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Aberrant Crypts in Human Colonic Mucosa: Putative Preneoplastic Lesions

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Abstract Aberrant crypts are recognized in methylene blue-stained, unsectioned, colonic mucosa by their increased size, elliptical lumenal opening, thicker epithelial layer, and increased pericryptal region. Aberrant crypt foci in rodents are observed as early as 2 weeks and for at least 9 months after a single dose of carcinogen, have a distribution that parallels that of tumors, and have an increased number of aberrant crypts per focus with time after the carcinogen dose. The ability to quantify these lesions in the entire colon of rodents in less than an hour suggests that aberrant crypts may provide a highly efficient *in vivo* bioassay for colon carcinogens. Since aberrant crypt foci appear to be the earliest identifiable putative precursors of colon cancer, they represent lesions that can be characterized further for the earliest genetic and biochemical alterations. In F344 rats, we have demonstrated that aberrant crypts have multiple histochemically-detectable enzyme alterations. Using similar techniques, we were the first to demonstrate aberrant crypts in unsectioned human mucosa. After embedding and sectioning, these microscopic aberrant crypts resemble rare lesions described earlier in the literature after extensive serial sectioning. In rats and humans, aberrant crypts may be histologically normal or display varying degrees of dysplasia and histochemically-detectable altered enzyme activities. These putative, preneoplastic lesions should reveal early changes that precede colon cancer and ways to alter their progression.

Key words: aberrant crypts, chemoprevention, enzyme-altered foci, intermediate biomarker, preneoplastic lesions, putative colorectal cancer precursor

Adenomas have long been recognized as neoplastic lesions that precede the development of most human colonic carcinomas [1]. We shall discuss the identification and characterization of microscopic putative preneoplastic lesions that can be observed in colonic mucosa. Since most of our understanding of the nature and development of these early lesions has been obtained from animal models, we shall consider the animal studies first and then the more recent work with human tissues. A better understanding of these early events in colon carcinogenesis should increase our understanding of the biology of this very common cancer and permit the use of these lesions as intermediate biomarkers.

COLONIC ENZYME-ALTERED FOCI

Our initial studies [2,3] to look for microscopic putative preneoplastic lesions in colonic mucosa were patterned after earlier studies in liver carcinogenesis [4,5] in which focal areas of changed enzymatic expression or "enzyme-altered foci" were detected prior to architectural changes in the livers of rodents treated with carcinogens. The hepatic enzyme-altered foci are generally thought to be putative preneoplastic lesions and the clonal derivatives of single altered cells that display a large number of phenotypic alterations (reviewed in [4]). Colonic mucosa was obtained from F344 rats that were injected sc with 6.8 mg/kg dimethylhydrazine (DMH) for 20 wk and sacrificed 1, 12, and 33 wk after the last injection, *i.e.*, 20, 31, and 52 wk from the start of injections. Segments of distal colonic mucosa were fixed and embedded at 4°C in glycol methacrylate; cut serially at 2-4-μm intervals; and stained with hematoxylin-eosin-azure (HEA), for PAS-reactive substances, and for the enzyme-histochemical demonstration of hexosaminidase, α-naphthyl butyrate esterase (ANBE), 5'-nucleotidase, acid phosphatase, alkaline phosphatase, and γ-glutamyl transpeptidase (GGT) activity [3,6].

Multiple phenotypic changes were observed in both the epithelial and stromal cells of these colons, but a single marker was not found to identify all foci. A marked decrease of histochemically demonstrable hexosaminidase activity in epithelial cells was the most frequent and useful marker to identify colonic enzyme-altered foci. Serial sections of these foci frequently displayed decreased ANBE activity in epithelial cells; a marked reduction of mucin-producing cells or smaller

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mucin-filled vacuoles; and increased GGT, alkaline phosphatase, 5'-nucleotidase, and acid phosphatase activities in the stromal cells. Some foci had decreased 5'-nucleotidase activity in what have been called "pericryptal fibroblast sheath cells" (see [6] for references).

