Use of the Lectin from *Amaranthus caudatus* as a Histochemical Probe of Proliferating Colonic Epithelial Cells


Department of Internal Medicine, Veterans Administration Medical Center, University of Michigan School of Medicine, Ann Arbor, Michigan 48105 [C. R. B.]; Department of Biological Chemistry, University of Michigan School of Medicine, Ann Arbor, Michigan 48109 [Y-F. C., S. J. J., I. J. G.]; Human Tissue Resource, Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland 21201 [J. H. R.]; Department of Internal Medicine, Wayne State University School of Medicine, Detroit, Michigan 48201 [G. D. L.]; and Department of Preventive Medicine, Creighton University School of Medicine, Omaha, Nebraska 68178 [H. T. L.]

ABSTRACT

A newly isolated lectin, *Amaranthus caudatus* agglutinin (also called amaranthin or ACA), which binds to the Thomsen-Friedenreich antigen (T-antigen) and its sialylated variants, was used as a histochemical probe for proliferating cells in sections of human colonic tissues. Binding inhibition studies revealed that ACA binds to different sites on histological sections when compared to peanut agglutinin, which also recognizes the T-antigen. ACA bound selectively to the cells at the base of the colonic crypt [46 ± 4% (SEM) of glands] which is the zone of proliferation in this tissue and preferentially labeled cytoplasmic and apical membrane glycoconjugates. Only 7 ± 2% of the upper portions of the colonic crypts were labeled (P < 0.001 compared to the base), and this was largely a result of extensive labeling in 2 of 23 samples studies. A marked increase in histochemical labeling by ACA was seen in adenomatous polyps and adenocarcinomas of the colon, in which 92 ± 7 and 97 ± 2% of the glandular units were labeled, respectively. Transitional mucosa and connective tissue adjacent to cancers were also labeled by ACA. Neuraminidase studies indicated that removal of sialic acid residues enhanced binding by peanut agglutinin, but not ACA, to glycoconjugates in cancer specimens. Specimens of colonic tissue from patients with familial adenomatous polyposis (FAP) were examined with ACA; 83 ± 7% of adenomatous glands and 60 ± 7% of glands in flat, normal-appearing tissue were labeled. Colonic tissues from persons at 50% risk for hereditary nonpolyposis colorectal cancer (HNPPC), FAP, and normal colons were studied and given “weighted average” labeling scores that ranged from 0–400 to accommodate variable intensity and distribution of labeling. Normal colons had a weighted average score of 65 ± 33; FAP tissues had a score of 224 ± 76 (P < 0.001 compared to normal colon) and HNPPC tissues had a score of 74 ± 70 (P < 0.05 compared to normal colon). A group of five HNPPC cases had scores of 203 ± 43 (P < 0.001 compared to the base), and this was largely a result of extensive labeling in 2 of 23 samples studied. ACA labels glycoconjugates in the proliferative region of normal human colonic epithelium and neoplastic lesions of the colon. The results of FAP and HNPPC tissues suggest that it may be useful for identifying foci of abnormal proliferation in familial colorectal cancer syndromes.

INTRODUCTION

A lectin has been isolated from the South American plant *Amaranthus caudatus* that binds to the Thomsen-Friedenreich antigen (T-antigen) and its sialylated variants (1). This lectin, amaranthin or ACA, 1 has a subunit molecular weight of approximately 33,000 and exists as a homodimer (M, = 63,500) with one binding site per monomer. The binding specificity of ACA has been determined by the ability of defined hapten oligosaccharides to inhibit the precipitation of asialoovine submaxillary mucin (1). These studies defined the critical domains in the T-antigen disaccharide necessary for lectin-carbohydrate interaction, as illustrated in Fig. 1.

Inasmuch as ACA was isolated by its interaction with immobilized T-antigen, it was anticipated that its binding specificity would be similar to that of PNA (2, 3). A series of synthetically modified oligosaccharides was used to determine the specific sites of interaction between ACA and the T-antigen (1). As illustrated in Fig. 2, ACA and PNA both recognized T-antigen but interacted with different portions of the disaccharide. These and other experiments have demonstrated that PNA would bind T-antigen even if the C-6 hydroxyl group of GalNAc was substituted (4) (Fig. 2) and that ACA would interact with T-antigen even if both the C-6 hydroxyl group of GalNAc and the C-3 hydroxyl group of galactose were substituted (1). This is of some importance, since the T-antigen may be sialylated at either of the two sugars (4), and previously it had been assumed that sialylation of the T-antigen would make it inaccessible for recognition by PNA (3).

