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Colonic and Gastric Mucus-associated Antigens: a Comparative Immunohistological Study in Precancerous and Cancerous Rat Intestinal Mucosa

CATHERINE DECAENS,* JEANNETTE NARDELLI, JACQUES BARA and PIERRE BURTIN
Laboratoire d'Immunochimie, Institut de Recherches Scientifiques sur le Cancer, B.P. 8, 94802 Villejuif Cédex, France

Abstract—*Gastric M1 antigens were previously shown to be oncofetal markers for the colon in man and in the rat. They were observed very early during carcinogenesis in goblet cells of precancerous colonic mucosa; using an immunohistological method, we found that M1 were produced in* 68% (41/60) *of colonic adenocarcinomas and in* 33% (21/63) *of duodenal adenocarcinomas. M3C antigen has been described as being associated with human colonic mucus; in the rat it is restricted to the proximal colon. We found that M3C was produced in* 91% (55/60) *of colonic adenocarcinomas and in* 15% (8/53) *of duodenal adenocarcinomas. Before tumor appearance, M3C was sometimes expressed by goblet cells of the distal colon. We could not find it during fetal life. We have concluded that mucus-associated antigens can characterize modifications in cell differentiation in rat colonic carcinomas.*

INTRODUCTION

SEVERAL antigens associated with high-molecular-weight components of gastrointestinal human goblet cells have previously been described: the colonic mucoprotein antigens, CMA [1] and WZ [2], and several gastrointestinal mucoprotein antigens [3–8]. The M1 antigens normally present in gastric mucosa shared common antigenic specificities in man and rat [9]; they were shown to be oncofetal markers for the colon in these two species [7, 10]. During chemically induced carcinogenesis in the rat, M1 antigens were expressed by colonic goblet cells very early during the precancerous stage; thus changes in the differentiation of goblet cells could be shown using these mucus-associated antigens [10]. Recently, M3C antigen was described as being expressed by goblet cells of human colonic mucosa [8]. We wondered whether M3C, like M1 antigens, was associated with a precancerous stage during carcinogenesis in rat and whether it could be a marker of cell differentiation in rat colonic carcinomas.

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*To whom correspondence and requests for reprints should be sent.

MATERIALS AND METHODS

Intestinal adenocarcinomas

These were obtained by weekly 1–2 DMH injections to 32 female Wistar rats for 28 weeks [11].

Rats were killed by cardiac puncture under ether anesthesia; intestines were opened and rinsed. After determination of their location and size, tumors were fixed in 95% ethanol [12] embedded in paraffin, and 3-μm-thick sections were cut with a R-Jung autocut.

Rat colon could be easily divided into a distal part with longitudinal folds and a proximal part with 'herring-bone' folds; this macroscopic difference corresponded well with histological ones.

One hundred and twenty-three tumors were obtained; 119 were well-differentiated tubular adenocarcinomas and four were mucinous carcinomas. All but one showed mucosecretion. All stages of invasion according to Gutmann's classification [13] were found: I, II, III and IV.

Sixty-three adenocarcinomas were found in the duodenum, 34 in the distal colon and 26 in the proximal colon.

Precancerous colonic mucosae

Twelve distal colonic mucosae were obtained by killing rats every week from week 8 to week 20 after the beginning of DMH injections; after dissection, they were coiled up into 'Swiss-rolls'. Neither macroscopic nor microscopic carcinomas were found within this period; however, changes in goblet cell differentiation—as shown by antigenic modifications associated with mucins—and in histological lesions in the mucosa were found, as previously described [10].

Fetuses and newborn rats

Rat fetuses 18, 19, 20 and 21 days old were obtained. Animals aged 1, 5 and 10 days were also used.

All tissue samples were fixed and treated for histology as described for adenocarcinomas.