Focal areas of altered enzyme activity were seen in the distal colons of all carcinogen-treated rats evaluated microscopically but only rarely in those of the untreated control rats [3]. The mean number of foci per sq cm in distal colons was much higher from carcinogen-treated rats than from untreated controls ($P = 0.01$) 1 wk after the last injection (20 wk after the first injection), and this number did not change significantly in the carcinogen-treated rats killed 31-52 wk after the first injection. The presence of enzyme-altered foci at 33 wk after the last injection with carcinogen suggests that these changes are long-term or permanent effects of the carcinogen and are not manifestations of toxicity. These foci ranged in size from single, normal-sized crypts to clusters of 4 or more crypts; histologically their morphology varied from normal to overtly dysplastic as illustrated previously [3].

Five of 20 rats killed 31-52 wk after their first dose of carcinogen had 6 tumors that were confirmed to be colonic carcinomas by histologic examination of HEA-stained sections. Many of the phenotypic changes seen in the enzyme-altered foci were histochemically demonstrable in these tumors (unpublished data). Hexosaminidase appears to be a good marker to follow the neoplastic process in rat colon since reduced hexosaminidase activity is expressed (a) in colonic epithelial cells, (b) by the highest proportion of enzyme-altered foci at all of the time periods analyzed, and (c) in foci which vary from normal to dysplastic morphology [3]. It is histochemically reduced in rat colonic tumors induced in our study (unpublished data) and was decreased biochemically in rat DMH-induced colonic tumors [7].

Putative preneoplastic changes reported previously in the colons of carcinogen-treated rodents have included alterations in glycoproteins, morphology, and proliferative activity (see [3] for references and discussion). Mayer *et al.* [8] described focal areas of altered activity for acid phosphatase and several enzymes involved in carbohydrate metabolism in rat colon, but all of their illustrated lesions with enzyme alterations displayed marked structural abnormalities as well.

ABERRANT CRYPTS (ACs) IN RODENTS: PUTATIVE PRENEOPLASTIC LESIONS

Our work with enzyme-altered foci was begun in 1987 and reported in 1988 [2,3]. In 1987, Bird [9] demonstrated that ACs could be identified microscopically in methylene blue-stained, unsectioned, formalin-fixed colons from carcinogen-treated mice and rats. These ACs differ from enzyme-altered foci in that they are larger than normal crypts in the field, have a thicker layer of epithelial cells, often have elliptically shaped lumina, and have an increased pericryptal zone that separates them from adjacent normal crypts [10]. ACs are seen as early as 2 wk after a single dose of carcinogen [10] and are induced in a dose-dependent manner [10-12]. The development of ACs appears to be a specific response of the colon to colon carcinogens since carcinogens that target other organs rarely induce ACs [10-12] and toxic noncarcinogenic compounds, such as cholic acid that induces cell proliferation and inflammation [9] or colchicine that induces nuclear aberrations [13], do not induce ACs.

Initially we asked the question whether these ACs are enzyme altered like the enzyme-altered foci that we had identified in grossly normal-appearing colonic mucosa from carcinogen-treated F344 rats [2,3]. For these studies [14] F344 rats were given 2 sc injections of 15 or 20 mg/kg azoxymethane (AOM) 1 wk apart and sacrificed 3 wk after the first injection. The colons were opened longitudinally, pinned out flat, fixed for 2 hr at 4°C in 2% paraformaldehyde, and stained with 0.2% methylene blue. ACs were identified by the method described by Bird [9], marked with permanent ink with a microprobe, and embedded in glycol methacrylate [3,6]. All 30 ACs identified in this manner had a marked reduction of histochemically demonstrable hexosaminidase activity [14]. To date we have observed a marked reduction of histochemically demonstrable hexosaminidase activity in over 300 ACs, and we have not observed ACs (as defined by Bird [9]) that lack this phenotype (unpublished data). As we have discussed previously [14], the strong linkage of reduced hexosaminidase activity with ACs suggests that this might be a marker to investigate the molecular event(s) associated with this early event in colon carcinogenesis. Linkage of esterase D deficiency with retinoblastoma facilitated the mapping of the retinoblastoma gene to chromosome 13 in humans (see [14] for references). This hypothesis is particularly intriguing since in humans both the gene for familial polyposis coli (FPC or APC) and the gene for hexosaminidase B have been mapped to the long arm of chromosome 5 (see [14] for references). ACs also have decreased histochemically demonstrable ANBE activity and increased PAS reactivity in many but not all lesions analyzed 3-4 wk after the first dose of carcinogen.

