Monoclonal Antibodies against Oncofetal Mucin M1 Antigens Associated with Precancerous Colonic Mucosae

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ABSTRACT

We obtained seven monoclonal antibodies (MAb) against a gastric mucin of an ALe patient. By immunoperoxidase on normal gastric mucosae, two MAbs (3-3A and 2-25LE) reacted exclusively with the A and Lewis-positive individuals, respectively; the five other MAbs (1-13 Mi, 2-11 Mi, 2-12 Mi, 9-13 Mi, and 58 Mi) stained the mucus cells of surface gastric epithelium independently of ABO or Lewis status. They did not stain normal colonic mucosa, but did stain fetal and precancerous colonic mucosa. Using serial sections, each anti-Mi MAb stained the same goblet cells in fetal and precancerous colon. Extensive search of other normal tissues showed that Mi antigens were restricted to the epithelium embryologically derived from the foregut (gastric and bronchial epithelium) and from Müllerian ducts (mucus cells of endocervix and prostatic utriculus). Some differences in the reactivities of the various anti-Mi MAbs were observed in subepithelial, submucosal, and muscularis mucosae cells, suggesting that each anti-Mi MAb characterized a different Mi epitope. A mixture of these five anti-Mi MAbs allowed the estimation of Mi mucus modification in the precancerous colonic mucosa with a sensitivity near to that obtained with polyclonal anti-Mi antibodies. Papain and mercaptoethanol treatments destroyed the Mi epitopes, at variance with the A- or Lewis-related antigens. Our results therefore suggest that the expression of Mi epitopes in precancerous colonic mucosae cannot be due exclusively to alterations in mucin glycosylation but may be related to the reexpression of antigens associated with native gastric mucin which is normally produced by the fetal colon during the sixth month of gestation.

INTRODUCTION

Several years ago (1), we described antigens common to human ovarian mucinous cyst fluids and gastric mucosae. These antigens, called Mi antigens (2), were later found in 29% of ovarian adenocarcinomas, especially those located on the proximal side (3), in 80% of gastric carcinomas (4), and in 100% of ovarian mucinous tumors, often in association with other M mucus-associated antigens (5). The fact that one particular histological type of ovarian mucinous tumor can express only one type of M antigen (5), the Mi antigens, more easily permitted their isolation. In the normal gastrointestinal tract, such mucus-associated Mi antigens were demonstrated in the mucus cells of surface gastric epithelium but not in the colonic mucosa; however, they were found in the fetal (6) and precancerous colon (7, 8); moreover, these antigens were expressed during rat colonic carcinogenesis (9), sometimes associated with histological lesions comparable to those observed in human colonic mucosae adjacent to adenocarcinomas (10). Recent observations have pointed out that mucus alterations during colonic carcinogenesis could be due to the saccharidic moiety of mucus; these include differences in the number of sugar sulfate groups (11), a resurgence of blood group antigens (12), and the accessibility of galactose to peanut agglutinin (13).

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The abbreviations used are: MAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; CEA, carcinoembryonic antigen.

MATERIALS AND METHODS

Tissues

Normal Tissues

Gastrointestinal Mucosa. Human gastrointestinal mucosae were obtained from kidney donors. Autopsies were performed on 40 patients (31 men and 9 women) within 5 min of death. Most patients were in their fourth decade of life (mean age 33.88 years) with ages ranging from 17 to 54 years. Tissue samples measuring about 10 x 1 cm were taken from 6 different areas of the gastrointestinal tract, as already described (15). We studied 25 samples of esophageal epithelium, 14 of them including subepithelial mucus glands, 24 samples of cardia mucosa, 35 samples of fundic mucosa, 35 samples of pylorus-duodenal junctions, 23 samples of jejunal mucosa, 28 samples of ileocolonic junctions, 21 samples of transverse colonic mucosa, and 36 samples of sigmoid mucosae. Seven duodenal samples contained Wirsung’s ducts. Rat colonic mucosae were resected 5 min after sacrifice. Mucosae were washed in PBS and cut into 2 parts corresponding to the proximal and distal colon.

