Immune-regulated Ion Transport of Mouse Distal Colon

Russell R. Broadbush, Michael J. Wargovich, and Gilbert A. Castro

INTRODUCTION

Recent molecular biological evidence (1) supports histological evidence (2) indicating an adenoma-to-carcinoma sequence in the development of colon cancer. The accumulation of mutations in oncogenes and deletions and mutations of tumor suppressor genes allows for the progression of hyperproliferative epithelium to adenoma and then to adenocarcinoma. In addition to these genetic events, epigenetic and microenvironmental changes also contribute to the development of neoplasia (3). For colon cancer, these latter changes have been much less intensively studied.

Compelling results suggest that local mucosal immunity is one such microenvironmental factor important in the pathogenesis of colorectal carcinoma. Colon adenocarcinomas often contain an inflammatory infiltrate, typically consisting of T lymphocytes with some macrophages and B lymphocytes (4). The presence of lymphoid infiltration or of lymphoid follicles near resected colon cancers is associated with an increased patient survival rate (5, 6). Levamisole, a nonspecific stimulator of many different arms of the immune system, in combination with 5-fluorouracil significantly improves disease-free survival and overall survival in patients with Stage C resected colon cancer (7). These observations underscore a delicate modulatory role of the immune system, especially the mucosal immune system of the colon, in colonic neoplasia. To enhance an understanding of the interaction between the mucosal immune system and neoplastic tissue, we investigated the effects of experimental colon carcinogenesis on immunity in the colon and small intestine.

Systemic administration of the procarcinogen DMH3 to C57 mice is a well-established laboratory model of colon carcinogenesis. DMH selectively induces colonic adenomas and adenocarcinomas (8, 9) while only rarely causing liver or small intestine tumors (10). Administration of DMH causes a continuum of morphological changes from normal colonic epithelium to carcinoma. One injection of DMH causes the development of aberrant crypts within 2 weeks (11), while 6 injections of DMH, 1 per week for 6 weeks, typically causes grossly visible adenomas and carcinomas of the colon within 4–6 months (8, 9). Within the same time frame as the histological development of aberrant crypts, there is electrophysiological evidence of inhibition of the Na+-K+-ATPase (12) and stimulation of Na+-H+ exchange (13) in the distal colon. These changes in ion transport are important, because Na+ influx into the cell (14) and increased intracellular pH (15) are early events in mitogenesis and may, at least partially, be the basis for the hyperproliferation observed in aberrant crypts (16).

The hyperploriferative lesions, aberrant crypts, adenomas, and adenocarcinomas of the colon induced by DMH are biologically and histologically quite similar to those seen in humans (17). Importantly, there is pathological evidence that DMH-induced colon tumors (18) resemble human tumors (2) in that both follow an adenoma-to-carcinoma sequence. Because of the potential progression of early changes to malignancy, the study of the premalignant hyperproliferative lesions and aberrant crypts is crucial in understanding the pathogenesis of colon cancer.

Immunity in the small intestine and colon of DMH-treated mice was measured by quantifying a vital physiological function, ion transport, that is regulated by the mucosal immune system (19). Immune-regulated intestinal ion transport has been described for a variety of host-antigen systems. Antigenic challenge of intestinal segments from

1 This work was supported by NIH Grant PO1-DK37260 and the University of Texas-Houston Health Science Center Mucosal Biology Program.

2 To whom requests for reprints should be addressed, at Department of Physiology and Cell Biology, University of Texas Medical School, P.O. Box 20708, Houston, TX 77225.