ACs are found predominantly in the distal colons of mice [10] and rats [14] where colonic tumors are found in these animals [15,16]. Most foci of ACs consist of 1-3 glands at the early time periods (2-4 wk after the first injection with carcinogen) [9,10,17]; many but not all of these foci become larger with multiple crypts with increasing time [10,12]. McLellan and Bird [18] analyzed the proliferative activity of 8 ACs from rats that received a single dose of DMH 19 wks earlier. "Compared to NC [normal crypts], AC contained higher ($P \leq 0.05$) numbers of cells and had higher labeling indices, ... [but] labelled cells were not present in the top third of the AC." In a review paper, Bird *et al.* [12] state that "The proliferative status of AC ... demonstrated a marked heterogeneity ... some AC exhibited marked increases in mitotic activity and S-phase cells, whereas others appeared to be quiescent with respect to proliferative status."

Foci of ACs, as viewed topographically in unsectioned colon, can vary appreciably in size even in the same animal (unpublished data). This has been illustrated by Bird and collaborators (a) with adjacent foci of ACs consisting of 2 crypts and >20 crypts in a mouse after 4 weekly injections of AOM and a high fat diet for 16 wk [10] and (b) with 4 foci in the same field with 2, 3, 5, and 13 crypts in a rat 19 wk after 1 dose of DMH

[12]. Histologically, ACs (as defined at 40X magnification and the staining of whole mounted colon [9]) vary morphologically (Fig. 1) from near normal or mild atypia [10,14], to dysplastic [10,12], to invasive cancer (Fig. 2). Note that the lesions illustrated in Figures 1 and 2 are both from F344 rats that received a single 30 mg/kg dose of AOM 36 wk prior to sacrifice. Both lesions were identified microscopically in unsectioned, methylene-blue stained colons, marked with permanent ink, and embedded in methacrylate. This invasive carcinoma that was identified as a focus of ACs with the methylene-blue assay is similar to the microcarcinomas described previously [16] in random histologic sections of "grossly normal, flat mucosal areas in rats that received" AOM. Bird *et al.* [12] point out that the pathologic features of ACs do "not depend on the size of the focus or on the number of crypts present in the focus." Since the histologic features of the ACs are not evident in the lesions identified topographically in the unsectioned tissue and the pathology is very variable as just illustrated, the terms "dysplastic crypts" or "microadenomas" should be reserved for those lesions that have been confirmed histologically to have those particular histopathological alterations. We prefer the term "aberrant crypts" for lesions identified topographically.

Lesions similar to those described by Bird [9] with methylene blue and light microscopy had been described earlier with scanning electron microscopy (SEM) [19,20] in rats treated with either DMH or AOM. Features described by SEM in the colonic mucosa from carcinogen-treated animals included "raised" [20] or "protuberant glands" [19] and "slit-like" orifices of the glands [19,20]. "With time the size of the lesions observed ('microadenomas') and the number of crypts per lesion increased" [20]. The disadvantage of SEM is that only small samples, e.g., one sq cm, of the colonic mucosa rather than the entire colonic mucosal surface can be readily evaluated. Other investigators [21,22] have inspected whole-mount preparations of colonic mucosa from carcinogen-treated animals for abnormalities with the light microscope. Kimura *et al.* [21] used 1% alcian blue solution with formalin-fixed tissue to identify colonic mucosal changes; histologic sections of their lesions demonstrated "mild, moderate, or severe dysplasia." Sandforth *et al.* [22] identified mucosal lesions, in glacial acetic acid-fixed specimens stained with Schiff's reagent, that appear very similar to those identified by Bird [9]) with methylene blue. Cytospectrophotometry with serial cryostat sections of unfixed tissue demonstrated "decreased activities of the brush-border enzyme diaminopeptidase IV (DAP IV), the lysosomal acid B-galactoidase (a. B-gal.) and the mitochondrial succinate dehydrogenase (SDH), thus showing a lack of enterocyte differentiation" [22]. It is not clear how the areas for cryostat sectioning and/or evaluation were identified. The methylene-blue method has the advantage that it uses a vital dye with either fixed or unfixed tissue [23] to screen large amounts of colonic mucosa rapidly for ACs, the earliest identifiable putative precursor of colon cancer. The lesions identified in this manner are now available for molecular and biochemical characterization either directly or in histochemical preparations with *in situ* methods.