Other Tissues. Bronchial epithelium were obtained from kidney donors; 4 samples of 4 different patients were resected and included alveolar as well as bronchial epithelium. Endocervical mucosae were obtained immediately after surgery from 20 patients undergoing hysterectomy for reasons other than neoplasia. They were provided by the Department of Pathology, University of Groningen (The Netherlands); 1 to 2 cm of tissue wall were cut off the endocervical bulk. Samples of liver (5 patients), pancreas (20 patients), gallbladder (2 patients), bladder (10 patients), prostate (4 patients, two of them containing prostatic utriculus), seminal vesicles (3 patients), and testicles (1 patient) were removed from kidney donors. Samples of kidney (5 patients), urethra (1 patient), and adrenal glands (1 patient) were removed surgically from patients with lithiasis.

Fetal and Newborn Colonic Mucosae. Colonic mucosae were obtained from 65 fetuses after spontaneous or therapeutic abortion occurring between 2 and 7.5 months of gestation and from 4 stillborn infants and 8 newborns (premature or term) who died within a few hours or days after birth.

Colonic Tumoral Tissue

Thirty colonic adenomas were obtained from the Centre Medico-Chirurgical de la Porte de Choisy, Paris, France. These were either resected from distal colonoscopic polypectomies of patients without detectable cancer (15 cases) or were removed from mucosae adjacent to distal adenocarcinomas which were surgically resected (15 cases). Human mucosae adjacent to distal adenocarcinomas were taken after surgery for cancer. The macroscopically normal colonic mucosae were resected as already described (8). Three strips, including the muscularis mucosae, were cut from the entire length of each specimen,
cells; consequently, 1 cm of mucosa contained approximately 6,000 colonie mucosae, we calculated that 1 cm of mucosa contained approx
adenomatous areas.

Preparation of Tissues

Samples of normal fetal gastrointestinal human mucosa and rat colonic mucosa were immersed in a box filled with 95% ethanol, as
already described (9). After a 2-h fixation, the mucosae were cooled into “Swiss rolls” (16). These Swiss roll cooled mucosa and the normal
and tumoral tissue samples were fixed in 95% ethanol for 24 h, processed
together, and then embedded in paraffin wax. Serial sections 3 μm thick were cut using an Autocut, Reichert-Jung, Heidelberg, Federal
Republic of Germany.

Preparation of M1 Antigens

We obtained 11 mucinous ovarian cysts of the pure endocervical type according to Fenoglio’s classification (17). The fluids of these cysts
were aspirated and then lyophilized (crude extracts). Preparation of M1 antigens was carried out by chromatography on Sepharose CL 6B
of these crude extracts, as already described (2). Mucin concentration was estimated using the orcinol method (18).

Antiserum

Polyclonal rabbit anti M1 serum was obtained as already described (2). It was absorbed by normal human plasma, RBC, and by colonic extract of an ALeb patient (50 mg dry powder/ml of antiserum) (19). With immunoperoxidase, this anti-M1 serum stained the mucus cells of the surface gastric epithelium and did not react with the other parts of the normal gastrointestinal tract.

Immunoperoxidase Method

Deparaffinized sections were incubated for 30 min with polyclonal rabbit anti-M1 serum at a 1/50 dilution in PBS, with the undiluted supernatant of hybridomas, or with ascites fluids of mice at 1/1000 dilution; then, immunoperoxidase reactions were performed using ami
noethylcarbazol as already described (20). Before microscopic exami
nation, cell nuclei were stained with 1% hematein for 1 min. The staining was inhibited by incubation of diluted rabbit anti-M1 antiserum or mouse monoclonal antibodies with lyophilized ovarian mucinous cyst fluid, 50 mg/ml (dry weight) from patient ALeb(a-b+). On mucosae
adjacent to adenocarcinomas, M1 staining was evaluated as already described (8): colonic glands were grouped by patches of 25 and the percentage of M1-positive glands in each patch was estimated. A gland was considered M1 positive when it contained at least one M1-positive goblet cell. A score of 1, 2, or 3, respectively, was established for 4 to 25, 25 to 50, or greater than 50% of M1-positive glands by patch. Patients were classified according to the presence and intensity of M1 modification at 1 cm from the tumors: type 1, no modification; type 2, patches of glands scoring 1 or 2; type 3, patches of glands scoring 3. In
adenomas, M1 staining was evaluated using a semiquantitative scoring system as already described (7): scores of 0, 1, 2, or 3 referred, respecti
vely, to 0 to 10, 10 to 50, 50 to 75, and more than 75% of M1-positive adenomatous areas.

