Journal of Korean Medical Science
Vol 4, No. 4, P 193-201, December 1989

In Vitro Immunohistochemical Localization of S-Phase Cells by A Monoclonal Antibody to Bromodeoxyuridine

Sang-Sook Lee, M.D., Tae-Seung Lee, M.D.,* June-Sik Park, M.D.**
Eun-Kyung Cho, M.D.** and Chai-Hong Chung, M.D.

Department of Pathology, Obstetrics and Gynecology, Keimyung University School of Medicine and Department of Otolaryngology, Kyungpook National University School of Medicine***

Bromodeoxyuridine, an analogue of thymidine, can be detected by means of monoclonal antibodies and utilized as a marker of the S-phase of the cell cycle. In vitro immunohistochemical application of the BrdU/anti-BrdU-MAb method permits a quantitative assessment of the proliferative activity of a tissue as well as the direct location of the actively replicating cells in histological sections. In this paper, a method for the detection of the labeling index of S-phase cells in normal and neoplastic tissues with in vitro BrdU labeling and standard immunohistochemical techniques using anti-BrdU-MAb and avidin-biotin peroxidase complex is described. We have employed this method in 47 human solid tumor samples, including squamous cell carcinomas of head and neck and cervix uteri, adenocarcinomas and malignant lymphomas, and also evaluated the possible application of the BrdU labeling index to estimate the cycling S-phase cells in neoplastic cell populations. In our data, the in vitro labeling index varied greatly in an individual case (3.56-29.2%) and from an area to an area within the same case. Squamous cell carcinomas of the head and neck showed higher LI than those of the cervix uteri. A case of metastatic carcinoma to the lung from ductal carcinoma of the breast had the highest LI (29.2%), in contrast to the low LI (3.6%) in the primary ductal carcinoma of breast.

Key Words: *Bromodeoxyuridine, S-phase, in vitro BrdU labeling, immunohistochemistry, proliferative activity, avidin-biotin peroxidase.*

INTRODUCTION

Bromodeoxyuridine (BrdU) is a thymidine analogue that is incorporated into nuclear DNA. BrdU has recently been available for the study of cell kinetics of the tumor, and the development of a monoclonal antibody makes it possible to immunohistochemically visualize a population of S-phase cells that can be expressed as the BrdU labeling index (LI) (Gratzner, 1982, Morstyn et al., 1983). Up to now, the evaluation of DNA synthesizing cells has mainly been based on autoradiography. Although this technique delivers satisfactory results, it suffers from the disadvantage of technical complications associated with the use of radioactive substances (Schutte et al., 1987).

The BrdU labeling technique offers the advantage of simple and safe handling and a comparatively short processing time. In vitro, the immunohistochemical application of the BrdU/anti-BrdU-MAb method of solid tumor permits a quantitative assessment of the proliferative activity of a tissue as well as the direct location of the actively replicating cells in histological sections (Risio et al., 1986); it also obviates risk to patients and expense. Until now, there has been few reports on the

Address for Correspondence: *Sang-Sook Lee, M.D. Department of Pathology, Keimyung University School of Medicine, #194 Dong-san Dong, Taegu, Korea 700-310.*
This study is in part supported by Keimyung University Research Grant (1989).

immunohistochemical detection of S-phase cells in vitro in the human biopsy specimens, using standard ABC technique.

In this study, we have used the BrdU/anti-BrdU method histologically, incubating the small samples of human tissues in vitro with a suitable quantity of BrdU. We then applied anti-BrdU-MAb directly to the histological sections to display the cycling S-phase cells with a positive anti-BrdU-MAb nucleus using standard immunohistochemical techniques (avidin-biotin peroxidase complex). We believe that in vitro BrdU-labeling may become a promising new tool for cell biologists and oncologists.

MATERIALS AND METHODS

We have examined various tumor samples from 47 patients with neoplasia (squamous cell carcinoma of head and neck, 16 patient; squamous cell carcinoma of cervix uteri, 19 patients; breast carcinoma, primary and metastatic, 1 patient, respectively; adenocarcinoma, primary, 5 patients; malignant lymphoma, 5 patients). A fresh tissue is required for determining LI, but it may be held in a refrigerator overnight. Several pieces of fresh human normal or pathological tissues, about 0.5cm×0.5cm in size, were sampled. They were cut into smaller tissue blocks, not larger than 1 mm^3, and placed immediately in the vial containing incubation medium. It is known that oxygenation of tissues is necessary for DNA synthesizing cells to incorporate 3H-thymidine in vitro (Steel and Bensted, 1965). The optimal oxygen pressure was described to be 3-4 atm (Fabricant et al., 1969). To obtain this condition, we filled the 8-ml vial with 4ml of incubation medium containing 10^{-3}M BrdU and 10^{-5}M 5-fluoro-2'-deoxyuridine (FUdR), and then plunged more than 10ml of pure oxygen into the capped vial, using disposable syringe. For in vitro labeling with BrdU, 10^{-5}M FUdR is included to lower intracellular thymidine phosphate pools by blockade of thymidylate synthetase and thereby to enhance the uptake of the BrdU (Meyer, 1982).

