A New Mucin-associated Oncofetal Antigen, a Marker of Early Carcinogenesis in Rat Colon

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ABSTRACT

We report the characterization of an IgG2a monoclonal antibody, (MAb) 660, prepared against rat gastric high molecular weight glycoproteins. By immunoperoxidase staining, MAb 660 reacted only with the mucous cells of surface gastric epithelium and with a few duodenal goblet cells close to the pylorus in normal adult rats. In fetuses, it reacted with intestinal and colonic goblet cells. The adult colon was always negative. The MAb 660 stained 100% (30 of 30) of chemically induced colonic carcinomas and 100% (7 of 7) of duodenal carcinomas. Several weeks before the appearance of tumors, histologically normal glands, then hyperplasia and dysplasia were precociously stained with MAb 660. The tissue distribution was different from that of blood group related antigens and M1 mucinocins. The recognized antigen was not sensitive to neuraminidase treatment.

After electrophoresis in polyacrylamide gel, staining with periodic acid-Schiff reagent and Western blotting showed that the MAb 660 recognized an epitope associated with high molecular weight glycoproteins. This epitope was unaffected by β-mercaptoethanol reduction-peridote treatment and neuraminidase and trypsin digestion. However, trypsin digestion performed after β-mercaptoethanol reduction destroyed the 660 epitope. These data suggest that the antibody could recognize the peptide moiety of the mucin rather than its carbohydrate moiety.

Thus, the new antigen identified by MAb 660 is a mucin-type glycoprotein with an oncofetal behavior in the rat colon and is precociously expressed by precancerous colonic mucosa.

INTRODUCTION

Changes have been observed in mucin-associated antigens during gastrointestinal carcinogenesis (1, 2). Many of these tumor-associated antigens were detected using polyclonal (3, 4) or monoclonal (5–9) antibodies and lectins (10–13); because mucins contain up to 85% carbohydrates arranged in oligosaccharide side chains, these tumor-associated antigens are often carbohydrate determinants, sometimes related to blood group antigens (14–17). Moreover a number of them are oncofetal (6, 9). Mucin-associated carbohydrate side chains, these tumor-associated antigens are often carbohydrate determinants, sometimes related to blood group antigens (14–17).

The aim of the work was to obtain monoclonal antibodies against colonic tumor markers in rat, M1 antigens among others.

We report here the production of a monoclonal antibody, MAb1 660, prepared against HMWG isolated from rat gastric surface epithelium. We describe the tissue distribution of the corresponding epitope by immunoperoxidase reaction in rats using a high molecular weight glycoprotein fraction in 0.1 M potassium phosphate, pH 7.4, with 10−3 M diisopropylfluorophosphate and 5 mM N-ethylmaleimide; after centrifugation at 500 × g, the supernatant was incubated for 30 h at 20°C with 400 units/ml of DNase I from bovine pancreas (Sigma) and 13 units/ml of RNase A from bovine pancreas (Sigma) with 0.01 M magnesium sulfate. After centrifugation, the soluble fraction was chromatographed on Sepharose CL-4B in PBS; the void volume was then rechromatographed on Ultrogel A4.2. The HMWG recovered in the void volume has a gross neutral sugar/protein ratio of 4/1. The M1 antigens were associated with this fraction.

Orcinol was used for neutral sugars dosage and the Coomassie Blue method was used for protein quantitation.

Fusion. BALB/c mice were given 3 i.p. injections of 50 μg of immunizing fraction in complete Freund's adjuvant at 15-day intervals. Three days prior to fusion, the fraction was given i.v. in PBS.

Fourteen fusions were performed according to the method of Buttin et al. (21), using 10⁶ spleen cells from one immunized mouse and 3 × 10⁶ Sp2O myeloma cells (M3x63.Ag 8).

After 1 to 2 weeks of culture, the medium from wells containing growing cells was reacted with paraffin sections of rat surface gastric epithelium using the indirect immunoperoxidase technique. Positive hybridomas were cloned twice by limiting dilution.

To produce ascites of selected clones, mice which had been given injections of pristane (Lab. Aldrich, Paris, France) 8 days before received 1.7 × 10⁶ cells i.p. Purification. Ascitic fluid was centrifuged to eliminate lipids and fibrinogen. The immunoglobulins were purified on a protein A-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). The purification was controlled by electrophoresis on Cellogel strips (Chemetron, Italy). The activity of purified antibodies was checked by a nitrocellulose immunofixation technique (22) and by staining of tissue sections by indirect immunoperoxidase.

Antibody isotype was determined by double immunodiffusion with class and subclass specific antisera (Nordic, The Netherlands; Sigma, St. Louis, MO).