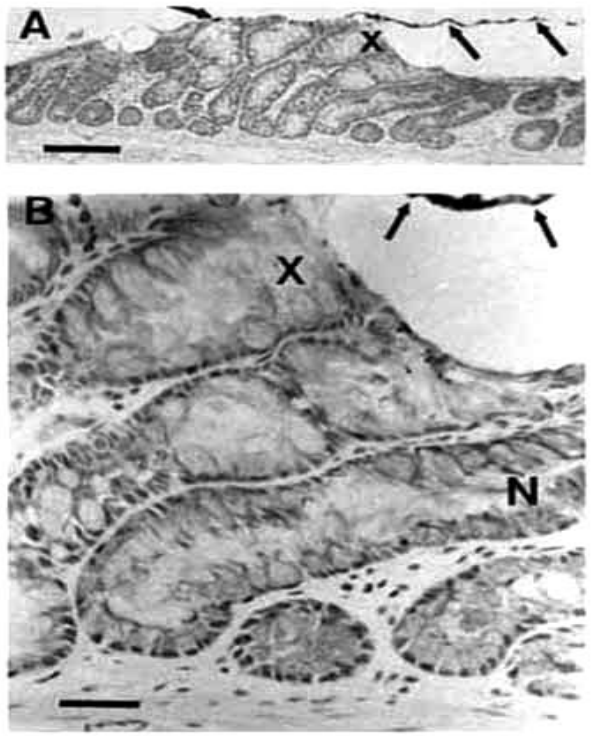

Fig.1. Aberrant crypts (ACs), detected in methylene-blue stained unsectioned colonic mucosa from an F344 rat 36 wk after 1 dose of 30 mg/kg AOM, were marked with permanent ink (arrows) and embedded. The ink with plastic separated (artifactually) during embedding. The ACs include the 4 crypts to the right of the left most arrow and display only mild atypia. An X indicates the same crypt in **A** and **B**, N indicates a normal crypt. **A**, x64, bar = 150 μm; **B**, x320, bar = 30 μm; stained with hematoxylin, eosin, azure (HEA).

ARE ENZYME-ALTERED FOCI AND ABERRANT CRYPTS THE SAME LESIONS?

As discussed above, ACs do express enzyme alterations [14,24]. However, there do appear to be some differences between these two lesions. The enzyme-altered foci in histologic sections appear the same size or smaller than adjacent normal glands [3]. ACs are identified because of their increased size in unsectioned colon [9]; in histologic sections, they frequently, but not always, display dilated lumina. This feature can be masked by the orientation of the crypt in the section. All ACs that we have evaluated in F344 rats have had histochemically demonstrable reduction of hexosaminidase activity, but a small percentage of enzyme-altered foci fail to demonstrate this phenotype [3]. The mucin in ACs stains more intensely with PAS 3-4 wk after the first carcinogen injection (the only time period evaluated to date, unpublished data) while the number of goblet cells and/or the size of the mucin-filled vacuoles is frequently decreased (56% of foci at 20 wk and 82% at 31-52 wk after the first carcinogen injection [3]) in enzyme-