Slices were incubated with BrdU in the presence of FUdR and hyperbaric oxygen for 2 hours at 37°C with agitation. We inserted the needle of empty syringe into the top of the vial, and allowed pressure to force plunger up. We recorded the amount of O_2 returned and also temperature of water bath and color of solution in vial. We aspirated supernatant, and washed tissue slices twice with Carnoy's fixative. The tissues were fixed in Carnoy's fixative for 12-24 hours and processed through paraffin. For a partial denaturation of double-stranded DNA, we incubated sections in 95% (v/v) formamide in 0.15M trisodium citrate at 70°C for 45 minutes, and three washes in PBS, pH 7.5 for 5 minutes. The primary antibody (anti-BrdU) is a mouse monoclonal (DAKO, U.S.A.). We applied the avidin-biotin peroxidase complex technique, using the Vectastain-ABC Kit PK-4002 (VECTOR, U.S.A.).

The treatment involved the following steps:

1) Incubation of the sections with normal horse serum for 30 min; 2) Incubation with anti-BrdU-MAb (Mouse IgG1) diluted 1:20 in 0.2 M PBS buffer, pH 7.2; 3) Incubation with biotinylated horse anti-mouse IgG1 antibody for 30 min; 4) incubation with avidin-biotin peroxidase complex for 45 min; 5) Incubation with 0.06% 3-3' diamino-benzidine tetrahydrochloride (DAB) (SIGMA, U.S.A.) and 0.03% H_2O_2 in 0.2M PBS buffer, pH 7.2; 6) Weak contrast staining with nuclear fast red and light green. Dehydration, clarification and mounting of sections were done. The treatment of some sections with DAB alone and omittion of one or more incubations in others were invariably negative.

The LI (labeling index), indicating S-phase cells, was determined by calculating the percentage of BrdU-labeled cells with respect to the total tumor cells observed. A total of more than 1,000 cells from 4-6 different microscopic fields (100× power field) of the same tumor were counted and expressed as percent labelled cells. When fragments were small enough to allow the BrdU to completely pentrate, counting was done throughout all the section; otherwise, the counting was limited to the periphery of the section.

RESULT

A variety of normal and malignant tissues including squamous cell carcinoma (head and neck, and cervix uteri primary), adenocarcinomas (breast, gastric, colon, overy and cervix uteri primary) and malignant lymphomas has been studied.

The immunoperoxidase staining for BrdU was confined to the nucleus. Nuclear immunostaining of S-phase cells was mostly seen in the superficial layers of the tissue slices to a depth of 80-100 microns, although occasional cases revealed BrdU-labeled nuclei in the full-thickness of the sections.

The nuclear details and the histological relationship of the positive BrdU cells in a neoplastic lesion, such as cellular foci with the different kinetic activity in the histological sections were well demonstrated.

Our results indicated that the in vitro labeling indices were greatly variable (3.56-29.2%) from a case to a case and from an area to an area (Table 1). Squamous cell carcinomas of the head and neck showed higher LI than those of the cervix uteri. A case of

Table 1. BrdU Labeling Indices of the Human Neoplasms.

	No.	Percent LI* Mean (range)
Squamous cell carcinoma		
Head and neck	16	17.9 (4.6-21.4)
Larynx	9	15.5
Tongue	4	21.4
Tonsil		6.2
Nasopharynx	1	4.6
Cervix uteri	19	10.3 (3.4-17.6)
Breast, carcinoma	1	3.6
Metastasis to lung	1	29.2
Adenocarcinoma, primary		
Stomach	1	14.4
Colon, transverse	1	21.8
Cervix uteri	2	6.2
Ovary	1	12.4
Malignant lymphoma	5	9.0

LI*: in vitro BrdU labeling index

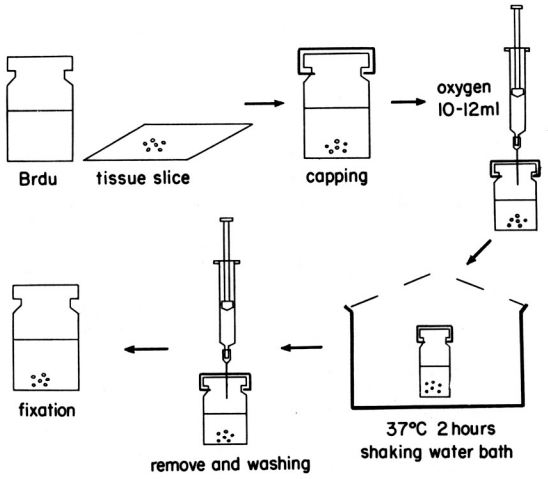

Fig. 1. Schematic drawing of in vitro BrdU labeling of human biopsy samples.