Colon Carcinogenesis

As described previously (18), colonic carcinomas were raised according to the method of Martin et al. (23), using weekly s.c. dimethylhy-
precancerous colon mucosae were obtained by killing 1 or 2 treated rats every week from the beginning of the experiment.

The carcinogen often induced duodenal carcinomas also.

Carcinomas were classified according to Gutmann's classification (24); stage I corresponding to carcinoma in situ; stage II corresponding to submucosa crossing; stage III corresponding to muscular invasion, and stage IV corresponding to metastasis.

Preparation of Tissues

The colons were opened, rinsed, and fixed in 95% ethanol as well as tumors (25); they were then embedded in paraffin and 3-μm-thick serial sections were cut with an R-Jung autocut (Heidelberg). The precancerous colons were also opened and then coiled up into “Swiss rolls” (26) and processed as the tumors. The other organs were studied in the same way.

One section from each sample was stained with hematoxylin/eosin/safranin.

Some sections were obtained from frozen tissues and processed without fixation.

Immunoperoxidase

Immunoperoxidase staining was performed as described previously (18). Briefly, after deparaffinizing, the tissue sections were incubated overnight at 4°C with supernatant of cell cultures. After 3 rinses with PBS, sheep antiserum against mouse IgG (H+L) labeled with peroxidase (Diagnostics Pasteur, Marnes La Coquette, France) was applied at a 1/100 dilution for 1 h; the sections were washed 3 times with PBS and peroxidase activity was revealed using aminothiacearbazol according to the method of Graham et al. (27). Before microscopic examination, cell nuclei were stained with hematein. The specificity of staining was controlled by different absorptions; lyophilized scrapings of rat gastric mucosa (50 mg dry weight for 1 ml of 660 supernatant) completely suppressed rat gastric mucosa and colonic adenocarcinoma staining. On the contrary, absorption with lyophilized rat liver or colon (50 mg dry weight for 1 ml of 660 supernatant) had no effect on gastric mucosa and colonic adenocarcinoma staining.

In some experiments, before incubation with antibody, the sections were preincubated with neuraminidase (1 unit/ml from Vibrio cholerae, Behring Institut, Germany) in 0.05 mol/liter sodium acetate and 154 mol/liter sodium chloride, pH 5.5, for 1 h at 37°C. After neuraminidase treatment, the sections were washed and allowed to incubate with the monoclonal antibody as described above. Several monoclonal antibodies reacting with specific oligosaccharide sequences were used to stain the rat gastrointestinal tract in order to compare their tissue distribution with that of 660: anti-A was raised by Bara (28), anti-B, anti-LewisX, and anti-LewisY were from Chemibiol, Edmonton, Alberta, Canada; anti-sialylated LewisX, i.e., 19-9, was provided by ORIS Industrie, France. Terminal fucose determinants were stained using a specific lectin of Lotus tetragonolobus (Miles) labeled with peroxidase using glutaraldehyde as described previously (19).

Western Blotting

Proteins separated by acrylamide electrophoresis were transferred to nitrocellulose paper by applying 100 mA for 17 h in 0.025 M Tris base, 0.2 M glycine, and 20% (v/v) methanol buffer. The nitrocellulose paper was then incubated for 2 h in 2% ovalbumin to minimize nonspecific adsorption and then incubated for 24 h at 4°C in constant agitation with the 660 ascitic fluid diluted 1/1000 after extensive washings with PBS, 1/250 dilution of sheep antiserum against mouse IgG labeled with peroxidase was applied for 2 h. After extensive washings, the peroxidase was revealed using H2O2 and chloronaphthol.

Enzymatic Digestions of Antigen 660

Neuraminidase. Neuraminidase from V. cholerae (Behringwerke) dissolved in 50 mM sodium acetate, pH 5.5, was added to the HMWG fraction for 1.5 h at 37°C; enzyme concentration was 0.02 unit/mg of protein.

Trypsin. Trypsin type XI (Sigma) in 0.2 M Tris-HCl, pH 8, was added to HMWG and to β-Mercaptoethanol reduced HMWG for 4 h at 37°C; enzyme concentration was 50 μg of trypsin/mg of protein.

β-Mercaptoethanol Reduction

β-Mercaptoethanol (0.2 M) was added to the fraction for 5 h at 37°C; possible contaminating proteases were blocked using 5 mM N-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride. Then 1 mg iodoacetamide was used to alkylate for 16 h in the dark. The reduced fraction was then dialyzed.

