ABSTRACT

The distribution of colonic mucoprotein antigen in various types of cancer was examined to determine whether the antigen might have a role as a tissue marker. An antiserum prepared against normal colon-derived colonic mucoprotein antigen and appropriately absorbed with mucinous ovarian cystadenocarcinoma fluid had reactivity limited to the colon and cecum when tested by immunoperoxidase against various normal adult tissue specimens. Among colon carcinomas, 60% of the tumors were stained, and a correlation with tumor differentiation was noted: seven of seven tumors were well differentiated; 21 of 35 tumors were moderately differentiated; and one of seven poorly differentiated tumors expressed the determinant. Of note was the absence of staining with gastric, pancreatic, lung, and endometrial mucin-producing carcinomas. Only one of five mucinous ovarian cystadenocarcinomas was positive for colonic mucoprotein antigen. The potential role of the mucin as a marker for colon carcinoma is discussed.

INTRODUCTION

In recent years, there has been a great deal of interest in the search for tumor-specific antigens and the development of immunological assays for the evaluation of the cancer patient. Unfortunately, although the literature is replete with proposed tumor antigens, true tumor-specific antigens remain elusive (3, 7). Thus far, most investigators have concentrated on the identification of tumor-specific antigens as opposed to the normal adult tissue components. A true tissue-specific antigen having an altered structure and/or distribution as a consequence of neoplasia might, however, prove more useful as a marker for specific forms of cancer.

Our laboratory has been interested in the analysis of organ-specific antigens of the colon. Previous studies have shown that a CMA2 can be obtained in an immunologically pure state by phenol-water extraction followed by ethanol precipitation and molecular sieve chromatography on agarose (4). The antigen, a high-molecular-weight mucin-type glycoprotein, was shown to be present within goblet cells and as a secreted product overlying the mucosal surface. By precipitin and immunohistochemical procedures, we were able to demonstrate that the mucin contained at least one determinant specific for the colon (5). This determinant was distinct from CEA and the blood group ABH and Lewis determinants. The present study was undertaken to examine the distribution of the antigen in neoplastic tissues as a step towards determining the usefulness of CMA as a marker for colon carcinoma.

MATERIALS AND METHODS

Tissues. Surgical and autopsy specimens were fixed with 10% buffered formalin prior to being embedded in paraffin. Pathology reports for each of the tissue specimens were kindly provided by the physicians of the Department of Pathology at the University Hospital.

Antisera. Rabbit anti-CMA antiserum was prepared as described previously (4). Absorption of the antiserum was performed as follows: the antiserum was heated to 56° for 45 min and then cooled on ice. One-tenth volume of packed human type AB RBC was added, and the suspension was kept on ice for 30 min. The suspension was then centrifuged at 2500 × g for 10 min, the supernatant was collected, and the absorption was repeated until no further agglutination occurred. The antiserum was then passed through an affinity column that was prepared by coupling 1 ml of normal human serum per 5 ml of Affi-gel 10 (Bio-Rad Laboratories, Rockville Centre, N. Y.). The antiserum was then absorbed with ovarian cystadenocarcinoma fluid. Cyst fluid was lyophilized and redissolved to 10 mg/ml in PBS. Four ml of 10 mg/ml cyst fluid were added to 1 ml of antiserum, and the mixture was incubated for 2 hr at 37° and then overnight at 4°. Before use, the precipitate was removed by centrifugation.

As controls for antigen specificity, aliquots of the antiserum (0.5 ml) were individually absorbed with CMA (200 μg) that was purified from each of the following: 2 sources of normal colon; one normal stomach; one primary colon carcinoma (moderately differentiated); and HCT-120, a xenografted colon carcinoma kindly provided by Dr. Daniel Dexter of Brown University. The antiserum:antigen mixtures were incubated at 37° for 2 hr and then overnight at 4°. Before use, the precipitate was removed by centrifugation.