In order to estimate the percentage of M1 goblet cells in the normal colonic mucosa, we calculated that 1 cm of mucosa contained approximately 100 glands and that 1 gland contained approximately 60 goblet cells; consequently, 1 cm of mucosa contained approximately 6,000 goblet cells; the number of cells could then be estimated from the length of mucosa evaluated using a centimeter graduated rule. We deduced
the percentage of M1 positive goblet cells by counting the number of positive cells per cm.

Monoclonal Antibodies

BALB/c mice were immunized with preparations of M1 antigens isolated from ovarian mucinous fluid of a pure endocervical type from an ALeb(a+b) patient. These M1 antigens were high molecular weight
components obtained after successive chromatographic purification on Sepharose CL 6B and CL 2B, as already described (2); 45 μg of proteins of these high molecular weight components were injected s.c. into the mice at days 1, 15, and 30. At day 40, three days prior to sacrifice, the mice having the highest titer of anti-M1 antibodies received an i.v. injection of 45 μg of high molecular weight components. After sacrifice, 107 splenic cells from immunized mouse and 2 x 104 to 4 x 106 myeloma Sp2/O cells were mixed and cell fusion was performed according to the method described by Buttin et al. (21). The superna
nants of hybridomas were screened by ELISA for the presence of anti
M1 antibodies against M1 antigen preparation. The most positive supernatants were then tested using the immunoperoxidase method on pyloric-duodenal mucosae of an ALLe patient and then on the paraffin section of an M1(+) score 3 adenoma of an OLe patient. Each antibody-secreting hybridoma was cloned 2 times by limiting dilution from each cloning. Clones were grown as ascites tumor by injection of 5 x 106 cells into the peritoneal cavity of male BALB/c mice primed with 2,6,10,14-tetramethylpentadecane.

Immunochromogen

For ELISA, vinyl plates were coated with M1 antigen preparation (10 μg/ml) in 0.1 M sodium bicarbonate buffer, pH 9.5 at 37°C for 2 h
and then incubated according to the usual method (22). Double diffusion studies were performed by Ouchterlony’s method in 1% agar in PBS. Crude extracts of ovarian cyst fluids were used at a concentration of 50 mg/ml (dry weight). After diffusion, plates were washed for 2 weeks in PBS and stained with amido black.

The ABO and Lewis status of each patient were determined when possible using the conventional hemagglutination technique or by im
munoperoxidase with sections of duodenal mucosa or the epithelium of ovarian mucinous cyst using monoclonal antibodies against A, B, Lea, or Leb antigens (Chembio Ltd., Edmonton, Canada).

Determination of the heavy chain isotype of monoclonal antibodies was carried out as previously described (23).

Papain and Mercaptoethanol Treatments

Thiol Reduction of Mucin. The mucin preparation (2 ml containing 5 mg of protein/ml) was reduced by treatment with 0.2 M β-mercaptoethanol in Tris buffer, 300 mM, pH 8.6 containing guanidine, 6 M, and 1 mM of phenylmethylsulfonyl fluoride (Sigma, St Louis, USA) for 72 h at 4°C; then, iodoacetamide, 0.4 M was added for 2 h at 4°C. Mucin samples were dialyzed exhaustively against PBS. Appropriate controls (without mercaptoethanol) were carried out to determine the effects of guanidine and iodoacetamide on mucin antigenicity.