Periodate Oxidation. The HMWG fraction was treated for 1 h at 20°C with periodic acid aqueous solution at 0.5, 1, 10, and 40 mM final concentrations.

Binding to Artificial Antigens with Blood Group-related Specificities

The technique used was described by Le Pendu et al. (31). Blood group-related artificial antigens were obtained through the kindness of Dr. R. U. Lemieux and Chemibiol, Ltd. Poly styrene tubes were coated with the artificial antigens diluted to 5 μg/ml in PBS by overnight incubation at room temperature. Bovine serum albumin, 3% in PBS, was used to prevent nonspecific bindings. The 660 ascitic fluid diluted 1/100 was added and incubated for 3 h. Binding was measured by the sequential addition of purified rabbit anti-mouse immunoglobulins and 125I-labeled protein A. Artificial antigens included A, B, H structures of types 1, 2, 3, and 4, LewisX, LewisY, X and Y antigens as well as the type I and type II oligosaccharide precursors.

RESULTS

MAB 660: Characterization, Purification, and Isoype

MAB 660 was selected for its reactivity on mucus cells of the surface epithelium of the rat fundus and antrum; hybridoma cells were then cloned twice.

MAB 660 from ascitic fluid was then purified on protein A-Sepharose CL-4B; electrophoresis on Cellogel strips indicated that purification of IgG was effective. Antibody activity was recovered in this IgG fraction as controlled by indirect immunoperoxidase as well as by the nitrocellulose immunofixation technique.

Immunodiffusion with specific class and subclass antisera indicated that the monoclonal antibody 660 was of IgG2a type.

Immunoperoxidase Localization

Staining of Normal Tissues. In the normal adult rat gastrointestinal tract, the staining was localized in the mucous cells of the surface epithelium of the antrum and fundus and in a few goblet cells of the duodenum near the pylorus. The staining was cytoplasmic. The other parts of the duodenum, as well as
jejunum, ileum, cecum, proximal and distal colon, pancreas, and Brunner's glands, were unstained.

Lung, ovary, liver, spleen, and kidney were also negative.

The use of frozen sections instead of paraffin-embedded ones showed the same pattern for the 660 epitope distribution, indicating that the immunodeterminant does not occur on a glycolipid.

Staining of Fetal Tissues. Colonic mucosa from 19-, 20-, and 21-day-old fetuses showed cytoplasmic staining of numerous goblet cells and also of the lumen; this was true for the proximal and distal parts of the colon. Some small intestinal goblet cells were positive in the cytoplasm and at the apex. Salivary glands were also stained. Faint staining was observed at the surface of a 20-day-old fetal stomach.

Staining of Cancerous Colonic Mucosae. All of the induced colonic carcinomas were stained with MAb 660 and sometimes very strongly (Fig. 1); 23 carcinomas were located in the distal part and 7 in the proximal part of the colon. All stages of invasiveness were positive from carcinomas in situ (11 of stage I) to carcinomas invading the muscular layers and metastasizing (6 of stage IV) and also carcinomas of intermediate stages (6 of stage II and 7 of stage III). Most of the carcinomas were adenocarcinomas. Three of 30 were signet ring cell carcinomas, at least partially, and 1 of 30 was a mucinous carcinoma (Fig. 2). Staining was observed in cancerous cells, signet ring cells, mucus deposits, lumen of glands, and mucinous carcinomas.

Staining was also observed in glands of 10 of 12 transitional mucosae (Fig. 3). Two cases of colonic metastasizing cancer cells invading lymph nodes were stained with MAb 660.

Staining of Duodenal Carcinomas. Seven duodenal adenocarcinomas of stages I, II, and III, localized at 0.1 to 4 cm of the pylorus, were stained with MAb 660. Three transitional mucosae near these adenocarcinomas showed positive glands.

Staining of Precancerous Colonic Mucosae. A gland was considered positive when one goblet cell was positive. Forty-five precancerous colonic mucosae from week 2 to week 34 after the first DMH injection were examined; see Table 1. From
Table 1 660 antigen expression in histologically normal glands, epithelial hyperplasia, and dysplastic glands during rat colon carcinogenesis

<table>
<thead>
<tr>
<th>Wk after the start of DMH treatment</th>
<th>No. of mucosae counted</th>
<th>Mean no. of glands/colonic sections producing 660 antigen</th>
<th>Range of 660 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>2–10</td>
<td>18</td>
<td>6</td>
<td>2–19</td>
</tr>
<tr>
<td>11–20</td>
<td>14</td>
<td>14</td>
<td>3–34</td>
</tr>
<tr>
<td>21–34</td>
<td>13</td>
<td>23</td>
<td>4–41</td>
</tr>
</tbody>
</table>

Fig. 4. Histologically normal colonic mucosa after 10 weeks of DMH injections, showing a gland with cells expressing 660 antigen. Immunoperoxidase. Bar, 10.25 μm.

