

Is Ki-67 a Better Proliferative Marker in the Colon than Proliferating Cell Nuclear Antigen?¹

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Abstract

Endogenous markers of proliferating cells have increasingly supplanted the use of incubation of biopsy tissues *in vitro* with tritiated thymidine or with bromodeoxyuridine, thus avoiding the potential variation resulting from the incubation procedure. Antibodies to proliferating cell nuclear antigen (PCNA) such as PC10 have been promoted as optimal for this purpose, although considerable variation in colonic proliferating cells with this antibody has been reported. We have compared the detection of colonic proliferating cells in normal mucosa and adenomata using the PC10 monoclonal antibody (mAb) to PCNA and the Mib-1 mAb to Ki-67 in formalin-fixed tissues using antigen retrieval solutions with microwaving. The PC10 antibody showed variable immunostaining of proliferating and nonproliferating cells with minor changes in primary antibody concentration or microwave conditions and between normal and adenomatous tissue. In contrast, Mib-1 immunostaining was quite constant with differing antigen retrieval and antibody conditions and similar staining of proliferating cells in colonic adenomas. Some loss of immunoreactivity occurred if the cut sections were not immunostained within approximately 1 week. These data suggest that whereas PCNA immunohistochemistry is satisfactory when carefully controlled in large chemopreventive studies, the Mib-1 mAb to Ki-67 is superior to PCNA antibodies in immunostaining proliferating cells in the formalin-fixed human colon.

Introduction

Measurement of the rates of cellular proliferation of normal, preneoplastic, and neoplastic tissues has been an important method of studying cancer development (1). Differences in

tumor proliferation rates have been used as a prognostic tool and have helped to design cancer therapeutic regimens. Increased or altered proliferation is believed to be an early event in the adenoma-carcinoma sequence in the colon (2). Differences in the rate and distribution of proliferating cells in the colon have been used as biomarkers of neoplasia risk (3). For example, an increase in the colonic total crypt labeling index or a shift of the zone of proliferation toward the crypt lumen (stage I abnormalities) has been consistently shown to be associated with an increased risk for colon neoplasia in, for example, familial polyposis, colorectal cancer, and sporadic adenoma (4, 5). Changes in the abnormal number and distribution of proliferating cells after the administration of putative chemopreventive agents have been used as markers of response to therapy (6).

Because the classical autoradiographic method using tritiated thymidine incorporation into nuclear DNA is labor-intensive, requires the use of radionucleotides with major disposal problems, and takes much time, alternative methods have been used recently. Such methods have concentrated on utilizing endogenous cellular proliferative markers that omit the potential variation resulting from *in vitro* biopsy incubations (7). The most common marker used heretofore has been the PCNA³ (8). Although methodological problems were recognized (8, 9), PCNA antibodies have recently been advocated as the prime marker for studies of rectal biopsy tissues during chemopreventive studies (10). An excellent correlation between PCNA detection and epithelial cell proliferation kinetics has been determined when studying ethanol-fixed rectal mucosa by some authors (11), but others have found a poorer correlation, particularly in colon cancer-susceptible patients (7, 12). Antibodies to another cell cycle-associated protein, Ki-67, initially could be measured only in frozen tissue specimens, which limited the use of pathological material, but recently, Ki-67 immunoreactivity has been localized using a particular mAb, Mib-1, which can be used in formalin-fixed sections (13). The present study was designed to evaluate more carefully potential pitfalls in the measurement of PCNA and Ki-67 in the formalin-fixed human colon, focusing upon methodological variation with changes in antibody concentrations and in antigen-retrieval methods.

Materials and Methods

Specimens. Blocks containing histologically normal colon were obtained from the margin of a segment of ascending colon removed during colonic resection for diverticulosis from a 70-year-old woman. Blocks of adenomatous polyps with adjacent transitional mucosa were also obtained by surgical resection in an additional 10 patients. The tissues were fixed rapidly

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³ The abbreviations used are: PCNA, proliferating cell nuclear antigen; mAb, monoclonal antibody; LI, labeling index.

in 10% buffered formalin, orientated, and embedded in paraffin for routine histopathology by the Department of Pathology at St. Luke's-Roosevelt Hospital Center. Before immunostaining was performed, new sections were cut from each block and stained with H&E. The sections were examined by an experienced histopathologist to ensure that tissues did not contain fixation artifacts. Sections were usually used for immunostaining within 3 days of sectioning.

