Comparison of Proliferating Cell Nuclear Antigen versus the More Standard Measures of Rectal Mucosal Proliferation Rates in Subjects with a History of Colorectal Cancer and Normal Age-matched Controls

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Abstract
Measurement of proliferation rates by the more standard in vitro uptake techniques of [3H]thymidine and 5'-bromo-2'-deoxyuridine (BrdUrd) labeling indices (LIs) were compared to proliferating cell nuclear antigen (PCNA) in rectal mucosal biopsies from 16 subjects with resected colorectal cancer and 14 normal age-matched controls. Correlation coefficients for BrdUrd versus PCNA, [3H]thymidine versus PCNA, and BrdUrd versus [3H]thymidine were 0.691, 0.876, and 0.770, respectively. No significant differences (P > 0.05) were detected in total mean LIs between the LI methods for the normal group. In contrast, total PCNA LIs were found to be significantly different in the resected cancer patients when compared to either BrdUrd (P = 0.005) or [3H]thymidine (P < 0.001). A significant difference (P = 0.010) in total PCNA LI but not in total [3H]thymidine or BrdUrd LI was also observed between normal controls and resected colorectal cancer subjects. Compartmental analysis of the cancer group versus the normals showed a different in compartments 1 and 3 for PCNA LIs only. The reproducibility of two PCNA LI counts was excellent (r = 0.9). In addition, the reliability of mean LIs were >0.8 with the exception of [3H]thymidine in the normal group (0.7). These study results demonstrate that PCNA LIs in human rectal mucosa biopsies is correlated highly with other more commonly used cellular proliferation measurements; however, PCNA LIs were found to be significantly higher than the other two methods in the resected colorectal cancer subjects.

Introduction
Measurement of cellular proliferation by [3H]thymidine, BrdUrd, and more recently, PCNA in normal appearing colorectal mucosa has been used extensively in both human and animal studies as an intermediate marker of risk for the subsequent development of colorectal neoplasia (1-17). Hallmark studies of Deschner et al. (2), Lipkin et al. (3, 4, 6), Newman et al. (13), and others (1, 5, 7, 8, 10, 11) established colorectal mucosal proliferation rates and crypt epithelial cell proliferation patterns, measured by [3H]thymidine incorporation, as the most widely used surrogate end point biomarker for estimation of colon cancer risk. An increase in total LI and/or expansion of the normal proliferative zone in the rectal mucosal crypts has been demonstrated consistently in subjects with familial polyposis, sporadic polyps, and colorectal cancer (2-4, 7, 8, 10). Hyperproliferation is considered to be one of the earliest events in the adenoma-carcinoma model (18-20).

The classical autoradiographic technique of [3H]thymidine incorporation into S-phase cells is expensive, labor intensive, and necessitates the use of radiolabeled nucleotides. In addition, exposure of autoradiographs takes up to 4 weeks for adequate development (21-23). Alternatively, BrdUrd (a pyrimidine analogue of thymidine) also labels cells in S-phase but may be detected immunohistochemically, eliminating many of the time consuming and technical difficulties encountered with [3H]thymidine (24-26). A number of workers have shown this phenomena using BrdUrd incorporation into rectal biopsies (24, 27-29). Use of BrdUrd measured by immunochemistry has essentially replaced [3H]thymidine, but this method still requires an incorporation step, introducing the possibility of in vitro artifacts and making it less adaptable to large chemoprevention trials.

Several recent studies have shown the percentage PCNA LIs and location of staining within the colorectal crypt to be comparable to those of [3H]thymidine and/or BrdUrd in normal and carcinogen-treated animals (16, 17, 30). Yamada et al. (17) found a strong correlation (r = 0.8) between PCNA and [3H]thymidine LIs and percentage S-phase, as determined by flow cytometry in normal and preneoplastic colonic mucosa. Risio et al. (28) found a good correlation (r = 0.7) between BrdUrd and PCNA LIs in rectal mucosal biopsies from humans and a significant difference in PCNA LIs between controls (10.8 ± 0.1% (SD)) and subjects with colonic neoplasia (14.8 ± 0.5%). In addition, Weisgerber et al. (29) found a significant correlation (r = 0.6) between PCNA and BrdUrd LIs in rectal biopsies obtained from normal subjects. The distribution of

Footnotes
1 To whom requests for reprints should be addressed, at University of Arizona Health Sciences Center, Arizona Cancer Center, 1515 North Campbell Avenue, Tucson, AZ 85724.
2 The abbreviations used are: BrdUrd, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; LI, labeling index.