Papain Digestion. The mucin sample (2 ml containing 5 mg of protein/ml) was incubated with 1 mg of water-soluble papain, 3.5 units/ml (Merck, Darmstadt, West Germany) in acetate buffer, 0.2 M, pH 5.6 containing cysteine and 5 mM EDTA at 65°C for 36 h. A control mucin sample was incubated similarly but without enzyme.

RESULTS

Generation and Screening of Monoclonal Antibodies

Sera obtained from 8 mice after injection of a human ALeb mucin preparation were analyzed for comparative titer of anti
M1 antibodies by ELISA using the M1 antigen preparation and by the immunoperoxidase method on sections of gastrointestinal mucosa. The 2 mice with the highest titers were selected for 2 cell fusions. In the first fusion, 109 of 170 wells showed growing hybrids, and the supernatants of 77 wells showed a reaction by ELISA with the ALLeb mucin preparation. The 30 strongest wells were then tested by immunoperoxidase on pyloric-duodenal sections of an ALLe patient; 27 of these 30 hybrid supernatants reacted strongly on such gastrointestinal mucosa. The strongest hybrid was selected and a clone designated 3-3A was established. Among the other 26 hybridomas, only 4 reacted strongly on the mucosae of an OLe patient and
3 gave strong positivity on some goblet cells of the adenoma of an OLe<sup>e</sup> patient, showing a pattern identical to that of the polyclonal anti-M1 serum. Four different clones, designated 1-13 M1, 2-11 M1, 2-12 M1, and 2-25 LE, were established. In a second fusion, approximately 300 of 480 cells showed growing hybrids. The 100 fastest growing hybridomas were tested directly by immunoperoxidase on a adenoma section of an OLe<sup>e</sup> patient. Eight of them were positive. After subsequent transfers, 6 of the hybrids had lost the ability to excrete specific antibodies. The 2 stable hybrids were initially cloned and designated 9-13 M1 and 58 M1. These 7 clones were subcloned once again and were expanded as ascites tumors.

Characterization of Monoclonal Antibodies

Among the 35 samples of gastric surface epithelium, MAb 3-3A stained only the mucosae of A patients independently of Lewis status (Table 1). This MAb 3-3A stained almost all columnar cells of the surface gastric epithelium (some rare negative cells were observed). In the fundic glands, the parietal cells were strongly stained by this MAb. MAb 2-25 LE also stained the surface gastric epithelium of only those Lewis-positive patients (33 of 35) showing the same pattern as MAb 3-3A. In contrast, antibodies 1-13 M1, 2-11 M1, 2-12 M1, 9-13 M1, and 58 M1 all stained the mucus cells of the surface gastric epithelium independently of ABO and Lewis status. Gastric glands were unstained. No differences in the gastric staining pattern could be observed between these 5 anti-M1 MAbs and rabbit polyclonal anti-M1 antibodies.

These 7 monoclonal antibodies were typed as IgG1. Their concentration of immunoglobulins in the ascites fluids is reported in Table 2, as is the staining titer of these antibodies, which was estimated by the immunoperoxidase method on a fundic mucosa of an ALe<sup>e</sup> patient.

By immunodiffusion, MAb 3-3A gave a strong precipitin line with ovarian mucinous cyst fluids of patients belonging exclusively to blood group A; in contrast, MAb 2-25 LE gave a precipitin line with the ovarian extracts of Lewis-positive patients independently of their ABO blood group. The 5 other MAbs, 1-13 M1, 2-11 M1, 2-12 M1, 9-13 M1, and 58 M1 did not precipitate in agar against the ovarian extracts containing M1 antigens, even when these 5 antibodies were pooled (Fig. 1).