Immunodiffusion and Immunoelectrophoresis. Immunodiffusion studies were performed by the method of Ouchterlony (14). Immunoelectrophoresis was carried out according to the procedure of Scheidegger (19). Standard low-molecular-weight agarose (Bio-Rad) was used at a concentration of 1% in 0.016 M sodium barbital (pH 8.8):0.014 M sodium acetate buffer. Antigen concentration ranged from 0.5 to 5.0 mg/ml (dry weight).

Immunoperoxidase. Tissue sections (4 μm) were placed on slides coated with gum arabic and dried overnight in an oven at 42°. After deparaffinizing the sections with xylene (2 changes for 10 min each) and hydrating the sections (absolute ethanol, 2 changes for 5 min each; 95% ethanol, 5 min; 70% ethanol, 5 min; PBS, 10 min), the endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol.

1 Supported by NIH Contract No1-CB-84259.
2 The abbreviations used are: CMA, colonic mucoprotein antigen; CEA, carcinoembryonic antigen; anti-CMA, anti-colonic mucoprotein antigen; PBS, phosphate-buffered saline (0.05 μ NaHPO4, in 0.15 μ NaCl, pH 7.4).
3 Received December 3, 1979; accepted November 5, 1980.
for 30 min. The tissue sections were then washed with PBS (3 changes for 5 min each), covered with 0.01% trypsin (DPCC treated; Sigma Chemical Co., St. Louis, Mo.) in 0.01 M Tris-HCl (pH 8.0) containing 0.01 M calcium chloride, and incubated for 1.5 hr at 37° in a moist chamber. The slides were washed free of trypsin with PBS (3 changes for 5 min each). Nonspecific background staining was then suppressed by coating the tissue section with 10% normal swine serum (diluted in PBS) for 20 min at room temperature. The suppressor serum was shaken off, the primary antiserum was applied, and the sections were incubated for 30 min at room temperature in a moist chamber. The primary serum was washed off with PBS (3 changes for 5 min each), peroxidase-conjugated swine anti-rabbit IgG (DAKO; Accurate Chemical, Hicksville, N.Y.) was applied, and the sections were incubated at room temperature for 30 min in a moist chamber. After washing off the secondary antiserum with PBS (3 changes for 5 min each), the tissue sections were covered with 0.01 M Tris-HCl (pH 7.6) for 20 min. The diamobenzidine reagent was prepared just prior to use by the dissolution of 1 mg 3,3'-diaminobenzidine tetrahydrochloride in 2 ml of 0.01 M Tris-HCl, pH 7.6, with the addition of 25 µl of 30% hydrogen peroxide. The tissue sections were incubated with this reagent for 10 min in the dark, washed in PBS (2 changes for 5 min each), and either mounted directly or lightly counterstained with hematoxylin.

Primary antisera dilutions were deliberately kept low so as to be able to detect small quantities of contaminating antibody-antigen reactions. The final dilutions used were as follows: rabbit anti-CMA semipurified by passage through normal human serum affinity column, 1:20; rabbit anti-CMA absorbed with ovarian cystadenocarcinoma fluid, 1:5; normal rabbit serum, 1:10; and normal rabbit serum absorbed with ovarian cystadenocarcinoma fluid, 1:2. The secondary antiserum was used routinely at a dilution of 1:20.