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labeled cells within the mucosal crypts was also similar in these subjects.

Proliferative indices have also been used to measure the biological effect of a variety of potential chemopreventative agents including, calcium (31-37), wheat bran fiber (38), urso-deoxycholate (39), and nutritional stress diets (13). In addition, various cellular proliferation markers are being evaluated in randomized, placebo-controlled, Phase II and III chemoprevention trials to determine whether chemopreventive agents such as calcium and wheat bran fiber demonstrate a modulating effect in subjects with resected colorectal adenomas and carcinomas (40).

Measurement of both \(^{3}H\)thymidine and BrdUrd LIs requires uptake into viable tissue. In contrast, the availability of mAbs to endogenous cell cycle-specific proteins such as Ki67 and PCNA are attractive alternatives for use in clinical studies. Use of Ki67 has been limited by the requirement for frozen tissue (41) until the recent availability of the MIB 1 antibody, which stains routinely fixed tissue (42). PCNA is a 36 KD protein that functions as an auxiliary protein to DNA polymerase-\(\sigma\) in DNA synthesis (43, 44). Expression of PCNA increases late in G\(_1\), is expressed maximally in S, and decreases in G\(_2\)-M (43-47). PCNA also may be involved in unscheduled/deregulated DNA synthesis (48, 49). Several mAbs are available to PCNA, but the PC10 clone (Oncogene Science, Uniondale NY) recognizes PCNA in routinely fixed, paraffin-embedded tissue. Fixation of tissue, choice of antibody, immunohistochemical technique, evaluation of labeled cells, and method of determining labeling indices are all important variables that should be standardized to assure consistency across studies (45, 47, 50, 51).

In the present study, in order to determine the applicability of PCNA LIs, we compared PCNA to the more standard methods of BrdUrd and \(^{3}H\)thymidine incorporation in flat rectal mucosal biopsies from 16 patients with resected colon cancers and 14 normal age-matched controls.

Materials and Methods

Patients. Sixteen patients with resected colon cancer and no evidence of recurrent disease or history of other severe acute or chronic disease took part in this study. Fourteen normal age-matched control subjects with normal colonoscopy exams and no previous history of colonic neoplasia also were recruited for this study. Patient characteristics are shown in Table 1. Informed written consent was obtained according to institutional and federal guidelines. During sigmoidoscopy, 6–9 samples of flat, normal appearing, rectal mucosa were taken 8–15 cm above the anal verge with standard forceps after 2 tap water enemas between 8 and 11 a.m. Due to technical problems (e.g., inadequate numbers of crypts, poor uptake of \(^{3}H\)thymidine into tissue, excessive background after autoradiography) in the laboratory data, \(^{3}H\)thymidine LIs were not available on all sets of biopsies.

Tissue Preparation. Immediately after removal, mucosal biopsy samples were transported to the laboratory in MEM (Sigma Chemical Co., St. Louis, MO), oriented mucosal side up under a dissecting microscope, and transferred onto premoistened metrical membranes (Gelman, Ann Arbor, MI). Two to 3 biopsies were then placed in 3-ml MEM supplemented with 10% fetal bovine serum (Whitaker, Walkersville, MD), 1 mM HEPES (Sigma), 1% penicillin/streptomycin (ICN Biochemicals, Irvine, CA), and 150 \(\mu\)l 1 mM BrdUrd (Sigma) in HPLC vials (Waters, Milford, MA). Capped vials were injected with 95% \(O_2\), 5% \(CO_2\) through self-sealing septums (Waters), and biopsies were incubated for 1 h at 37\(^{\circ}\)C in a shaking waterbath. Biopsies for BrdUrd were fixed in 70% ethanol. A second set of 2–3 biopsies was placed in 3 ml of supplemented MEM with 15 \(\mu\)l of \(^{3}H\)thymidine (20 Ci/mM) (DuPont New England Nuclear, Wilmington, DE) under the same conditions as BrdUrd, with the exception of a 90-min incubation and fixation in 10% buffered formalin. The last set of 2–3 biopsies was not incubated but was immediately fixed in 70% ethanol for PCNA immunohistochemistry.