The PNA lectin has been an important probe used by histochemists interested in colorectal neoplasia (5, 6). PNA does not bind readily to mucins found in the normal colon but binds to mucin secreted by colon cancers (5–8). In addition, PNA binds to glycoconjugates present in a variety of premalignant lesions in the colon (9–13). Cooper (6) reported that the treatment of histological sections of human colonic tissue with neuraminidase resulted in the “unmasking” of cryptic T-antigens to which PNA subsequently bound (6); however, this has not been the experience of all investigators (5). Moreover, a series of oligosaccharides isolated from the mucin of normal human colonic tissues revealed no evidence of the T-antigen or its sialylated variants (14, 15). Thus, it is not clear whether a variant form of T-antigen may be synthesized in normal colonic tissues. The availability of a new probe for the T-antigen which was capable of recognizing variations in the T-antigen that would not be recognized by PNA led to our use of ACA as a histochemical probe of human colonic tissues. Specimens of normal human colon, neoplastic human colon, and a series of potentially premalignant lesions were examined using this lectin to determine whether a variant form of T-antigen was responsible for some of the above described controversies. We have found that ACA binds selectively in the portion of the colonic crypt where epithelial cell proliferation ordinarily occurs.

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2 To whom requests for reprints should be addressed, at GI Section (11 ID), VA Medical Center, 2215 Fuller Road, Ann Arbor, MI 48105.

3 The abbreviations used are: ACA, amaranthin or *Amaranthus caudatus* agglutinin; PNA, peanut agglutinin; FAP, familial adenomatous polyposis; HNPPC, hereditary nonpolyposis colorectal cancer; T-antigen, Thomsen-Friedenreich antigen; Gal, galactose; GalNAc, N-acetylgalactosamine; PBS, phosphate-buffered saline; BPA, Bauhinia purpurea agglutinin; RCA1, Ricinus communis agglutinin; Neu5Ac, N-acetyl neuraminic acid (a sialic acid); DBA, Dolichos biflorus agglutinin.

4 Unpublished data from the dissertation of S. J. R.

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Sectioned for lectin histochemistry. These protocols were approved by victims with no pathological abnormality of the colon, were immediate autopsies. Adenomatous colon polyps from resected specimens were obtained from the immediate autopsies at the University of Maryland Hospital (G. D. L.), fixed in formalin, and embedded in paraffin for tissue sectioning. Rectal biopsy specimens were obtained from individuals who were first degree relatives of patients with HNPCC, as identified by Dr. H. T. Lynch. These patients were the siblings or offspring of index patients who fit the criteria for Lynch syndrome I or II (16). However, since there is no definitive genetic or phenotypic marker for this familial syndrome, it is not possible to be certain that these patients represent a single homogeneous genetic abnormality. The specimens were fixed in formalin and embedded in paraffin for tissue sectioning.

**Materials and Methods**

Lectin Preparation. ACA was purified from the seeds of *A. caudatus* by fractionation on DEAE-cellulose followed by affinity chromatography using Sarsorb-T beads (Calbiochem, Calgary, Alberta, Canada) (Galβ1,3GalNAc-O-Sarsorb) (1). The lectin was biotinylated as follows. Biotinyl-N-hydroxysuccinimide dissolving in dimethylformamide was added to highly purified ACA that had been dissolved in PBS with 0.1 M NaHCO3, pH 8.3, at a protein concentration of 5.15 mg/ml. using a 5:1, mol:mol ratio. GalNAc, final concentration 10 mg/ml, was added during biotinylation to protect the sugar-binding sites of the lectin. The solution was gently stirred at room temperature for 6 h and thoroughly dialyzed against PBS and purified by affinity chromatography on Sarsorb-T beads. The binding activity of biotinylated ACA was compared with that of nonbiotinylated ACA by hemagglutination using the same amount of protein, in which the titers were 1:32 and 1:16, respectively.

Biotinylated PNA was purchased from Vector Laboratories (Burlingame, CA).

Tissue Procurement. Specimens of adenocarcinoma of the colon and adenomatous colon polyps from resected specimens were obtained from the Pathology Service at the Ann Arbor Veterans Administration Medical Center. These specimens were obtained during surgery or colonoscopic polypectomy, fixed in buffered formalin, and embedded in paraffin for routine pathological examination. Additional 4-μm sections were cut for lectin histochemistry after the pathological diagnosis was confirmed (C. R. B.).

