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Histochemical Study of Colonic Cancer in Experimental Colitis of Rats

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A reliable test for premalignant lesions in the development of colonic cancer in chronic ulcerative colitis has been needed. Thus, we designed this cytochemical study, using a model of experimental colitis and colonic tumors induced in Wistar male rats by the feeding of dextran sulfate sodium. The colitis had histologic similarities to ulcerative colitis in man. The percent frequency of polypoid lesions (dysplasia or dysplasia with carcinoma in situ*) in the cecum and ascending colon was about 25% at three months and 90% at six months of dextran sulfate feeding. The cytochemical findings by high-iron diamine–Alcian blue staining and* Ulex europeus *agglutinin binding were chronologically paralleled by histological changes in the colonic mucosa, and the binding pattern of peanut agglutinin was not different between normal and dextran-treated animals. Moreover, some cytochemical changes that occurred during the inflammatory responses were not present in dysplastic or malignant lesions. Thus, the histochemical tests were not useful for detecting of premalignant lesions earlier than by conventional histology. Nevertheless, the dextran sulfate model of colitis in the rat appears suitable for study of cancer development in ulcerative colitis.*

KEY WORDS: ulcerative colitis; dysplasia; colon cancer; high-iron diamine–Alcian blue staining; *Ulex europeus* agglutinin; dextran sulfate sodium.

The risk of developing colonic cancer in patients with ulcerative colitis (UC) is high (1–4). The cancers may be preceded by the development of epithelial dysplasia, so rectal or colonic biopsies for identification of dysplasia often are performed (2, 5–9). The histologic criteria of dysplasia, however, are subjective, and earlier, more reliable markers of premalignancy would be useful. Experimental colitis may be valuable in the investigation of the relationship between inflammation and dysplasia and/or cancer in UC. Among several agents that have been used for the induction of experimental colitis or tumors in animals (10–16), carrageenan or dextran sulfate are remarkably effective. For example, colonic epithelial dysplasia in association with chronic inflammation has developed in rabbits treated with degraded carrageenan (10, 11), and the feeding of dextran sulfate sodium (DSS) has induced colitis and colonic tumors in rats (12–14), and colitis or epithelial dysplasia in Syrian hamsters and mice (15, 16).

Recently, histochemical studies to identify premalignant colonic epithelium have been described (17–19). In one report (19), *Ulex europeus* agglutinin (UEA) binding was found to be a consistent feature of premalignant colonic mucosa in dimethylhydrazine-treated rats. In this study, we applied histochemical methods to try to identify premalig-

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nant colonic lesions in a chronic ulcerative colitis associated with developing carcinoma in rats given DSS in their drinking water.

MATERIALS AND METHODS

Chemicals. DSS (mol wt 54,000), provided by Meito Sangyo Co., Ltd., Nagoya, Japan, was dissolved in distilled water at a concentration of 2% (w/v).

Animals. Male Wistar rats, weighing 100–120 g, were used. An experimental group was fed standard meal (Oriental East Co. Ltd., Tokyo, Japan) with drinking water containing 2% (w/v) DSS until sacrificed. A control group was fed the same diet but with drinking water lacking DSS. Eight animals each were sacrificed at two weeks and at one, two, three, and four months after starting the experiments, and 12 rats were sacrificed at six months.

Histological Techniques. After the rats were killed, the large intestine was rapidly removed, opened longitudinally, and observed macroscopically. Specimens from the cecum, ascending colon, descending colon, and rectum were fixed in Bouin solution (75 ml saturated aqueous picric acid, 25 ml formalin, 5 ml glacial acetic acid) and embedded in paraffin. The stomach, small intestine, liver, pancreas, and spleen of each rat also were removed and embedded. The sections were cut and deparaffinized by treatment with xylene, then 100% ethanol, then water. For routine histologic examination, the slides were stained with hematoxylin–eosin. The colonic tumors were classified histologically according to Rosengren's criteria (20). Periodic acid–Schiff (PAS) staining was used to identify neutral mucin, and high-iron diamine–Alcian blue (HID-AB) staining (21) was used to identify sialomucins and sulfomucins.

Reactivity of the tissues with fluoresceinated peanut agglutinin (FITC-PNA) and fluoresceinated *Ulex europeus* agglutinin (FITC-UEA) was evaluated as follows. Tissue sections were deparaffinized and covered with FITC-PNA or FITC-UEA (Vector Laboratories, Inc., Burlingame, California) at a concentration of 50 μg/ml in 10 mM HEPES, 50 mM NaCl, 1 mM $MgCl_2$, and 0.04% sodium azide, pH 7.5 (HEPES buffer), for 30 min in the dark at room temperature. For specificity control, FITC-PNA solution incubated with 300 mM D-galactose (Sigma) and FITC-UEA incubated with 300 mM L-fucose (Sigma) in HEPES buffer were used. The slides were washed in phosphate-buffered saline, mounted in 90% glycerol/10% phosphate-buffered saline, and observed and photographed with a Nikon fluorescence microscope.