Neuraminidase treatment of normal gastric mucosa and of some colonic carcinoma sections prior to 660 incubation produced no change in the staining.

Comparison of the 660 Epitope with Specific Oligosaccharidic Sequences

Tissue Distribution of Blood Group-related Antigens and Terminal Fucose Determinants, in Comparison with That of 660 in Normal Adult Gastrointestinal Tract. As can be seen in Table 2, the tissue distribution of 660 staining by immunoperoxidase did not coincide with those of A, B blood group determinants or with Lewis*, Lewis*, Lewis* sialylated, and terminal fucose revealed by the lectin of *L. tetragonolobus* labeled with peroxidase.

Binding to Artificial Antigens. By solid phase radioimmunoassay, the 660 ascitic fluid showed no reactivity with the following blood group-related oligosaccharides: A, B, H structures of type 1, 2, 3, and 4; Lewis*, Lewis*, X and Y antigens; as well as the type I and II oligosaccharide precursors.

Analysis of 660 Antigenicity by SDS-PAGE and Western Blotting

The immunizing fraction used to produce MAb 660, obtained by sequential chromatographies after nucleic acid digestion with inhibition of proteases, was composed of high molecular weight components. These components were analyzed by SDS-PAGE in 5% polyacrylamide gels with 3% stacking gels; two bands...
were visualized using PAS for carbohydrates: a band in the 3% stacking gel with a very high molecular weight; a second band at the top of 5% acrylamide gel with an apparent molecular weight greater than 10^6 (Fig. 6a). Coomassie blue did not stain these components, the Coomassie blue-binding sites being probably masked by carbohydrates in these heavily glycosylated glycoproteins. No other components were stained with Coomassie blue. As shown by Western blot, the two PAS-positive bands contained the 660 antigenicity (Fig. 6b).

Reduction of the fraction by β-mercaptoethanol decreased the molecular weight of the band appearing in the 3% gel; however, the antigenicity was recovered specifically in the band appearing in the 5% gel (Fig. 7).

**Analysis of 660 Antigenicity after Enzymatic Digestions**

**Neuraminidase.** After treatment with neuraminidase, no change was observed. No loss of PAS- or MAb 660-positive bands and no decrease in electrophoretic mobility (Fig. 6) was seen.

**Trypsin and Trypsin after β-Mercaptoethanol Reduction.** The electrophoresis were made on 0.1-cm-thick gels in these experiments; the pattern of migration was the same as that obtained with 0.3-cm-thick gels, except that it gave broader bands in the 5% polyacrylamide gel.

Digestion by trypsin seemed to be without effect on the two PAS-positive bands (Fig. 7a) as well as on silver staining of the fractions (data not shown); the MAb 660 reactivity was still present on these two bands although slightly reduced particularly for the band appearing in the 5% gel (Fig. 7b).

When trypsin digestion was performed on the β-mercaptoethanol-reduced fraction, the two PAS-positive bands were still present but somewhat fainter (Fig. 7a). The reactivity with MAb 660 had completely disappeared (Fig. 7b).

We have verified that this treatment, i.e., β-mercaptoethanol reduction followed by trypsin digestion, did not modify the saccharidic moiety of the HMWG fraction. The reactivity with monoclonal anti-blood group A was unchanged, as evidenced by dot blot (Fig. 8).

**Analysis of 660 Antigenicity after Periodic Acid Oxidation.** It can be seen by dot blot that oxidation with periodic acid has little effect on the 660 antigenicity (Fig. 9). For comparison, reactivity with a monoclonal anti-blood group A specificity decreases regularly from 10 mM to 40 mM periodic acid oxidation.

**DISCUSSION**

We report the distribution of the 660 antigen in the rat gastrointestinal tract tissues, as defined by a monoclonal antibody using the indirect immunoperoxidase technique. In normal adults, 660 antigenicity is associated with gastroduodenal mucous cells. The rest of the intestine as well as other organs

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Table 2  **Tissue distribution of blood group-related antigens and terminal fucose determinants in comparison with that of 660 epitope and M1 and M3C mucosa-associated antigens**