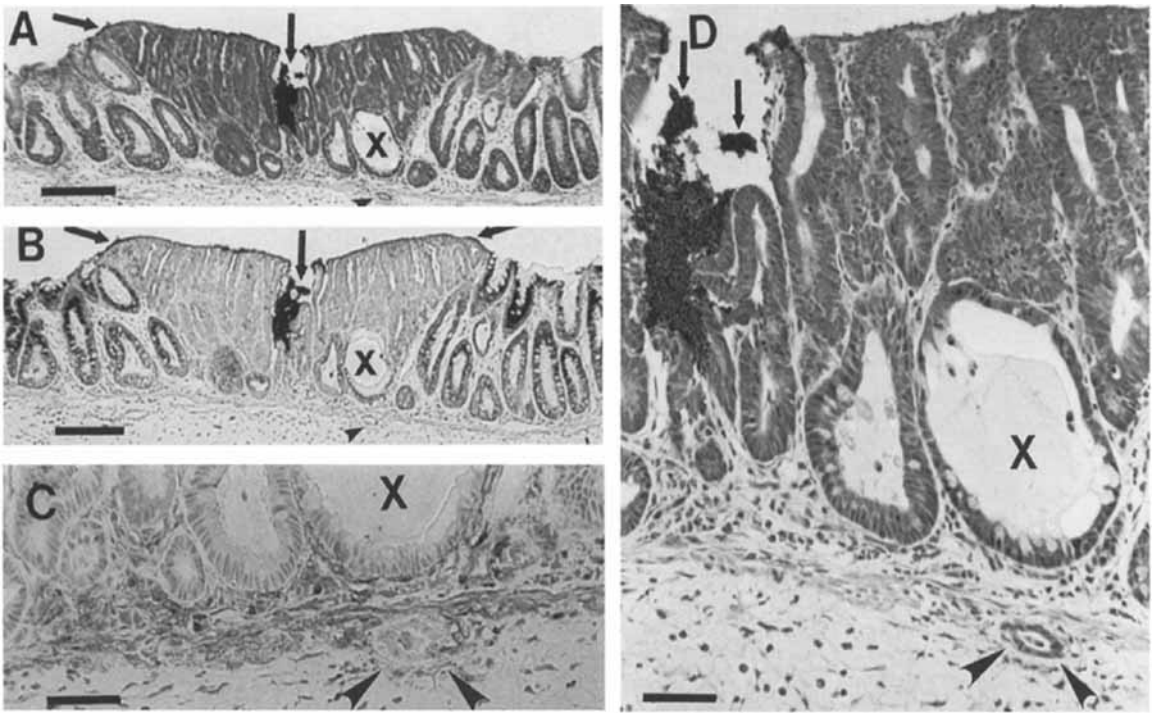

Fig. 2. ACs, detected in methylene-blue stained unsectioned colonic mucosa from an F344 rat 36 wk after 1 dose of 30 mg/kg AOM, were marked with permanent ink (arrows) and embedded. These ACs display carcinoma (arrowheads) that has invaded the muscularis mucosa. **A**, x64, bar = 150 μm; HEA. A serial section (**B**) displays in the ACs marked reduction of the red precipitate (black in the photomicrograph) that depicts hexosaminidase activity (methyl green counterstain, x64, bar = 150 μm). In another serial section (**C**), the dark brown precipitate that depicts 5'-nucleotidase activity clearly delineates the muscularis mucosa (methyl green counterstain, x160, bar = 60 μm). **D** is a higher magnification (x160, bar = 60 μm) of the section in A. An X indicates the same lumen in the 4 views.

altered foci. There appear to be some crypts that express enzyme alterations that would not be detected by the aberrant crypt assay. Whether these lesions are different stages of the same process or two independent putative precurors of colon cancer remains to be established.

ABERRANT CRYPTS: INTERMEDIATE BIOMARKERS FOR COLON CANCER

The advantages of using the aberrant crypt assay to screen for compounds that are specific for colon carcinogenesis and for compounds that might inhibit or promote this process have been discussed by many [3,9-14,25,26]. Evidence that ACs are intermediate *in vivo* biomarkers for colon cancer includes the apparent specificity of the development of ACs after exposure to colonic carcinogens as discussed above. Since ACs appear in animals very soon after the administration of carcinogen and there are multiple lesions per animal (up to 100/rat colon), this assay requires many fewer animals and a much shorter period of time (4-16 wk rather than 9-12 months) as compared with experiments that use tumor formation as an endpoint. In addition the aberrant crypt assay is easy to learn [9] and allows the total number of microscopic lesions in the entire colon of an experimental animal to be assessed in a short period of time (less than 1 hr for a rat colon). As pointed out by McLellan and Bird [26], to find these microscopic lesions "in histological sections is analogous to looking for a needle in a haystack."