Immunoperoxidase Staining of Human Gastrointestinal Mucosae

Adult Mucosae. With immunoperoxidase, both monoclonal and polyclonal anti-M1 antibodies gave an identical staining pattern for the gastrointestinal tract. The mucus cells of the surface gastric epithelium and some rare duodenal goblet cells near the pylorus were strongly stained. The remaining gastrointestinal tract did not react with anti-M1 antibodies (jejunum, 0 of 17; ileum, 0 of 24); however, some mucus cells of Wirsung's duct near the duodenal mucosa (7 of 7 cases) were positive. Special studies were performed on histologically normal colonic mucosae (Table 3). Approximately 10 m of normal colonic mucosae from 36 kidney donors, coiled into Swiss rolls, were studied; 25 of 36 individuals showed less than 0.01% M1-positive goblet cells, 7 of 36 individuals contained between 0.1 and 0.01% M1-positive cells, and 4 of 36 between 0.3 and 0.1%. These M1-positive cells were principally located in the upper part of the colonic glands. Small mucinous hyperplasia, containing M1-positive goblet cells also located in the upper part of the gland, was observed in 3 patients and such alterations were not counted in the above estimations. An identical M1 pattern was observed using an anti-M1 MAb alone or a pool of the 5 anti-M1 MAbs. No particular differences were observed between the left and right colon in terms of M1 expression.

Fetal Colon. Like polyclonal anti-M1 antibodies, the pool of the 5 anti-M1 MAbs stained the fetal colonic mucosae, but less strongly. The intensity of staining was weaker when an anti-M1 MAb was incubated alone. The expression of M1 was slightly higher in the left than in the right colon. M1(+ ) goblet cells were observed from the fourth month of gestation (Table 4). The M1 expression was maximum during the sixth month showing Lieberkühn glands containing nearly 95% M1-positive cells (Fig. 2A). Goblet cells located near the surface epithelium were most strongly stained. After the sixth month, few mucosae were M1 positive; however, some rare M1-positive goblet cells located in the upper part of the glands were strongly stained (Fig. 2C). Such a pattern was observed in the mucosae adjacent to adenocarcinomas (Fig. 2, B and D). Meconium deposits, when observed, were strongly stained.

Immunoperoxidase Staining of Other Human Tissues

Only MAbs 9-13 M1 and 58 M1 reacted with the mucus cells of subvesophageal glands (15 of 15). The ducts of these glands were negative. Monoclonal and polyclonal anti-M1 antibodies stained goblet cells of tracheal epithelium (4 cases); moreover, MAbs 1-13 M1 and 9-13 M1 faintly stained some mucus cells of the submucosa. The anti-M1 MAb (except for MAb 2-12 M1) stained the mucus cells of endocervical epithelium (20 cases), but less strongly than did polyclonal anti-M1 antibodies. Differences in reactivities among the 5 anti-M1 antibodies are reported in Table 5. The 5 MAbs, like the polyclonal antibodies, also strongly stained the mucus cells of the prostatic urticulus (2 of 2 cases) and were negative on the male and female urogenital tract, including the urethra (1 case), bladder (10 cases), prostate (4 cases, except for the urticulus), kidney (5 cases), seminal vesicle (3 cases), and subrenal glands (2 cases). On the other hand, liver (10 cases) and pancreas (except for

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**Table 1** Reactivity of the 7 monoclonal antibodies against gastric fucumucins and the anti-M1 polyclonal antibodies with normal gastric mucosae

<table>
<thead>
<tr>
<th>Donor</th>
<th>Lewis phenotype</th>
<th>Monoclonal antibodies*</th>
<th>1-13 M1, 2-11 M1, 2-12 M1, 9-13 M1, 58 M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Le(a+b)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A</td>
<td>Le(a+b)</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>A</td>
<td>Le(a-b)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>Le(a+b)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>Le(a+b)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Le(a-b)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>O</td>
<td>Le(a+b)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>O</td>
<td>Le(a+b)</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

* Number of individuals having an identical phenotype.