Full-thickness specimens of normal colonic epithelium were obtained from the Immediate Autopsy program at the University of Maryland Human Tissue Resource (J. H. R.). These tissues came from accident victims with no pathological abnormality of the colon, were immediately fixed in formalin after death, and embedded in paraffin and sectioned for lectin histochemistry. These protocols were approved by the University of Maryland Human Volunteer Research Committee.

Full-thickness specimens of colonic tissue were obtained from colorectum specimens of familial adenomatous polyposis at Johns Hopkins Hospital (G. D. L.), fixed in formalin, and embedded in paraffin for tissue sectioning. The tissue specimens contained normal-appearing flat mucosa, as well as a polypoid, adenomatous tissue.

Rectal biopsy specimens were obtained from individuals who were first degree relatives of patients with HNPCC, as identified by Dr. H. T. Lynch. These patients were the siblings or offspring of index patients who fit the criteria for Lynch syndrome I or II (16). However, since there is no definitive genetic or phenotypic marker for this familial syndrome, it is not possible to be certain that these patients represent a single homogeneous genetic abnormality. The specimens were fixed in formalin and embedded in paraffin for tissue sectioning.

Lectin Histochemistry. Paraffin-embedded tissue sections, 4 μm, attached to glass slides were deparaffinized by sequential incubations in xylene (twice), absolute ethanol (thrice), 95% ethanol, 70% ethanol, and water and finally equilibrated in PBS, pH 7.0, containing 0.1% bovine serum albumin (Sigma). The slides were blotted dry adjacent to the tissue sections, and biotinylated lectin was added at a concentration of 10 μg/ml (for ACA) or 20 μg/ml (for PNA) in a quantity sufficient to completely cover the hydrated tissue (100–200 μl). The lectin was rinsed off after a 30-min incubation by eight 2-s dips into PBS. The slides were again blotted, and the tissue was covered with streptavidin-horseradish peroxidase (Zymed Laboratories, South San Francisco, CA) at a concentration of 50 ng/ml for 30 min. The slides were rinsed as mentioned above, and the pigmented reaction product was developed by the addition of a freshly prepared chromogen reagent solution prepared within 2 h of use as follows. 3,3′-Diaminobenzidine was aliquoted at 5 mg/ml in 0.01 M Tris saline, pH 7.5, and frozen at −20°C until use. For use, the aliquot was thawed, diluted 1:10 in 0.01 M Tris saline, pH 7.5, and 3.3 μl 30% H2O2 was added to each 5 ml. This solution was allowed to stand for 10 min, and 100–200 μl was applied to the tissue sections that had been incubated earlier with biotinylated lectin and streptavidin-horseradish peroxidase. After 10 min, the slides were rinsed with albumin-free PBS. The tissues were briefly counterstained for 50 s with Gill's 3 hematoxylin (Sigma), rinsed with water, serially dehydrated using graded alcohols and xylene and embedded in Permount (Allied-Fisher Scientific, Livonia, MI).

The specificity of lectin binding was evaluated by preincubating the biotinylated lectin with inhibitory hapten sugar for 1–2 h prior to its application to the tissue. In each instance, the reagents were prepared so that the final concentration of lectin on the slide was either 10 or 20 μg/ml and the hapten sugar was as indicated in "Results." Galactose, lactose, GalNAc, and galactose-β1,3-N-acetylgalactosamine-α-O-benzyl were obtained from Sigma.

Sialidase digestions were performed on the tissue sections after hydration. *Vibrio cholerae* neuraminidase (Serva, Heidelberg) was diluted in 50 mM citrate-phosphate buffer, pH 4.5, at a concentration of 0.43 millunits/ml, and 100–200 μl was incubated on the slide at 37°C for 16 h. Newcastle disease virus neuraminidase (viral suspension;...
United States Department of Agriculture, Agriculture Research Service, Athens, GA) was diluted in 50 mM cacodylate buffer, pH 6.0, at a concentration of 0.5 milliunits/ml, and 100-200 µl was added to the tissue for a 2-2.5-h incubation at 37°C. Controls for these experiments utilized the same buffers without neuraminidase.