3 The abbreviations used are: DMH, 1,2-dimethylyhydrazine; PGE2, prostaglandin E2; 5-HT, 5-hydroxytryptamine; Isc short-circuit current; KRB buffer, Krebs-Ringer bicarbonate buffer; TGF-β, transforming growth factor β; cAMP, cyclic AMP; cGMP, cyclic GMP.
laboratory rodents sensitized to dietary proteins (20, 21) or parasites (22, 23) results in an immediate (type I) hypersensitivity reaction, expressed as net epithelial anion secretion. In Ussing chambers, net anion secretion is manifested electrophysiologically as a rise in transmural I_{sc}. Applying similar principles, we have recently described a method to quantify colonic mucosal immune function in situ (24). Briefly, segments of colon from mice immunized by infection with the intestinal nematode parasite *Trichinella spiralis* undergo an increase in I_{sc} (ΔI_{sc}) after challenge with *T. spiralis*-derived antigen in Ussing chambers. The antigen-induced ΔI_{sc} is inhibited by furosemide, indicating that the ΔI_{sc} is most likely due to net Cl⁻ secretion by the colon. *T. spiralis* antigen bridges IgE bound to mucosal mast cells, triggering the release of anaphylactic mediators that stimulate epithelial ion secretion. Ion secretion and the resulting colonic ΔI_{sc} therefore serve as sensitive indicators of local immune function. In this study, antigen-stimulated changes in colonic I_{sc} in DMH-treated mice were used as a physiological correlate of mucosal immunity to investigate the impact of early stages of colon carcinogenesis on the local immune system.

**MATERIALS AND METHODS**

**Animals**

Male CF-1 mice (Harlan Sprague-Dawley, Houston, TX), 22–24 g initial body weight, were used as experimental hosts. The mice were housed in cages with standard bedding and were given a laboratory pelleted formula diet and tap water ad libitum. On the day of carcinogen administration, DMH was dissolved in 1 mM sodium EDTA and the pH was adjusted to 6.5 with 1 N NaOH. Mice were given i.p. injections of 20 mg/kg of DMH, housed in microisolators (Lab Products Inc., Maywood, NJ) under a chemical fume hood for 6 h, and then returned to standard cages in the Animal Care Facility.

Mice were immunized by infection with 400 muscle stage larvae of *T. spiralis*. The number of intestinal worms established following a primary or challenge infection with *T. spiralis* was quantified according to established methods (25). Infective larvae were collected from the skeletal muscle of parasitized mice according to the method of Castro and Fairbairn (26). *T. spiralis* antigen was prepared from infective larvae (27), analyzed for protein (28), and stored in aliquots at -20°C.

**Ion Transport Measurements**

Antigen-induced and secretagogue-induced ion transport in the jejunum and colon were measured in Ussing chambers (23) 8 weeks after mice were given the primary *T. spiralis* infection. Mice were killed by cervical dislocation, and the small intestine and colon were surgically removed and rinsed free of luminal contents with KRB buffer (pH 7.4) as described previously (23). The jejunum and colon were slit longitudinally, placed in ice-cold KRB buffer, and gassed with a mixture of 95% O₂-5% CO₂ until the tissues were mounted in Ussing chambers. The colon was divided into four segments, designated near-proximal, mid-proximal, mid-distal, and far-distal, beginning at the cecum and extending distally to the anus. Jejunal segments and the four colonic segments were mounted as full-thickness, flat sheets between two Ussing half-chambers with an aperture of 0.516 cm². Each segment was bathed on the mucosal and serosal surfaces with 10 ml KRB buffer gassed with 95% O₂-5% CO₂ and maintained at 37°C. Colonic and jejunal segments were voltage clamped at zero transmural potential difference using a VCC-600 voltage current clamp (Physiologic Instruments, San Diego, CA). A continuous record of I_{sc} with respect to time was recorded on a BD-41 Kipp Zonen recorder (Delft, the Netherlands).