Immunochemistry

Rabbit anti-M3C serum was prepared against human colonic mucosa as described [8]. Briefly, high-molecular-weight components were obtained from mucosal scrapings by chromatography on Cl 6B and then on Cl 2B Sepharose columns and used for rabbit immunization. The antiserum obtained was absorbed with a panel of A, B and O red blood cells, with normal human plasma polymerized with glutaraldehyde and with crude extract of normal gastric mucosa (500 mg of lyophilized powder for 1 ml of antiserum) to remove antibodies against antigens common to gastric and intestinal mucosae. To obtain organ specificity the antiserum was then absorbed with duodenal crude extract (500 mg/ml of the antiserum). The antiserum was ultracentrifuged at 40,000 rev/min for 1 hr in a Spinco L 65 centrifuge (Beckman, U.S.A.). Using an immunoperoxidase method, this antiserum then reacted with goblet cells of human colonic mucosa, more intensively in the distal part and not at all with other parts of the gastrointestinal tract; the corresponding antigen was then named M3C.

Before use in the rat, the antiserum was absorbed with rat distal colonic crude extract (5 mg/10 μl of the antiserum).

The specificity of the reaction was controlled by absorptions with lyophilized scrapings of rat caecum and proximal colon (500 mg each dry weight for 1 ml of anti-M3C serum) or with human colonic mucosa.

Anti-M1 serum was used as previously described [10]; briefly, it reacted with mucous cells of the surface epithelium of normal gastric mucosa.

An indirect immunoperoxidase method was used. Anti-M1 and anti-M3C sera absorbed as described were diluted 20-fold in PBS (0.9% NaCl in 0.01 M potassium phosphate buffer, pH 7.4) and applied for 3 hr on the section; after three rinses with PBS, sheep antiserum against rabbit IgG (H+L) labeled with peroxidase (Institut Pasteur Production, France) was applied at the 1/100 dilution for 45 min; the section was washed three times with PBS and peroxidase activity was revealed using aminoethylcarbazole [14]. Before microscopic examination, cell nuclei were stained with hematoxylin.

During the precancerous stage each gland with at least one stained cell was counted as a positive gland for the corresponding antigen.

Adenocarcinomas were considered positive when at least one gland of the tumor was positive.

RESULTS

Expression of M1 and M3C in adult rats, fetuses and young rats

As previously described [10], M1 antibodies reacted with mucous cells of the gastric surface epithelium in adults; they reacted with colonic goblet cells of 19- and 20-day-old fetuses; after birth, M1 antigens completely disappeared from the colon.

In adult rats anti-M3C serum reacted with goblet cells of the colonic mucosa, though more extensively in the proximal part; to obtain a more specific reaction, the anti-M3C was absorbed with rat distal colonic crude extract; thereafter it reacted only with goblet cells of rat proximal colonic mucosa and caecum. The reactivity with human colon was unchanged after this last absorption with rat extract.

Absorption by lyophilized scrapings of rat caecum and proximal colon, as well as scrapings of human colon, completely suppressed rat colonic mucosa staining. Staining of human colonic mucosa was also suppressed by absorption with scrapings of human colon, but we could not obtain complete absorption in human colon with scrapings of rat caecum and proximal colon.

Distal colonic mucosa was tested with anti-M3C serum in 19-, 20- and 21-day-old fetuses; the seven tested mucosae were M3C-negative. On the same animals, sections of stomach, duodenum and small intestine were also M3C-negative. In contrast, at 1 day, as at 5 and 10 days after birth, the proximal colon showed intense M3C-positive goblet cells; no staining could be observed for stomach, small intestine and distal colon.

Expression of M1 and M3C antigens by intestinal adenocarcinomas

Distal colon. As can be seen in Table 1, 55% (19/34) of distal colonic adenocarcinomas were M1-positive and 83% (29/34) were M3C-positive, whatever the stage of invasion (Fig. 1). Eleven

adenocarcinomas were of stage I, and all but one were mucosecreting.

Proximal colon. Of proximal colonic adenocarcinomas 84% (22/26) produced M1 antigens (Fig. 2) and 100% (26/26) produced M3C antigen (Fig. 3, Table 2).

Duodenum. Of the duodenal adenocarcinomas 33% (21/63) were M1-positive and 15% (8/53) were M3C-positive (Table 3, Fig. 4).