Immunohistochemistry. All biopsies were processed routinely at a temperature not exceeding 59\(^{\circ}\)C and embedded in paraffin, and 3-\(\mu\)m serial sections were cut. Just prior to staining, slides were deparaffinized through a series of graded alcohols and immersed in methanol with 3% \(H_2O_2\) to block endogenous peroxidase activity. Slides for BrdUrd were hydrolyzed in 4 N HCl for 20 min and neutralized with 0.1 N Na\(_2\)B\(_4\)O\(_7\) (pH 8.5) for 5 min. Immunostaining was performed with the use of a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as follows: a 30-min incubation with horse serum to reduce nonspecific background; anti-BrdUrd antibody (Boehringer Mannheim, Indianapolis, IN) at 1:200 dilution in PBS with 2% BSA (Sigma) for 1 h; biotinylated horse anti-mouse IgG for 30 min; and avidin-biotin-peroxidase conjugate (ABC complex) for 30 min. Each step with the exception of the first was followed by two PBS rinses. The complex was visualized with 0.25 mg 3,3-diaminobenzidine/ml in PBS, and 0.06% \(H_2O_2\) (Sigma) was added just prior to use. Slides were then stained lightly with hematoxylin, dehydrated, and coverslipped. PCNA immunostaining was identical to BrdUrd, with the exception of no hydrolysis step, and an anti-PCNA (PC10) antibody (Oncogene Science) was used at 1:800. In addition, the biotinylated antibody supplied with the Vectastain kit was substituted with a biotinylated F(ab')\(_2\)-IgG (Dako, Capinteria, CA) diluted at 1:400, and the Vectastain ABC complex was used at 1:12 dilution.

Autoradiography. Autoradiography was performed on biopsies incubated with \(^{3}H\)thymidine with the use of standard techniques (10, 26). Briefly, slides were dipped in NTB-2 photographic emulsion (Eastman Kodak, Rochester, NY), ex-

<table>
<thead>
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<th>Table 1 Characteristics of study subjects</th>
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</table>

Table 1 Characteristics of study subjects

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posed for 1 month at 4 °C, developed in D-19 (Kodak), fixed in 
Rapid Fix (Kodak), and counterstained with hematoxylin and 
esin.

Quantitation of Colonic Crypts. Only those crypts that were 
sectioned longitudinally from base to lumen, with bases adja-
cent to the muscularis mucosa, and with a continuous single 
layer of epithelial cells were included in the count. Crypts were 
quantified directly into a computer program, which stored 
the total number of labeled and unlabeled nuclei, as well as the 
label position per crypt column. A crypt column was defined as 
the column of cells divided at the midpoint of the crypt base to 
the luminal surface. Subjects with at least 12 columns were 
included in the analysis, although ideally 20 crypts (40 crypt 
columns) were quantitated. Crypts were analyzed at ×600. The 
count for BrdUrd included all positive (brown) crypt nuclei. For 
PCNA, only those cells showing distinct, intense positive 
(brown) staining were included in the count (positive nuclei 
were 3+ and 4+ in staining intensity, standardized to our 
laboratory). Ten of the slide sets were recounted for PCNA to 
sure counting reproducibility. Criteria for [3H]thymidine-
positive cells required that at least three grains overlay each 
nucleus.

Statistical Analysis. Total LIs for each method were calcu-
lated on a subject basis by summing labeled cells over crypts 
counted and dividing by the sum of total cells. Mean LIs 
computed in this manner are the weighted average of crypt LIs 
where the weight is proportional to the number of cells in the 
crypt. The weighted average will adjust for unbalanced contri-
butions of individual crypt LIs. Crypt compartmental data were 
obtained by dividing crypt columns into five equal longitudinal 
compartments, wherein compartment 1 starts at the crypt base 
and compartment 5 ends at the luminal surface. Compartmental 
LIs were calculated as described for total LIs.