Scoring the Tissue Sections. The stained sections were examined with a light microscope using a ×25 and ×40 objective lenses and ×10 eyepiece. Evaluation of lectin binding required the use of several approaches. First, the binding specificity was confirmed by coincubation of lectin with hapten sugars known to bind the lectin maximally. Maximum labeling by the brown reaction product was arbitrarily assigned a score of 4+ (8). No staining of the slide compared to control sections (i.e., in which lectin was omitted from the staining procedure) was assigned a score of 0. Intermediate scores of 1* (trace or weak staining) and 2* (stronger staining, but definitely not less than control slides) and 2-3* (stronger staining) were assigned. Although subjective, the histochemical scoring system using lectins has been demonstrated to correlate well with an objective measure of the binding of lectins to glycoproteins extracted from colonic tumors (8). This method was used to confirm the binding specificity to known "positive" tissues and to evaluate changes in lectin binding after neuraminidase treatment. A somewhat more simplified system (using a global 0-3* score) was used for documenting carbohydrate-binding specificity, as described below.

A second method for scoring tissues was used to survey normal colonic tissues, adenomatous polyps, and cancers. For these studies, individual epithelial "units" were counted as either positive or negative; i.e., each crypt of Lieberkühn or neoplastic gland was considered an independent scorable unit, and the tissue score was expressed in terms of the percentage of units that showed any degree of definite labeling (1*-4*). The normal colonic crypt was further subdivided into the lower one-half and upper one-half for independent scoring because of the nonuniform distribution of labeling between the proliferative region (at the base) and the region of mature, differentiated cells (at the top).

Finally, an examination of the HNPCC tissues revealed subtle variations in labeling that required a more sophisticated method of scoring. These tissues were histologically normal but showed variations in intensity of labeling when compared to the normal colonic tissues. Therefore, each individual crypt of Lieberkühn was given a score from 0-4*, and the labeling was expressed as a "weighted average," or the sum of scores/100 crypts, which could range from 0 (all 100 crypts not labeled) to 400 (all 100 crypts maximally labeled). A modification of this approach has been used to evaluate histochemical results using other lectins and produces reproducible scores on tissue sections (17).

For some experiments, a simplified 0-3 scoring system was used, in which a global estimate of absent (0), trace (1*), intermediate (2*), and intense (3*) staining was given for the whole section. Essentially, the tissues were scored globally, and the strongly labeled tissues (i.e., 3* and 4*) were grouped together under a 3* score. This method was used for two experiments, the hapten inhibition studies (Table 1) and the comparison of ACA and PNA (Table 4), in which only relative degrees of labeling were relevant.

RESULTS

Lectin-binding Specificity

Specimens of well-differentiated colonic cancers were incubated with biotinylated lectins. Working concentrations on the slide were 10 µg/ml for ACA and 20 µg/ml for PNA. The hapten sugars Gal, GalNAc, and Galβ1,3GalNAc-α-0-benzyl (T-antigen) were used to inhibit lectin binding to the tissues. Using the biotinylated lectins, ACA produced intense labeling on colon cancers; for the purposes of the inhibition studies the scores were recorded from 0-3* since the cancers were uniformly labeled, and a simplified scoring system was most useful. Glycoconjugates secreted into the malignant glands or lumina were the predominant locations of labeling; cytoplasmic labeling was less intense in all specimens. Biotinylated PNA labeled these tissues much less intensely and produced relative scores of 1*-2* on the 15 cancer specimens as the maximal degree of labeling. The ability of hapten sugars to inhibit labeling with these lectins is indicated in Table 1. Labeling by ACA was inhibited by concentrations ≥250 mM GalNAc and ≥100 µM T-antigen but required 400 mM lactose or galactose for partial inhibition of binding. PNA was inhibited by ≥10 mM lactose or galactose, ≥100 µM T-antigen, and ≥100 mM GalNAc.

Survey of Tissue Labeling with ACA

Lectin histochemistry was analyzed with biotinylated ACA, and tissue labeling was expressed in terms of the percentage of epithelial units showing any degree of definite reaction product (1*-4*).