RESULTS

Gross Appearances of Colonic Mucosa

Within three days after beginning the ingestion of DSS, all animals passed soft mucus and bloody stools. After two weeks of DSS, the colonic mucosa appeared edematous, and multiple erosions were scattered along the entire colon. These changes were more marked in the cecum and the ascending colon than in the descending colon or the rectum. Small elevated lesions (about 2–4 mm diameter) were observed on the edematous mucosa of the cecum in two of eight rats after three months (Table 1). After four and six months, the wall of the entire colon became thick, and the colon was shortened. Tumors were observed in three of eight animals sacrificed at four months and in 12 of 13 rats at six months. The largest polyp was about 20 mm in diameter and 10 mm in height. Most of the animals had solitary polyps, but two of 13 rats after six months had multiple tumors in the cecum and the ascending colon. One of these two animals had four polypoid tumors in the cecum and the ascending colon and one polyp in the rectum. The stomach, small intestine, pancreas, liver, and spleen had no macroscopic or microscopic changes at any time point.

TABLE 1. TIME, NUMBER OF RATS AFTER START OF DSS AND INCIDENCE OF TUMORS

Months after start of DSS	*No. of animals sacrificed*	*No. of rats with tumors*	
		Adenoma	*Adenocarcinoma*
1	8	0	0
2	8	0	0
3	8	2	0
4	8	0	3
6	13	1	11

Histology

Control animals had neither inflammatory changes nor loss of colonic surface epithelium at any time. After two weeks to one month of DSS treatment, the colonic mucosa had various degrees of inflammatory cells, including lymphocytes, plasma cells, macrophages, and polymorphonuclear leukocytes. Loss of surface epithelium and diminished numbers of glands also were evident (Figure 1A). There was no distinctive pattern of infiltrate. The mucosal height was not increased. After three months of DSS treatment, more infiltrates and focal loss of surface epithelium were present, and regenerated crypts were elongated and dilated (Figure 1B). Crypt abscesses also were focally present in the damaged glands. At this time, two polypoid lesions in eight rats were classified as the mild to moderate grade dysplasia (Figure 2A). Tubular irregularity was common, and the nuclei lost polarity and were hyperchromatic and pseudostratified. Dysplastic glands were seen also in the entirely flat mucosa, in which the crypts were tortuous but not enlarged (Figure 2B).

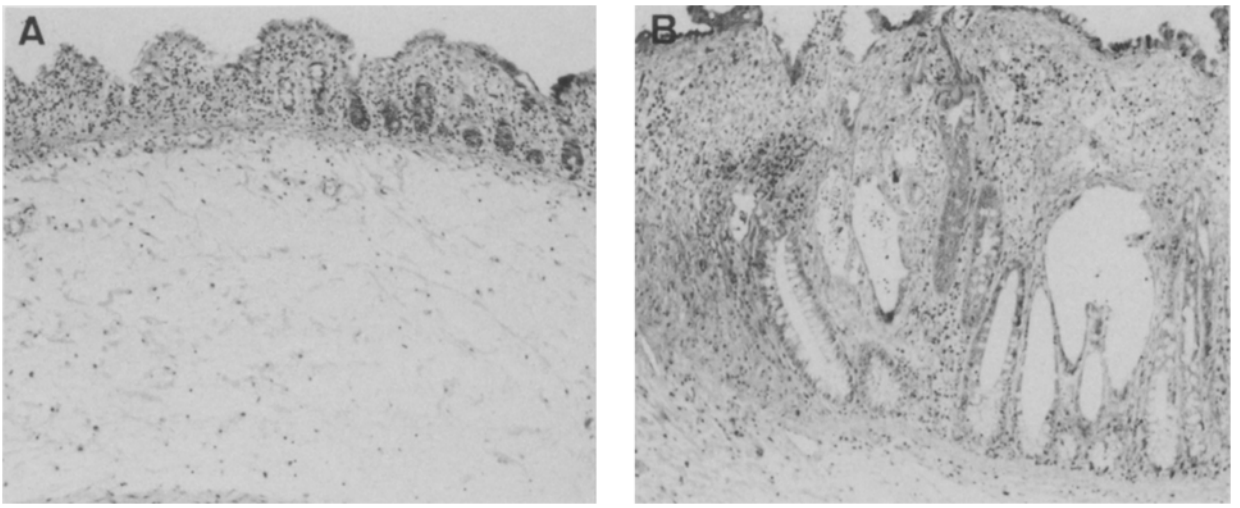

Fig 1. (A) Histopathology of the cecum of a rat after one month of DSS (×100). Erosion, loss of glands, and cellular infiltrates are present. (B) Histopathology after three months (×100). Marked cellular infiltrates, loss of glands, and dilated, distorted glands with flattened epithelium are present.