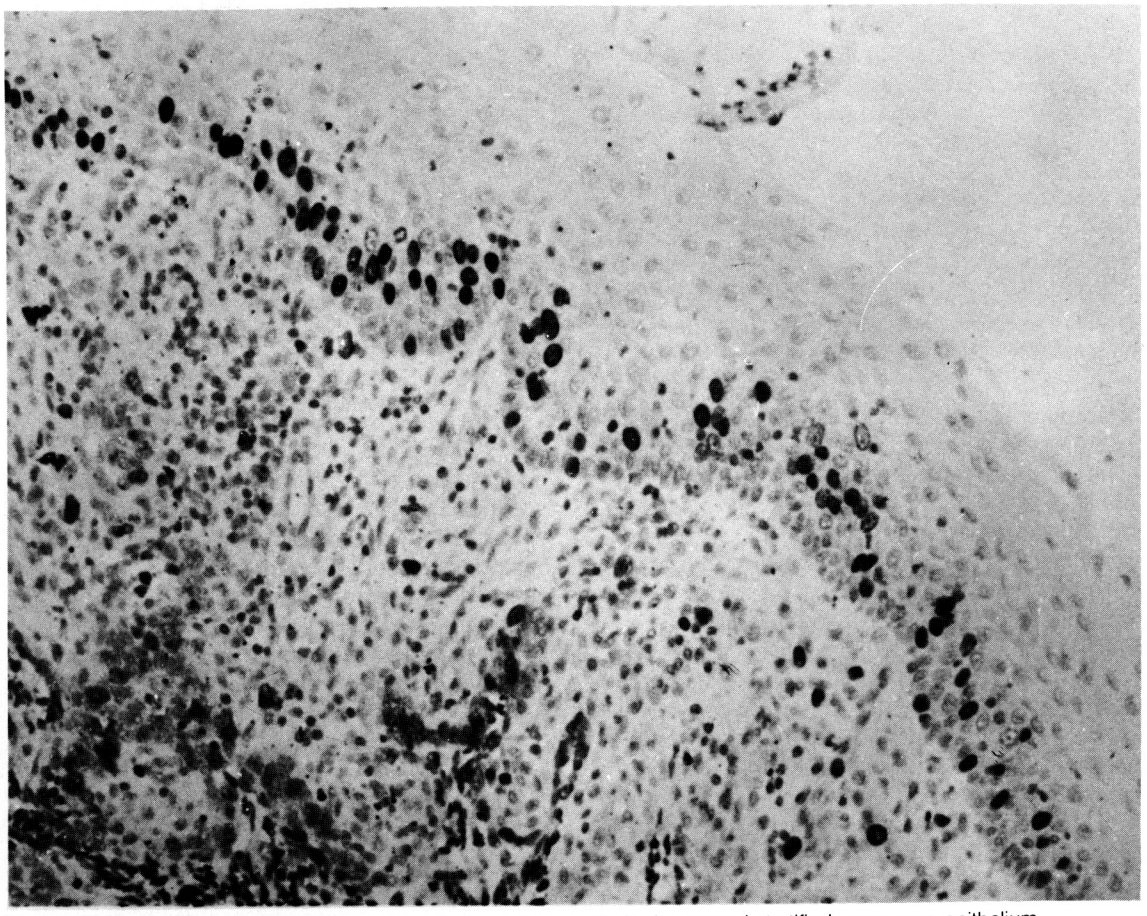

Fig. 2. S-phase cells were distributed in the para-and basal cells in the normal stratified squamous epithelium.

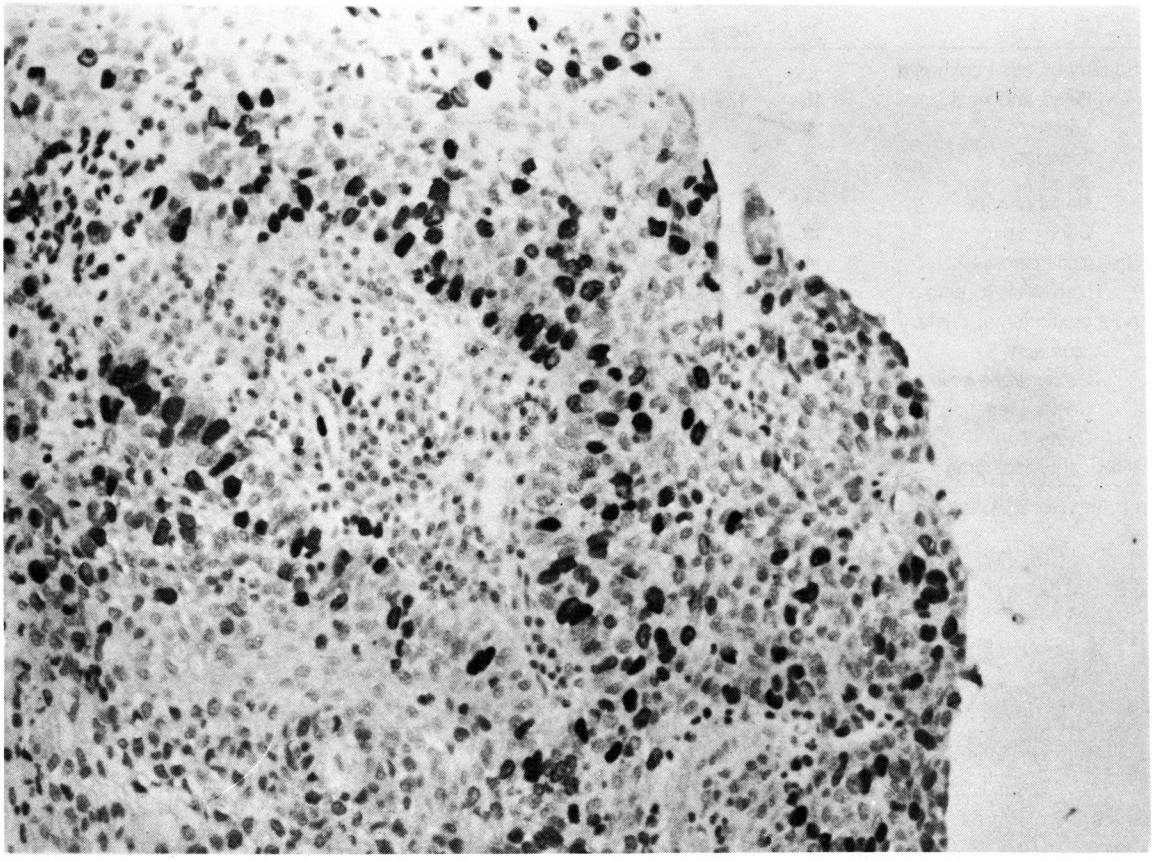

Fig. 3. In the squamous cell carcinoma of larynx, the covering neoplastic epithelium contained disorderly arranged, S-phase cells in the whole thickness.

