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Advances in Brief

Distribution of Intestine-associated Lymphoid Tissue, Aberrant Crypt Foci, and Tumors in the Large Bowel of 1,2-Dimethylhydrazine-treated Mice[1]

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Abstract

Six-week-old male CF-1 mice were fed the AIN-76 diet, given eight weekly s.c. injections of either the colon carcinogen 1,2-dimethylhydrazine or saline, and killed 24 weeks after the last injection. Parameters measured in the large bowel included the incidence and locations of all intestine (gut)-associated lymphoid tissue (GALT) sites; the locations, incidence, and sizes of all aberrant crypt foci (ACF); and the incidence, locations, and types of all overt tumors. In saline-treated mice the distribution of GALT along the length of the large bowel was bimodal, with a majority peak of lymphoid nodules occurring in the distal large bowel and a minority peak occurring in the proximal large bowel. No ACF or tumors were present in the large bowel of the saline-treated mice. In 1,2-dimethylhydrazine-treated mice the majority of ACF were present in the middle third of the colon, between the two peaks of GALT, but the majority of the tumors were found over the GALT in the distal colon. There was a significant positive linear regression relationship between the numerical distribution of GALT and the numerical distribution of tumors along the length of the large bowel. There was no significant relationship between the distribution of ACF and the distribution of (*a*) tumors or (*b*) GALT along the length of the large bowel. Thus the numerical density of lymphoid nodules, not the numbers or distribution of ACF, was the significant predictor of the distribution of tumors in the large bowel of 1,2-dimethylhydrazine-treated mice. It is proposed that lymphoid nodules in the distal large bowel play a promotional role following initiation of colon carcinogenesis and that ACF have little if any malignant potential in the mouse.

Introduction

ACF,[3] described by Bird (1) in methylene blue-stained whole mount preparations of colons from mice treated with a colon carcinogen and carcinogen-induced 'intraepithelial foci of neoplasms' described by Shamsuddin (2), in histological sections of the large bowel, have been proposed as putative premalignant lesions in mice (1, 3), rats (4), and humans (5, 6). The ACF, which are induced by colon carcinogens (4, 7-9), arise shortly after exposure to the carcinogen and present pathological changes indicative of premalignant states (10-13). Individuals diagnosed with colon cancer have higher numbers of ACF in their colonic mucosa than persons without colon cancer (4, 5). Although ACF are thought to be biomarkers of colon carcinogenesis, Pretlow *et al.* (14) have cautioned that the acceptance of ACF as a biomarker of colorectal cancer depends on subsequent investigations designed to validate that the numbers of ACF correlate with the incidence of colorectal cancer. Two laboratories have reported that there is no correlation between numbers of ACF and the colorectal cancer incidence after dietary interventions to alter colorectal cancer incidence (15, 16).

Hardman and Cameron (17) and others (18-23) have recently reported that lymphoid nodules play a promotional role in tumor formation in the large bowel in rats and possibly in humans. Is the number and the location of ACF and/or is the number and the location of lymphoid nodules (GALT) a significant predictor of the location of DMH-induced colonic tumors along the length of the large bowel in the mouse? The answer to this question should provide a more complete understanding of the relationship between the normal distribution of GALT in the large bowel prior to carcinogen administration and the location and incidence of ACF and of tumors following carcinogen administration. The results of this study provide data on the distribution of GALT, ACF, and tumors in the large bowel of mice treated with DMH and give an answer to the question posed above.

Materials and Methods

Animals. Fifty male CF-1 mice, 28 days old, were obtained from Harlan and housed five/cage in plastic Micro-Isolater cages with raised wire floors. All mice were housed in the same room and acclimated to a room temperature of 25°C and a 12-h light-dark cycle. All animals had *ad libitum* access to semipurified AIN-76 diet (24) and deionized water during the entire experiment. This protocol was approved by the Institutional Animal Care and Use Committee.