**Table 2** Class, subclass, concentration, and titer of antibodies in mouse ascites fluids

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Class</th>
<th>Concentration (mg/ml)</th>
<th>Titer (x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-3A</td>
<td>IgG1</td>
<td>2.9</td>
<td>1/10</td>
</tr>
<tr>
<td>2-25LE</td>
<td>IgG1</td>
<td>4.4</td>
<td>1/10</td>
</tr>
<tr>
<td>1-13 M1</td>
<td>IgG1</td>
<td>3.0</td>
<td>1/20</td>
</tr>
<tr>
<td>2-11 M1</td>
<td>IgG1</td>
<td>2.9</td>
<td>1/20</td>
</tr>
<tr>
<td>2-12 M1</td>
<td>IgG1</td>
<td>1.0</td>
<td>1/15</td>
</tr>
<tr>
<td>9-13 M1</td>
<td>IgG1</td>
<td>2.9</td>
<td>1/20</td>
</tr>
<tr>
<td>58 M1</td>
<td>IgG1</td>
<td>2.5</td>
<td>1/20</td>
</tr>
</tbody>
</table>
MAbs AGAINST ONCOFETAL MUCIN M1 ANTIGENS

Fig. 1. Immunodiffusion in agar showing reactivity of the 7 monoclonal antibodies against mucin from mucinous ovarian fluid extract of different ABO and Lewis phenotypes. \( \text{ALe}^a, \text{ALe}(a-b^+) \) mucin; \( \text{ALe}^-, \text{ALe}(a-b^-) \) mucin; \( \text{OLe}^a, \text{OLe}(a-b^+) \) mucin; \( \text{OLe}^-, \text{OLe}(a+b^-) \) mucin.

Table 3 M1 antigens in normal colonic mucosae

<table>
<thead>
<tr>
<th>% of M1(+) goblet cells</th>
<th>No. of patients with identical % of M1(+) cells</th>
<th>Length of mucosae (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>25/36</td>
<td>700</td>
</tr>
<tr>
<td>0.1–0.01</td>
<td>7/36</td>
<td>240</td>
</tr>
<tr>
<td>0.3–0.1</td>
<td>4/36</td>
<td>90</td>
</tr>
</tbody>
</table>

Table 4 M1 antigens in human fetal colon (ratio of the number of M1-positive colonic mucosae and number of mucosae according to the month of gestation using a pool of the 5 anti-M1 MAbs)

<table>
<thead>
<tr>
<th>Mo of gestation</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/2</td>
<td>0/1</td>
<td>1/4</td>
<td>6/9</td>
<td>17/20</td>
<td>3/6*</td>
<td>4/11*</td>
<td>1/12*</td>
<td></td>
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</tbody>
</table>

Some rare isolated but very strongly stained M1 goblet cells could be observed (see Fig. 2).

Positive reaction from ± to +++ represents increasing intensity of immunoperoxidase.

some mucus cells of large ducts in the head of the pancreas (20 cases) were M1 negative. Saliva extracts could not absorb the gastric reactivity of the anti-M1 antibodies.

Rat Mucosae

Anti-M1 MAbs 1-13 M1, 2-11 M1, 2-12 M1, 9-13 M1, and 58 M1 did not react with the gastrointestinal mucosa and precancerous colon, at variance with the polyclonal anti-M1 antibodies.

Precancerous Colonic Mucosae

One anti-M1 MAb alone stained these mucosae less strongly than a pool of 2 or more anti-M1 MAbs. The pool of the 5 anti-M1 MAbs generally stained the same areas and the same goblet cells as did polyclonal anti-M1 antibodies. We classified M1 alterations as described in “Materials and Methods.” The results (Fig. 3) show that this pool of anti-M1 MAbs gave the same classification of M1 modifications in 80% of adenomas and in 80% of histologically normal mucosae adjacent to adenocarcinomas.

Papain and Mercaptoethanol Treatment

Using immunoperoxidase, 15-μg of mucin preparations (hexoses) totally absorbed the staining given by 1 μg of a pool of 5 monoclonal anti-M1 antibodies; 0.15 μg of the same mucin preparation absorbed 1 μg of anti-A MAb. Guanidine and iodoacetamide treatment induced a slight loss of M1 activity, since 30 μg were then necessary to absorb the same quantity of antibodies. In contrast, M1 activities were totally destroyed by papain or by mercaptoethanol treatment and even by heating at 65°C for 36 h; on the contrary, blood group A activity was only slightly impaired by these enzymes or reducing agents.