Immunohistochemistry. Four- μ m-thick sections were cut and placed on Superfrost Plus-coated microscopic slides (Fisher Scientific, Pittsburgh, PA). The two proliferation-associated antigens were examined in serial sections of the same tissue block using mAbs to PCNA and to Ki-67. Sections were deparaffinized in xylene, hydrated through a series of graded ethanols (100, 95, and 70%) to distilled water, and then exposed to microwave pretreatment [in 10 mM citrate buffer (pH 6) at 850 W] for varying periods of time to enhance antigenicity. Endogenous peroxidase activity was then quenched by a 5-min incubation with 3% H₂O₂ in double-distilled water, followed by rinsing in PBS [0.1 M K₂HPO₄ and KH₂PO₄ (pH 7.4)-150 mM NaCl]. Nonspecific antibody binding was blocked by incubation for 45 min with 10% normal horse serum in PBS.

Anti-PCNA Immunohistochemistry. Anti-PCNA antibody (PC10; Signet Labs, Dedham, MA) was then incubated with sections overnight in PBS at 4°C at concentrations varying from 1:50 to 1:1600. The next day, the sections were washed three times for 5 min in PBS followed by incubation with the second antibody (biotinylated anti-mouse IgG; Vector Laboratories, Burlingame, CA) at 1:200 in PBS for 45 min. After three additional PBS washes, slides were incubated with avidin-biotin complex in 5% BSA for 45 min and washed three times in PBS. The sections then were rinsed in 0.1 M acetate buffer (pH 6) and then rinsed with the streptavidin complex with nickel diaminobenzene as chromogen for 20 min, using D-glucose (2 mg/ml) and glucose oxidase (Sigma; crude) at a concentration of 4.75 units/ml. After washing in distilled water three times, the sections were counterstained with 2% Harris hematoxylin, rinsed, and dehydrated using a graded alcohol series to xylene, and the sections then were mounted using Permount (Fisher Chemicals, Fairlawn, NJ).

Anti-Ki-67 Immunohistochemistry. Anti-Ki-67 antibody (Mib-1) was obtained from Immunotech (Marseilles, France) and incubated with sections overnight at concentrations varying from 1:50 to 1:1600 in PBS at 4°C. The next day, the sections were handled as described for anti-PCNA and mounted using Permount. In one study, microwave pretreatment was performed in 10 mM citrate buffer (pH 6) and in 0.05 M glycine HCl buffer (pH 3.5) in parallel to determine whether changing the retrieval solution improved the immunostaining intensity.

Quantitation. After scanning sections at low power to select representative areas, quantitative analysis was performed by one observer in representative areas of the normal colonic epithelium using light microscopy at $\times 400$ magnification. The number of positively stained nuclei was then expressed as a percentage of the total number of epithelial cells counted, the LI. For PCNA, only heavily stained nuclei were designated as positive as determined by the observer. For Ki-67, all clearly stained nuclei were counted. For both normal and adenomatous mucosa, regional LIs were also calculated, as described previously (14), by determining the location of each positively stained cell within the crypt, which is divided into five equal portions from the base (crypt compartment 1) to the luminal surface (crypt compartment 5).

In evaluating adenomatous polyps, only surgically re-

Table 1 Effect of microwave condition on colonic crypt PCNA LIs

Slides of normal colonic epithelium were incubated with differing microwave conditions using the PCNA primary antibody at a concentration of 1:1000 as described in "Materials and Methods." Data presented are the mean \pm SEM of two studies. Microwave times as shown, once ($\times 1$) or twice ($\times 2$). The differences between 2.5 \times 2 min and 5 \times 1 min were not statistically different. All other conditions were significantly ($P < 0.05$) different from each other.

| Crypt compartment | LI (%) | | | |
|-------------------|---------------------------|----------------|--------------|---------------|
| | Microwave condition (min) | | | |
| | 2.5 \times 1 | 2.5 \times 2 | 5 \times 1 | 20 \times 1 |
| 1 | 25.5 (1.8) | 92.6 (4.4) | 88.0 (2.9) | 100 (0) |
| 2 | 34.6 (5.6) | 71.3 (3.2) | 70.0 (4.4) | 100 (0) |
| 3 | 0.0 | 62.6 (4.4) | 60.3 (5.8) | 90 (3.8) |
| 4 | 0.0 | 6.6 (2.3) | 7.3 (1.5) | 74.5 (2.6) |
| 5 | 0.0 | 0.0 | 0.0 | 20.1 (1.8) |
| Total crypt | 10.6 (1.1) | 44.3 (1.6) | 43.2 (1.9) | 82.1 (1.3) |

sected specimens were examined to avoid diathermy and crushing artifacts from endoscopy. The regional distribution of labeled cells was assessed in crypts that could be visualized entirely, from crypt surface to base.