The gland lumens were dilated, and the epithelial cell nuclei were larger and hyperchromatic. The goblet cells contained little mucin. By six months, both the numbers of macroscopic lesions and the severity of the histopathological lesions increased. More cellular infiltrates and damaged epithelia were seen. At this time, glands were more elongated and distorted than at three months. The polypoid lesions were larger, and all of three polyps at four months and 11 of 12 polyps at six months were dysplastic with adenocarcinoma *in situ*. The dysplastic glands had various degrees of atypia, varying from slight, moderate, and pronounced up to pronounced atypia with suspected signs of malignancy. Adenocarcinoma *in situ* showed pronounced epithelial and intraglandular atypia (cribrous formation and buddings), but no signs of infiltration to deeper layers (Figure 3A and B).

Histochemistry

Periodic Acid–Schiff. In control rats, the droplets of goblet cells and the luminal surface mucin were stained; there was no difference in staining intensity or patterns between the right and left colon. In DSS-treated rats, there was no dramatic change in

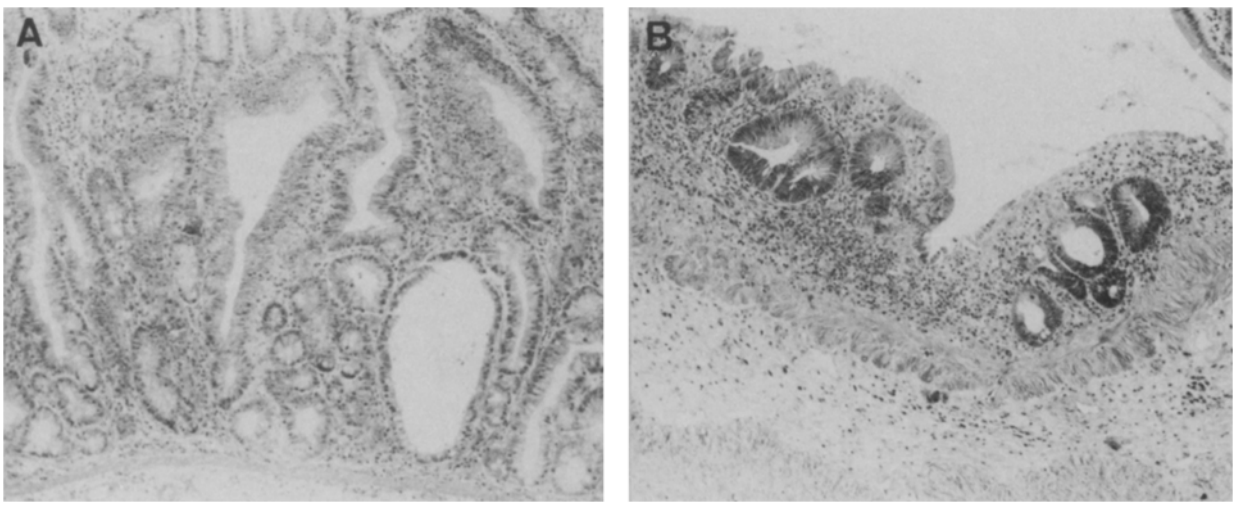

Fig 2. (A) slightly elevated polypoid area of the cecum of a rat treated with DSS for three months (×100). The lesion is considered dysplastic, with distorted glands lined with hyperchromatic and pseudostratified epithelial cells. (B) An entirely flat area of mucosa of the cecum in a rat treated with DSS for three months. Again, the glands are dysplastic (×100).