Segments of intestine were allowed to stabilize for 30 min after mounting in the chambers to obtain a steady-state I_{sc}. The basal I_{sc} is an index of net active ion transport across the serosal and mucosal surfaces. The tissues were stimulated with antigen or secretagogues added to the serosal bathing fluid. *T. spiralis* antigen was added at a concentration of 50 µg protein/ml of Ussing chamber fluid. PGE₂ and 5-HT were added at final chamber concentrations of 10⁻⁵ M and 10⁻⁴ M, respectively. After addition of antigen or secretagogue, the maximum change in I_{sc} (ΔI_{sc}) from the basal state was calculated and expressed as µA/cm².

**Histology**

Colonic segments from tumor-bearing mice were slit longitudinally and rinsed free of luminal contents with ice-cold KRB buffer. The segments were fixed in 10% buffered formalin, embedded in paraffin, and cut into 5-µm sections. Sections were stained with hematoxylin and eosin before examination by light microscopy.

**Experimental Design**

**Ussing Chamber Studies.** To investigate the effects of early stages of DMH-induced colon carcinogenesis on the development of mucosal immune responsiveness, 6 weekly injections of DMH were superimposed on a primary *T. spiralis* infection. Group 1 mice were infected with *T. spiralis* 1 week after the first DMH injection and then given the remaining 5 weekly DMH injections. I_{sc} responses to *T. spiralis* antigen and secretagogues were then examined 8 weeks after the primary infection (4 weeks after the sixth injection of DMH). Infected mice given 6 weekly injections of EDTA carrier solvent served as immune controls. To determine the effects of early stages of DMH-induced colon carcinogenesis on an established mucosal immune response, group 2 mice were given a primary *T. spiralis* infection, allowed 8 weeks to develop full immunity, and then given 6 weekly injections of DMH. I_{sc} responses to stimulation with *T. spiralis* antigen and secretagogues were then examined 6 weeks after the sixth injection of DMH. Immune mice receiving 6 weekly injections of EDTA served as controls.

**Worm Rejection Studies.** To determine the effects of early stages of DMH-induced colon carcinogenesis on functional immunity, worm rejection from the small intestine was measured. Groups of mice were immunized with a primary *T. spiralis* infection of 400 larvae after the second (group 3) or sixth (group 4) DMH injection. The effects of one injection of DMH were examined by administering DMH either 1 day before the primary infection (group 5) or 1 day before the secondary challenge infection (group 6). For all 4 groups, a challenge infection of 400 larvae was given 30 days after the primary infection. The number of worms that established in the small intestine was determined 2 days after the challenge infection (25).

**Materials**

PGE₂ and 5-HT were purchased from Sigma (St. Louis, MO). DMH was purchased from Aldrich (Milwaukee, WI). PGE₂ was solubilized in ethanol and 5-HT was solubilized in H₂O.

**Statistics**

Student’s *t* test was used to compare the means of two experimental groups (29). When more than two means were compared, a single analysis of variance was used, and the Duncan test was used to analyze multiple comparisons between the means (29). The results are expressed as mean ± SE. *P* = 0.05 or less was considered significant.
RESULTS

Segments of mid-distal and far-distal colon from group 1 mice (6 weekly DMH injections superimposed on a primary *T. spiralis* infection) demonstrated decreased responsiveness to *T. spiralis* antigen (Fig. 1A). However, DMH treatment did not significantly affect antigen-induced $I_{sc}$ responses in the proximal colon or jejunum (Fig. 1, B and C). Collectively, these results suggest that early stages of DMH-induced colon carcinogenesis suppress mast cell-dependent immune reactions of the distal colon but not of the proximal colon or jejunum.