Regression analyses were performed to assess the degree of 
correlation between LI methods. Paired t tests were used to 
test for a difference in LI methods within groups. The cancer 
and normal groups were compared using the Welch-Aspin (52) 
version of the t-test. A random effect model was used through-
out the entire analysis (53). Between subject variation and 
within subject variation were taken into account to evaluate 
variance of LI due to subject and biopsy nested within subjects. 
Boxplots and mean LI plots were generated for mean total and 
compartmental LIs. Reliability (of a single measurement) was 
estimated for the three labeling index methods and Spearman-
Brown’s formula (53) was used to compute the reliability of 
replicate measurements.

Results
To determine the applicability of PCNA LIs for the measure-
ment of rectal mucosal cell proliferation in prevention trials, we 
compared PCNA (intensely stained nuclei) to the more standard 
methods of BrdUrd and [3H]thymidine uptake in a group of 
resected colorectal cancer patients and a group of age-matched 
controls. We also compared the three LI methods in this group 
of resected colorectal cancer patients to the normal age-
matched controls.

Fig. 1 shows the regression analyses comparing the three 
LI methods for the normal and resected colon cancer subjects. 
The correlations were r = 0.876 for [3H]thymidine versus 
PCNA, r = 0.770 for [3H]thymidine versus BrdUrd, and r = 
0.691 for BrdUrd versus PCNA. Table 2 summarizes the mean 
number of cells per crypt column, mean number of labeled cells 
per crypt column, and mean number of crypt columns quanti-
tated for the three methods. There were no significant differ-
ces (P > 0.05) detected between the 3 LI methods in the 
normal group for total number of cells per crypt column or 
the number of labeled cells per crypt column, while fewer 
crypts columns were obtained for PCNA than for the other 
two methods. Also shown in Table 2 are data obtained from the
16 resected colorectal cancer patients. The mean number of cells per crypt column and the mean number of crypt columns counted were not significantly different between the 3 methods, but a significant difference was detected in the mean number of labeled cells per crypt column between PCNA (6.17 ± 1.80) and both BrdUrd (4.88 ± 1.35, P = 0.03) and \[^3H\]thymidine (4.94 ± 1.27, P = 0.04). The mean number of labeled cells for BrdUrd versus \[^3H\]thymidine was not significantly different (P = 0.9).

Boxplot graphs for the normal group and resected cancer group are shown in Fig. 2. Mean % LIs for the three LI methods in the normal group were not significantly different (P > 0.05) (7.01 ± 0.60%, 7.76 ± 0.61%, and 7.26 ± 0.56% for BrdUrd, PCNA, and \[^3H\]thymidine, respectively). In contrast in the resected cancer group no significant differences (P > 0.05) were found between total LIs for BrdUrd (8.17 ± 0.67%) and \[^3H\]thymidine (8.53 ± 0.60%) LIs, while a significant difference was demonstrated between PCNA (10.12 ± 0.69%) and both BrdUrd (P = 0.005) and \[^3H\]thymidine (P < 0.001) LIs.

Graphs depicting the five crypt compartments for each LI method are shown in Fig. 3. The PCNA LIs were significantly different from the BrdUrd and \[^3H\]thymidine LIs for compartments 1 and 2 (P < 0.01) for both the normal group (Fig. 3A) and the resected cancer subjects (Fig. 3B).

Table 3 summarizes the total and compartmental LIs for the 3 different LI methods in the 14 normal controls and 16 subjects with resected colon cancer. There was no statistical difference between the two study groups for mean total BrdUrd (control 7.01 ± 0.60%, resected 8.17 ± 0.67%) or \[^3H\]thymidine (control 7.26 ± 0.56%, resected 8.53 ± 0.60%) LIs; however, there was a significant difference (P = 0.010) in the mean total PCNA LI between the controls (7.76 ± 0.61%) and resected cancer patients (10.12 ± 0.69%), as well as between the two groups with respect to LIs in crypt compartments 1 (P = 0.012) and 2 (P = 0.044), while for compartment 2 the P value was 0.053.