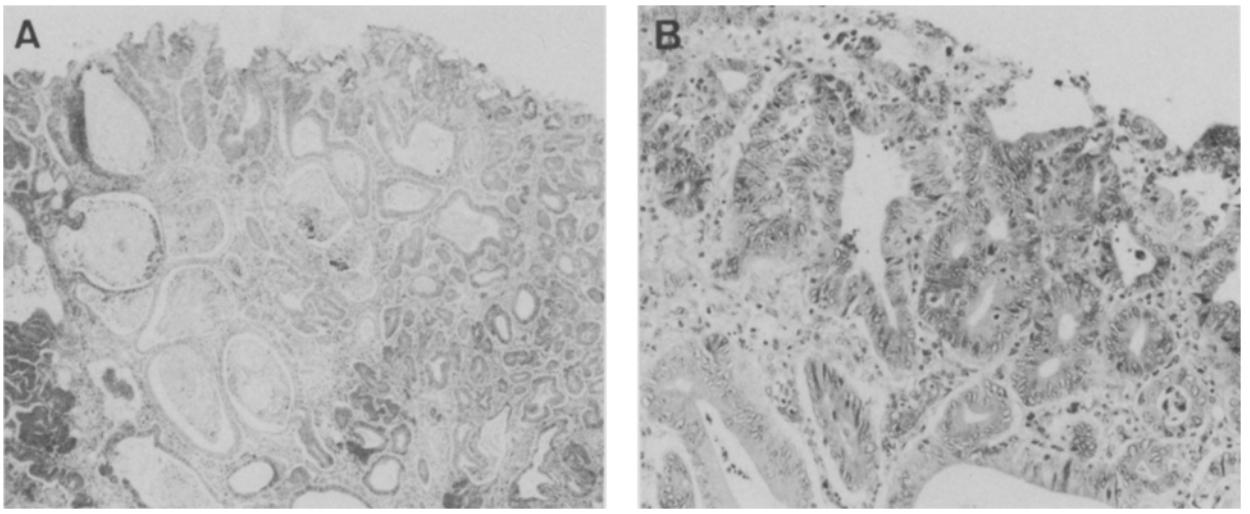

Fig 3. A polypoid lesion in the cecum of a rat after six months of DSS treatment. (A) Histopathology reveals dyplasia with carcinoma *in situ* (×20). (B) Higher magnification of A. Carcinoma *in situ* (×100).