Experimental Design. Upon arrival, the mice were assigned cages and earmarked for identification. After 14 days of acclimation they were randomly assigned to either the saline-treated (control) group or the DMH-treated group. The 25 control animals received eight weekly s.c. injections of sterile PBS plus the vehicle, EDTA (0.18% with pH 6.5). The DMH-treated group of 25 mice received eight weekly s.c. injections of DMH (12 mg/kg body weight; volume, 0.1 ml/100 g body weight). Twenty-four weeks after the final injection the mice were sedated in a CO_2 chamber and decapitated.

Processing of Colon and Tumor Specimens. The abdominal cavity was opened, the colon was removed, and its wet weight was determined using a Sartorius model E2000D single-pan electronic balance (Brinkman Instruments, Long Island, NY). The colon was opened longitudinally, pinned flat, serosal side down onto corkboard, and fixed in 10% neutral buffered formalin with 0.1 M phosphate buffer (pH 7.3) for 2 h. Colon length was measured with a millimeter scale and recorded. Tumor presence and distance from the anus were determined and recorded. Tumors were excised, placed in individual containers of 10% formalin, and processed for histology. Four-μm-thick sections of the entire tumor were cut, mounted on glass sides, and stained with hematoxylin and eosin. Slides of each tumor were examined by a board-certified pathologist and classified as an adenoma or an adenocarcinoma. After the tumors were removed, the colon specimens were transferred to 70% ethanol and stored at room temperature for 4 weeks.

Staining and Counting of Aberrant Crypt Foci and GALT. In this study colonic lymphoid tissue (GALT) in the intestinal wall of each mouse was studied in the fixed colons. The fixed tissue was removed from the cork board, kept moist with PBS, transilluminated, and observed with the aid of a dissecting microscope (×25). A ruled grid in the same viewing field was used to measure the location of each lymphoid nodule site with reference to distance from the anus. The number of GALT sites along the length of the colon was scored and recorded. In several

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[3] The abbreviations used are: ACF, aberrant crypt foci; GALT, intestine (gut)-associated lymphoid tissue; DMH, 1,2-dimethylhydrazine; PBS, phosphate-buffered saline.

cases identity of GALT as seen in whole mounts was verified by histological examination as seen in Figs. 1 and 2.

Methylene blue (1.0% in PBS, pH 7.1) was used to aid visualization of the ACF. Each colon specimen was removed from the cork board and placed luminal side up in a Petri dish. The colon was flooded with stain for 30 s; then excess stain was rinsed off with PBS. The number, size classification, and location of all ACF along the length of the colon were counted and recorded; examples of ACF are illustrated in Fig. 3.

Statistical Analysis. The average incidence of each of these three variables (GALT, ACF, and tumors) was calculated for each of twelve 10-mm segments of the large bowel beginning at the distal end and progressing proximally. Using pairs of variables the numerical distributions of GALT, ACF, and tumors along the length of the large bowel were evaluated by linear regression analysis to determine if significant correlations exist between these variables. The null hypothesis was that the sets of observations were drawn from populations with the same distributions. Thus, $P \leq 0.05$ was considered to indicate statistically significant differences or noncongruent distributions. These statistical procedures were performed on an IBM PS/2 model 57 SX computer using the WinStar statistical package.

Results

Distributions of GALT, Tumors, and ACF along the Length of the Mouse Large Bowel. Each lymphoid nodule or GALT site was 1-2 mm across (Fig. 1). The numbers and location of all GALT sites found in each mouse are reported in Table 1. Histological sections through several of the GALT sites confirmed that they were lymphoid nodules (Fig. 2). The data in Table 1 reveal that each of the 24 mice contained 3 to 7 nodules in the distal large bowel and that 21 of the 24 mice contained one or more lymphoid nodules in the proximal large bowel. Because the distance from the anus to the ileum varied among the mice, the data in Table 1 were normalized to be expressed as the percentage of total distance from the anus to the ileum. The frequency distribution of GALT sites along the length of the large bowel is presented in Fig. 4. This figure reveals a bimodal frequency distribution with a major peak of GALT occurring between 0 and 40% of the distance from the anus to the ileum and a minor peak of GALT occurring between 60 and 80% of the distance from the anus to the ileum. The position of lymphoid nodules in the proximal colon has been described previously in mice (25), but a bimodal distribution of GALT sites along the length of the large bowel does not appear to have been reported previously.

