484	3455
Labeling index (total)++		19.5%	18.0%
Intestinalized gland			
Labeled cells	Counted No.	838	1062
	Site*+	42 – 116	50 – 135
Nonlabeled cells	Counted No.	2541	3138
Labeling index (total)++		25.2%	24.2%

* The site of labeled cells was indicated by the cell order of counted cells from the surface of the gland.

\+ : $P < 0.05$ for difference in sites of labeled cells between ^{3}H—thymidine autoradiography and BrdUrd immunohistochemistry

++ : $P < 0.01$ for difference between LI of pyloric gland and that of intestinalized gland, but not significant for difference between the LI calculated by ^{3}H—thymidine autoradiography and that by BrdUrd immunohistochemistry

(Table 1). Informed consent for administration of BrdUrd was obtained from each patient. The method used in this study is described by Hoshino et al[9]. Briefly, the patients were given a 60-min i.v. infusion of BrdUrd, 200 mg/m², at the time of surgery but before gastric resection. Non-cancerous and non-ulcerated regions of the antral mucosa were cut into rectangular pieces and embedded in paraffin. They were cut serially and mounted on glass slides treated with tissue adhesives. Tissue sections were deparaffinized for 15 min in xylene, rinsed 3 times (5 min each time) in 0.01 M phosphated buffered solution (PBS), hydrated for 30 min in 1N HCl, and incubated for 20 min at 37°C in PBS containing 0.05% proteinase type VII (Sigma, U.S.A). The sections were then stained by avidin biotin peroxidase method using Vectastain ABC kit: they were incubated for 16 hr at room temperature with 1 : 60 dilution of purified anti-BrdUrd monoclonal antibodies (Bection Dickinson, U.S.A) in bovine serum albumin, for 30 min at room temperature with biotinilated antimouse IgG (horse); and for 30 min at room temperature with ABC. The tissue sections were rinsed with PBS at each stage. Finally, the slides were reacted with 0.03% 3,3'-diaminobenzidine (DAB) and PBS containing 1% H_2O_2. Light counter stains were performed by hematoxylin.

3. Analysis of Results

Black-silver grains in the nuclei (Fig. 1) and dark brown colored nuclei (Fig. 2) were considered as labeled cells in autoradiography and immunohistochemistry, respectively, and a zone of labeld cells was considered as a proliferative zone of the mucosa[8,11]. The site of the labeled cells was indicated by the number of counted nuclei from the surface of the gland. The labeling index (LI) was defined as the percentage of the labeled cells counted in a total number of over 1,000 nuclei in the generative cell zone between the level of the uppermost labeled cells and that of the lowermost cells in each specimen[8].

RESULTS

The schematic representation of the results of this study is shown in Fig. 3. In short, the metaplas-

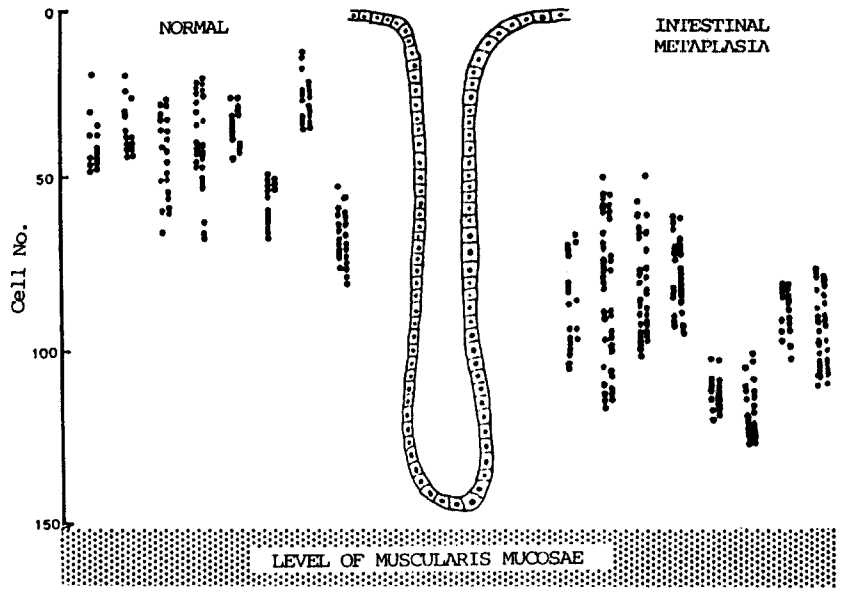

Fig. 3. *A schematic representation of the sites and distribution of labeled cells in a specimen from a 46-year-old male, obtained by in vivo bromodeoxyuridine immunohistochemical method. The site of labeled cells was indicated by the number of nuclei counted from the surface of the gland. The labeling index, the percentage of the labeled cells counted in a total number of over 1000 nuclei in the generative cell zone between the level of the uppermost labeled cells and that of the lowermost cells in each specimen in this case was 19.4% in the normal pyloric glands and 22.0% in the intestinalized glands.*

tic gastric glands showed a lower level of proliferative zone and a higher LI. The difference was statistically significant ($p<0.01$, Table 2).