Statistics. Differences in overall and regional LIs were compared by two-way ANOVA, with multiple comparisons of means (Newman-Keuls method). All statistical tests were two-sided. $P < 0.05$ was considered to show statistical significance.

Results

Effect of Microwave Pretreatment. Microwave pretreatment permitted retrieval of PCNA immunostaining using the PC10 primary antibody in formalin-fixed sections from both normal and adenomatous tissues. PCNA immunoreactivity could be identified at a primary antibody concentration as low as 1:800–1000. Immunostaining was not possible in sections from formalin-fixed tissues in the absence of microwave pretreatment. Using the Mib-1 primary antibody, immunostaining was possible at an antibody concentration as low as 1:1000, in contrast to the 1:100 concentration necessary in the absence of microwave pretreatment.

Microwave pretreatment conditions markedly affected the intensity and extent of immunostaining using the PC10 primary mAb. As shown in Table 1, altering the time of microwave exposure greatly affected the crypt LIs in the total crypt as well as the distribution of positively immunostained cells in the differing crypt compartments. When sections were exposed to the citrate retrieval solution for 2.5 min under standard microwave conditions, the total crypt LI was 10.6%, with labeled cells present only in the lowest 40% of the crypt. When sections were microwaved for 5 min under two differing conditions, a total of approximately 44% of all crypt cells were labeled, with a distribution as high as compartment 4. There were no significant differences between the two 5-min microwaving conditions. After 20-min microwaving, the total crypt LI reached 82%, with labeling throughout the crypt. Substituting a glycine buffer-based retrieval solution with various microwave conditions did not result in any staining of proliferating cells.

In contrast, using the Mib-1 mAb formalin-fixed tissues and pretreating with a citrate retrieval solution microwaved for between 2.5 and 20 min, there were no differences in the total crypt LI or in the distribution of labeled cells in the crypt (Table 2). When sections were microwaved for as long as 40 min, a modest increase in the number and distribution of immunostained cells was detected (Table 2).

Table 2 Effect of microwave condition on colonic crypt Mib-1 LIs

Slides of normal colonic epithelium were incubated with differing microwave conditions using the Mib-1 primary antibody at a concentration of 1:200 as described in "Materials and Methods." Data are presented as the mean \pm SEM of two studies. Microwave time as shown, once ($\times 1$) or twice ($\times 2$).

| Crypt compartment | LI, (%) | | | |
|-------------------|---------------------------|--------------|---------------|-------------------------|
| | Microwave condition (min) | | | |
| | 2.5 \times 2 | 5 \times 2 | 10 \times 2 | 20 \times 2 |
| 1 | 61.6 (4.7) | 63.4 (0.7) | 50.3 (0.1) | 84.1 (3.9) ^a |
| 2 | 58.8 (6.4) | 56.5 (8.8) | 57.1 (8.2) | 82.3 (6.4) |
| 3 | 20.6 (8.1) | 19.8 (10.0) | 23.3 (7.9) | 55.7 (9.5) |
| 4 | 2.9 (8.1) | 2.5 (2.5) | 6.5 (3.9) | 20.7 (9.6) |
| 5 | 0.0 | 0.0 | 0.0 | 2.2 (2.2) |
| Total crypt | 28.1 (1.9) | 28.4 (2.3) | 27.9 (3.1) | 48.1 (5.4) ^a |

^a Significantly different from other conditions ($P < 0.05$).