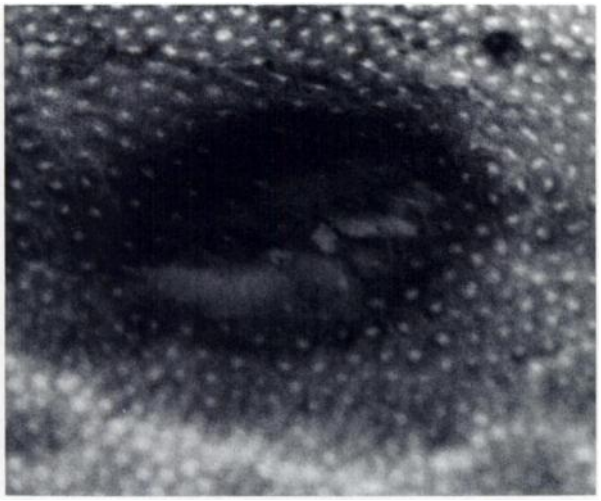

Fig 1. Photomicrograph of a transilluminated, methylene blue-stained, whole mount of descending colon from a mouse which did not receive DMH. An example of GALT is shown surrounded by normal crypts. The GALT is the oval, darkly stained object in the center of the photomicrograph. This GALT was confirmed by histology to be lymphatic nodular tissue (see Fig. 2).

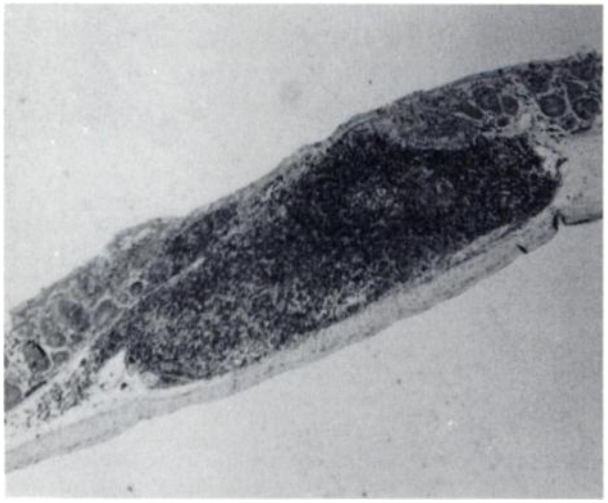

Fig 2. Photomicrograph of a hematoxylin and eosin-stained 4-μm section of descending colon cross-sectioned through a lymphatic nodule that was previously identified as GALT in the methylene blue-stained whole mount of the colon as illustrated in Fig. 1. Thus GALT, as recognized in whole mounts, was confirmed by histology to be lymphatic nodular tissue.

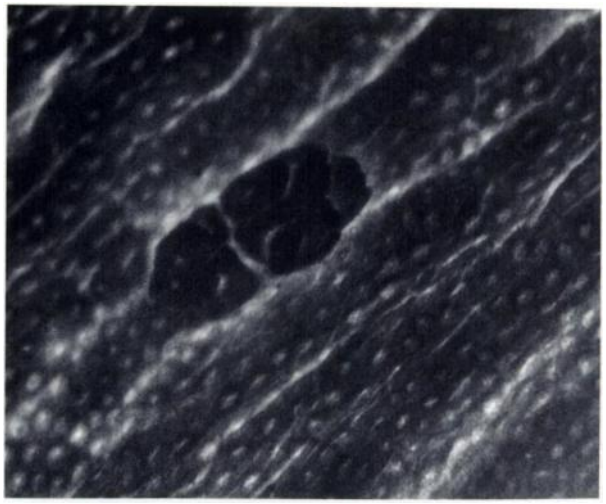

Fig 3. Photomicrograph of a transilluminated, methylene blue-stained, whole mount of descending colon from a mouse which had been treated with DMH. Examples of ACF are shown on a normal crypt background. Note the increased density of staining around the crypt mouth and the altered morphology of the mouths of the crypts in the ACF.