1. Autoradiography

Autoradiographs of the pyloric mucosa not involved with metaplastic change showed the labeled cells to be confined to the region of the isthums between the base of the pits and the upper part of the pyloric gland (the middle one-third of the mucosa) (Fig. 4). The cell order, the number of counted nuclei from the surface of the gland of the labeled cells, was distributed between 19 and 72 (Table 2). The LI varied from 15.3 to 26.6%, and the total LI was 19.4% (Table 3).

In the autoradiographs of the intestinalized mucosa, a zone of the labeled epithelial cells was found in the lower third of the mucosa (Fig. 5). The cell order of the labeled cells of metaplastic glands was distributed between 42 and 116 (Table 2). The LI varied from 20.6 to 29.4%, and the total LI was 25.2% (Table 3).

2. Immunohistochemistry

The pattern of distribution of labeled cells revealed by in vivo BrdUrd immunohistochemistry (Fig. 6a, 6b, and 7a, 7b) was very similar to that revealed by in vitro ^{3}H-thymidine autoradiography.

The cell order of the labeled cells in the pyloric mucosa varied from 13 to 84 and in the intestinalized mucosa between 50 and 135 (Table 2). The LI varied from 12.5 to 22.2% in the pyloric mucosa and between 21.8 and 27.2% in the intestinalized mucosa. The total LI of the pyloric mucosa was 18.0% and that of the intestinalized mucosa 24.2% (Table 3).

DISCUSSION

Tritiated thymidine autoradiography is widely used to study cellular kinetics of the gastrointestinal mucosa. Although ^{3}H-thymidine has been

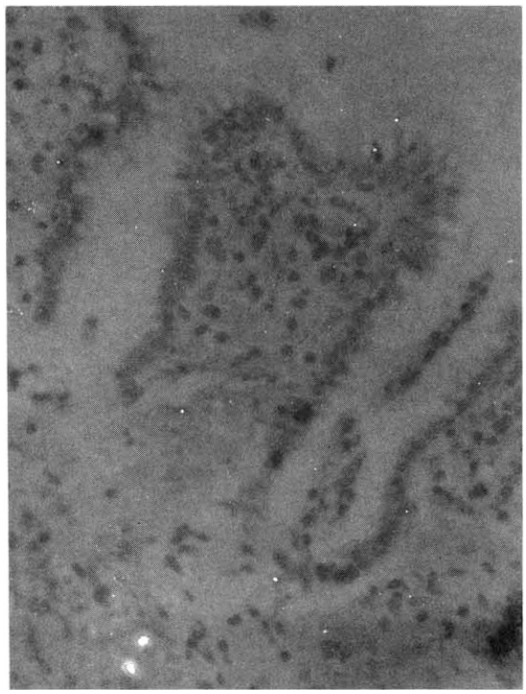

Fig. 4. *Autoradiograph of normal pyloric mucosa (from a 62-year-old male) obtained by in vitro incubation with ^{3}H-thymidine for 60 min. Labeled epithelial cells are confined to the region of the isthmus of the pyloric gland. H.E. X 200.*