Effect of Antibody Concentration. The LI of PC10-positive cells was also greatly dependent upon antibody concentration (Table 3). Using concentrations of the primary antibody ranging from 1:100 to 1:1600, the total crypt LI ranged from 6.9–52.2%, with greater immunostaining of cells in the upper half of the crypt with increasing antibody concentration (Figure 1). In contrast, the numbers and distribution of Ki-67-positive epithelial cells within individual sections were not influenced significantly using Mib-1 antibody concentrations ranging from 1:50 to 1:800 (Table 4; Figure 1). There was also a striking difference between the intensity of PC10 immunostaining of proliferating cells within adenomatous polyps and in adjacent transitional normal mucosa. At a primary antibody concentration of 1:1000, proliferating cells in normal mucosa were readily stained, whereas no stain was seen in adenomatous polyps. At concentrations of 1:200 and 1:400, proliferating cells in both polyps and normal mucosa were readily apparent.

Effect of Time between Cutting Sections and Immunostaining. When sections of formalin-fixed paraffin-embedded tissue were cut and the immunostaining was delayed for up to 1 month, there was no effect upon PC10 immunoreactivity. However, a delay in immunostaining sections did alter the immunoreactivity for Mib-1. A delay in Mib-1 immunostaining of 5 days did not change the whole crypt LI, the distribution of crypt-labeled cells, or the intensity of the immunostaining. However, when sections were kept for 9–21 days, there was a gradual loss of immunoreactivity with time, resulting in labeled-cell distribution favoring compartment 1 and 2 (data not shown).

Discussion

The present study asks how dependably proliferating cells in formalin-fixed normal and adenomatous colonic tissues can be stained using antibodies to either PCNA (PC10) or Ki-67 (Mib-1). A citrate buffer retrieval solution, microwaved for short time-periods, allows for PC10 immunostaining of such formalin-fixed tissues and permits a reduction in the concentrations of the primary antibody needed for satisfactory staining. However, the data demonstrates several problems using the PC10 mAb against PCNA to accurately determine the number and distribution of colonic proliferating cells. Immunostaining of epithelial cells from normal colon was possible with dilutions of the concentrated PC10 primary antibody of about 1:1000. However, the 1:1000 concentration resulted in no staining of proliferating cells in adenomatous polyps; instead, a minimum

Table 3 Effect of PC10 antibody concentration on colonic crypt PCNA LIs

Slides of normal colonic epithelium were pretreated under constant microwave conditions (for 5 min \times 1) and incubated with differing concentrations of primary antibody using the PC10 mAb as described in "Materials and Methods." Data are presented as the mean \pm SEM of three studies. The differences between concentrations were all significant from each other except where indicated by asterisk.

| Crypt compartment | LI (%) | | | |
|-------------------|------------------------|-------------|-------------|------------|
| | Antibody concentration | | | |
| | 1:100 | 1:400 | 1:800 | 1:1600 |
| 1 | 89.1 (1.6) | 73.5 (1.0) | 83.0 (1.1) | 31.9 (3.4) |
| 2 | 73.6 (1.8) | 60.5 (0.8) | 43.3 (2.6) | 0.0 |
| 3 | 58.7 (2.9) | 26.1 (3.2)* | 24.7 (3.7)* | 0.0 |
| 4 | 46.7 (2.9) | 9.4 (2.3)* | 4.0 (1.5)* | 0.0 |
| 5 | 12.6 (0.9) | 0.0* | 0.0* | 0.0* |
| Total crypt | 52.2 (1.5) | 37.1 (1.2) | 30.7 (1.0) | 6.9 (0.8) |

concentration of 1:400 was necessary. In addition, varying the PC10 antibody concentration increased the whole crypt LI and resulted in a shift of immunostained proliferating cells to more superficial portions of the crypt. Furthermore, relatively modest differences in the length of exposure of the retrieval solution to microwaving also resulted in dramatic differences in the total crypt LI and in proliferating cell distribution within the normal colonic crypt. Thus, PCNA immunostaining with the PC10 mAb may be feasible only in ethanol-fixed tissues and under tightly controlled conditions.

In sharp contrast, altering the concentration of the Mib-1 primary antibody from 1:50 to 1:800 resulted in no significant difference in the total LI except in one individual crypt compartment in normal colonic mucosa or in adenomas. Altering the length of microwave exposure of the retrieval solution from 2.5 to 20 min also did not result in any significant change in the immunostaining of proliferating cells. Normal colon and sections from adenomatous polyps were readily stained using similar conditions.