The two antigens were found in tubular adenocarcinomas as well as in mucinous carcinomas.

Expression of M3C and M1 antigens in distal precancerous colonic mucosa

During DMH treatment several changes were observed in distal colonic mucosa before tumor appearance; in a previous study antigens associated with gastric mucus, called M1 antigens and which were early oncofetal markers for the colonic mucosa, were found in histologically 'normal' colonic glands and histological lesions; as carcinogenesis continued, the number of positive glands increased. In 12 distal precancerous mucosae we compared the production of M1 and M3C antigens (see Table 4, Figs 5, 6).

The number of M3C-positive glands was always smaller than the number of M1-positive glands: the mean number of positive glands per colonic section was 1.5 for M3C and 19 for M1 antigens. Sometimes the two antigens were expressed in the same gland and they could also be expressed in the same cell, as shown on serial sections.

Table 1. *Expression of M1 and M3C antigen in distal colonic adenocarcinomas*

Stage of tumors according to Gutmann's classification	M1-positive adenocarcinomas / total adenocarcinomas	M3C-positive adenocarcimonas / total adenocarcinomas
I	4/10	7/10
II	9/18	16/18
III	5/5	5/5
IV	1/1	1/1
Total	19/34 = 55%	29/34 = 83%

Table 2. *Expression of M1 and M3C antigens in proximal colonic adenocarcinomas*

Stage of tumors according to Gutmann's classification	M1-positive adenocarcinomas / total adenocarcinomas	M3C-positive adenocarcimonas / total adenocarcinomas
I	3/6	6/6
II	5/5	5/5
III	7/8	8/8
IV	7/7	7/7
Total	22/26 = 84%	26/26 = 100%

Table 3. *Expression of M1 and M3C antigen in duodenal adenocarcinomas*

Stage of tumors according to Gutmann's classification	M1-positive adenocarcinomas / total adenocarcinomas	M3C-positive adenocarcimonas / total adenocarcinomas
I	1/17	3/17
II	2/12	4/13
III	3/10	6/13
IV	2/14	8/20
Total	8/53	21/63

Table 4. *Number of M3C- and M1-positive histologically 'normal' glands on precancerous colonic mucosa*

Weeks from the first DMH injection	Total No. of M3C+ glands / No. of colonic mucosae	Total No. of M1+ glands / No. of colonic mucosae
8–10	0/1	17/1
11–15	5/4	42/4
16–20	12/7	167/7
	$m^* = 1.5$	$m^* = 19$

m^* = mean number of positive glands from week 8 to week 20.

The number of M1-positive glands per colonic mucosa increased regularly until week 20; moreover, every mucosa of treated rats was affected. On the contrary, the number of M3C-positive glands showed only a small increase from week 8 to week 20, with half of the 12 precancerous mucosae being M3C-negative.

Near Peyer's patches, seen on several sections, we noticed numerous glands with architectural dysplastic or hyperplastic-like modifications; some of these glands produced M1 antigens and a greater number produced M3C antigen.

DISCUSSION

The M3C antigen is expressed by goblet cells of the caecum and the human colon, especially the distal part. Using an antiserum prepared against this human antigen, we found that it was restricted to the proximal colon and caecum of the rat. After absorption of the antiserum with human colonic mucosa the staining of the rat colon mucosa was abolished. However, there was still staining of human colonic mucosa after absorption of anti-M3C with rat caecum and proximal colon, though to a lesser extent. Thus, as was previously shown for the other mucus-associated antigens called M1, the M3C antigen present in man was also found in the rat, though some of the antigenic determinants of the human antigen were not recovered in the rat.

In a precancerous stage during chemical carcinogenesis in the rat, M3C antigen was shown to be present in some histologically normal colonic glands of the distal part; treatment with the carcinogen produced precocious changes in goblet cell differentiation, as previously shown with the M1 antigens [10]. The study of the mucus-associated antigens therefore appeared to be very useful for investigating changes in differentiation during precancerous stages. However, the change in differentiation leading to M3C production was not found in every precancerous colonic mucosa and the number of M3C-positive glands seemed to increase only slightly, if at all, with DMH injections, contrary to M1 antigens. Thus M3C, although sometimes present in precancerous colonic mucosa, is not as good a marker as M1 during early carcinogenesis.