One of the first demonstrations that ACs can be used to assay the promotional phase of colon carcinogenesis was that of McLellan and Bird [10]. One wk after 4 weekly ip injections of 5 mg/kg AOM, some mice were killed; half of the remaining mice were fed a high fat diet (20% by wt), a known promoter of colon cancer; and the other half were continued on a low fat diet (5% by wt). Mice on a high fat diet for 16 wk had significantly more foci of ACs and larger foci compared to foci that developed in mice on a low fat diet [10]. Disulfiram, a known inhibitor of chemically-induced colon cancer, completely inhibited the formation of ACs with DMH but did not inhibit the formation of ACs with its metabolite, AOM [26]. It should be pointed out that although these studies further support the hypothesis that ACs are putative preneoplastic lesions of colon cancer, neither study correlated the development of ACs with the tumor incidence in the same animals under exactly the same conditions. Our limited experiments [17,27] suggest that some data obtained with the aberrant crypt assay do correlate with data obtained relevant to the development of carcinomas under the same conditions when selenium [17] or phytic acid [27] were added to the diet 1 wk after a single dose of 30 mg/kg AOM. With the phytic acid studies [27], however, it was the

sizes of the aberrant crypt foci rather than the number of foci at 12 wk after treatment that were predictive. Until additional studies include both short-term (aberrant crypt) and long-term (tumor) data, the most meaningful parameters of the aberrant crypt assay will not be known.

Corpet *et al.* [28] used this same assay to evaluate the effect of cooking one or more components (sugar, caesin, and fat) of a diet on the promotion of the formation of ACs that they called "microadenomas." A diet in which 20% was cooked sucrose or 40% was casein and beef tallow cooked together promoted the formation of ACs "in initiated mice and rats, and would appear [my underlining] to contain promoters for colon cancer" [28]. In similarly designed experiments, Caderni *et al.* [29] looked at the effects of dietary carbohydrates (sucrose and starch) on the formation of ACs that they called "dysplastic crypts" in rats initiated with DMH. Their "overall results suggest [my underlining] that starch in high-fat/low calcium/low cellulose diets has a protective role against DMH-colon carcinogenesis in the rat" [29]. Both of these studies acknowledge by their tentative conclusions that, although there is good evidence that ACs are putative preneoplastic lesions of colon cancer, this assay cannot be used as an intermediate biomarker for colon cancer until the results of the aberrant crypt assay are verified with tumor data generated in the same study with several different experimental systems. Since both of these studies [28,29] used the method developed by Bird [9] to identify similarly described lesions in unsectioned colon and neither study [28,29] examined the pathology of the identified lesions in histological sections, the term "aberrant crypts" is preferred over "dysplastic crypts"or "microadenomas" as we have discussed above. Wargovich [30] recently presented data on several candidate chemopreventive agents for ACs that might be chemopreventive agents for colon cancer.

ABERRANT CRYPTS IN HUMAN COLONIC MUCOSA

As evidence was accumulating that ACs are putative preneoplastic lesions of colon cancer in rodents and might be useful as *in vivo* biomarkers to evaluate chemopreventive agents, we asked the question whether similar lesions also occur in humans. Using grossly normal-appearing colonic mucosa resected from patients along with their colon cancers, our laboratory [24,31] appears to have been the first to demonstrate that ACs can be detected in whole mounts of human colonic mucosa with the methylene-blue technique developed by Bird [9]. Colonic mucosa, located within 15 cm of the tumor, was obtained from 22 consecutive resections for sporadic colon cancer. Twelve of the specimens were from the right colon, one from the transverse colon, and nine from the left colon or rectum; all were placed immediately in 0.9% saline at 4°C in the operating room. The patients had a mean age of 69 ± 12 (S.D.) yr and included 10 females and 12 males. Surgically resected colonic mucosa was also obtained from a patient with Gardner's syndrome. Since normal colonic mucosa was available from resections of only 2 patients without predisposing conditions for colon cancer during this time, additional colonic mucosa was obtained from autopsy patients who never had colon cancer. All tissues were obtained for us by the Tissue Conservation Core Facility of the Case Western Reserve University Cancer Center.