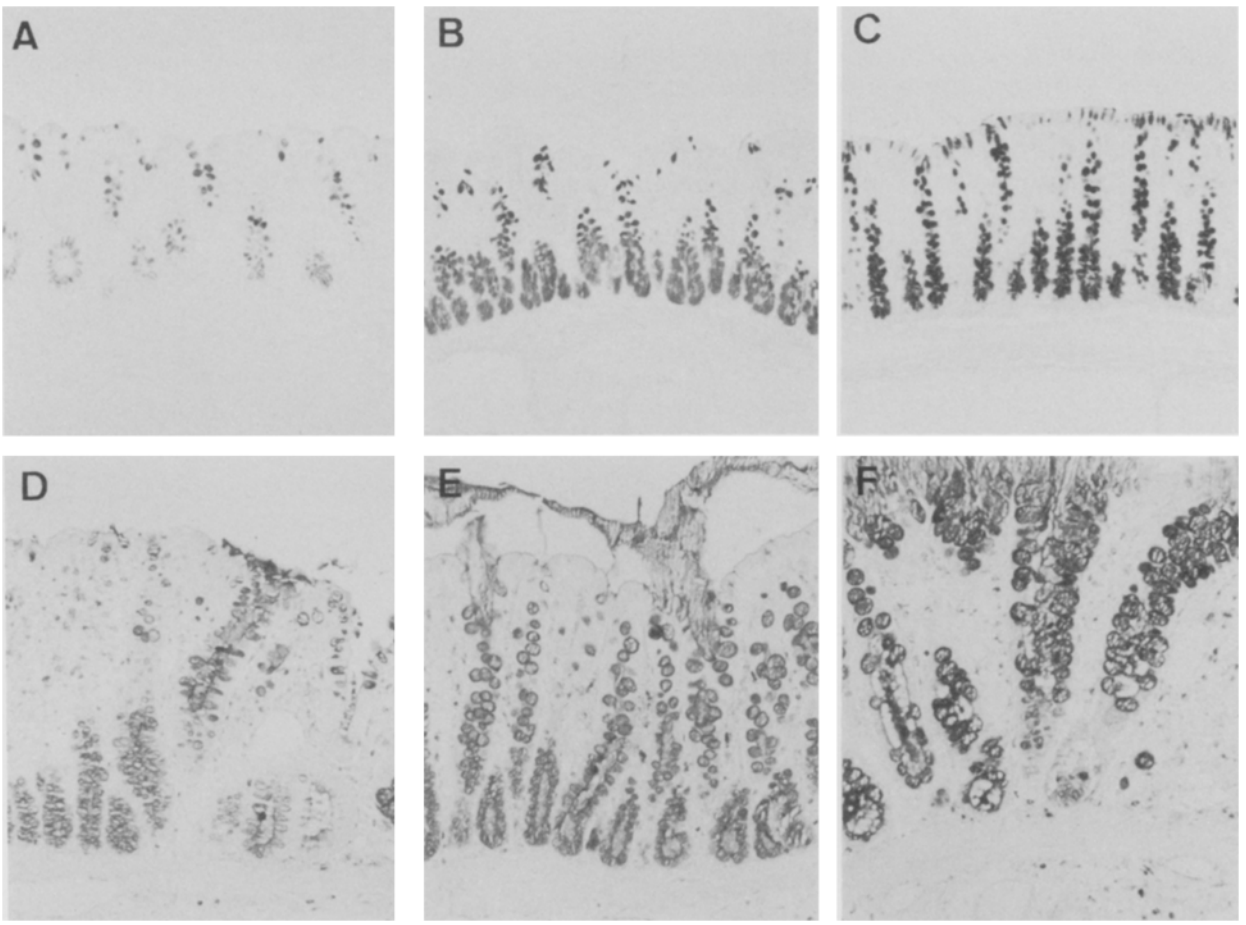

Fig 4. HID-AB staining of colonic mucosa (×100). A–C: control rat; D–F: after three months of DSS treatment. (A) Cecum—sulfomucin is present in the upper three fourths and sialomucin in the base of glands. (B) Ascending colon—sialomucin is present in the lower half and sulfomucin in the upper half of the crypts. (C) Descending colon—sulfated mucin is present nearly throughout the gland. (D) Cecum—glandular mucin is nearly entirely sialylated. (E) Ascending colon—again, mucin is sialylated. (F) Descending colon—glands are composed of the cells containing both kinds of mucin.

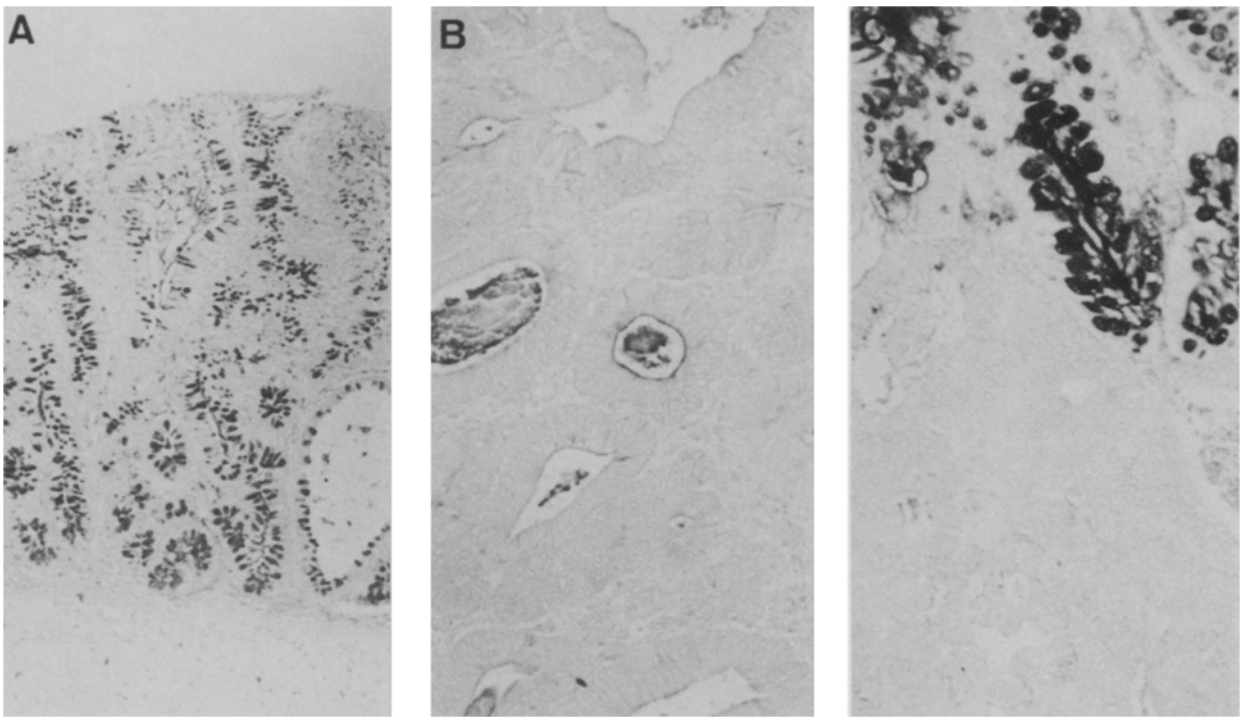

Fig 5. HID-AB staining. (A) Dysplasia in cecum after three months of DSS treatment (×100). Goblet cell droplets contain mainly sulfated mucins. (B) Dysplasia with carcinoma *in situ* in cecum after six months of DSS treatment (×200). Absence of stained mucus. (C) Dysplasia with carcinoma *in situ* in rectum after six months of DSS treatment (×200). The malignant cells are not stained.