In the proximal colon M3C was present in normal mucosa as well as in 100% of adenocarcinomas; M1 was produced in 84% of adenocarcinomas. In distal colon, which is M3C-negative when normal, 87% of adenocarcinomas were M3C-positive and 55% were M1-positive; we were not able to show M3C in fetal colon, and thus this antigen cannot be considered as an oncofetal marker; its expression resembled an ectopic one for this part of the colon.

It should be noted, however, that one day after birth, more than 50% of the proximal colonic goblet cells were intensively stained with anti-M3C serum, indicating a very marked change in differentiation. We have already observed such rapid variation in expression, with M1 antigens appearing in the gastric mucosa and with sialomucins disappearing in the colonic mucosa of 1-day-old rats.

Thus M3C antigen, which is present in 51/55 colonic adenocarcinomas and is produced by 35–80% of the cancerous glands, can (like M1 antigen) be regarded as a marker of colonic adenocarcinomas.

In contrast, only 15% of duodenal adenocarcinomas had M3C-positive cells, occupying an average of 5–10% of the total area. Again, we did not find M3C in fetal duodenum. Of these carcinomas 33% had M1-positive cells.

We noted the frequent association of M3C antigen with goblet cells of glands near to or present in Peyer's patches; changes in the differentiation of goblet cells had already been observed in Peyer's patches when colonic mucosa was submitted to chemical carcinogen: the goblet cells often produced M1 antigens and sialomucins [10]. Some authors have noted the close proximity of carcinogen-induced colonic adenocarcinomas to lymphoid follicles [15, 16].

Altered differentiation is often associated with carcinogenesis; a pool of stem cells is initiated by the carcinogen; they begin to proliferate for a prolonged period during their migration in the colonic crypts without repressing their DNA synthesis by lack of normal regulatory controls [17]. Some of these cells then undergo modified differentiation, leading, for example, to production of tumor-associated molecules, usually of an embryo-fetal type, like M1 antigens. However, the expression of M3C in distal colonic as well as duodenal adenocarcinomas did not seem to be of an oncofetal type. It more closely resembled an ectopic feature, as described for hormone production in non-endocrine tumors [18]. This ectopic production could be the consequence of gene depression or of abnormalities in regulation of gene expression [18]. The presence of M3C in distal colonic adenocarcinomas could reveal a close similarity of differentiation between distal and proximal colon tumors.

In conclusion, the antigen M3C, associated with human colonic mucin, was found in the proximal colon and caecum of the adult rat in 92% of colonic adenocarcinomas and in 15% of duodenal adenocarcinomas; it could not be found in the fetal gastrointestinal tract; it was sometimes present in precancerous colonic mucosa. M3C thus appeared to be strongly associated with