DISCUSSION

M1 antigens have been defined, using rabbit polyclonal antibodies, as being associated with the columnar mucus cells of surface gastric epithelium; they are absent from normal colon (2) but not from fetal (6) and precancerous colon (7, 8). Up to now, no allotypic antigenic determinants have been found, which is at variance with the ABO, Lewis, and WZ antigens (24). We have obtained 5 hybridomas secreting antibodies which reacted with antigens having this immunohistopathological M1 definition; consequently, we conclude that MAbs 1-13 M1, 2-11 M1, 2-12 M1, 9-13 M1, and 58 M1 characterize...
Table 5 Differences in reactivity of the 5 anti-M1 monoclonal antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Tissue</th>
<th>1-13 M1</th>
<th>2-11 M1</th>
<th>2-12 M1</th>
<th>9-13 M1</th>
<th>S8 M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subesophageal mucus cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Endocervical mucus cells</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Submucous glands of broncheal epithelium</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
</tbody>
</table>

* Positive reaction from + to ++++ represents increasing intensity of immunoperoxidase.

Using both monoclonal and polyclonal antibodies we have demonstrated that M1 antigens are normally expressed by epithelium which is embryologically derived from the foregut (mainly from the gastric mucosa and in small amounts from the tracheal epithelium) and from the Müllerian ducts (mainly the mucus cells of prostatic utriculus and in small amounts from the mucus cells of the endocervix). The presence of gastric mucin in the prostate is not surprising, since other gastric antigens such as pepsinogens have already been described in this gland (25) and in tumors of the epithelium derived from the Müllerian ducts (26); moreover, in the human fetal colon, such M1 antigens are mainly expressed during the sixth month of gestation. This observation is at variance with our earlier results (6) but can be easily explained by the fact that our polyclonal rabbit antibodies contained antibodies against the ALeb antigen (19) and, up to now, have characterized M1 antigens as well as ALeb antigens present in the colonic mucosae.

Fig. 2. Comparison of M1 positive glands of fetal colonic mucosa and histologically normal mucosa adjacent to colonic adenocarcinomas stained with the pool of anti-M1 monoclonal antibodies. Fetal colon, sixth month of gestation (A), shows colonic glands containing most M1-positive cells (arrows indicate the M1(-) cells of these glands). The staining of the gland in A are similar to the gland observed in mucosa adjacent to adenocarcinomas (arrows, B, O, M1(-) glands). Fetal colonic mucosa (C), eighth month of gestation, showing positive M1 goblet cells in upper part of the glands (arrows), as observed in mucosa adjacent to adenocarcinoma (arrows, D). × 250.
MABs against oncofetal mucin M1 antigens

of newborns. M1 antigens as well as CEA have an oncofetal behavior which is restricted to a particular organ. The CEA is a normal component of colonic mucosa restricted to the colonic columnar epithelium cell (27) and is not found in normal gastric mucosa (28); however, it can be detected in fetal and cancerous gastric mucosa; likewise, M1 antibodies are normal components of gastric mucosae absent from the normal colon but present in the fetal or precancerous colon. In the same way that CEA is an oncofetal antigen restricted to the stomach, M1 antibodies can be regarded as oncofetal markers restricted to the colon. In addition, the presence of fetal antigens associated with precancerous tissues (7, 9) is a new and interesting observation recently confirmed in the pancreas (29).

Using a pool of 5 monoclonal anti-M1 antibodies, in 80% of cases it is possible to estimate M1 mucus modifications in precancerous colonic mucosae with the same sensitivity as with rabbit polyclonal antibodies; however, in 20% of cases, the score is generally lower than with the rabbit anti-M1 serum. The M1 pattern in the fetal Lieberkühl gland is comparable to that observed in the glands of precancerous colonic mucosae; and some patterns showing glands which contain most of the M1 (+) goblet cells are observed exclusively in precancerous and fetal colon, but are never seen in the 10 m of normal colon studied here.