metastatic carcinoma to the lung was highest in the LI (29.2%). In normal stratified squamous epithelium, S-phase cells were distributed in the para-and basal cells (Fig. 2). But in squamous cell carcinoma, the covering neoplastic epithelium contained the disorderly arranged S-phase cells in the whole thickness of the tumor epithelium (Fig. 3). The S-phase cells were mostly confined to the periphery of the invasive tumor nests in the well-differentiated, basaloid squamous cell carcinoma (Fig. 4), but became disorderly arranged in the poorly differentiated squamous cell carcinoma (Fig. 5). There was no BrdU staining in the nuclei showing mitotic activity. The labelled cells in adenocarcinomas were distributed haphazardly along the entire length of tubular structures or in the sheets of tumor cells (Fig. 6). In malignant lymphoma, several lymphoid cells were positive (Fig. 7).

DISCUSSION

Evaluation of the proliferative activity of neoplastic cell population is one of the most essential parameter of the biology of neoplasms for prognostic and therapeutic purposes (Risio et al., 1986). For this purpose either autoradiography after incubation with tritiated thymidine (Meyer and Conner, 1977) or flow cytometric measurement (Dean et al., 1982) was mainly used. However, these techniques which lack optimal cytologic resolution are rather time consuming or require complex mathematical data processing (Tazzari et al., 1988).

More recently, immunologic methods for measuring the proliferation rates of cells have been proposed. In particular,monoclonal antibodies which recognize antigens expressed during the cell cycle (e.g., OKT9 and Ki67) have been used in order to assess the

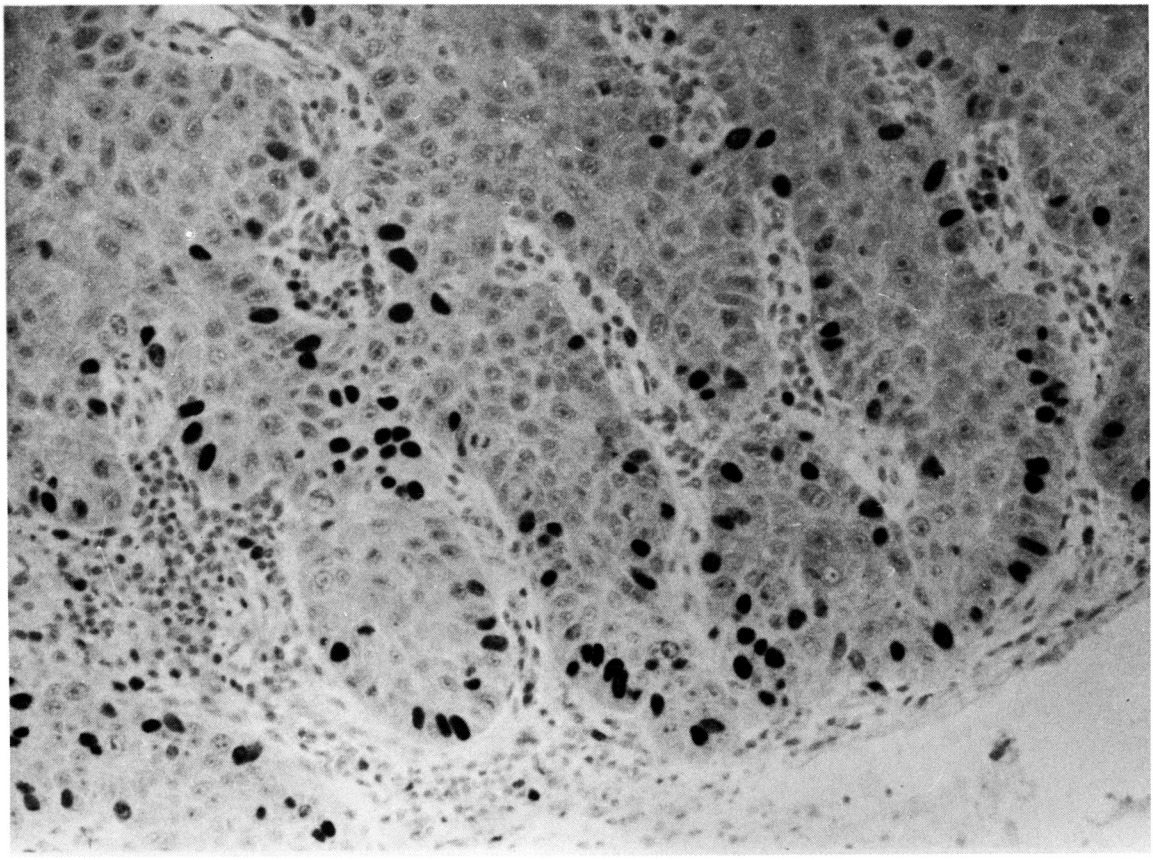

Fig. 4. S-phase cells were mostly confined to the periphery of the invasive tumor nests in the well-differentiated, basaloid squamous cell carcinoma of larynx.

growth fraction (Gerdes et al., 1984, Pileri et al., 1987).