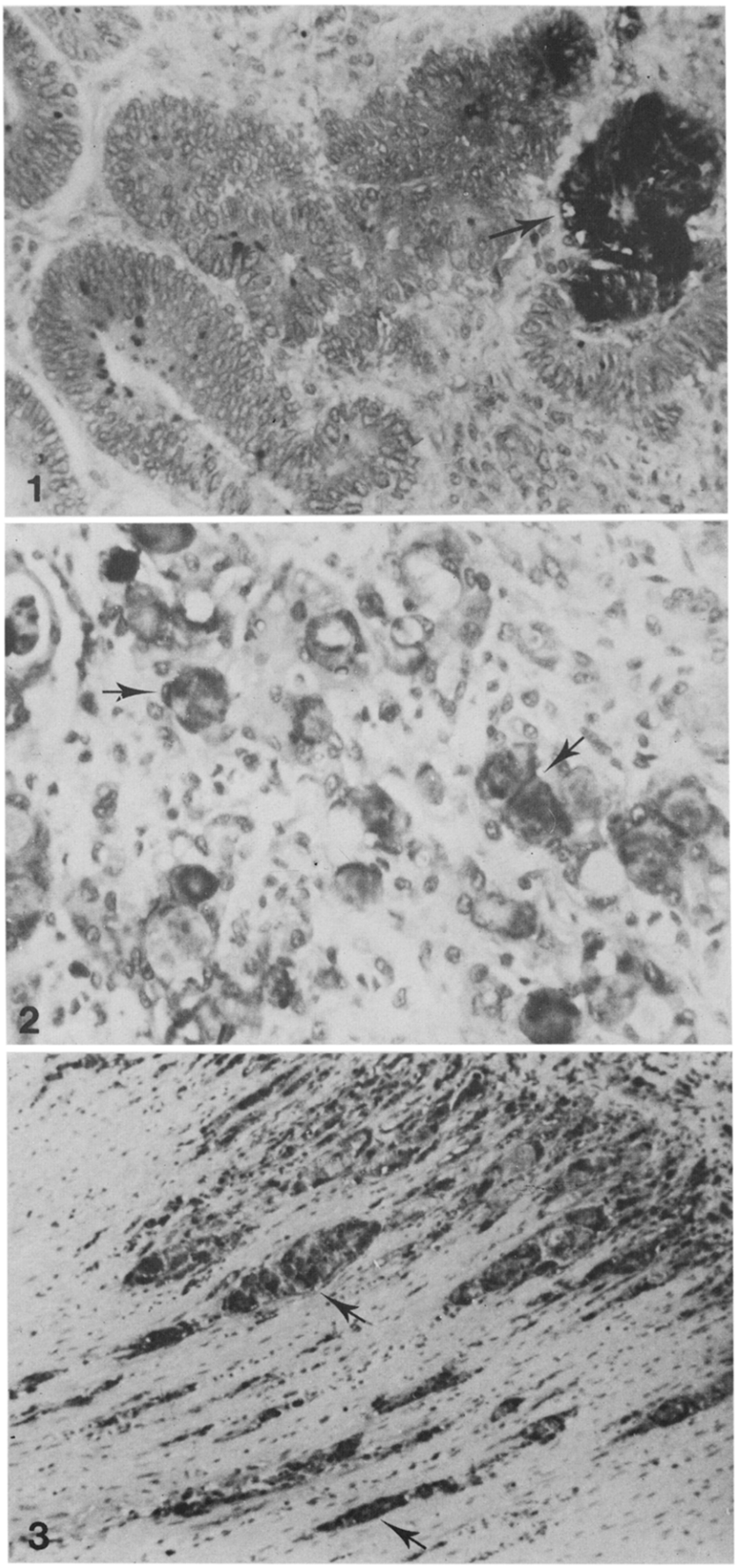
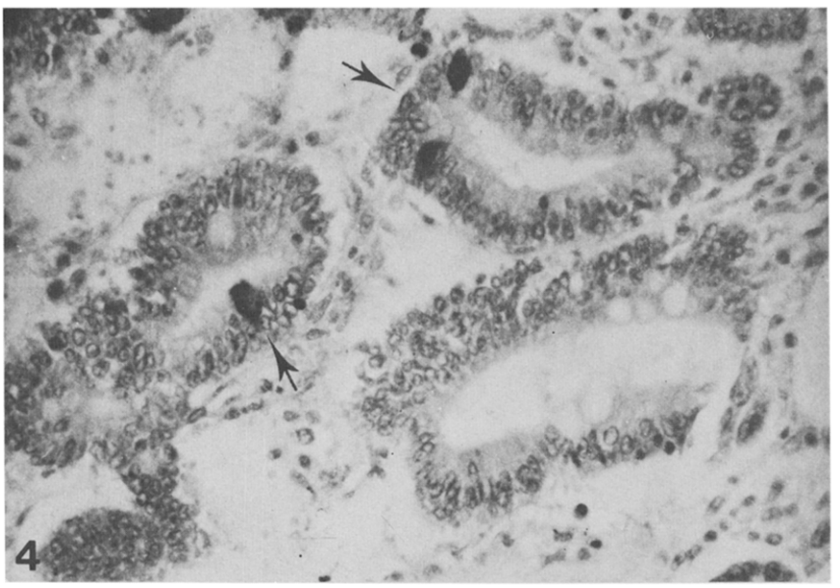
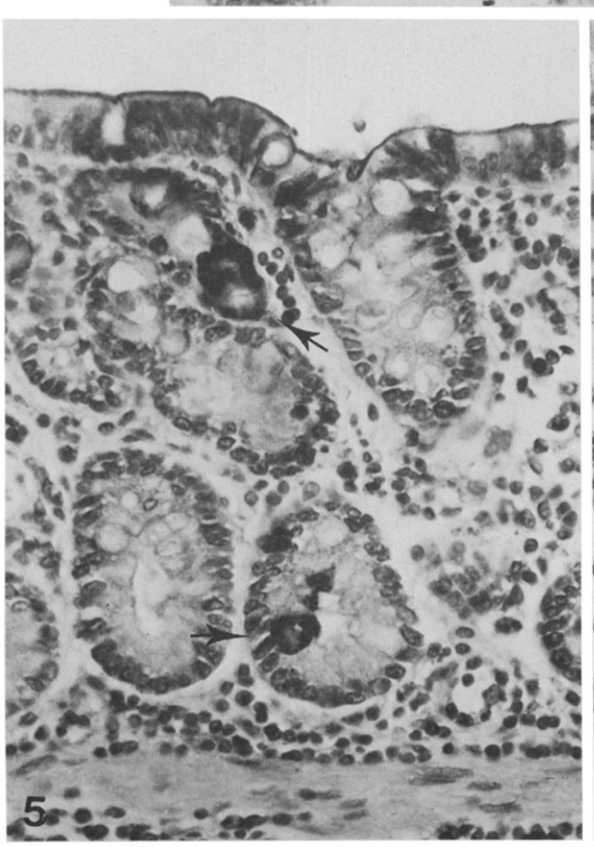


