

## Cell Proliferation in Rat Colon Measured with Bromodeoxyuridine, Proliferating Cell Nuclear Antigen, and [<sup>3</sup>H]Thymidine

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### Abstract

**Epithelial cell proliferation was studied in the normal colonic mucosa of 5-week-old Sprague-Dawley rats, comparing [<sup>3</sup>H]thymidine incorporation (group 1) with two newer proliferation markers, bromodeoxyuridine (group 2) and proliferating cell nuclear antigen (group 3). Microautoradiography (group 1) or immunoperoxidase assays (groups 2 and 3) were carried out. Cells were counted for positive reaction and position along 50 colonic crypt columns/animal. No significant differences were found in number or distribution of labeled epithelial cells in proliferative compartments in crypt columns of normal colonic mucosa; labeled cells were mainly in the lower 60% of colonic crypts. Thus, in this model, bromodeoxyuridine and proliferating cell nuclear antigens were comparable to [<sup>3</sup>H]thymidine as reliable markers of proliferating epithelial cells in rat colon.**

### Introduction

Measurements of cellular proliferation have revealed abnormal proliferative patterns in normal-appearing colonic mucosa of individuals at increased risk for the development of colorectal cancer. In patients with familial polyposis, who have a genetically increased risk to develop colon cancer, an expansion of the proliferative compartment has been found (1). This abnormality of epithelial cell proliferation can be considered a preneoplastic biomarker and has been observed to a lesser extent in the mucosa of patients with sporadic adenomas, nonfamilial colon cancer, and hereditary nonpolyposis colorectal cancer (2, 3). Similarly, corresponding changes have been demonstrated to develop in animals after carcinogen treatment prior to tumor formation (4). Thus, epithelial cell proliferation as an intermediate biomarker is being increasingly studied in order to assess the effects of

chemical agents. More recently dietary intervention regimens attempting to inhibit the development of colorectal cancer have been evaluated (5-8).

[<sup>3</sup>H]dThd<sup>3</sup> labeling of S-phase cells and detection of labeled cells by autoradiography is the classical way of identifying proliferating cells in tissue sections. More recently monoclonal antibodies and immunohistochemical techniques have become available for the assessment of cellular proliferation. BrdUrd, a pyrimidine analogue of thymidine, is also incorporated into DNA during cell replication and can be detected by an anti-BrdUrd monoclonal antibody (9). PCNA, a M<sub>r</sub> 36,000 nuclear protein, is expressed in proliferating cells and functions in replication as an auxiliary protein of DNA polymerase  $\delta$ . Its synthesis sharply increases late in G<sub>1</sub> phase, immediately preceding DNA synthesis, and it continues to increase during S phase and declines throughout G<sub>2</sub> and M (10). Little attention has been given so far to the question of whether these newer markers really match [<sup>3</sup>H]dThd labeling and might therefore be potential substitutes. In this study we compared [<sup>3</sup>H]dThd, BrdUrd and PCNA as markers for epithelial cell proliferation in normal rat colon.

### Materials and Methods

Twenty female Sprague-Dawley rats were purchased at 3 weeks of age and kept under standardized conditions in the animal facility. They were allowed to adapt for 2 weeks and then treated as follows: seven animals (group 1) received i.p. injections of 1  $\mu$ Ci/g [<sup>3</sup>H]dThd (Amersham), six animals (group 2) received an i.p. injection of 0.02 mg/g BrdUrd (Sigma), and the remaining seven animals (group 3) received an injection of an equal volume of 0.9% saline. All animals were sacrificed by cervical dislocation 1 h after the injection. The colons were removed, and pieces of tissue from the sigmoid were fixed for 24 h and subsequently processed and embedded in paraffin. The fixatives used were 10% buffered formalin (groups 1 and 2) and 95% ethanol (group 3), respectively. Four- $\mu$ m serial sections were cut from paraffin blocks and mounted on gelatin-coated slides.