Bromodeoxyuridine, a pyrimidine analog of thymidine, may be identified in the nucleus after its incorporation into S-phase cells during DNA synthesis in a culture medium by means of a monoclonal antibody anti-bromodeoxyuridine, recently produced by Gratzner (1982). Bromodeoxyuridine has also been widely used to estimate the proliferative activity of neoplastic populations, including solid tumors, especially of brain tumors, breast carcinomas, leukemias and lymphomas (Hoshino et al., 1983, Meyer, 1986, Raza et al., 1985, Danova et al., 1987). The percentage of BrdU positive elements represents a reliable determination of the S-phase fraction in a given cell population. It has been shown that the results obtained with this technique are comparable to those by the autoradiographic method using ^{3}H-thymidine (Hamada, 1985, Raza et al., 1984, Boccadoro et al., 1986); according to Dolberare et al. (1983), anti-BrdU-MAb is so sensitive that it can recognize cells in the very early S-phase which could not be detected by autoradiographic method.

The BrdU/anti-BrdU-MAb method can be employed in vivo, studying BrdU distribution in normal and neoplastic cells in animals (Schutte et al., 1987) or in humans (Hoshino et al., 1983, Nagashima et al., 1987). But in vivo studies in humans by using BrdU intravenously have been limited for reasons of risk to patient and expense. The in vitro BrdU labeling technique presented here offered the advantage of simple and safe handling and a comparatively short processing time, so that information on individual patients can be obtained within at least two days after taking a tumor sample. It also obviates the patient's risk and expense. As previously reported by Sasaki and Takahashi (1980), the in vitro labeling indices were

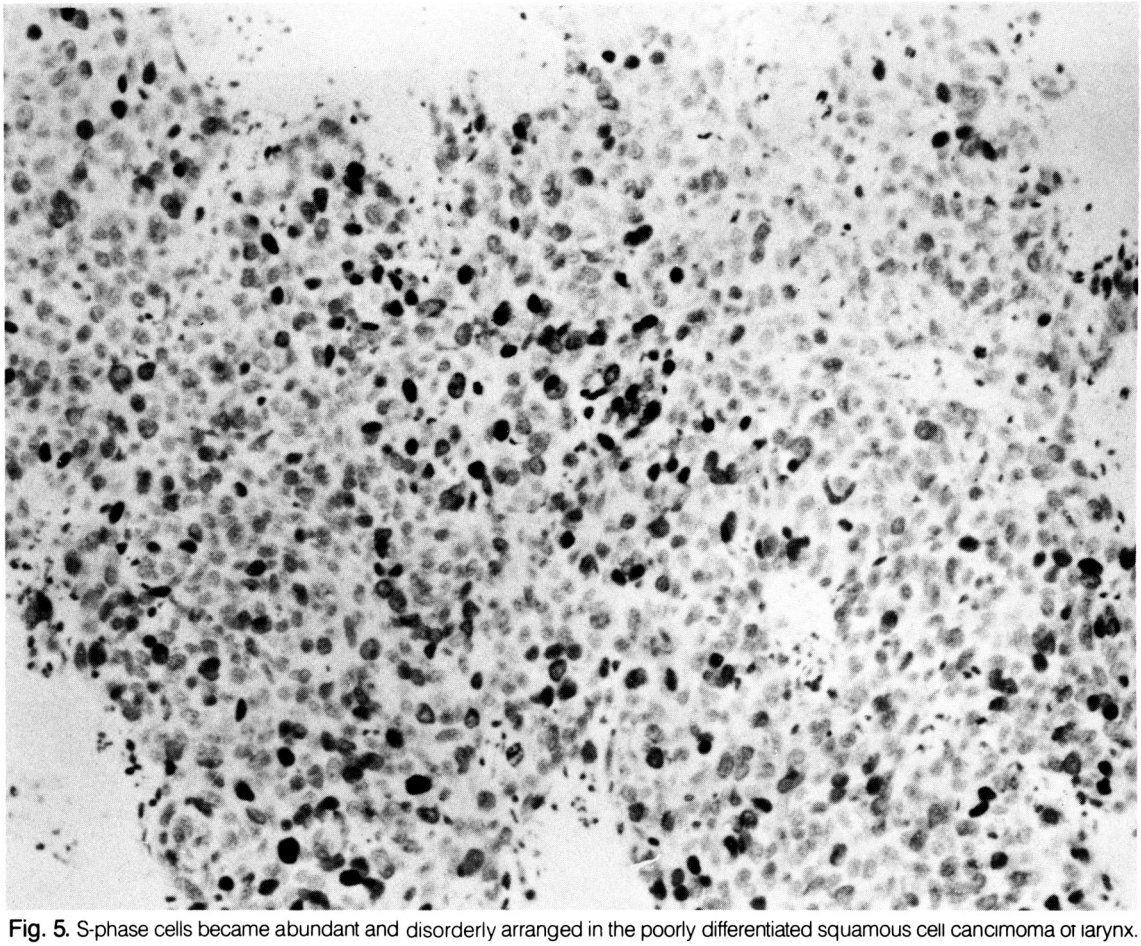

Fig. 5. S-phase cells became abundant and disorderly arranged in the poorly differentiated squamous cell cancimoma of larynx.

in good agreement with the in vivo labeling indices. Moreover, no difference was found between in vivo and in vitro labeling in the S-phase cells in the damaged mouse liver (Shimizu, 1987). Until now, there has been few reports of immunohistochemical detection of S-phase cells in vitro in the human biopsy specimens, by using standard ABC technique.