Normal Colon. Twenty-three specimens of normal colon were obtained from 16 “immediate autopsy” cases and were free of histological abnormality. Full-thickness, well-oriented sections were available in each case. Initial examination of these tissues indicated that ACA labeling was faint (1* for most specimens) and preferentially occurred at the base of the crypt of Lieberkühn in the apical cytoplasmic region, microvillar membrane, and glycocalyx but was less frequently seen in the upper one-half of the crypt, which occurred in only 2 of 16 of the normal colons (Fig. 3). Goblet cell mucin, connective tissue, and lamina propria were not stained. The epithelial crypt unit was evaluated separately for staining of the base or lower one-half and upper one-half of the crypt. Labeling was seen in the lower one-half or base of the crypt in all colonic specimens and was present in 46 ± 4 (SEM)% of all crypts in the 23 specimens studied. The upper one-half of the crypts showed staining in 7 ± 2% of all crypts and consisted of secreted material in the lumen of the gland in some cases but definitely labeled (1*-2*) the apical membrane and cytoplasm in 2 of 23 of the specimens. In these two cases, >30% of the epithelial units had 1*-2* labeling. The difference in labeling between upper and lower crypt regions was statistically significant (P < 0.001). More important, however, 18 of 23 normal colonic specimens showed no labeling in the upper portion of the crypt.

No labeling of mucosal glycoconjugates in the normal colon was observed with PNA. Treatment with V. cholera or Newcastle disease virus neuraminidase did not produce an increase in labeling by PNA.

Neoplastic Colon. Twenty-five specimens of colonic adenocarcinoma sections. Labeling was scored on a simplified scale from 0-3* for this experiment, as described in "Materials and Methods.

Table 1 Inhibitory hapten sugars

<table>
<thead>
<tr>
<th>Lectin</th>
<th>No sugar</th>
<th>T-antigen (µM)</th>
<th>GalNAc (mM)</th>
<th>Gal (mM)</th>
<th>Lactose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>200</td>
<td>400</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>ACA (10 µg/mL)</td>
<td>3*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3*</td>
</tr>
<tr>
<td>PNA (20 µg/mL)</td>
<td>1-2*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1-2*</td>
</tr>
</tbody>
</table>
carcinoma were studied with ACA, and 15 specimens were studied with PNA. In all cases, labeling by ACA was more intense and more diffusely distributed than that seen with PNA, which was weaker in intensity, generally localized to secreted material, and focal within most of the tumors. Labeling of cytoplasmic glycoconjugates was commonly seen with ACA (Fig. 4). Every cancer specimen showed extensive labeling with ACA, and in a limited study of 13 cancer specimens, 97 ± 2% of the malignant glands showed 1*-3* labeling of the secreted or cytoplasmic glycoconjugates. This was significantly greater than that seen in the normal colon (P < 0.001).

Cancer specimens were digested with neuraminidases from V. cholerae (which hydrolyzes both \( \alpha_2,3\)- and \( \alpha_2,6\)-linked sialic acid (Neu5Ac)) and Newcastle disease virus (which preferentially hydrolyzes \( \alpha_2,3\)-linked Neu5Ac) (18, 19). Treatment with V. cholerae neuraminidase greatly enhanced the intensity and distribution of PNA binding, exposing binding sites in secreted and cytoplasmic glycoconjugates. However, the use of Newcastle disease virus neuraminidase had much less effect on the intensity of labeling by PNA. Both enzymes enhanced PNA staining in connective tissue. Neither sialidase enhanced labeling with ACA.

The mucosa immediately adjacent to colonic cancer, called "transitional mucosa," is known to express abnormal glycoconjugates in spite of its nonneoplastic pathological appearance (5, 20). This mucosa demonstrated 1* labeling in the upper and lower crypt region using ACA. Of interest, the collagen, blood vessels, and muscle surrounding invasive cancers were variably labeled in 22 of 25 tumors, which was never seen in surrounding normal colonic epithelium.

A small group of six adenomatous polyps was studied using ACA. The lectin bound to 82 ± 7% of the glands in the adenomatous polyps and stained cytoplasmic as well as secreted glycoconjugates (Fig. 5). A larger group of adenomatous polyps confirms this observation.5

5 E. A. Boohaker and C. R. Boland, manuscript in preparation.

FAP. A total of 28 specimens of colonic tissue from 12 patients with FAP was examined with ACA. Each specimen contained flat, normal-appearing epithelium and small adenomatous polyps. Epithelial units from flat and polypoid tissue were scored separately. Polypoid tissues demonstrated labeling in 83 ± 7% of the glands, which is similar to that seen in the small series of sporadic adenomatous polyps, all of which were larger than those present in the FAP sections. The flat tissue in FAP demonstrated labeling in 60 ± 7% of the glands (P = 0.055 compared with polypoid tissue), and labeling was inappropriately seen in the upper portion of the crypt as frequently as in the lower portion (Fig. 6). Labeling in FAP tissues was significantly greater than that seen at the apical portion of the crypt in normal colons (P < 0.001) but was not different from that at the basal portion of the crypt (0.05 < P < 0.10); however, the intensity of labeling was greater in FAP sections in all portions of the crypt. Secreted glycoconjugates demonstrated an increase in staining intensity. The histochemical results are summarized in Table 2.