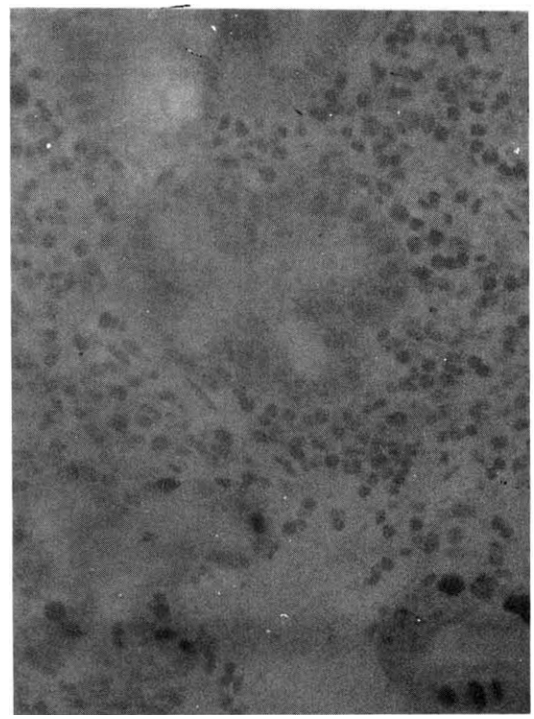

Fig. 5. *Autoradiograph of intestinalized mucosa (from a 56-year-old male) obtained by in vitro labeling with ^{3}H-thymidine. A zone of the labeled epithelial cells is seen at the lower level of the mucosa. H.E X 400.*

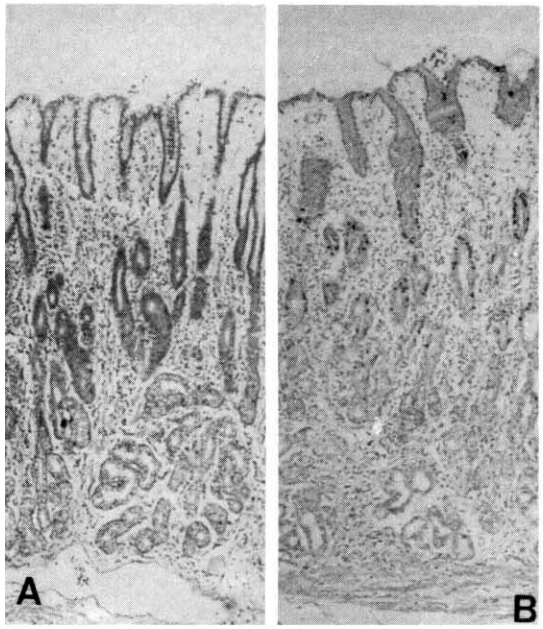

Fig. 6. *Photomicrograph of normal pyloric mucosa (from a 46-year-old male) obtained by hematolxylin-eosin stain (6A) and in vivo bromodeoxyuridine immunohistochemical method (6B). Labeled epithelial cells are confined to the isthmus region of the gland (6B). X 100.*

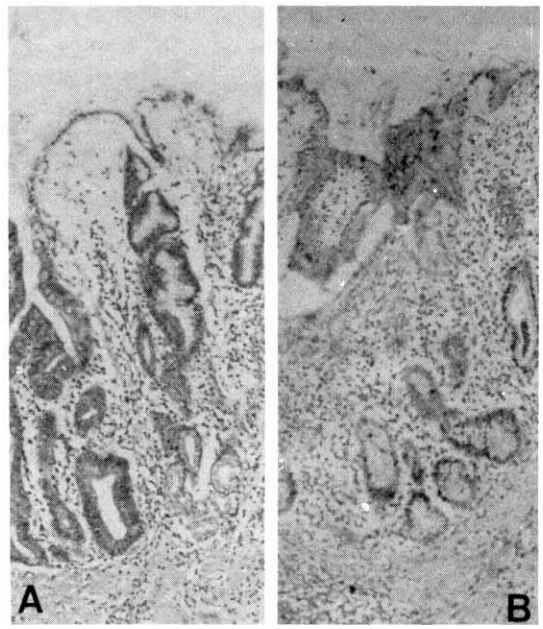

Fig. 7. *Photomicrograph of intestinalized mucosa (from a 46-year-old male) obtained by hematoxylin-eosin stain (7A) and in vivo bromodeoxyuridine immunohstochemcial method (7B). Labeled cells are seen at the lower level of the mucosa (7B). X 100.*

administered intravenously in a limited number of patients[7,11,12], a systemic administration of the isotope is almost impossible in humans because of the potential radiation hazard to normal tissue[13]. So the only acceptable method of obtaining specimens is through the "tissue or organ culture" method. In the present study, the specimens were labeled by brief in vitro incubation with ^{3}H-thymidine. But application of this technique still has problems: It is tedious to perform and requires several months to complete.