In our study, it is clear that an immunohistochemical study of cell kinetics can be performed on paraffin-embedded material, as indicated in the preliminary studies in surgical biopsies obtained from patients with melanoma or brain tumors after prior intraoperative intravenous infusion of BrdU (Hoshino et al., 1983). We used the BrdU/anti-BrdU method histologically, incubating small samples of human tissues in vitro with a suitable quantity of BrdU. We then applied anti-BrdU-MAb directly on the histological sections to display the actively replication cells with a positive anti-BrdU-MAb nucleus using standard immunohistochemical techniques (avidin-biotin-peroxidase complex).

In our data, the in vitro labeling index varied greatly in an individual case (3.56-29.2%) and from an area to an area within the same case. Squamous cell carcinomas of the head and neck showed higher LI than those of the cervix uteri. A case of metastatic carcinoma to the lung from ductal carcinoma of the breast had the highest LI (29.2%), in contrast to the low LI (3.6%) in the primary ductal carcinoma of breast.

Concerning the basis for the marked variability in the labeling index, we have no explanation. But MacDonald (1951) suggested that the cancer cells in individual cases may be endowed with inherent proliferation activity. Data are available from the most types of human malignancy; 4% (using 3H-thymidine LI) for primary breast cancers, 11% for colorectal cancers, 13% for squamous cell carcinomas, 5% for sarcomas and 21% for intermediate grade lymphomas; many solid tumors in human contain at most 10% cells

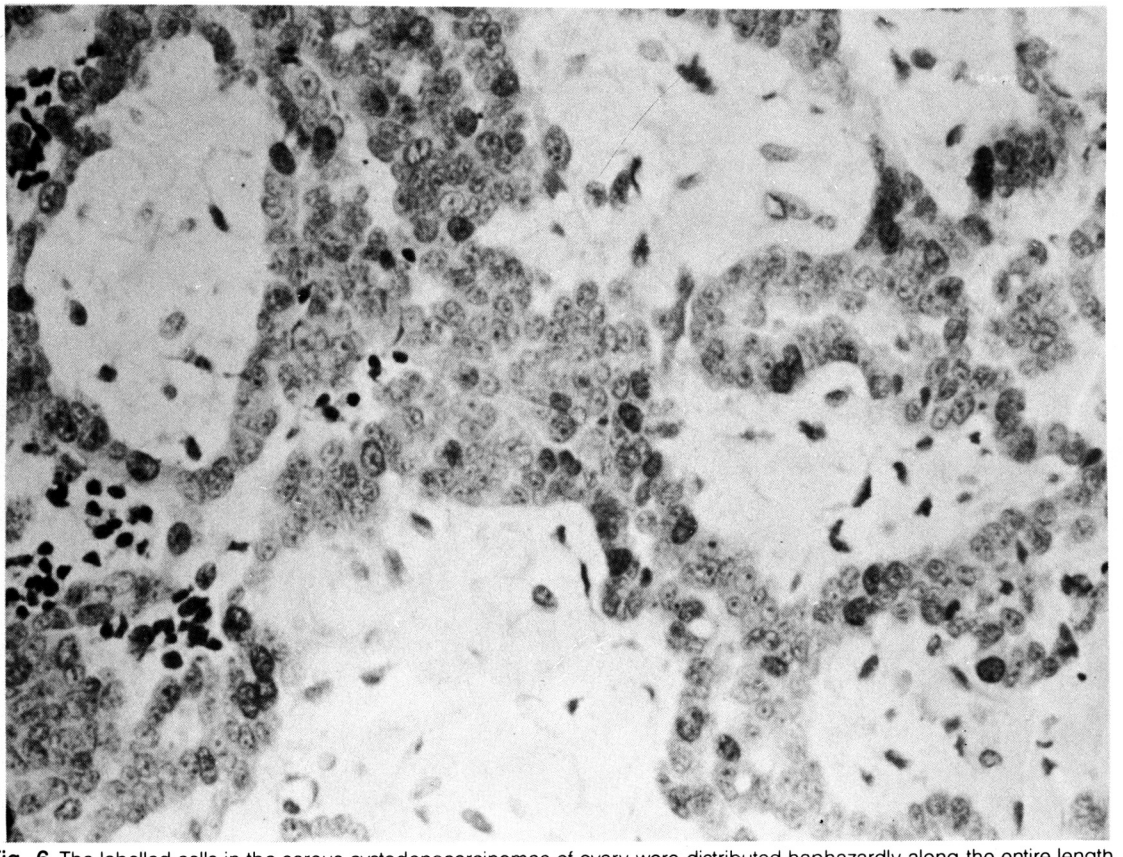

Fig. 6. The labelled cells in the serous cystadenocarcinomas of ovary were distributed haphazardly along the entire length of tubular structures or in sheets of tumor cells.

that are in DNA synthesis, although the higher proportions of S-phase cells are found in some rapidly growing tumors such as high grade lymphomas (Meyer, 1982, Wolberg and Ansfield, 1971). Proliferating cells in normal bone marrow and intestinal crypts have the values of labeling index in the ranges of 30% to 70% and 12% to 18%, respectively. Thus, tumors do not have a higher proportion of S-phase cells than some normal tissues (Tannock, 1989). In our study the immunoperoxidase staining for BrdU was confined to the nucleus. The nuclear immunostaining of S-phase cells was mostly seen in the superficial layers of the tisue slices to a depth of 80-100 micrometers, although occasional cases revealed BrdU-labeled nuclei in the full-thickness of the sections.