Inhibition of antigen-induced $\Delta I_{sc}$ in the distal colon could be due to either a breakdown in local mucosal immune reactions or the inability of the colonic epithelium to secrete anions after receiving signals generated by immune cells. The basal $I_{sc}$ of the distal colon from *T. spiralis*-infected mice (19.1 ± 3.8 $\mu$A/cm², $n = 7$) was not significantly different from the basal $I_{sc}$ of group 1 DMH-treated mice (27.1 ± 4.8 $\mu$A/cm², $n = 6$). Therefore, the sensitivity of the epithelium to physiological stimulation was determined by measuring the $\Delta I_{sc}$ evoked by the addition of exogenous PGE$_2$ and 5-HT, which are both mast cell-derived mediators and epithelial Cl$^-$ secretagogues. These secretagogues were chosen because we have previously shown that PGE$_2$ and 5-HT are important mediators of the colonic $\Delta I_{sc}$ in response to *T. spiralis* antigen stimulation (24). DMH treatment had no significant effect on epithelial responsiveness to either PGE$_2$ or 5-HT (Fig. 2), suggesting that the epithelium retains the ability to respond to immune agonists.

The epithelial $I_{sc}$ responses to PGE$_2$ and 5-HT indicate that the decreased antigen-induced $\Delta I_{sc}$ observed in the distal colon is secondary to a defect in the immune processes that contribute to the formation of a local type 1 hypersensitivity reaction. Such processes include antigen presentation by macrophages, B cell production of IgE, T cell secretion of cytokines, and mast cell release of chemical mediators. Because in group 1 the DMH injections were occurring as the host was mounting a primary immune response to *T. spiralis*, this defect could involve any of these components necessary for the expression of type I hypersensitivity. To facilitate the identification of the immune defect, group 2 mice were immunized with a primary *T. spiralis* infection and then 8 weeks postinfection given 6 weekly injections of DMH. With this protocol antigen presentation, T cell and B cell activation, and IgE production have all occurred before the onset of DMH injections. The group 2 protocol therefore examines the effect of DMH treatment on mast cell communication with the colonic epithelium.

Distal and mid-proximal colon $I_{sc}$ responses to *T. spiralis* antigen were decreased in group 2 mice (Fig. 3, A and B). However, antigen-induced $\Delta I_{sc}$ in the near-proximal colonic segment and the jejunum was unaffected by DMH treatment (Fig. 3, B and C). Therefore, as in group 1 mouse, early stages of DMH-induced colon carcinogenesis resulted in suppressed antigen-stimulated $I_{sc}$ responses in the more distal portions of the colon, but antigen responsiveness in the proximal colon and jejunum remained intact. As in group 1, the colonic basal $I_{sc}$ and the $\Delta I_{sc}$ elicited by exogenous Cl$^-$ secretagogues were not inhibited by DMH (results not shown). These observations suggest that the defect in antigen responsiveness in the distal colon is in mast cell functioning or in the transductive pathway connecting the mast cell-derived signals to the colonic epithelium.

A group of DMH-treated mice was examined for tumors 30 weeks after the final dose of DMH (Fig. 4). Normal colonic mucosa is arranged in orderly crypts containing differentiated goblet cells packed with mucin (Fig. 4A). Adenomas were characterized by crypts with an increased number of mitotic figures and a decreased number...
of mucin-containing goblet cells. Some adenomas were severely dysplastic, containing nests of anaplastic cells without the normal crypt architecture (Fig. 4B). Adenomas developed only in the distal portions of the colon of DMH-treated mice; no tumors developed in the proximal colon or small intestine. Therefore, the suppression of antigen-induced responsiveness in the distal portions of the colon, as observed in group 1 and 2 mice, occurred only in the distal colon, the portion of the colon that eventually developed adenomas.

A possible carcinogenic mechanism of DMH is methylation of DNA (30). Maximum DNA damage in the colon occurs 6–12 h after one injection of DMH, with repair occurring over the next 72 h (31). To determine if one acute exposure to DMH is sufficient to inhibit responsiveness to antigen, mice were infected with T. spiralis and challenged in Ussing chambers with T. spiralis antigen. Columns, mean; bars, SE; n, number at base of column. Asterisk, significant difference (P < 0.05) as compared with respective immune control.