These data would suggest that the Mib-1 antibody is preferred over the PC10 primary antibody, except in cases in which only very similar ethanol-fixed tissues and conditions are utilized. It seems likely that data showing that the PC10 primary antibody against PCNA is accurate for chemopreventive studies using normal "flat" colonic mucosa (11) are because homogeneous tissues were used. However, when immunostaining proliferating cells in multiple formalin-fixed tissues (normal, abnormal, normal peneoplastic, and neoplastic tissues), a Mib-1 primary antibody against Ki-67 is clearly superior. Our study did observe one limitation with the use of Mib-1 immunostaining in that sections should be stained quite rapidly after they have been cut from the formalin-fixed paraffin block, as reported for other antigens (15). Maintaining cut sections refrigerated and away from the light failed to prevent such a loss of Mib-1 immunoreactivity with time after sectioning (data not shown). The study is also limited in that intra- and inter-observer reproducibility was not examined.

The objective of a proliferative marker is to localize and quantify replicating cells and to exclude quiescent and postmitotic cells. Immunostaining colonic tissue with the anti-PCNA antibody clearly identifies nonproliferative cells and may reflect the fact that the PCNA gene product is present in all cells (16) and throughout the cell cycle. This cellular PCNA occurs in two forms, one in which concentration does not change during the cell cycle and another that is synthesized in parallel, with DNA increasing in late G₁ and reaching a peak during the