Fig. 1. Several glands in a tubular adenocarcinoma of distal colon (stade I) producing M3C antigen. Immunoperoxidase × 225.

Fig. 2. Signet ring cells in an adenocarcinoma of proximal colon (stade IV) producing M1 antigen. Immunoperoxidase × 360.

Fig. 3. Invading the muscular layer, glands of an adenocarcinoma, mucinous in part, of proximal colon (stade IV) producing M3C antigen. Immunoperoxidase × 90.





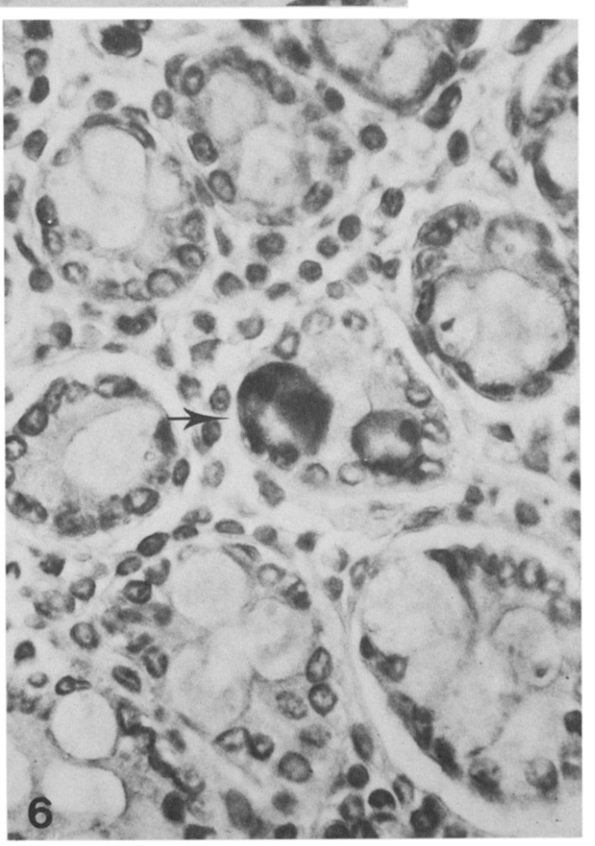


Fig. 4. *Two glands in an adenocarcinoma of duodenum (stade II) producing M3C antigen. Immunoperoxidase* × 225.