To help illustrate the relationships between the numerical distribution of ACF, of tumors, and of GALT along the length of the large bowel, the data on percentage of total number of ACF, of tumors, and of GALT in each of the 12 segments along the length of the large bowel were plotted. Fig. 4 shows the distribution of GALT, ACF, and tumors along the length of the large bowel of the mice. The distal third of the large bowel contained 66.6% of the GALT, 98% of the tumors, and 12.5% of the ACF. The middle third of the large bowel contained 12% of the GALT, 2% of the tumors, and 86% of the ACF. The proximal third of the large bowel contained 21% of the GALT, no tumors, and 1% of the ACF. Only 3.6% (18 of 535) of the ACF, with >5 crypts/focus, were classified as large. Thirteen of these 18 large ACF were found in the middle third of the colon and 4 large ACF were located in the distal third where 98% of the tumors were found.

The relationships between the distributions of GALT, tumors, and ACF along the length of the colon were further determined by linear regression analysis. The data used in the analyses are listed in Table 2. The positive correlation coefficient between GALT and tumors was

Table 1 *Number and location of GALT sites along the length of the large bowel*

The number and location of 113 GALT sites along the length of the large bowel were scored in each of 24 male CF-1 mice given eight weekly injections of saline and killed for analysis 24 weeks after the last injection. The data are organized in 10-mm segments from the anus to the ileum. No GALT were observed in the colon between 101 and 120 mm proximal to the anus.

	Distance (mm) from anus											
Mouse	10	20	30	40	50	60	70	80	90	100	110	120
1	1	3	1	2	0	0	0	2	1	0	0	0
2	0	1	2	1	1	0	0	1	0	0	0	0
3	1	2	1	0	1	0	0	0	0	0	0	0
4	0	2	1	3	1	0	0	0	0	0	0	0
5	1	1	1	0	1	2	0	0	1	0	0	0
6	1	1	1	1	1	0	0	0	0	1	0	0
7	1	1	1	0	1	0	0	0	1	0	0	0
8	1	1	0	1	0	0	0	0	0	1	0	0
9	0	1	1	2	0	0	0	0	1	0	0	0
10	1	1	1	1	1	1	0	0	0	0	0	0
11	0	1	2	2	1	0	0	1	0	0	0	0
12	0	0	2	0	0	0	0	0	2	0	0	0
13	1	1	2	2	1	0	0	0	0	1	0	0
14	1	1	1	1	1	0	0	1	1	0	0	0
15	1	2	2	1	1	0	0	1	0	0	0	0
16	1	1	1	1	0	0	0	0	1	0	0	0
17	0	2	0	0	1	0	0	1	0	0	0	0
18	1	2	1	0	0	0	0	2	0	0	0	0
19	2	0	1	1	1	0	0	0	1	0	0	0
20	1	1	1	1	0	0	0	2	2	1	0	0
21	1	1	0	0	1	0	0	0	0	0	0	0
22	1	1	3	0	1	0	0	0	1	1	0	0
23	1	1	2	1	0	0	0	2	0	0	0	0
24	1	1	2	2	0	0	1	2	0	0	0	0
Totals	19	29	30	23	15	3	1	15	11	5	0	0