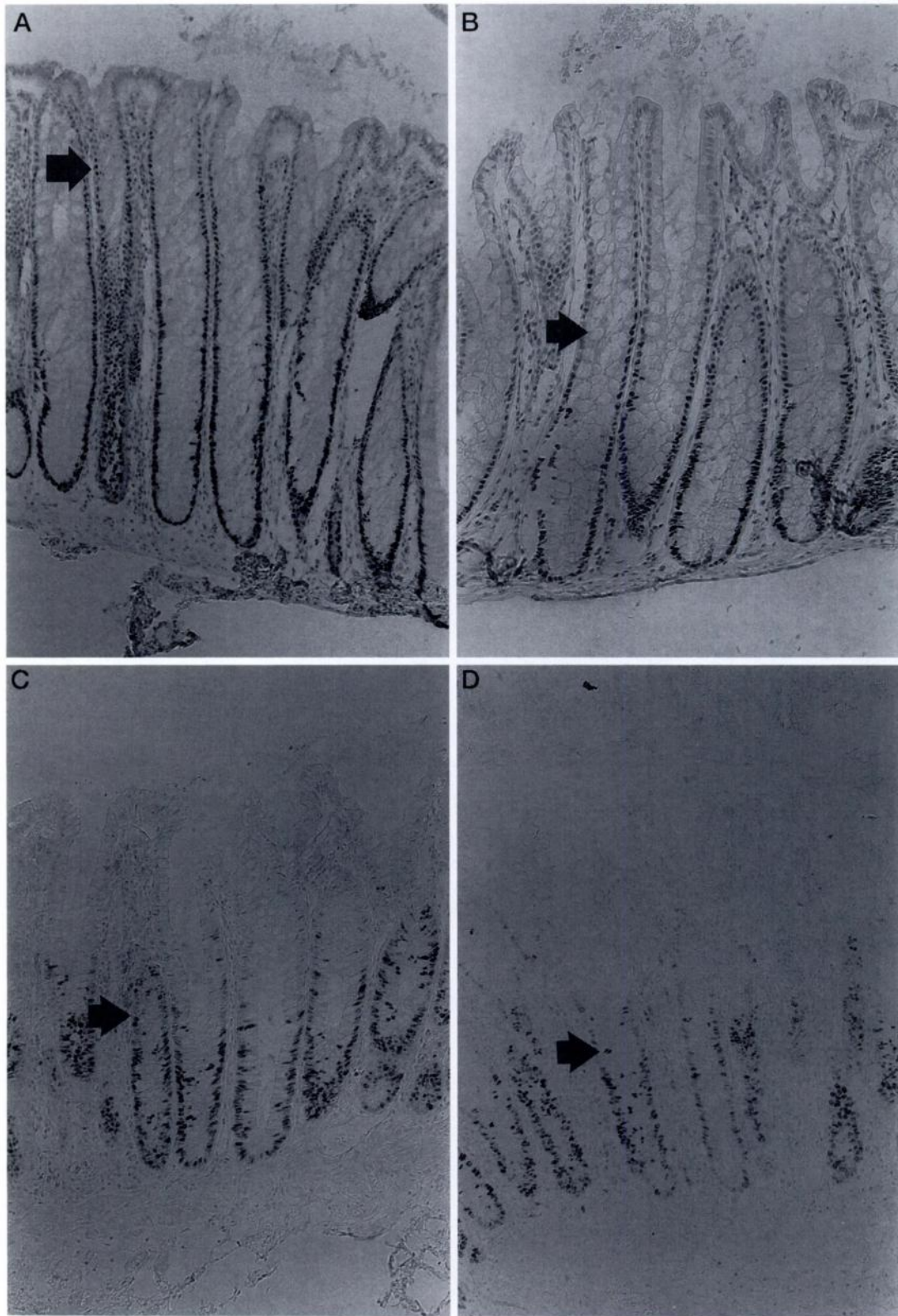


Fig. 1. Photomicrograph of the effects of antibody concentration upon labeling of rectal mucosal proliferating cells using PC10 or Ki-67 antibodies. *Top*, immunohistochemistry using PC10 mAb to PCNA at concentrations of 1:100 (A) and 1:400 (B). Note the increased labeling in the upper crypt when antibody concentrations of 1:100 are compared to concentrations of 1:400. *Bottom*, immunohistochemistry using Mib-1 mAb to Ki-67 at concentrations of 1:100 (C) and 1:400 (D). Note the similar labeling at the two concentrations throughout the crypt. *Arrows* indicate the upper limit of labeled cells.

Table 4 Effect of Mib-1 antibody concentration on colonic Mib-1 LIs

Slides of normal colonic epithelium were pretreated under constant microwave conditions (for 5 min \times 1) and incubated with differing concentrations of primary antibody using the Mib-1 antibody as described in "Materials and Methods." Data are presented as the mean \pm SEM in two studies. In one study, an antibody concentration of 1:50 also gave very similar results.

| Crypt compartment | LI (%) | | | |
|-------------------|------------------------|-------------|------------|-------------------------|
| | Antibody concentration | | | |
| | 1:100 | 1:200 | 1:400 | 1:800 |
| 1 | 52.4 (6.3) | 63.4 (4.5) | 60.0 (8.8) | 75.0 (4.3) |
| 2 | 45.6 (3.8) | 56.5 (8.8) | 54.8 (6.6) | 88.5 (3.3) ^a |
| 3 | 20.2 (3.9) | 19.8 (10.0) | 25.9 (6.2) | 27.9 (11.2) |
| 4 | 7.4 (5.6) | 2.5 (2.5) | 1.4 (1.4) | 0.0 |
| 5 | 0.0 | 0.0 | 0.0 | 0.0 |
| Total crypt | 25.0 (2.1) | 28.4 (2.3) | 28.6 (3.5) | 31.4 (3.0) |

^a Differences between concentrations were significant ($P < 0.05$).

S phase (17). These represent different forms of PCNA, which can be distinguished by their solubility properties (18). Detergent-extracted PCNA seems to be involved in DNA excision repair (19) in which cellular concentration does not change during cell cycling, whereas tightly bound, nonextracted PCNA is synthesized as an auxiliary protein of DNA polymerase δ (20) and reflects other measures of DNA replication such as bromodeoxyuridine incorporation into the nucleus. Immunohistochemical analysis of total PCNA in tissues will detect both forms of this protein and alter the apparent distribution of positive cells in the colon.

The Mib-1 mAb is one of three that were identified by cloning the *Ki-67* gene and raising antibodies against synthetic peptides of *Ki-67* (21). Mib-1 is expressed throughout the cell cycle except in G_0 , is rapidly metabolized, and has a very short half-life (in contrast to the half-life of PCNA; Ref. 22). Mib-1 is not affected by the DNA repair process; therefore, it is correlated solely with proliferation. The Mib-1 mAb also stains colonic neoplastic adenomatous polyps and cancer as well as adjacent normal colonic tissue.

In conclusion, the present study demonstrates the superiority of the Mib-1 antibody to Ki-67 in detecting proliferating cells in normal and neoplastic formalin-fixed colonic tissues. Antigen-retrieval systems, including appropriate citrate solutions and microwave conditions, permit reproducible measurements of the LI and the distribution of proliferating cells in normal colonic mucosa, adenomatous polyps, and cancer. Future studies should establish the utility and reproducibility of using the Mib-1 antibody in studies of chemoprevention to prevent the formation of colonic adenomatous polyps as well as change in polyp growth.

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