PAS staining; mucin was consistently positive in the inflamed mucosa as well as dysplastic mucosa. Cancer cells lacking mucin, however, were not stained by PAS.

High-Iron Diamine–Alcian Blue. In the normal colon, the HID-AB staining patterns of the epithelial mucins by HID-AB differed in various regions (Figure 4A–C). In the cecum, the upper three fourths of the crypts contained sulfated mucin; sialomucin was present only at the crypt base. In the proximal colon, sialomucin predominated in the lower half of the crypts, whereas the upper half contained sulfomucins. In the distal colon, sulfomucin was present throughout the glands. After three to six months of DSS feeding, sialylated mucin increased in the entire colon (Figure 4D–F). The cecum and the ascending colon contained a predominance of sialylated mucin, with only scattered positive staining of sulfated mucin in the upper regions of crypts of the ascending colon. The descending colon had much greater increase of sialylated mucin, and the glands were composed of the cells containing both kinds of mucin. Regenerated glands were predominantly sialylated. In the dysplastic mucosa, increased numbers of mucus droplets contained mostly sulfated mucin, and only the crypt base was faintly positive with sialylated mucin (Figure 5A). Cancer cells lacking mucin were not stained by HID-AB (Figure 5B and C), although mucin on the surface and in the lumen was faintly stained. Goblet cells around the margins of dysplastic areas or malignant tumors contained mainly sialylated mucins, and the mucin in cells adjacent to tumors was also mainly sialylated.

Lectin Binding. In normal rats, the binding sites of *Ulex europeus* agglutinin differed between the cecum and proximal colon compared to the distal colon (Figure 6A–C); in the cecum and proximal colon, UEA-positive cells, in which the mucin was stained, were localized in the lower half of the crypts, especially the crypt base. None of the cells in the distal colon was stained. In DSS-treated rats, in the cecum and proximal colon (Figure 6D and E), UEA-positive cells were present not only in the crypt bases but also in the upper part of the glands. In the distal colon (Figure 6F), positive cells were present also from the surface to the bottom of the regenerated glands, but epithelial cells in glands that appeared regularly aligned and unaffected were not stained. In the dysplastic mucosa, the glands