Microautoradiography was performed on [<sup>3</sup>H]dThd-tagged tissue as described elsewhere (3). Briefly, the slides were dipped into NTB2 photographic emulsion (Kodak), developed after 4 weeks of exposure, and counterstained with hematoxylin and eosin. Immunoperoxidase assays were carried out for BrdUrd and PCNA

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<sup>3</sup> The abbreviations used are: [<sup>3</sup>H]dThd, [<sup>3</sup>H]thymidine; BrdUrd, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen.

detection: tissue sections were deparaffinized in xylene and immersed in 1.2% H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase activity. For BrdUrd immunohistochemistry (group 2) after hydration through graded alcohols the slides were treated with 2 N HCl for 1 h. This DNA denaturation is necessary as the anti-BrdUrd antibody binds only to incorporated BrdUrd on single-stranded DNA. Enzyme digestion with 0.2% pepsin in 0.01 N HCl for 30 min at 37°C was followed by incubation with 10% normal horse serum to reduce unspecific background staining. Anti-BrdUrd monoclonal antibody (Becton-Dickinson) diluted 1:500 with phosphate-buffered saline was administered to the sections for 12 h at 4°C. Biotinylated horse anti-mouse IgG (Vector Laboratories) was used as secondary antibody at 1:100 dilution for 1 h. The slides were then incubated with avidin-biotin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories) at 1:100 dilution for 45 min. The peroxidase reaction was eventually performed by submerging the slides in 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in phosphate-buffered saline to which 0.03% H<sub>2</sub>O<sub>2</sub> had been added immediately prior to use. Finally, the slides were lightly counterstained with hematoxylin, dehydrated, and mounted in Permount (Fisher Scientific). PCNA immunohistochemistry (group 3) was performed accordingly, with omission of the DNA denaturation and enzyme digestion steps. Ten % normal goat serum was used for background reduction followed by a 12-h incubation with anti-PCNA 19A2-IgM monoclonal antibody (American Biotech) diluted 1:4000 at 4°C. Biotinylated goat anti-mouse IgM (Vector Laboratories) was used as secondary antibody. All incubations were carried out in a humidified chamber at room temperature unless otherwise indicated, and between incubations, slides were extensively washed with three changes of phosphate-buffered saline.

In counting immunohistochemically stained nuclei, positive cells were stained brown using diaminobenzidine as a chromogen. With hematoxylin counterstain, immunohistochemically negative nuclei were stained blue, providing strong contrast. In black-and-white photographs positive cells appeared gray. In this study nuclei stained brown were easily recognized under the microscope, and even nuclei stained light brown were counted.

## Results

In group 1 cells having incorporated [<sup>3</sup>H]dThd presented with black silver grains over the nucleus after microautoradiography (Fig. 1a). Immunoperoxidase staining (group 2 and 3) resulted in a distinct brown color over labeled nuclei (Fig. 1, b-d). In all three groups 50 colonic crypt columns/animal were studied. The crypt column is a column of cells from the midpoint of the crypt base to the surface of the crypt, providing a pathway for cell migration and differentiation. The number of labeled cells was recorded, as well as their position along the longitudinal crypt column, which was further subdivided into five compartments, compartment 1 being at the base of the crypt and compartment 5 at the colonic lumen. From these data labeling indices were computed as number of labeled cells/total number of cells. The data thus obtained are summarized in Table 1 and the results displayed graphically in Figs. 2 and 3.

No significant difference was found for any of the parameters studied by  $\chi^2$  analysis and Student's *t* test, respectively. The number of epithelial cells per crypt column was  $33.41 \pm 0.660$  (SE) (group 1) versus  $33.04 \pm 0.812$  (group 2) versus  $32.79 \pm 0.658$  (group 3). This parameter was not influenced by the different methods used and therefore indicates good comparability of the three groups. No difference was found in the number of labeled cells per crypt column:  $2.98 \pm 0.281$  (group 1);  $3.02 \pm 0.276$  (group 2); and  $3.11 \pm 0.298$  (group 3). The labeling index was  $0.089 \pm 0.008$  (group 1),  $0.092 \pm 0.009$  (group 2), and  $0.095 \pm 0.010$  (group 3), revealing no significant differences between the groups studied. The distribution of labeled cells within the crypt column was also quite comparable with the majority of labeled cells being in the lower 60% of the crypt.

## Discussion

Our study was undertaken to evaluate different markers for studying epithelial cell proliferation in the colon. [<sup>3</sup>H]dThd labeling of cells which are in the S phase of the cell cycle at the time of the experiment and detection of labeled cells by autoradiography have been extensively used in numerous cell kinetic studies. In particular, in the colon of humans and rodents, altered properties of cell proliferation have been observed in normal-appearing mucosa by [<sup>3</sup>H]dThd labeling. Specifically, an expansion of the proliferative compartment has been shown to be a preneoplastic biomarker (5,6). Thus, [<sup>3</sup>H]dThd labeling can be considered the gold standard for studying preneoplastic proliferative changes of the colonic mucosa. This method, although well established, has the disadvantage that an exposure time of several weeks is needed for microautoradiography, and radioactive material is used as a DNA precursor. The latter point makes *in vivo* studies in humans virtually impossible.