In order to demonstrate the counting reproducibility of PCNA LIs, blinded repeat counts were performed on 2 separate occasions on the same microscopic sections obtained from 10 subjects with a history of resected colorectal cancer. The mean (±SD) LIs for counts 1 and 2 were 10.2 ± 4.2% and 9.7 ± 3.3%, respectively, and the Pearson correlation coefficient for the two counts was r = 0.92.

Shown in Table 4 are variance component estimates for the 3 LI methods in the normal controls and resected cancer subjects. For PCNA, the estimated variation in the mean percentage LI related to differences in subjects, biopsies, and crypts was 28.9, 8.7, and 62.4% in the normal group and 36.8, 3.7, and 59.5% in the resected cancer group, respectively. The highest variance (13.5%) for a biopsy effect was seen for \[^3H\]thymidine in the normal group. Variance due to subject ranged from 18.6% for \[^3H\]thymidine in the normal group to 36.8% for PCNA in the resected cancer group. Variance due to differences in crypts ranged from 58.9% to 67.9%. Reliability for each LI method also was determined and is given in Table 5. Reliabilities of LIs per subject in the normal group were 0.902, 0.704, and 0.814 for BrdUrd, \[^3H\]thymidine, and PCNA, respectively. Similarly in the resected cancer group the reliability was 0.813 for BrdUrd, 0.865 for \[^3H\]thymidine, and 0.875 for PCNA.

Discussion

PCNA differs from BrdUrd and \[^3H\]thymidine LIs in that the protein is expressed endogenously in cells throughout the cell cycle but is expressed maximally in S phase. Additionally, PCNA is a stable protein with a half-life of approximately 20 h and may be detectable for 24–48 h after cells have stopped dividing (19, 21). Two populations of PCNA exist. One is likely associated with DNA replication sites, and the other is localized in the nucleoplasm. Both of these PCNA populations are preserved by formalin fixation, but the population located in the nucleoplasm is not observed after methanol fixation, sug-
Table 3  Mean total and compartmental labeling indices in normal rectal biopsies from 14 normal age-matched controls and 16 resected colon cancer subjects*  

<table>
<thead>
<tr>
<th></th>
<th>Total LI</th>
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<tr>
<td>PCNA (intensely labeled) labeling indices</td>
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<tr>
<td>Normals</td>
<td>7.76 ± 0.61</td>
<td>11.35 ± 1.31</td>
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<tr>
<td>Resected cancer</td>
<td>10.12 ± 0.69</td>
<td>16.20 ± 1.44</td>
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<td>BrdUrd labeling indices</td>
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<tr>
<td>Normals</td>
<td>7.01 ± 0.60</td>
<td>9.16 ± 0.98</td>
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<tr>
<td>Resected cancer</td>
<td>8.17 ± 0.67</td>
<td>10.38 ± 1.07</td>
</tr>
<tr>
<td>[3H]Thymidine labeling indices</td>
<td></td>
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<tr>
<td>Normals</td>
<td>7.26 ± 0.56</td>
<td>9.23 ± 1.29</td>
</tr>
<tr>
<td>Resected cancer</td>
<td>8.53 ± 0.60</td>
<td>10.16 ± 0.75</td>
</tr>
</tbody>
</table>

a SDs were calculated as follows:

\[
\frac{1}{n} \bar{\sigma}_j^2 + \left( \frac{\bar{\sigma}_j^2}{\frac{1}{n} \sum_{i=1}^{b} \sum_{i=1}^{k} w_i^2} \right) \bar{\delta}_j^2,
\]

where \( \bar{\sigma}_j^2 \) is the estimator of between person variation, \( \bar{\sigma}_j^2 \) is the estimator of within person variation, and \( w_i^2 \) is the weight for \( j \)th crypt of the \( i \)th person, \( k \) is the number of crypts for the \( i \)th person, and \( n \) is the number of persons.

\*Statistically different from normal \( (P = 0.010) \).
\*\* \( P = 0.012 \).
\*\* \( P = 0.053 \).
\*\* \( P = 0.044 \).
\* All \( P \) values > 0.05.