There are several lines of evidence that the inhibited antigen-induced immune competencies in the distal colon were not due to systemic immunosuppression by the carcinogen: (a) antigen-stimulated immune responsiveness in the more proximal regions of the colon (Figs. 1B and 3B) and of the jejunum (Figs. 1C and 3C) were not decreased by DMH treatment; (b) a functional immune response, worm rejection from the small intestine, was not affected by DMH (Table 1). Expulsion of primary and secondary T. spiralis infections is delayed in other experimental models of immunosuppression, such as athymic nude mice (33), athymic nude mice (34), W/Wv mast cell-deficient mice (35), mice exposed to sublethal irradiation (36), and mice treated with cortisone (37). Furthermore, in studies of intestinal immune-regulated ion transport, antigen-induced immune competencies in the distal colon are absent in athymic nude mice (38) and 70% reduced in mast cell-deficient W/Wv mice (39). Intact immune responses in the proximal colon and jejunum and intact immune expulsion of a secondary infection all suggest that the suppressive effects of DMH-induced carcinogenesis on mucosal immunity are not generalized but instead are localized to the distal colon.

The dichotomy between the distal colon and the proximal colon regarding antigen responsiveness is also observed in other biological aspects of both DMH-induced and human colon tumors. For example, DMH treatment causes tumors predominantly in the distal, rather than the proximal, colon (17). In the present study, DMH-treated mice allowed to survive for 30 weeks developed adenomas only in the distal portions of the colon (Fig. 5); no tumors were observed in the proximal colon. Furthermore, the tumors induced in the distal colon are histologically different than those in the proximal colon (40). Following injection of DMH there is inhibition of the Na+-K+-ATPase (12) and stimulation of Na+-H+ exchange (13) in the distal colon. Despite these changes in membrane transport, the epithelium of the distal colon maintained normal responsiveness to exogenous Cl- secretagogues (Fig. 2), suggesting that carcinogenesis in the distal colon disrupted mast cell communication with a normally secreting epithelium.

Human colon carcinomas also exhibit a proximal-distal dichotomy. Allelic deletions of chromosomes 17p, 18, and 5q are all more frequent in distal tumors than in proximal tumors (41, 42). Increased
Colon carcinogenesis suppresses mucosal immune function

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Experimental rodent studies of colorectal carcinoma suggest that there is a biological difference between proximally and distally located tumors. Furthermore, the initiation and progression of proximal and distal colon cancers may involve different genetic mechanisms. It is possible that the differences in the biology and genetic mechanisms of distal versus proximal colon tumors are the basis for the suppression of antigen-induced ion transport in the distal colon.

One exposure to DMH, which is known to be sufficient to cause DNA methylation (30) and subsequent DNA repair (31), is not associated with suppression of antigen responsiveness in the distal colon (Fig. 5). However, decreased antigen-induced \( \Delta I_{sc} \) responses in the distal colon (Figs. 1A and 3A) are temporally associated with previous reports of Na\(^+\)-K\(^+\)-ATPase inhibition (12), Na\(^+\)-H\(^+\) exchange stimulation (13), and the histological appearance of aberrant crypts (11) in DMH-treated mice. Furthermore, the observed suppression of colonic mucosal immune function (Figs. 1 and 3), the changes in epithelial membrane transport (12, 13), and the development of aberrant crypts (45) all preferentially occur in the distal, rather than the proximal, colon. Altered Na\(^+\)-K\(^+\)-ATPase (14) and Na\(^+\)-H\(^+\) exchange (15) provide signals for increased cell proliferation, which is suggested to be necessary for the formation of aberrant crypts (16). It is possible that suppressed mucosal immunity is also a component of the progression

Fig. 4. Histological sections of distal colon from control (A) and DMH-treated (B) mice. DMH treatment causes an increase in mucosal thickness and colon circumference due to crypt hyperplasia. A, normal colonic mucosa with differentiated, mucin-containing goblet cells in characteristic crypts; B, section through an adenoma, 30 weeks after the final dose of DMH. The tumor contains prominent cysts, crypts with goblet cells, less-differentiated crypts lacking goblet cells, and nests of anaplastic cells. Adenomas were not observed in the proximal colon of DMH-treated mice. H & E, \( \times \) 200.