RESULTS

Prior to our analysis of neoplastic tissues, the organ specificity of the rabbit anti-CMA antiserum was examined by an immunoperoxidase procedure with the following normal adult tissues as substrates: salivary gland; esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; gall bladder; pancreas; bronchus; trachea; lung; bladder; liver; heart; spleen; kidney; endometrium; ovary; and cervix. Antiserum, which had been absorbed with type AB RBC and subsequently passed through an affinity column prepared by coupling normal human serum to agarose, was found to react not only with the colon but also with other mucin-producing organs as well. At this point, the antiserum was made colon specific by absorption with ileocecal mucin as described previously (5). In subsequent analyses, however, it was noted that the ileocecal mucin-absorbed anti-CMA, although colon specific, reacted with mucinous-type ovarian cystadenocarcinomas. An effort was made to inhibit the ovarian cancer-associated reactivity by absorption of the antiserum with ovarian cystadenocarcinoma fluids (4 cases, each lyophilized and redissolved to 10 mg/ml of PBS) as well as cyst tissue homogenates (2 cases, each homogenized 1 g of wet tissue per 2 ml of PBS). Only 2 of the 4 cyst fluids examined were capable of inhibiting ovarian cancer-associated reactivity, whereas neither of the cyst tissue homogenates inhibited the reactivity. Further, it was noted that, by absorption of the normal human serum affinity-purified antiserum with the ovarian cystadenocarcinoma fluid, the antiserum had reactivity restricted to the colon and cecum. By immunodiffusion, a single arc was observed when the antiserum was reacted with the homologous antigen (Fig. 1). No precipitin arcs were observed with normal human serum, purified gastric mucin, CEA (kindly provided by Dr. J. Primus of the University of Kentucky), and colon-specific antigen-p (kindly provided by Dr. D. Goldenberg of the University of Kentucky). Because of these results, all further analyses utilized antiserum absorbed with ovarian cyst fluid rather than ileocecal mucin. The immunoperoxidase reaction obtained with normal colon is shown in Fig. 2. Mucin droplets within goblet cells were stained.

Of 49 colon carcinoma specimens examined, 29 were found to have detectable antigen by this procedure. A correlation of staining with histological grade of tumor (Table 1) demonstrated that all well-differentiated tumors and 60% of the moderately differentiated tumors contained the antigen. Poorly differentiated tumors, on the other hand, except for one did not stain with the anti-CMA antiserum. No correlation was noted between tumor staining and Dukes' classification, nor with geographical location of the tumor within the colon.

In many of the well- and moderately differentiated tumors, a heavy stain associated with cell debris was noted within the gland lumen. Although inflammatory cells were present in this area, the normal rabbit serum control tissue section proved to be negative for endogenous peroxidase activity. The predominant staining pattern in the colon cancers was mucin droplets within the cytoplasm. The well-differentiated tumors were characterized by typical goblet cells in which the staining pattern differed little from the normal colon. Within the moderately differentiated tumor group, a range of staining patterns was displayed. A number of tumors were seen in which the cellular organelles appeared to maintain a somewhat orderly orientation; the cells were tall and columnar with basal nuclei. No typical goblet cells could be discerned (Fig. 3). Upon staining with anti-CMA antiserum, abnormal apical mucin droplets and extrusion of mucin into the gland lumen were evidenced (Figs.

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Table 1

<table>
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<th>Histological grade of differentiation</th>
<th>Total</th>
<th>Well</th>
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<th>Poor</th>
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<tr>
<td>Positive</td>
<td>29</td>
<td>7</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>20</td>
<td>0</td>
<td>14</td>
<td>6</td>
</tr>
</tbody>
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Fig. 1. Immunodiffusion pattern showing reaction of ovarian cyst fluid-ab sorbed anti-CMA (Well 1) with the homologous CMA (Well 2). No reaction was observed between the antiserum and gastric mucin (Well 3).
Another group of tumors, exhibiting less cellular orientation, displayed what appeared to be pools of mucin within the cytoplasm of scattered cells (Figs. 6 to 8). A small number of tumors (4 moderately differentiated and one poorly differentiated) exhibited a diffuse, granular cytoplasmic stain (Fig. 9). No staining was evident in any of the tumors when normal rabbit serum absorbed with ovarian cyst fluid was used as the primary antiserum. In addition, absorption of the antiserum with mucin preparations from 2 normal colons, one primary colon carcinoma, and one xenografted colon carcinoma completely inhibited the observed staining reactions. Absorption of the antiserum with gastric mucin had no effect upon the observed reactions.