Suggesting that the PCNA population preserved by alcohol is associated with DNA replication sites (21). PCNA is under both transcriptional and posttranscriptional control, and overexpression in tumors and adjacent tissue may be influenced by autocrine and paracrine factors (19, 28). There is also a role for PCNA in unscheduled DNA synthesis (22, 23).

Both BrdUrd and PCNA immunohistochemical methods represent clear improvements over the standard autoradiographic [3H]thymidine technique. Problems with the [3H]thymidine method, such as grains overlaying nuclei in the autoradiographs, often obscure adjoining nuclei, making quantitation difficult. Conversely, BrdUrd immunostaining results in distinct and easily discernible staining of labeled cells but still requires the incorporation of a precursor into viable tissue. Inadequate tissue uptake of BrdUrd and [3H]thymidine often results in edge labeling in tissue sections, making quantitation difficult. Evaluation of PCNA immunostaining is more problematic than that of BrdUrd due to the varying nuclear staining intensities encountered; nevertheless, the fact that the PCNA LI method does not require an incubation step (to allow uptake of a label) more than compensates for its more difficult detection.

Two recent studies of colonic mucosa from normal and carcinogen-treated rodents have demonstrated excellent correlations between BrdUrd and/or [3H]thymidine and PCNA for both total labeling indices and the distribution of labeled cells in crypt compartments (15, 25). A third study by Risio et al. (27) found a significant correlation between BrdUrd and PCNA LIs in the normal rectal mucosa of controls \((r = 0.7; P < 0.0001)\) but noted a failure of the PCNA method to discern the hyperproliferative compartmental shift observed with BrdUrd in subjects with colonic neoplasia. In our study the number and distribution of intensely labeled nuclei stained by the anti-PCNA (PC10) antibody and fixed in 70% ethanol are not significantly different from BrdUrd and [3H]thymidine total LIs in the rectal mucosa from normal subjects, while rectal mucosal PCNA LIs were significantly different from those of BrdUrd and [3H]thymidine in the resected cancer group. Additionally, when PCNA LIs from 14 control subjects were compared to 16 resected colon cancer patients, there was a significant difference in total LI for the PCNA method only, with the resected colon cancer patients having a significantly higher LI. Also, when PCNA labeling data were analyzed by crypt compartment, statistical significance was detected in compartments 1...
PCNA vs. \([^3]H\)-Thymidine vs. BrdUrd in Rectal Mucosa

Table 4  Variance components for BrdUrd, \([^3]H\)thymidine, and PCNA LIs

<table>
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<tr>
<th>Method</th>
<th>BrdUrd*</th>
<th>%b</th>
<th>([^3]H)thymidine*</th>
<th>%b</th>
<th>PCNA*</th>
<th>%b</th>
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<tr>
<td>Subject</td>
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<td>2.43</td>
<td>18.6</td>
<td>4.11</td>
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<td>1.77</td>
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<td>Subject</td>
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* REML was used to estimate components of variance.  
\(^b\) Percentage of total variability.

Table 5  Reliability for BrdUrd, \([^3]H\)thymidine, and PCNA LIs in normal controls and in resected colorectal cancer subjects

<table>
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<th>Method</th>
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</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of subjects</td>
<td>14</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>No. of biopsies</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Average no. of crypts</td>
<td>6.300</td>
<td>7.150</td>
<td>5.430</td>
</tr>
<tr>
<td>Reliability of a single crypt</td>
<td>0.368</td>
<td>0.163</td>
<td>0.293</td>
</tr>
<tr>
<td>Reliability for mean LI</td>
<td>0.902</td>
<td>0.704</td>
<td>0.814</td>
</tr>
<tr>
<td>Resected cancer subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of subjects</td>
<td>16</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>No. of biopsies</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Average no. of crypts</td>
<td>5.750</td>
<td>5.260</td>
<td>5.040</td>
</tr>
<tr>
<td>Reliability of a single crypt</td>
<td>0.305</td>
<td>0.318</td>
<td>0.367</td>
</tr>
<tr>
<td>Reliability for mean LI</td>
<td>0.813</td>
<td>0.865</td>
<td>0.875</td>
</tr>
</tbody>
</table>

* Reliability of a single measurement was estimated for the 3 LI methods, and Spearman-Brown’s formula was used to compute reliability of replicate measurements.

and 3, although the LI for each crypt compartment was higher in the cancer group than in the control group. In contrast, we did not detect a higher LI or shift in the rectal mucosal proliferative zone for either BrdUrd or \([^3]H\)thymidine labeling in this small group of resected colorectal cancer patients versus normal controls.