Recently, Gratzner[14] developed a monoclonal antibody that can identify nuclei containing Brd-Urd. Anti-BrdUrd monoclonal antibody can be detected by immunohistochemical methods[15]. This is an important breakthrough for studies of cell kinetics. BrdUrd, like ^{3}H-thymidine, is incorporatd into nuclear DNA during DNA synthesis but is neither radioactive nor myelotoxic at the doses to be used for in vivo labeling studies[15,16]. The immunohistochemical method of detecting anti-BrdUrd immunohistochemistry was mainly used to evaluate the proliferative activity of human tumors

Table 3. Individual Labeling Indices (LIs) of Pyloric Glands and Intestinalized Glands

Patient (Age/Sex)	Pyloric Gland	Intestinalized Gland
In vitro ^{3}H–thymidine autoradiography		
1. 66 F	15.3%	29.4%
2. 62 M	16.6%	*
3. 55 F	20.8%	20.6%
4. 56 M	26.6%	26.5%
Total **	19.4%	25.2%
In vivo bromodeoxyuridine immunohistochemistry		
1. 55 F	12.5%	21.8%
2. 50 M	18.0%	27.2%
3. 46 M	19.4%	22.0%
4. 68 M	22.2%	24.2%
Total **	18.0%	24.2%

* LI could not be calculated because of an insufficient number of labeled cells.

** $P < 0.01$ for difference between LI of pyloric glands and that of intestinalized gastric glands.

and then to elucidate the prognosis of individual patients[9,10], but application of this method to cell kinetic study of intestinal metaplasia is rare.

In the present study, the authors used both in vitro ^{3}H-thymidine autoradiography and in vivo BrdUrd immunohistochemistry for elucidation of the proliferative kinetics of the intestinal metaplasia found around the gastric cancer. The results of both methods were comparable: The labeling pattern and the rate of labeling were very similar.

Both methods revealed that the labeled cells were confined to the middle third (isthmus region) of the mucosa in the normal pyloric mucosa and to the lower third of the mucosa in the intestinalized gastric mucosa. This labeling pattern indicates that in the human pyloric mucosa the cells proliferate within the isthmus region and that cell proliferation takes place deep in the mucosa in the intestinalized mucosa. It is generally accepted that in mammalian gastrointestinal mucosa, proliferation of epithelial cells is confined to a certain level of the mucosa: In the stomach, it is a neck area[11,17] and in the intestine it is a lower crypt[18,19]. Therefore the proliferative pattern of intestinalized gastric glands is just like that of normal intestinal glands.

However, the cell order of the labeled cells counted from the surface of the gland studied by BrdUrd immunohistochemistry was higher (i.e., labeled cells were located deeper in the gland) than that obtained by ^{3}H-thymidine autoradiography. This difference might be due to the difference of the method itself or to the materials used. Hattori and Fujita[8] point out that the generative cell zone of the metaplastic gland shifts from the isthmus to the base of the gland following atrophy of the pyloric gland. So the difference of the cell order might be due to the difference of the materials (i.e., degree of intestinalization) rather than to the difference of the method itself, because the difference between the LIs calculated by the 2 methods were statistically insignificant.

The LI of the intestinalized mucosa was significantly higher than that of the pyloric mucosa. This can be explained by the fact that the renewal time of the intestinal epithelium is faster than that of the gastric epithelium[18,19]. Winawer and Lipkin[7] calculated an LI using in vivo ^{3}H-thymidine autoradiography in a patient and report that the LI of a normal gastric gland is 14% and that of a metaplastic gland 19%. Hattori and Fjuita[8] report that the LI of a normal pyloric gland varies from 20 to 25% and that of am intestinalized gastric gland from 28 to 34%. Hattori[20] reports that the LI of a normal pyloric gland varies from 16 to 26% and the LI of an intestinalized gland 12 to 26%. In the present study, the LI of the normal pyloric mucosa was 19.4% by in vitro ^{3}H-thymidine autoradiography and 18.0% by in vivo BrdUrd immunohistochemistry. The LI of the intestinalized gastric mucosa was 25.2% by autoradiography and 24.2% by immunohistochemistry.

Recently several investigators noted that the intestinal metaplasia of the stomach can be divided into several subtypes, and the significance as a precancerous condition is confined to certain sybtypes[21~25]. In the present study, chemical subtyping of the intestinal metaplsia was not done. Morphologically, most of them were complete types showing Paneth cells. The difference of proliferative kinetics among the different subtypes of intestinal metaplasia requires further investigation.

In conclusion, BrdUrd immunohistochemistry is expected to be used widely in the fields of cell kinetics because of its advantage over ^{3}H-thymidine autoradiography: in vivo use and its capacity of rapid processing with very similar results to autoradiography. Both methods showed that the proliferative kinetics of intestinalized gastric glands was similar to that of normal intestinal glands rather than pyloric glands, i.e., a lower level of proliferative zone and higher LI were present.

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