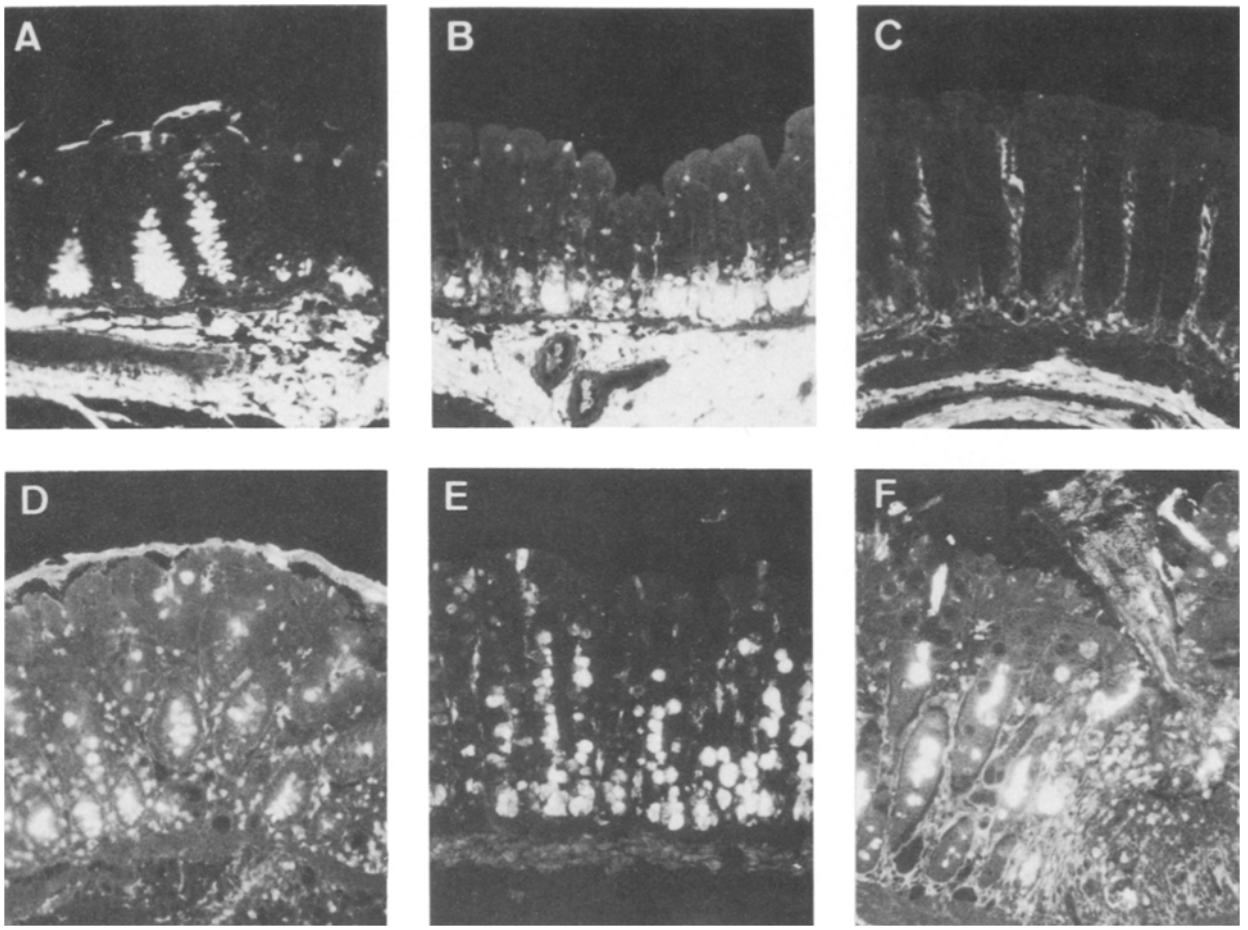

Fig 6. FITC-UEA staining of rat colon (×130). A–C: Control rat; D–F: after three months of DSS treatment. (A) Cecum. (B) Ascending colon. (C) Descending colon. (D) Cecum—stained crypts. (E) Ascending colon—stained crypts. (F) Descending colon—stained regenerated glands.

were mainly composed of mucus-containing cells, and mucin was strongly stained with FITC-UEA (Figure 7A). On the other hand, the adenocarcinomas *in situ* were composed mostly of mucus-lacking cells, which were stained variably (Figure 7B and C); in the positive cells, the cytoplasm was stained. The mucosa adjacent to the tumors also was stained.

The binding pattern of the lectin peanut agglutinin (PNA) was not different between normal and DSS-treated rats. As in nontreated animals, there was faint staining in the cytoplasm of goblet cells in dysplastic areas and adenocarcinoma *in situ* of DSS-treated rats.

DISCUSSION

Experimental colitis has been induced by carrageenan or DSS in rabbits (10, 11), guinea pigs (21–23), hamsters (15), and mice (16), and DSS administration has induced intestinal or colorectal tumors in rats (12, 13). Okayasu et al (16) reported that oral administration of DSS in drinking water induced acute and chronic ulcerative lesions in mice, and these lesions resembled those in human colonic ulcerative colitis. In this work, we have induced similar lesions in colonic mucosa of rats by the same agents. In the hamster models (15) and our model, the right colon was more severely damaged than the left, while the opposite side was more damaged in mice (16); The reason for these differences is unknown, but it can be speculated that DSS retained in the right colon, especially the cecum, of rats might act as a chronic irritant to the colonic epithelium. Furthermore, long-term ingestion of carrageenan by rabbits (10, 11) and of DSS by mice (6) might induce dysplastic changes in the colonic