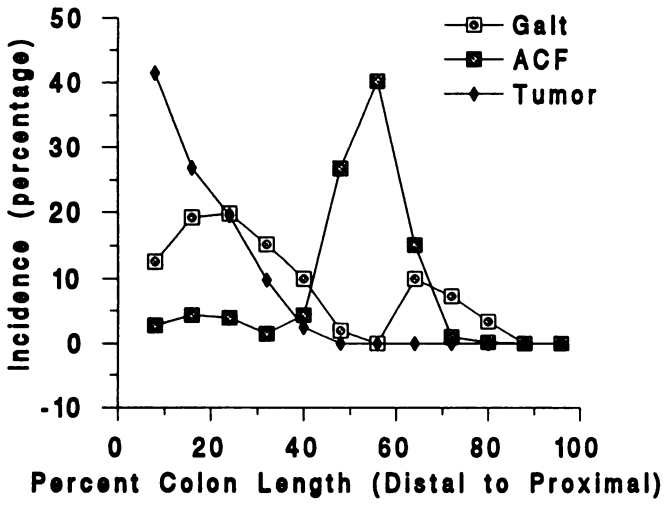

Fig 4. Distribution of ACF, of tumors (both adenomas and adenocarcinomas), and of GALT along the length of the large bowel of mice killed 24 weeks after the last of 8 weekly injections of either saline or DMH. The data on GALT distribution were from the 24 saline-injected mice; 113 GALT were identified and used in this distributional analysis. From the 25 DMH-injected mice, 535 ACF and 41 tumors were identified. Of the 41 tumors all were adenomas except 2 adenocarcinomas (one located at 8% and the other at 16% of the distance from the anus to the ileum). The percentage of the total number of ACF, tumors, and GALT is shown as a function of the percentage of distance from the anus to the ileum in the large bowel. The figure illustrates congruence in the occurrence of DMH-induced tumors and the locations of GALT in the distal colon, but lack of congruence in the distribution of ACF and the locations of tumors.

0.688 and with 10 degrees of freedom was significant at $P = 0.013$. The negative correlation coefficient between ACF and tumors was −0.259 and with 10 degrees of freedom was not significantly different from a slope of zero ($P = 0.417$). The negative correlation coefficient between GALT and ACF was −0.326 and with 10 degrees of freedom was not significantly different from a slope of zero ($P = 0.301$).

A summary of the distributions of GALT, ACF, and tumors indicate that: (*a*) the distribution of ACF was not congruent with the distribution of tumors in the large bowel; (*b*) the distribution of tumors was significantly correlated with the distribution of GALT in the large bowel; and (*c*) the distribution of ACF was not congruent with the distribution of GALT in the large bowel. The overall distribution of GALT was demonstrated to be a significant predictor of tumor location, but the overall ACF distribution was not demonstrated to be a significant predictor of tumor location as determined by linear regression analysis. That no tumors formed over GALT in the proximal colon indicates that the GALT at this particular site was not spatially related to formation of overt tumors in the proximal colon of the mice.

Discussion

The main findings of this study are: (*a*) the significant correlation between the distributions of lymphoid nodules and overt tumors; (*b*) the lymphoid nodules in the proximal colon apparently have greatly reduced tumor promotion potential compared to the lymphoid nodules in the distal colon; and (*c*) the lack of significant correlation between the distributions of ACF and overt tumors. These facts indicate that it is the presence of lymphoid nodules in the distal colon of the mouse, not the numbers or distribution pattern of ACF, that predicts the distribution of overt tumors in the large bowel of DMH-treated mice. The absence of DMH-induced tumors in the region of the proximal colon where GALT occurs and the high incidence of GALT and tumors in the distal colon may be related to the reported differences in the cell types identified between the proximal and the distal lymphoid nodules of the mouse colon (25).

In a recent report Hardman and Cameron (17) concluded from their research on rats that preexisting lymphoid nodules in the wall of the large bowel of DMH-treated rats are strongly promotional to carcinogenesis in nearby colonic crypt epithelium. The significant overall correlation between the numerical distribution of GALT and overt tumors along the length of the large bowel in mice adds further support to the conclusion that lymphoid nodules (GALT), specifically those in the distal colon, are promotional to the carcinogenic process in nearby colonic crypt epithelium.

Do lymphoid nodules promote carcinogenesis in nearby colonic crypt epithelium in humans? Lymphoid nodules are known to be present in the wall of the large bowel in humans (26-28). Langman and Rowland (29) reported that the density of large bowel lymphoid follicles in humans is 1.5 to 2 times higher in the rectum than in the rest of the large bowel, regardless of age or sex. This lymphoid nodule

Table 2 *Incidence of GALT, ACF, and tumors along the length of the large bowel*

Male CF-1 mice 8 weeks of age were divided into non-DMH-treated and DMH-treated groups, given weekly injections of 12 mg DMH per kg body weight or saline for 8 weeks, and killed for analysis 24 weeks after the last injection. The number and location of 113 GALT along the length of the large bowel were scored in 24 non-DMH-treated mice. The number and location of 535 ACF and 41 tumors (adenomas and adenocarcinomas) along the length of the large bowel were scored in 25 DMH-treated mice. The data are organized in 10-mm segments from the anus to the ileum. No overt tumors were present in the colon between 51 and 120 mm proximal to the anus. No GALT or ACF were observed in the colon between 101 and 120 mm proximal to the anus.