The strips of mucosa were peeled from the submucosa, pinned out flat, fixed at 4°C for 2 hr in 2% paraformaldehyde, and stained with 0.2% methylene blue for 3-5 min. Tissue is now held for 30-60 min in 1% paraformaldehyde at 4°C, rather than phosphate buffer as described previously [31], prior to evaluation for ACs. Some of the ACs were marked with permanent ink and embedded in methacrylate as described earlier for rat ACs [14].

Foci of ACs were identified in the grossly normal colonic mucosa in all (9 of 9) resections from the left colons; none (0 of 1), from the transverse colon; and 33% (4 of 12) from the right colons of patients with sporadic colon cancer in the same regions. An average of 16.3 ± 7.1 (S.D.) sq cm of mucosa per patient or a total of 358 sq cm of mucosa were evaluated from these patients. Foci of ACs were not detected in the surgically resected left colon of a patient with clinically redundant colon or in 253 sq cm from right colons of 13 autopsies of patients without colon cancer. One focus was seen in 253.3 sq cm from the left colons of 13 autopsies of patients without colon cancer. A very small focus of ACs (2.8×10^{-4} sq cm) was detected in the left colon from a patient with diverticulitis and an inflammatory polyp.

The foci of ACs in human colonic mucosa [31] clearly resemble those observed previously in rodents. The foci observed in these human tissues had a mean size of 1.4 ± 2.0 sq mm (range, 0.001-8.1 sq mm); varied from single crypts to large plaques of >30 crypts; and appeared, microscopically, to be slightly elevated above the mucosal surface. The lumina of most ACs were oval or slit-shaped rather than circular; some were dilated and smooth while others were serrated or had invaginations. We have not attempted to correlate the luminal patterns of ACs observed in the methylene-blue stained whole-mount preparations of human mucosa with the pathological features observed in histologic sections; Roncucci *et al.* [23] have recently done so. In their study, only 3 of 29 ACs with round lumina, 0 of 14 with serrated lumina, and 9 of 10 with elongated lumina were dysplastic; 7 of 14 with serrated lumina were hyperplastic [23]. While it is tempting to speculate that the "The presence of slit-like lumen in the crypts of ACF [aberrant crypt foci] on the mucosal surface ... identify[ies] microadenomas" [23], it seems best to us to reserve the term microadenoma for only those lesions that have been confirmed to have this histopathology on histologic examination until much more data are obtained.

AC foci were much more frequent in colonic mucosa from patients with colon cancer than in our patients without colon cancer (Table I). The difference between these two populations is even greater when the area occupied by the altered crypts is compared than when the numbers of AC foci per sq cm of mucosa are compared (Table I). The AC foci were 10-fold more frequent and occupied a 10-fold greater area in mucosa from the Gardner's syndrome patient than from patients with sporadic colon cancer. A similar increase of ACs in familial

TABLE I. Aberrant Crypts (ACs) in Human Colonic Mucosa†

	Colon		Gardner's
	with ca[a]	without ca	Sydrome
Patients (n)	22	28	1
Patients with ACs (%)	59	7	100
Frequency*	0.15 ±0.19	0.007 ±0.03	2.10
Area Altered**	0.24 ±0.50	0.8×10^{-5} $\pm 3.4 \times 10^{-5}$	2.86

†A mean of 16.3 ± 7.1 sq cm of mucosa/patient were examined; [a]ca, cancer; *AC foci/cm^2; **Mean % ±S.D.