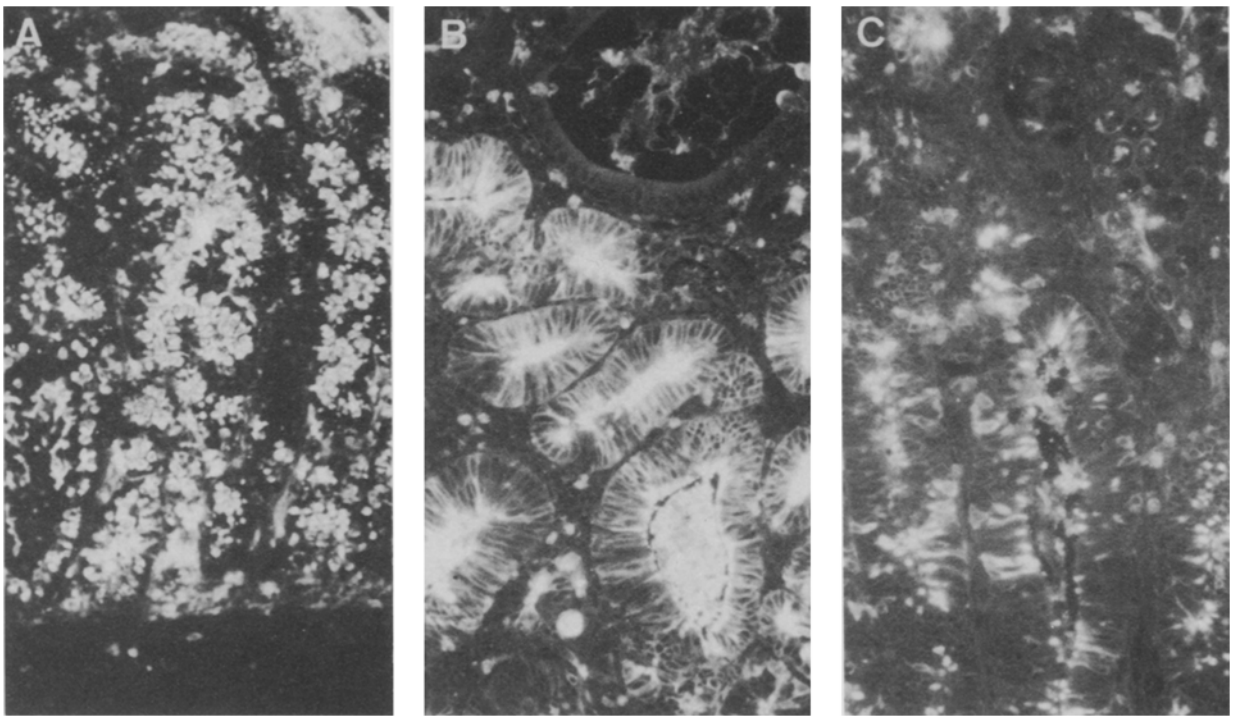

Fig 7. FITC-UEA staining of rat colon. (A) Dysplasia in cecum after three months of DSS treatment (×130). Staining of goblet cell mucins. (B) Dysplasia with carcinoma *in situ* in cecum after six months of DSS treatment (×260). Both negative and positive cells are present. (C) Dysplasia with carcinoma *in situ* in rectum after six months of DSS treatment (×260). Malignant cells are variously stained.

mucosa. In the present study of rats, we induced adenocarcinomas *in situ* as well as dysplasia after a long-term existence of ulcerative lesions by exposing the colonic mucosa to DSS. The frequency of appearance of the tumors was more on the right side (11 of 13 rats after six months) than the left (1 of 13 rats after six months), suggesting that chronic stimulation by DSS of the colonic mucosa and long-standing inflammation led to the development of carcinoma.

Several attempts have been made to detect premalignant changes at an early stage of colonic cancer. We applied some chemical markers to find if one or more of these would detect premalignant changes of the colonic mucosa in the rat DSS model. The results of the present study revealed: (1) regional differences in UEA binding and the staining pattern by HID-AB in the colonic mucosa of normal rats, (2) increased stainability of sialylated mucin and UEA bindings in inflammatory mucosa, (3) increased sulfated mucins and positive staining by FITC-UEA in the cells of dysplastic epithelium, and (4) loss of mucins in the cells of adenocarcinoma *in situ*, which showed no staining by HID-AB and mixed stainability of positive and negative cells by FITC-UEA. However, the data did not show any differences in PAS positivity or PNA binding between the colonic mucosa of normal rats and dysplasia or adenocarcinoma *in situ*. The regional differences of carbohydrate content (25) and the staining patterns by HID-AB (26) or FITC-UEA (27) between the proximal and the distal colon in the colonic mucosa of normal rats have been described; our results support those data. Histochemical changes in malignancy also have been reported. Boland and Ahnen (17) found the lectin peanut agglutinin to bind to mucin secreted by DMH-induced neoplasms in CF-1 mice. Shioda et al (19) reported that *Ulex europeus* agglutinin bound consistently to the premalignant colonic mucosa in DMH-treated rats. Fillipe (26) described the changes of HID-AB staining in DMH-induced colonic tumors of rats. Clinical studies in the colonic wall of patients with UC also showed the histochemical changes (18, 28–33). However, the usefulness of lectins or mucin histochemistry to detect premalignant changes is still controversial. In our experiments, an increased stainability in FITC-

UEA and sialomucin was seen in the inflamed colonic mucosa of rats treated with DSS. These findings suggested that chemical components of the mucins in the colonic epithelium are altered by the administration of DSS. However, since changes observed in FITC-UEA or HID-AB staining in the inflamed segments were not observed in dysplastic or malignant lesions, neither of these markers was useful for detecting the premalignant changes in this animal model.

Despite our failure to find a marker of premalignancy in the rat DSS model of colitis, this model still appears to be valuable for examining the mechanisms of cancer development in ulcerative colitis.

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