HNPPC. HNPPC is a more problematic tissue to evaluate. Rectal biopsy specimens were obtained from individuals at risk for this syndrome, but no markers have been available to definitively identify truly affected patients. These tissues were all histologically normal, and lectin staining was scored from 0–400 using the "weighted average" described in "Material and Methods." An examination of the HNPPC tissues revealed that a subset exhibited an inappropriate increase in labeling with ACA (compared with colonic tissues from the Immediate Autopsy Program) showing both an increase in intensity and labeling of the cytoplasm in both the upper and lower portions of the crypt (Fig. 7). For these tissues, each crypt was considered an independent "unit" and was scored regardless of the cellular location within the crypt.

The weighted average for normal colon was 65 ± 33. The score for HNPPC tissues was 74 ± 70 (P < 0.05 compared with normal colon). However, it was apparent that the HNPPC scores were not uniformly distributed and contained a group of

Fig. 3. Lectin histochemistry-ACA staining of normal colon. A, low power. This photomicrograph shows three colonic crypts in which ACA selectively binds to glycoconjugates in the basal portion of the crypt. The reaction product appears black in this photomicrograph. B, low power. This is a photomicrograph of normal colonic mucosa processed identically to that seen in A, except that the biotinylated lectin (ACA) was omitted from the procedure. This indicates the low level of background labeling in normal tissue. C, this is a higher power view of the lower portion of a crypt of a normal colonic crypt stained with ACA. Note the black reaction product in the apical portion of the cytoplasm and microvillar membranes and the absence of staining in the middle and upper portions of the crypt.
outliers with high scores (Fig. 8). Five specimens in the group had a score of $203 \pm 43$ ($P < 0.001$ compared with normal colon). When 11 of the FAP tissues were scored in this manner, a weighted average of $224 \pm 76$ was obtained ($P < 0.001$ compared with normal colon), which was not different from that seen in the HNPCC outliers (Table 3).

Direct Comparison of Biotinylated ACA and PNA. Since prior work with PNA on familial colon cancer had been undertaken using the fluorescein-conjugated lectin (17), we compared biotinylated ACA and PNA on paired sections, using the simplified, global 0–3* scoring system as was used for hapten sugar inhibition studies (Table 1). This experiment demonstrated that biotinylated PNA does not bind to normal or FAP colon tissues, rarely binds to HNPCC, and binds 1–3* in colon cancers, as demonstrated in Table 4.

DISCUSSION

ACA is a newly isolated, T-antigen-binding lectin that we adapted for histochemical studies of colorectal neoplasia. Data obtained during the characterization of the lectin suggested that its recognition specificity might differ from that of PNA, another lectin that binds T-antigen (1). We found that ACA has unique tissue-labeling characteristics compared to PNA, which suggests that variable forms of T-antigen may be produced in the colon under different biological circumstances. Lectins are particularly valuable as histochemical probes because of their ability to generate a reliable and uniform preparation of a stable agglutinin, the possibility for documentation of binding specificity with a high degree of precision (1, 21), and the facile use of lectins on formalin-fixed, paraffin-embedded tissue sections (5–13, 17, 22).