More recently, the use of monoclonal antibodies applied to immunohistochemical techniques has provided alternatives to radiolabeling the tissue in order to study cell proliferation. BrdUrd is a pyrimidine analogue of thymidine that can be incorporated into replicating DNA instead of thymidine. Monoclonal antibodies against BrdUrd have been developed (9), and it was shown indeed that BrdUrd incorporation parallels thymidine incorporation into S-phase cells (11-13). BrdUrd can be injected into animals and has been injected into humans for tumor cell kinetic studies (14). For prevention studies in the healthy population, however, *in vitro* techniques have to be used. Recently, *in vitro* incubation of colonic biopsies with BrdUrd and detection of labeled cells by immunohistochemistry have been demonstrated by Risio *et al.* (15).

PCNA is a *M<sub>r</sub>* 36,000 nuclear protein. It has been discovered and characterized by Miyachy *et al.* as an autoantibody in the serum of a subgroup of patients with lupus erythematosus (16, 17). Independently, Bravo *et al.* (18, 19) found a similar protein in HeLa cells. The latter group called this protein cyclin because of its predominant synthesis in cycling cells. It was later demonstrated by Mathews *et al.* (20) that PCNA and cyclin are identical. PCNA expression increases at the end of G<sub>1</sub> phase immediately preceding DNA synthesis, and it reaches a maximum during S phase and declines through G<sub>2</sub> (21). As shown by Celis *et al.* (22, 23) and Bravo and