Limitation of BrdU incorporation as a distance from the interface between the tissue and the incubation medium was demonstrated in most cases as also shown in autoradiographic studies in vitro with tritiated thymidine (Titus and Shorter, 1963). The depth of thymidine labeling is increased by hyperbaric oxygenation (Fabrikant et al., 1969) or by cold preincubation (meyer and Connor, 1977). Gradient of labeling intensity from the surface to the interior of the tissue, as described by Meyer and Connor (1977) with autoradiographic method was also found in some cases.

It also offers the advantage of studying, in a neoplastic lesion, cellular foci with the different kinetic activity in the histological sections and directly correlating these with standard histological and cytological morphologic parameters. In our study, the S-phase cells were distributed in the para-and basal cells in normal stratified squamous epithelium, but became abundant and disorderly distributed throughout the entire thickness of the covering neoplastic epithelium in squamous cell carcinoma. In the well-differentiated,

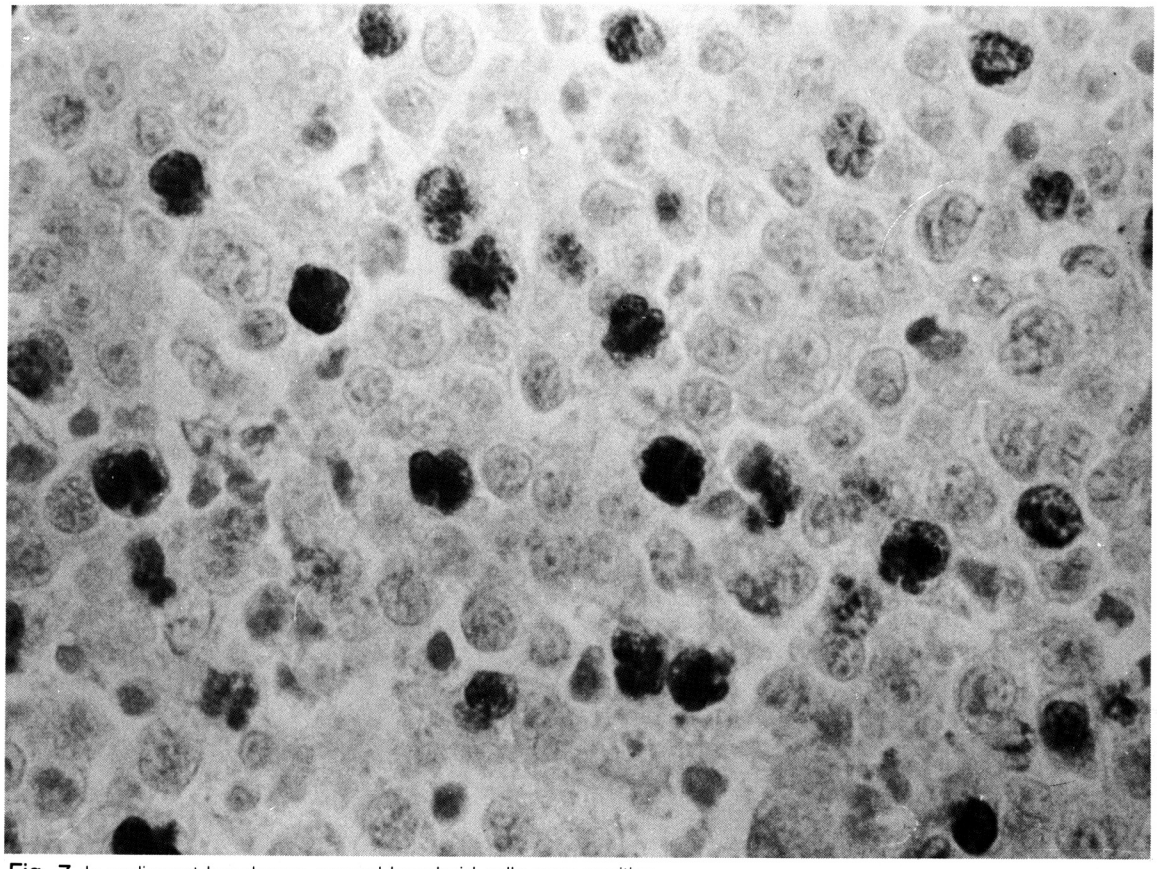

Fig. 7. In malignant lymphoma, several lymphoid cells were positive.

basaloid squamous cell carcinoma, S-phase cells were mostly confined to the periphery of the invasive tumor nests, but became more abundant and disorderly arranged in the poorly differentiated squamous cell carcinoma. There was no BrdU staining in the nuclei showing a mitotic activity. The labelled cells in adenocarcinomas were distributed haphazardly along the entire length of tubular structures or in the sheets of tumor cells. In malignant lymphoma, several lymphoid cells were positive.

Most procedures for the identification of BrdU labeled cells are based on the use of monoclonal antibody that recognize BrdU in single stranded DNA. The limitation of the immunochemical techniques is variability when different cell types are compared. We considered the variability to be due to differences in chromatin structure affecting the denaturation of DNA required for making BrdU accessible to the antibodies. In order to expose the binding sites for the antibodies, in situ denaturation of cellular DNA is needed prior to immune reaction (Schutte et al., 1987). In the initial paper on immunocytochemical detection of S-phase cells by Gratzner et al. (1982), acid denaturation of DNA was used. An alternative technique, based on thermal denaturation of DNA. appeared to yield more intense immunostaining (Dolbeare et al., 1985).