The most important and novel aspect of ACA histochemistry was our observation that it binds to epithelial cell glycoconjugates at the basal portion of the crypt of Lieberkühn, the proliferative zone in normal colonic mucosa. Labeling preferentially occurs in cytoplasmic, mucin-type glycoconjugates (although not necessarily mucins per se), on the apical membranes and in secretions at the base of the colonic crypt but typically is not expressed as the cells mature and migrate toward the luminal surface of the epithelium. In a small number of specimens, labeling also was seen in the upper portions of the colonic crypt, but this was exceptional. We have previously reported that β-linked galactosyl residues are preferentially expressed in goblet cell mucins from the lower one-half of the colonic crypt using BPA and RCA1 (5). A major limitation of these two lectins is that their binding specificity is relatively broad, and epithelial cell glycoconjugates are labeled by both lectins throughout the colonic crypt, limiting their utility to further identify proliferation-associated structures. ACA has a more restricted range of labeling in the colon but shares with BPA and RCA1 preferential binding to glycoconjugates in the lower one-half or proliferative region of the colonic crypt. This occurs in spite of the apparent paradox that BPA and RCA1 bind to cytoplasmic and secreted glycoconjugates possessing terminal galactosyl residues (21), whereas ACA is a T-antigen-binding lectin that has a critical interaction with GalNAc (1). BPA, RCA1, and ACA have a labeling pattern in the human colon that is complementary to DBA and soybean agglutinin; these latter lectins label goblet cell mucin in the upper one-half of the crypt in those epithelial cells that have migrated into the zone of differentiation (5). DBA and soybean agglutinin appeared histochemically to bind preferentially to goblet cell mucin. BPA and RCA1 label mucins in the lower crypt region but, as previously mentioned, also labeled cytoplasmic structures throughout the crypt region (5). In this study, we found that ACA labels cytoplasmic and membrane-bound structures and tends not to label goblet cell mucin in the normal colon. Secreted glycoconjugates are prominently labeled in neoplastic lesions. Therefore, entirely different molecular species may account for the labeling differences between ACA and BPA/RCA1.

We observed ACA binding in virtually all neoplastic lesions of the colon, including small adenomas. This is of particular...
interest since PNA binds to colonic neoplastic lesions at a time later in their natural history. PNA tends not to label adenomatous polyps <1 cm in diameter and binds to an increasing percentage of neoplastic glands in polyps that are larger, more villous, or more dysplastic (9, 22). The appearance of glycoconjugates that bind PNA is therefore a relatively late phenomenon in colorectal neoplasia, occurring in the same time frame as genetic mutations in the ras oncogene and deletions on chromosomes 5, 17, and 18 (23, 24). On the other hand, glycoconjugates that bind ACA occur earlier in the natural history of neoplasia, in the time frame reported for gene hypomethylation (25).

ACA-binding glycoconjugates are present in the proliferative region of the normal colon. It is not known whether the structure(s) recognized at the bottom of the colonic crypt is identical to those found in neoplastic tissue, which is a critical issue in need of clarification. ACA prominently labels secreted glycoconjugates in cancers (whereas cytoplasmic structures are the primary sites of labeling in the normal colon), suggesting that modifications in glycosylation may be associated with changes in glycoprotein trafficking.

The identification of T-antigen-like structures is highly dependent upon the methods used for their detection. Fluorescein isothiocyanate-conjugated PNA does not bind to glycoconjuga-
Table 2 Summary of ACA histochemistry I

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% of epithelial units labeled</th>
</tr>
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<tbody>
<tr>
<td>Normal colonic epithelium (N = 23)</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Upper one-half of crypt</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>Lower one-half of crypt</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>Well-differentiated colonic cancer (N = 25)</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>Adenomatous colonic polyps (N = 6)</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>Familial adenomatous polyposis (N = 28)</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>Flat tissue</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>Polypoid tissue</td>
<td>83 ± 7</td>
</tr>
</tbody>
</table>

The percentage of epithelial units showing any definite lectin labeling is expressed as the mean ± SEM.

Fig. 7. Binding of ACA to normal-appearing colonie tissues from patients with HNPCC. This is a section of normal-appearing tissue from a patient at risk for HNPCC. This tissue was selected from the group outliers who had an increase in ACA binding compared to the rest of the group. The labeling of epithelial cell glycoconjugates in this section is similar to that seen in FAP; omission of lectin gave minimal background labeling, as shown in Fig. 6B.

colon, although a cryptic variant of T-antigen may be present with chain extension or substituents on the C'-2, C'-3, C'-4, or C'-6 positions of the galactose residue that would interfere with recognition by PNA (Fig. 2). A candidate variation of the T-antigen that may be present in the normal colon is the disialylated tetrasaccharide: Neu5Acα2,3-Galβ1,3(Neu5Acα2,6) GalNAc, which has not yet been identified in the colon. However, the failure of neuraminidase to uncover PNA-binding sites in the normal colon would argue against this. Additional forms such as a monosialylated trisaccharide (in which only the α2,3-linked sialic acid remains) or the nonsialylated native T-antigen appear to be expressed in neoplastic epithelium. This speculation was supported by the experiments in which cancer specimens were treated with neuraminidase. Removal of α2,3-linked sialic acids with Newcastle disease virus neuraminidase enhanced labeling by PNA but had no effect on labeling by ACA. Additional characterization of the mucin-type oligosaccharides from colonic neoplasia is necessary to confirm this hypothesis.