	Colon parameter (mean±SE)		
Segment (mm)	GALT	ACF	Tumor
10	0.792 ± 0.104	0.600 ± 0.191	0.680 ± 0.214
20	1.208 ± 0.134	0.920 ± 0.305	0.440 ± 0.183
30	1.250 ± 0.150	0.840 ± 0.335	0.320 ± 0.095
40	0.958 ± 0.175	0.320 ± 0.150	0.160 ± 0.075
50	0.625 ± 0.101	0.920 ± 0.523	0.040 ± 0.040
60	0.125 ± 0.092	5.720 ± 1.170	0
70	0.042 ± 0.042	8.600 ± 0.983	0
80	0.625 ± 0.168	3.240 ± 1.001	0
90	0.458 ± 0.120	0.200 ± 0.100	0
100	0.208 ± 0.085	0.040 ± 0.040	0
110	0	0	0
120	0	0	0

distribution in humans correlates with the higher incidence of adenocarcinomas and adenomas found in the rectum, as compared to the rest of the large bowel of humans (30-33), and further supports the hypothesis that lymphoid nodules are promotional to carcinogenesis in nearby colonic crypt epithelium. Support for the idea that lymphoid cells promote colon carcinogenesis in humans also stems from the following findings: (*a*) 56% of microscopic adenomas found in colonic mucosa resected from colon cancer patients were located over lymphoid follicles (34); (*b*) in humans, the colonic crypts near lymphoid follicles have cells which were less differentiated than were the cells in those colonic crypts which were further away from the lymphoid follicles (23); and (*c*) inflammation of the large bowel increases risk for colorectal cancer. It has been reported that there is an increased incidence of cancer in persons who: (*a*) have chronic infection of *Schistosoma japonicum* in the large bowel (35); (*b*) have chronic inflammatory disease of the large bowel, such as Crohn's disease (36); and (*c*) have ulcerative colitis (37). Thus, these observations from mice (this report), from humans (26-29), and from rats (17) all support the hypothesis that lymphatic nodules are promotional to large bowel carcinogenesis.

The lack of correlation in the location of ACF and tumors in the large bowel of mice is not intended to convey to the reader that measurement of the numbers, size, and distribution of ACF in mice is of little or no value as a biomarker of colon carcinogenesis. The numbers of ACF found in the large bowel are undoubtedly indicators of exposure to the known colon carcinogens thus far tested (1, 7) and in this regard, ACF can be said to indicate carcinogen exposure. However, because the numbers of ACF do not appear to be a significant predictor of overt tumor formation along the length of the large bowel in the mouse model system, the use of the total number of ACF in the large bowel of the mouse is not necessarily a valid indicator of the overall risk of overt tumor formation. Indeed, the current and a past experimental study from this laboratory (16) could not confirm numbers of ACF as a reliable indicator of cancer risk throughout the large bowel of rats or mice. The results of a study of the formation of ACF in the colon of rats treated with DMH (15) and fed beet fiber also demonstrate a lack of significant correlation between ACF and tumor occurrence in the large bowel. The lack of correlation between ACF and tumor occurrence could be explained in three ways: (*a*) only a few of the ACF represent true premalignant lesions progressing to colon cancer; (*b*) factors associated with the GALT are so promotional to carcinogenesis that the few ACF which form in the region progress to tumors at a much higher rate than elsewhere in the colon; or (*c*) ACF and the overtly detectable tumors represent two parallel and independent events that are initiated by the colon carcinogen (*i.e.*, ACF are seldom if ever premalignant lesions). Thus, use of the total number of ACF per large bowel as the only biomarker of colon cancer risk may fail as a reliable screen for putative cancer preventive procedures.

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