With this technique, cell kinetic studies of human solid tumors after an in vitro labeling procedure can be performed, provide important information related to the optimal scheduling chemotherapeutic or radiotherapeutic treatment.

REFERENCES

Boccadoro M, Redoglio V, Gavarotti P, et al.: *Multiple myeloma plasma cell kinetics: rapid and reliable evaluation using 5-bromo-2-deoxyuridine (BrdUrd) DNA incorporation by an anti-BrdUrd monoclonal antibody. Tumori 72:135-137, 1986.*

Danova M, Wilson G, Riccardi A, et al.: *In vivo administration of bromodeoxyuridine and flow cytometry for cell kinetic studies in human malignancies. Haematologica 72:115-119, 1987.*

Dean PN, Gray JW, Dolbeare FA: *The analysis and interpretation of DNA distributions measured by flow cytometry. Cytometry 3:188-195, 1982.*

Dolbeare F, Gratzner H, Pallavicini M, et al.: *Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc Natl Acal Sci USA 80:5573-5577, 1983.*

Dolbeare F, Beisker W, Pallavicini MG, et al.: *Cytochemistry for bromodeoxyuridine/DNA analysis: Stoichiometry and sensitivity. Cytometry 6:521-530, 1985.*

Fabricant JI, Wisseman CL, Vitak M: *The kinetics of cellular proliferation in normal and malignant tissues. Radiology 92:1309-1320, 1969.*

Gerdes J, Lemke H, Baisch H, et al.: *Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 13:1710-1715, 1984.*

Gratzner HG: *Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science 218:474-475, 1982.*

Hamada S: *A double labeling technique combining ^{3}H-thymidine autoradiography with BrdUrd immunocytochemistry. Acta Histochem Cytochem 18:267-270, 1985.*

Hoshino T, Nagashima T, Murovic J, et al.: *Cell kinetic studies of in situ human brain tumors with bromodeoxyuridine. Cytometry 6:627-632, 1983.*

MacDonald I: *Biological predeterminism in human cancer, cited by* Sasaki K, Takahashi M, Ogino T, et al.: *An autoradiographic study on the labeling index of biopsy specimens from gastric cancers. Cancer 54:1307-1309, 1984.*

Meyer JS, Conner RE: *In vitro labeling of solid tissues with tritiated thymidine for autoradiographic detection of S-phase nuclei. Stain Technol 52:185-196, 1977.*

Meyer JS: *Cell kinetic measurements of human tumors. Hum Pathol 13:874-877, 1982.*

Meyer JS: *Cell kinetics in selection and stratification of patients for adjuvant therapy of breast carcinoma. NCI Monogr 1:25-28, 1986.*

Morstyn G, Hsu SM, Gratzner KH, et al.: *Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. J Clin Invest 72:1844-1850, 1983.*

Nagashima T, Hoshino T, Cho KG: *Proliferative potential of vascular components in human glioblastoma multiforme. Acta Neuropathol 73:301-305, 1987.*

Pileri S, Gerdes J, Rivano MT, et al.: *Immunohistochemical determination of growth fractions in human permanent cell lines and lymphoid tumours: a critical comparison of the monoclonal antibodies OKT9 and Ki-67. Br J Haematol 65:271-276, 1987.*

Raza A, Ilar K, Preisler HD: *Double labeling and in vitro versus in vivo incorporation of bromodeoxyuridine in patients with acute nonlymphocytic leukemia. Cytometry 6:633-640, 1985.*

Raza A, Preisler HD, Mayers GL, et al.: *Rapid enumeration of S-phase cells by means of monoclonal antibodies. N Engl J Med 310:991, 1984.*

Risio M, Coverlizza S, Poccardi G, et al.: *In vitro immunohistochemical localization of S-phase cells by a monoclonal antibody to bromodeoxyuridine. Bas Appl Histochem 30:469-477, 1986.*

Sasaki K, Takahashi M: *Preservation of cell cycle characteristics in solid tumor in vitro. Cancer Res 40:4810-4812, 1980.*

Schutte B, Reynders MMJ, Bosman FT, et al.: *Studies with anti-Bromodeoxyuridine antibodies: II. Simultaneous immunocytochemical detection of antigen expression and DNA synthesis by in vivo labeling of mouse intestinal mucosa. J Histochem Cytochem 35:371-374, 1987.*

Shimizu A, Inoue, T, Kanisawa N: *Analysis of hepatic cell kinetics by BrdU-anti-BrdU method (Abstr). Third Annual Meeting of the Society of Toxicological Pathology 1987, p.77 (in Japanese).*

Steel GG, Bensted JPM: *In vitro studies of cell proliferation in tumors-1. Critical appraisal of methods and theoretical considerations. Eur J Cancer 1:275-279, 1965.*

Tannock IF: *Principles of cell proliferation: Cell kinetics, in* DeVita VT, Hellman S, Rosenberg SA (eds): *Cancer: Principles and Practice of Oncology, ed 3. Philadelphia, J B Lippincott, 1989, chap 1.*

Tazzari PL, Gobbi M, Tassi C: *In situ staining of bromodeoxyuridine postive cells in normal and neoplastic colony-forming units grown on plasma clots. J Immunol Methods 113:215-219, 1988.*

Titus J, Shorter R: *Labeling of human tumors with tritiated thymidine. Arch Pathol 79:324-328, 1963.*

Wolberg WH, Ansfield FJ: *The relation of thymidine labeling index in human tumors in vitro to the effectiveness of 5-fluorouracil chemotherapy. Cancer Res 81:448, 1971.*