FAP is a genetic disease in which the normal restraints on epithelial cell proliferation eventually fail, the colon develops a large number of adenomatous polyps, and colonic cancer inevitably ensues. Prior investigators have used thymidine incorporation studies (27) and measurement of ornithine decarboxylase (28) to demonstrate expression of the proliferative zone and defective regulation of cell division in the normal-appearing, preadenomatous tissue. Both these techniques require the

gates in the normal colon. However, using a lectin and antibody technique (6) or a polyclonal antibody to T-antigen for immunohistochemical studies (22), labeling at the base of the crypt has been reported in a small proportion (approximately 5%) of normal colons (reviewed in Ref. 26). This has created confusion regarding the synthesis of T-antigen by normal colonic epithelium, which has been exacerbated by a detailed study of mucin oligosaccharides from the normal colon that failed to detect the T-antigen or any "cryptic" variations (14, 15). It is now apparent that T-antigen is not abundantly expressed in the normal colon, although a cryptic variant of T-antigen may be present with chain extension or substituents on the C'-2, C'-3, C'-4, or C'-6 positions of the galactose residue that would interfere with recognition by PNA (Fig. 2). A candidate variation of the T-antigen that may be present in the normal colon is the disialylated tetrasaccharide: Neu5Acα2,3-Galβ1,3(Neu5Acα2,6) GalNAc, which has not yet been identified in the colon. However, the failure of neuraminidase to uncover PNA-binding sites in the normal colon would argue against this. Additional forms such as a monosialylated trisaccharide (in which only the α2,3-linked sialic acid remains) or the nonsialylated native T-antigen appear to be expressed in neoplastic epithelium. This speculation was supported by the experiments in which cancer specimens were treated with neuraminidase. Removal of α2,3-linked sialic acids with Newcastle disease virus neuraminidase enhanced labeling by PNA but had no effect on labeling by ACA. Additional characterization of the mucin-type oligosaccharides from colonic neoplasia is necessary to confirm this hypothesis.

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Table 3 Summary of ACA histochemistry II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Weighted average (0-400)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal colon (N = 14)</td>
<td>65 ± 33</td>
</tr>
<tr>
<td>HNPCC (N = 32)</td>
<td>74 ± 70*</td>
</tr>
<tr>
<td>HNPCC outliers (N = 5)</td>
<td>203 ± 43*</td>
</tr>
<tr>
<td>FAP (N = 11)</td>
<td>224 ± 76*</td>
</tr>
</tbody>
</table>

* Significant difference from normal colon, P < 0.05.
* Significant difference from normal colon, P < 0.001.
availability of fresh colonic epithelium for their application. Lectin histochemistry may be performed on fixed tissues, including previously obtained archival specimens, because of the stability of carbohydrate structures during the fixation and embedding procedures. The labeling by ACA of small adenomas and flat, normal-appearing colonic tissue in FAP is consistent with the previously described defects in proliferation, which distinguishes ACA from PNA. We have recently reported that PNA does not label the flat mucosa or small adenomas in FAP (17). Collectively, these data suggest that early events in colorectal neoplasia, i.e., those associated with defective control of proliferation, may be associated with a form of T-antigen recognized by ACA but that later events, i.e., those seen in larger polyps and cancers, are associated with forms recognized by both ACA and PNA. In any event, ACA and PNA can bind to slightly different carbohydrate structures, which may differ primarily by their state of sialylation.

Potentially the most important histochemical finding with ACA occurred with the tissues obtained from individuals at risk for HNPCC. A subset of these tissues showed labeling with increased intensity and in inappropriate locations compared to normal colons. When studied with PNA, we have found no labeling in HNPCC tissues (17). However, a subset of patients at risk for HNPCC was found who had a significant reduction in the weighted average for labeling with DBA, the lectin that selectively binds to goblet cell mucin in the upper one-half, or one of differentiation, in the normal colon. When ACA was used, a subset of outliers had labeling scores similar to those seen in FAP specimens. If our ascertainment were perfect, only one-half of our group of patients at risk for HNPCC would be seen in FAP. Since there are no markers available to identify patients at risk for HNPCC, this potential use deserves additional investigation.

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REFERENCES


Use of the Lectin from *Amaranthus caudatus* as a Histochemical Probe of Proliferating Colonic Epithelial Cells


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