<table>
<thead>
<tr>
<th>Location</th>
<th>A</th>
<th>B</th>
<th>Lewis* sialylated 19-9</th>
<th>Terminal fucose 660</th>
<th>M1 antigens</th>
<th>M3C antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fundus</td>
<td>+ some mucous cells</td>
<td>+ surface</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Antrum</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pancreas</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brunner glands</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Duodenum</td>
<td>goblet cells; few glands</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Jejunum, ileum</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Caecum</td>
<td>-</td>
<td>enterocytes +</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>goblet cells; +++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distal colon</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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**Fig. 6.** Oxidation and enzymatic digestion of HMWG. Fractions (5 μg of proteins/lane) were separated by SDS-PAGE on a 5% polyacrylamide gel (12 cm long and 0.1 cm thick) with a 3% stacking gel followed by PAS staining (a) or Western blotting procedures (b). Lane 1, HMWG; Lane 3, HMWG after neuraminidase; Lane 4, HMWG after trypsin digestion.

**Fig. 7.** Reduction and trypsin digestion of HMWG. Fractions (8 μg of proteins/lane) were separated by SDS-PAGE on a 5% polyacrylamide gel (12 cm long and 0.1 cm thick) with a 3% stacking gel followed by PAS staining (a) or Western blotting procedures (b). Lane 1, HMWG; Lane 3, HMWG after β-mercaptoethanol reduction; Lane 3, HMWG after trypsin digestion; Lane 4, HMWG after β-mercaptoethanol reduction followed by trypsin digestion.
The 660 epitope, an oncofetal marker in the colon, is frequently expressed in precancerous colonic mucosa, can also produce the 660 epitope.

Thus the 660 epitope, an oncofetal marker in the colon present in all colonic carcinomas tested and precociously present in precancerous colonic mucosa, can be considered as a marker of goblet cell differentiation, associated with early modifications occurring in colonic mucosa subjected to chemical carcinogenesis.

We wondered whether the 660 epitope could be associated with a mucin and if it could have been a blood group oligosaccharide. The fraction used to produce MAb 660 corresponded to heavily glycosylated high molecular weight proteins like mucins (32). In addition, the tissue distribution (salivary glands, stomach) and the cytoplasmic staining of mucous and goblet cells of these tissues by MAb 660 provided another argument for the association of the epitope with mucins. We could, indeed, demonstrate by Western blotting experiments that the 660 antigeneity is linked to very high molecular weight carbohydrate-rich components with a pattern of electrophoretic migration, PAS reactivity, and absence of Coomassie blue staining similar to those described for mucins (33).

Many mucin antigenic markers are related to blood group antigens; moreover, the importance of these antigens as onco-developmental markers has been emphasized (6). However, according to its tissue distribution and reactivity, 660 antigen is different from several blood group antigens, A, B, Lewis', Lewis', sialylated Lewis', and also terminal fucose determinant. Neuraminidase digestion does not modify the immunoperoxidase staining or the electrophoretic migration and immunoblot reactivity, indicating that O-2-3-linked sialic acid is not involved in 660 antigenic epitope; we cannot exclude, however, O-a-cetylated sialic acid. Oxidation with periodic acid alters the epitope very little. The preservation of MAb 660 reactivity after 6-mercaptoethanol reduction indicates that the epitope is not conformational. The 660 epitope is also destroyed by trypsin digestion but only after 6-mercaptoethanol reduction, which indicates that the epitope is masked in the native glycoprotein. This trypsin treatment had no damaging effect on the saccharide part of the HMWG as shown following blood group A activity as control. These various data suggest a peptidic nature of the epitope although more precise biochemical data are required to ascertain this point.

The 660 epitope has numerous analogies with previously described M1 antigens (18). However, the 660 epitope and the M1 antigens are not identical: 100% of colonic carcinomas expressed 660 epitope instead of only 68% for M1 antigens (19); on serial sections, the immunoperoxidase staining for both antigens did not systematically correspond to the same glands in precancerous mucosa or to the same area in a given tumor. The most important difference is the behavior in humans: the 660 epitope is absent of human surface gastric epithelium; it is indeed, demonstrate by Western blotting experiments that the 660 antigeneity is linked to very high molecular weight carbohydrate-rich components with a pattern of electrophoretic migration, PAS reactivity, and absence of Coomassie blue staining similar to those described for mucins (33).

Thus the 660 epitope, an oncofetal marker in the colon present in all colonic carcinomas tested and precociously present in precancerous colonic mucosa, can also produce the 660 epitope.
marker present in precancerous mucosa. The MAb 660 should enable further characterization and purification of this mucin-like antigen.

ACKNOWLEDGMENTS

We would like to thank P. Mouradian for her excellent technical assistance and P. Echinard-Garin and all the persons taking care of the animals. We also thank D. Chardaire for typing the manuscript.

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