The resected colorectal cancer patients included in the present study had a mean time from resection of 4.7 ± 3.3 years (range, 0.3–11.5 years), with evidence of a recurrent colon neoplasia (adenoma in one case and unspecified in the other) prior to this study in only two patients. No attempt was made to analyze these patients separately due to the small number. Scalmani et al. (11) compared patients with previously resected colon cancer to normal controls and found that the total and compartmental \([^3]H\)thymidine LIs were similar in the two groups, with the exception of those patients with recurrent polyps. These patients had significantly higher total LIs, as well as higher mean LIs in compartments 3, 4, and 5, when compared to those without recurrence. The authors concluded that after resection of colonic neoplasia, the colonic mucosa becomes similar to that of normal controls, while in a subset of patients the mucosa may remain in the hyperproliferative state.

Both the normal age-matched controls and the resected colorectal cancer groups had a relatively wide range of LIs using all 3 LI methods, suggesting significant heterogeneity within the groups. Histograms and QQ plots for LIs were generated on each subject to assure that the assumption of normality was appropriate. Although there was not a significant difference between normal controls and subjects with resected colorectal cancer with respect to PCNA LIs, we may not have had sufficient statistical power to detect differences between these two groups using the BrdUrd or \([^3]H\)thymidine LIs methods.

The results of this study show that the intratechnician reproducibility of quantitating intensely labeled PCNA is excellent \((r = 0.9)\). Reliability of LIs per subject averaged over crypts and biopsies was greater than 0.8 for the 3 LI methods with the exception of \([^3]H\)thymidine in the normal group \((0.7)\). Lyles et al. (51) found that for PCNA LIs, 20% of the variability was due to subject, 30% was due to a biopsy effect within subjects, and 50% was attributed to crypts within biopsies. In the present study we found that for PCNA LIs the variance due to subject was 28.9% in the normal group and 36.8% in the cancer group. Variability due to a biopsy effect was 8.7% in the normal group and 3.7% in the cancer group, and variance due to crypts was 62.4 and 59.5% in the normal and cancer group, respectively. Many factors are likely to account for the variation in variance components estimates between our study and the previous study. These factors are likely to include differences in many technical aspects of LI measurement, as well as differences in the study populations. Determining the sources of variation within LI measurements will allow for a more accurate assessment of sample size requirements for clinical studies (51) and may in addition establish requirements for the number of biopsies and crypts that must be evaluated for each subject.

Further studies will be needed to fully assess the role of PCNA in normal, preneoplastic, and neoplastic colonic tissue. Clearly the choice of antibody, fixation, immunohistochemical technique, and definition of positive nuclear staining will all affect the intensity of the non-S-phase cells and must be standardized \((19, 24)\). However, measurement of PCNA in the normal colonic mucosa of subjects at risk of developing colorectal cancer in Phase II and III chemoprevention trials has several important advantages over the standard proliferation methods. The processing procedure for the PCNA LI method requires minimal technical expertise and no specialized equipment, the technical problem of inadequate uptake of precursor into the rectal mucosal biopsy tissue is eliminated, and use of radioisotopes is not required. In addition, the immunohistochemical analysis of PCNA LIs is available within days rather than the 2–4 weeks required for \([^3]H\)thymidine. The advan-
tages that the PCNA LI offers makes it a more feasible intermediate marker for large colon cancer prevention trials.

References
PCNA vs. [3H]-Thymidine vs. BrdUrd in Rectal Mucosa


Comparison of proliferating cell nuclear antigen versus the more standard measures of rectal mucosal proliferation rates in subjects with a history of colorectal cancer and normal age-matched controls.

J Einspahr, D Alberts, T Xie, et al.