Fig. 5. *Histologically normal mucosa after* 13 weeks *of DMH injections, showing* 2 *glands with cells producing M3C antigen. Immunoperoxidase* × 225.

Fig. 6. *Histologically normal mucosa after* 12 weeks *of DMH injections, showing* 1 *gland with cells producing M*1 *antigen. Immunoperoxidase* × 360.

colonic adenocarcinomas with an ectopic production in the distal colon.

M1 antigens, which are oncofetal markers for the colon, were found in 68% of colonic adenocarcinomas.

These two mucus-associated antigens are therefore interesting markers of cell differentiation in rat colonic adenocarcinomas.

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REFERENCES

1. Gold D, Miller F. A mucoprotein with colon specific determinants. *Tissue Antigens* 1978, **11**, 362–371.
2. Zweibaum A, Oudea P, Halpern B, Veyre C. Presence in colic glandular cells of various mammalian species of an antigen with human blood group substance. *Nature* 1966, **5019**, 159–161.
3. Rapp W, Windisch M, Peschke P, Wurster K. Purification of human intestinal goblet cell antigen (GOA), its immunohistological demonstration in the intestine and in mucus producing gastrointestinal adenocarcinomas. *Virchows Arch (Pathol Anat)* 1979, **382**, 163–177.
4. Ma J, De Boer WGRM, Ward HA, Nairn RC. Another oncofetal antigen in colonic carcinoma. *Br J Cancer* 1980, **41**, 325–328.
5. Goldenberg DM, Pant KD, Dahlman HL. Antigens associated with normal and malignant gastrointestinal tissues. *Cancer Res* 1976, **36**, 3455–3463.
6. Kawasaki H, Abe T, Akagi Y *et al.* Antigenic profiles of carcinoma cells of gastrointestinal tract as detected by immunofluorescence technique. *Kurume Med J* 1977, **24**, 65–80.
7. Bara J, Loisillier F, Burtin P. Antigens of gastric and intestinal mucous cells in human colonic tumours. *Br J Cancer* 1980, **41**, 209–221.
8. Nardelli J, Bara J, Rosa B, Burtin P. Intestinal metaplasia and carcinomas of the human stomach: an immunohistological study. *J Histochem Cytochem* 1983, **31**, 366–375.
9. Decaens C, Bara J, Waldron-Edward D, Labat-Robert J. Specific biochemical and immunological properties of some water-soluble glycoproteins produced by rat gastric mucosal scrapings *in vitro*. *Int J Biochem* 1981, **13**, 261–271.
10. Decaens C, Bara J, Rosa B, Daher N, Burtin P. Early oncofetal antigenic modifications during rat colonic carcinogenesis. *Cancer Res* 1983, **43**, 355–362.
11. Martin MS, Martin F, Michiels R *et al.* An experimental model for cancer of the colon and rectum. *Digestion* 1973, **8**, 22–34.
12. Sainte-Marie G. A paraffin embedding technique for studies employing immunofluorescence. *J Histochem Cytochem* 1962, **10**, 250–256.
13. Gutman RA, Bertrand I, Peristiani TJ. *Le Cancer de l'Estomac au Début.* Paris, Doin, 1939.
14. Graham R, Lundholm U, Karnovsky M. Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. *J Histochem Cytochem* 1965, **16**, 150–152.
15. Ward J. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. *Lab Invest* 1974, **30**, 505–513.
16. Rogers A, Herndon B, Newberne P. Induction by DMH of intestinal carcinoma in normal rats and rats fed high or low levels of vitamin A. *Cancer Res* 1973, **33**, 1003–1009.
17. Lipkin M. Phase 1 and phase 2 proliferative lesions of colonic epithelial cells in disease leading to colonic cancer. *Cancer* 1974, **34**, 878–888.
18. Imura H. Ectopic hormone production viewed as an abnormality in regulation of gene expression. *Adv Cancer Res* 1980, **33**, 39–75.