Fig. 5. Effect of 1 DMH injection on antigen-induced \( \Delta I_{sc} \) in the distal colon. Mice were immunized by infection with T. spiralis and 8 weeks later given one injection of DMH or EDTA carrier solvent. Segments of distal colon were challenged in Ussing chambers with T. spiralis antigen 6-12 h after the DMH/EDTA injection. Columns, mean; bars, SE; n, number at base of column.

c-myc expression is correlated with cancers in the distal colon (43). Somatic instability at microsatellites, which consist of (CA)\(_n\) repeats, on chromosomes 5q, 15q, 17p, and 18q occurs more frequently in tumors of the proximal colon (44). Collectively, the human and experimental rodent studies of colorectal carcinoma suggest that there is a biological difference between proximally and distally located tumors. Furthermore, the initiation and progression of proximal and distal colon cancers may involve different genetic mechanisms. It is possible that the differences in the biology and genetic mechanisms of distal versus proximal colon tumors are the basis for the suppression of antigen-induced ion transport in the distal colon.

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from normal colonic epithelium to the development of aberrant crypts. These results do not imply that type I hypersensitivity per se is important in the host defense against colonic neoplasia. Rather, this report demonstrates that early stages of colon carcinogenesis are capable of suppressing local immune function, which in the present study is dependent upon a type I hypersensitivity reaction against T. spiralis. The mechanisms by which this is accomplished could prove to be important in explaining how developing colonic neoplasms evade host antitumor immune responses.

The inhibition of antigen-induced ion transport in the distal colon of mice during early stages of tumorogenesis (Fig. 3A) is likely due to a defect in mast cell function or in the transductive pathway connecting the mast cell to the colonic epithelium. Various changes in tissue biochemistry could affect mast cell function. Mast cell phenotype and function are, at least in part, determined by the microenvironment or tissue factors present in the immediate vicinity of the mast cell (46). PGE₂ levels are elevated both within colon tumors and within normal appearing colonic mucosa from carcinogen-treated rats (47). In samples of human colon carcinoma, tissue-fixed macrophages secrete excessive amounts of PGE₂ (48), and TGF-β is overexpressed in epithelial cells throughout the crypts (49). Prostaglandins and TGF-β inhibit mediator release from mast cells (50, 51); therefore it is possible that carcinogenesis results in elevated levels of colonic prostaglandins and TGF-β, which in turn stabilize mast cells to prevent mediator release. Additionally, DMH-induced carcinogenesis decreases tissue levels of cAMP (52) and protein kinase C (53) and increases levels of cGMP (52). Protein kinase C and cAMP are important second messengers in mast cell function (54), and altered levels may lead to mast cell stabilization, constant degranulation and mediator depletion, or down-regulation of the high affinity surface receptors for IgE. These possibilities, under current investigation in our laboratory, are summarized in Fig. 6. The mechanism by which DMH changes levels of these important regulatory factors is not known. DMH may act directly by inducing mutations of genes that control the synthesis of these factors. Alternatively, the carcinogen may act indirectly by altering the production of other regulatory molecules that in turn modulate the actions and synthesis of prostaglandins, TGF-β, cAMP, cGMP, and protein kinase C. It is important to note that prostaglandins (55) and TGF-β (54) are potent inhibitors of many different immune reactions and that protein kinase C, cAMP, and cGMP are second messengers for many different cell types. Therefore, the results of such future studies should be relevant to other aspects of mucosal immune responsiveness in addition to type I hypersensitivity.

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Early Stages of 1,2-Dimethylhydrazine-induced Colon Carcinogenesis Suppress Immune-regulated Ion Transport of Mouse Distal Colon

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