TABLE II. Aberrant Crypts (ACs) in Different Regions of Human Colonic Mucosa†

	Right colon		Left colon	
	with ca[a]	without ca	with ca	without ca
Patients (n)	12	13	9	15
Patients with ACs (%)	33	0	100	13
Frequency*	0.023 ±0.037	0	0.33 ±0.17	0.013 ±0.039
Area Altered**	0.063 ±0.14	0	0.50 ±0.71	1.4×10^{-5} $\pm 4.6 \times 10^{-5}$

†A mean of 16.3 ± 7.1 sq cm of mucosa/patient were examined; [a]ca, cancer; *AC foci/cm^2; **Mean % ±S.D.

adenomatous polyposis was reported by Roncucci *et al.* [32]. The increased frequency of AC foci in these hereditary conditions that predispose to colon cancer further supports the hypothesis that ACs are putative precursors of colon cancer. Our observation [31] of more AC foci in the mucosa of the left colon than in that of the right colon from patients with sporadic colon cancer in the respective locations (Table II) was recently confirmed [23].

A limited number of human ACs were embedded in methacrylate and evaluated histochemically and histologically [31]. The histochemically demonstrable changes in hexosaminidase and α-naphthyl butyrate esterase activities in ACs in human colonic mucosa were less marked and in the opposite direction compared to those observed in F344 rats. Hexosaminidase activity was somewhat elevated in some and not changed in other human ACs; increased hexosaminidase activity had been demonstrated previously in our laboratory in extracts of human colonic cancers [33]. ACs in humans, like those in the rat, had increased PAS-staining material and variable morphology from normal to dysplastic [31].

As discussed above, the pathology of some ACs in histologic sections reveals dysplasia and resembles the microscopic adenomatous polyps that have been studied extensively in familial polyposis [34-38]. Similar microscopic adenomas have been observed rarely in colonic mucosa from patients with spontaneous colon cancer but often only after examining hundreds to thousands of sections [39-42]. Step-sectioning the entire resected specimens from 18 patients with single spontaneous colon cancers, Kuramoto and Oohara [42] found 22 microscopic adenomas, 3 of which were "microscopically minute [3-5 mm in diameter] cancers of a flat nonpolyploid type..." The advantage of the methylene-blue technique with segments of either fresh [23] or fixed material that is not embedded is not the discovery of new lesions, but the ability now to evaluate large areas of colonic mucosa in a relatively short period of time for all lesions present.

Some [43,44] have alluded to the use of methylene blue *in vivo* and suggest that it might be helpful to identify small, nearly flat lesions that are difficult to see ordinarily by endoscopy. Muto *et al.* [43] looked at 33 small "flat adenomas," all 1 cm or less in diameter, with 3 obtained in surgical specimens and the remaining (30) removed during colonoscopy. "Thirteen adenomas with severe atypia contained microcarcinomas....Since they may not be demonstrated on routine barium-enema examination, meticulous colonoscopic observation along with staining would be the best way to detect these small flat adenomas" [43]. In Figure 2 of their paper, Muto *et al.* [43] demonstrate that the 5 mm lesion shown in their previous figure is "strikingly demonstrated using less air insufflation and dye (methylene blue) spread." Although in a later study [44], 7 "of 32 small, depressed lesions biopsied" were adenomas and none were carcinomas, they also suggested using methylene blue for better visualization of uncertain lesions and the value of air insufflation. The finding of minute cancers (less than 1 cm in diameter) in "flat adenomas" by both Muto *et al.* [43] and Kuramoto and Oohara [42] suggests the importance of these small lesions and how the methylene blue technique might aid in their detection.

In conclusion, aberrant crypt foci detected with methylene blue are putative preneoplastic lesions of colon cancer, some of which, at least in the F344 rat, already demonstrate invasion. The ability to readily identify multiple precursor lesions may facilitate the characterization of pathological and molecular changes that occur in these earliest identifiable lesions as they progress to cancer in both animals and humans. Evaluation of ACs *in vivo* in humans may greatly facilitate the identification of colon cancers that begin in "flat mucosa"; evaluation of ACs *in vivo* or *in vitro* may also provide epidemiological data regarding human colon cancer. The observation of ACs in humans similar to those in rodents after treatment with carcinogen lends further support to the use of these animal systems as models for human colon cancer. The animal models should be very helpful to screen for chemopreventive agents for colon cancer after the validity of the system is verified with assays that quantify both ACs and tumors in the same system under several experimental conditions.

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