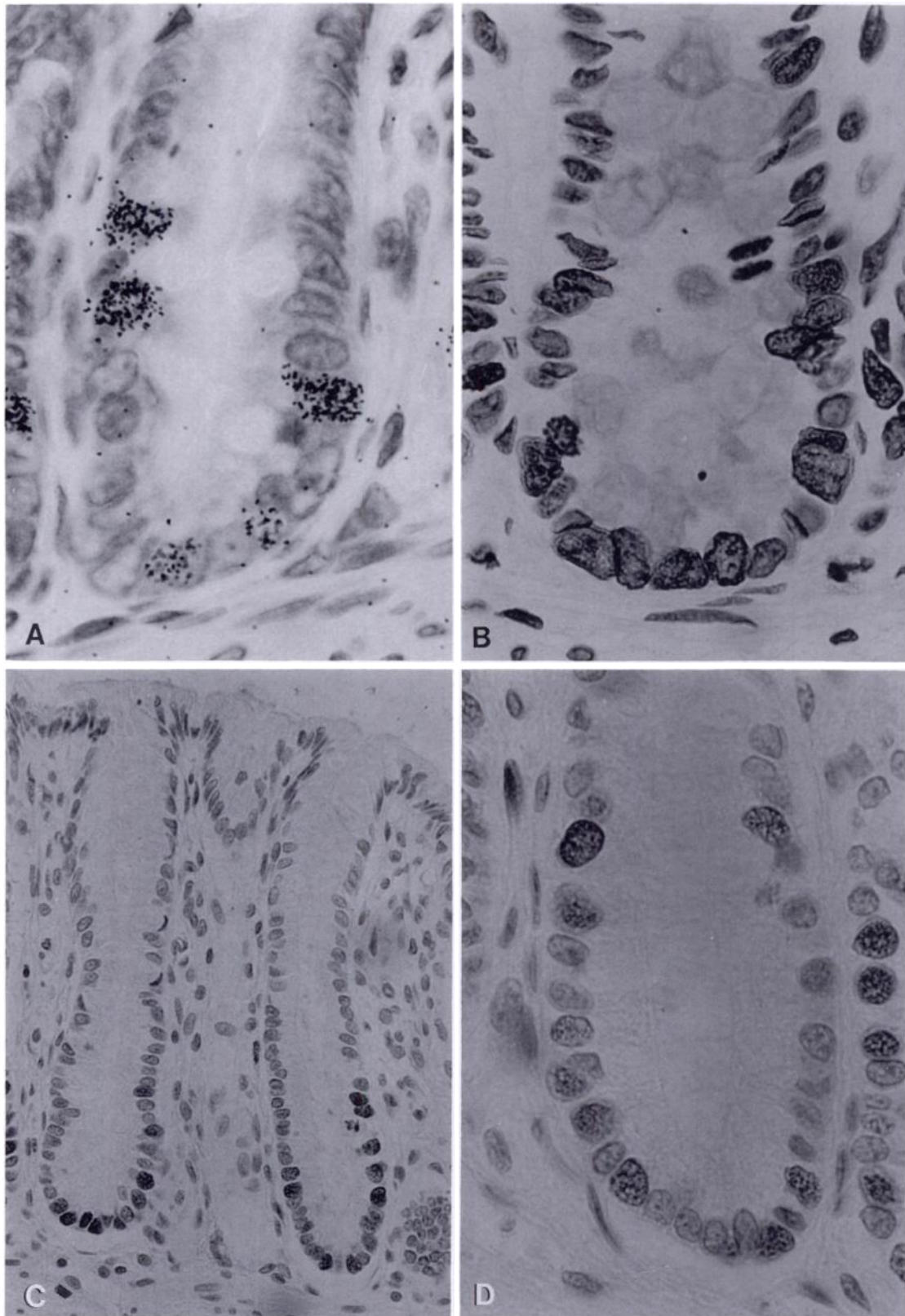


Fig. 1. Colonic crypts with proliferating epithelial cells labeled by different markers. A, [ $^3\text{H}$ ]dThd, H&E,  $\times 100$ ; B, BrdUrd, hematoxylin,  $\times 100$ ; C, PCNA, hematoxylin,  $\times 40$ ; D, PCNA, hematoxylin,  $\times 100$ .

Table 1 Summary of data obtained by analyzing 50 crypt columns/animal

	Groups Studied		
	[ <sup>3</sup> H]dThd	BrdUrd	PCNA
Empirical data			
No. of animals in group	7	6	7
Total no. of crypt columns assayed	350	300	350
Totals of cells for the crypt as a whole (labeled cells/all epithelial cells)	1042/11692	905/9912	1087/11477
Totals of cells by crypt compartment (labeled cells/all epithelial cells)			
Compartment 1	476/2203	466/1867	521/2166
Compartment 2	444/2345	350/1979	460/2299
Compartment 3	121/2326	861/1988	103/2288
Compartment 4	1/2345	3/1979	2/2299
Compartment 5	0/2473	0/2099	1/2425
Computed data			
Labeling index by crypt compartment (mean ± SE over the subjects in a group)			
Compartment 1	0.215 ± 0.018	0.251 ± 0.032	0.240 ± 0.019
Compartment 2	0.190 ± 0.011	0.178 ± 0.016	0.201 ± 0.022
Compartment 3	0.052 ± 0.011	0.044 ± 0.005	0.046 ± 0.011
Compartment 4	0.000 ± 0.000	0.001 ± 0.001	0.001 ± 0.001
Compartment 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Whole crypt	0.089 ± 0.008	0.092 ± 0.009	0.095 ± 0.010
No. of cells per crypt column (mean ± SE)			
Labeled cells	2.98 ± 0.281	3.02 ± 0.276	3.11 ± 0.298
All epithelial cells	33.41 ± 0.660	33.04 ± 0.812	32.79 ± 0.658

Macdonald-Bravo (24), there is a close correlation between the number of PCNA-positive cells and [<sup>3</sup>H]dThd-labeled cells, and the nuclear distribution of PCNA during the S phase parallels that of [<sup>3</sup>H]dThd and BrdUrd (22–24). These data suggest that the function of PCNA is closely related to DNA replication. It has in fact been shown that PCNA is identical to an auxiliary protein of DNA polymerase  $\delta$  (25), and it is required for simian virus 40 replication (26).