In fetal, precancerous, and normal gastrointestinal tracts, both monoclonal and polyclonal antibodies showed a comparable staining pattern, with some differences in intensity; nevertheless, in other tissues, anti-M1 MAbs did not show the same reactivity among themselves, nor did they react like polyclonal anti-M1 antibodies. Indeed, an important difference between the 2 kinds of antibodies was the lack of reactivity of anti-M1 monoclonal antibodies to rat gastrointestinal mucosae, thus demonstrating that rabbit antisera contains antibodies against M1 epitopes which are not recognized by any mouse monoclonal antibodies we have thus far obtained; moreover, differences in reactivity were observed for each anti-M1 monoclonal antibody: MAbl 9-13 M1 strongly stained the mucus cells of the subesophageal glands; S8 M1 did so, but only faintly; 2-12 M1 did not stain mucus endocervical cells. Such differences in immunoperoxidase reactivities could be explained by differences in the carbohydrate moieties of the mucins which, in some tissues, may prevent accessibility of the antibody to the M1 epitopes; it is suggested that these 5 MAbs characterize 5 different M1 epitopes. Preliminary results using immunoradiometric inhibition techniques confirm this view.3

In a recent study using rabbit immunization, it was revealed that a polyclonal antiserum against the ovarian mucin of an ALe(a–b+) patient contained 2 kinds of antibodies: (a) antibodies against blood group-related antigens such as the ALe(b) (19), localized on the saccharide moiety of the mucin and consequently considered as "sequential antigens"; and (b) antibodies against M1 antigens, related to the overall tertiary structure of the mucin (30) [as was also observed with small intestinal (31), colonic (32), and endocervical mucin (33)] and, for this reason, referred to as "conformational antigens." Likewise, using mouse immunization, we found antibodies against (a) "sequential antigens," such as blood group-related Lewis and A antigens, which are papain and mercaptoethanol resistant; these are characterized by MAbs 3-3 A and 2-25 LE, respectively; and against (b) "conformational M1 antigens" which are destroyed using papain or mercaptoethanol treatment and even by heating at 65°C for 36 h. The so-called "sequential antigens" are probably repeated on the surface of mucin and then give strong precipitin lines using immunodiffusion, as already described for Lea sialylated mucins (15). In contrast, the pool of 5 anti-M1 MAbs does not give a precipitin line against M1 mucin using immunodiffusion, probably because surface density of M1 epitopes per mucin molecule is weak; however, these anti-M1 MAbs give strong reactions using ELISA or immunoperoxidase because the concentration in M1 mucin is very high in ovarian mucinous fluids and gastric tissues. M1 epitopes may be located either at the end of the saccharide chain or on the peptidic core. The destruction of these epitopes, using papain or mercaptoethanol, lends support to their peptidic nature; however, some of them may also be associated with the saccharide portion; they could implicate antigenic determinants built with sugars which are located at the ends of 2 different saccharide chains near each other in the native protein and which are destroyed when the tertiary structure of the molecule is slightly modified. Biochemical analysis, including glycosidase treatments, is necessary in order to more clearly demonstrate the nature of the M1 epitopes.

Modulation of gastric M1 mucin antigens occurs during colonic carcinogenesis; indeed, M1 antigens are always expressed in the precancerous mucosae in association with intestinal M3 antigens (7), as observed in the fetal colon (6). Such a fetal association, which is not found in small noninvasive carcinomas (7), can be found in 29% of colonic adenocarcinomas (2), although some of them, especially those located in the right colon, expressed M1 antigen pattern differentiation not found in the fetal colon (3).

In conclusion, our immunohistopathological investigations suggest that the mucin modification in the precancerous colon could be explained not only by an alteration in the glycosylation of the saccharide chain (11–14) but also by the reexpression of antigens associated with native gastric mucin; these are characterized by M1 epitopes mainly expressed in the fetal colon during the sixth month of gestation.

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Fig. 3. Comparison of the score of M1 modification obtained by immunoperoxidase in adenomas (left) and in histologically normal mucosa adjacent to colonic adenocarcinomas (right) using a pool of 5 anti-M1 MAbs (M in abscissa) and polyclonal anti-M1 antibodies (P in ordinate).
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