Under the conditions chosen in our study colonic epithelial cell proliferation as assessed by BrdUrd and PCNA immunohistochemistry is quite comparable to the results obtained by [<sup>3</sup>H]dThd labeling. In the case of

BrdUrd this confirms previous reports of its ability to selectively label replicating cells in the S phase (27), and our results indicate that indeed it is a reliable, easy-to-use marker for epithelial cell proliferation in the colon. The situation is more complicated for PCNA. As mentioned before, PCNA synthesis increases in late G<sub>1</sub> and on a biochemical level is even detectable throughout the cell cycle in proliferating cells (24). Thus, PCNA expression seems to be less specific for the S phase as compared to [<sup>3</sup>H]dThd and BrdUrd and a considerably higher number of labeled cells could be expected. In fact, most of the histological studies of tissue sections that have been undertaken so far have reported a higher percentage of PCNA-positive cells than S-phase cells as detected by flow cytometry (28) or a considerably higher PCNA labeling index as compared to the [<sup>3</sup>H]dThd labeling index (29, 30). This has been attributed so far to G<sub>1</sub> cells expressing sufficient amounts of PCNA to be detected by the monoclonal antibodies. In view of these observations we were surprised to find that in our study the proliferation parameters obtained from PCNA immunohistochemistry were not different from [<sup>3</sup>H]dThd and BrdUrd labeling. However, Galand *et al.* (31, 32) recently reported comparable results in rat and human colon, too. This discrepancy may be due to the fact that different tissues and more importantly different fixatives have been used in the different studies. There is evidence now from some studies that indeed the fixative is of crucial importance for the degree of PCNA detection. It has been shown, for example, that two populations of PCNA can be distinguished in the nuclei of 3T3 cells, one of which is broadly detectable during the cell cycle

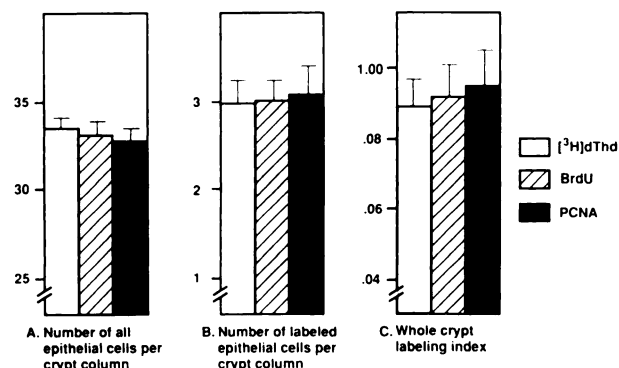


Fig. 2. Graphic display of whole-crypt proliferation parameters obtained by [<sup>3</sup>H]dThd, BrdUrd, and PCNA labeling. Bars, SE.

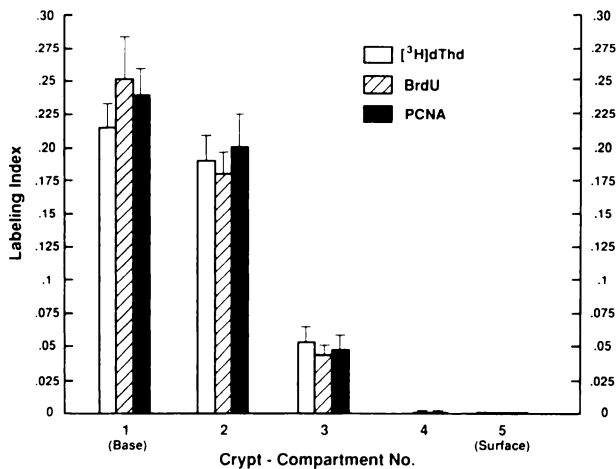


Fig. 3. Profiles of labeling indices in five colonic crypt compartments. Bars, SE.

in formalin-fixed cells; however, after treatment with detergent only a smaller PCNA fraction remains detectable which is tightly associated with DNA replication sites. Interestingly, absolute methanol, if used as a fixative, is also able to extract the loosely bound PCNA fraction, so that only the DNA-bound fraction remains detectable (24). This finding indicates a possibility of selectively detecting the PCNA fraction expressed in the S phase by use of alcohol as a fixative. This may be indeed the fact in our experiment (95% ethanol) as well as in Galand's study (methanol) and provide a possible explanation of the high comparability of PCNA and thymidine labeling indices in these studies.

In conclusion, under appropriate conditions BrdUrd and PCNA immunohistochemistry can be used as markers for colonic epithelial cell proliferation, giving results comparable to thymidine labeling. In the case of PCNA the fixative used is of crucial importance for the extent of PCNA expression. We recommend alcohol fixation to selectively label S-phase cells in the colon. Immunoperoxidase assays have the advantage that no radioactivity is used and no tedious exposure is necessary. PCNA, in addition, has the particular advantage that no DNA precursor is needed, facilitating *in vivo* studies in humans on a large scale. This is of special importance in view of the increasing numbers of studies on dietary intervention and chemoprevention that are now getting under way.

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