In Table 2, we have listed the results from staining tissues other than colon carcinoma. Within the nonmalignant colon tissue group, all tissues gave positive reactions. It is noteworthy that, in all cases examined, “normal” tissue directly adjacent to the colon carcinomas was always positive despite some of the abnormally appearing gland structures. Adenomas being particularly abundant in goblet cells gave strong positive reactions. Also of interest was that, in inflammatory bowel disease, edges of the溃疡ous lesions appeared to stain quite heavily for CMA. Extruded mucin overlying the lesions was also evident.

Within the gastrointestinal cancer group, none of the 14 gastric adenocarcinomas (3 well, 7 moderate, and 4 poorly differentiated) was positive for CMA, and neither were the pancreatic or esophageal tumors. Of the 3 cecal tumors examined, one gave a positive reaction similar to that observed for the colon cancer depicted in Fig. 6, although the number of such positive cells was considerably lower. Non-gastrointestinal cancers that were examined proved to be negative for CMA except for one mucinous ovarian cystadenocarcinoma. This particular tumor, a papillary carcinoma, contained no discernible goblet cells; however, it gave a faint positive reaction morphologically similar to that seen with the colon carcinoma depicted in Fig. 4. Four other mucinous-type ovarian cystadenocarcinomas (including 2 well-differentiated papillary tumors each having an abundance of goblet cells) and an ovarian mucinous cystadenoma were negative for CMA.

**DISCUSSION**

In 1965, Gold and Freedman (6) described a perchlorate-soluble membrane glycoprotein, the CEA, as a gastrointestinal system-specific tumor antigen. Studies with CEA stimulated considerable interest in this area, and many claims were made for the identification of tumor-associated antigens. To date, however, neither CEA nor any other tumor antigen has withstood rigorous scrutiny as being able to discriminate completely between normal and neoplastic tissue (3, 7). In addition, none of the antigens reported to be associated with colon carcinoma (2, 13, 16, 18, 20, 21) has been shown to be completely restricted to colonic tissue. On the basis of this, we have turned our attention to the identification of organ-specific antigens of the normal adult colon and an examination of the fate of these antigens during oncogenesis.

Previous studies have established the organ specificity of the CMA (5). An antiserum prepared against CMA, when appropriately absorbed, had reactivity limited to the colon. In the present study, we observed that the antiserum was reactive with 60% of the colon carcinomas examined and that, with the exception of one cecal adenocarcinoma and one mucinous cystadenocarcinoma of the ovary, the antiserum was not reactive with noncolonic neoplasms. In addition, the presence of CMA within colon carcinomas appeared to correlate with morphological differentiation of the tumor; 100% of the well-differentiated tumors, 60% of the moderately differentiated tumors, and only 15% (one of 7) of the poorly differentiated tumors expressed the antigenic determinant.

A variety of staining patterns could be discerned, which probably were dependent upon the ability of the cells to sequester mucin within membrane-bound granules. Most of the tumors exhibited a heavy granular stain that was associated with cellular debris within the glandular lumen. This was consistent with ultrastructural studies which have found that cytoplasmic organelles extruded into the lumen apparently in an apocrine secretory fashion (9). Well-differentiated tumors exhibited a homogenous staining of mucin granules in the apical region of the cell. As cellular structure became disoriented, as in the moderately differentiated tumors, pleomorphic mucin granules were noted, and, with further disorientation of cellular features, sequestering of mucin appears not to have occurred, giving rise to pools of mucin within the cytoplasm.

Of the noncolonic neoplasms examined in the present study, it was noteworthy that mucin-producing tumors of the stomach, pancreas, lung, and endometrium were not stained by the specific antisera. Only 2 noncolonic tumors gave positive staining reactions, a cecal adenocarcinoma and a mucinous cystadenocarcinoma of the ovary. That the former was positive was not surprising considering that the antisera used in this study was reactive with normal cecum. The cross-reactivity between colon and mucinous ovarian cystadenocarcinomas, however, deserves attention. Anti-CMA antisera absorbed with ileocecal mucin had reactivity restricted to the colon but was found to be reactive with mucinous-type ovarian cystad-
enocarcinomas. After absorption of the antiserum with cyst fluid, reactivity was abolished with 4 of the ovarian tumors and was considerably diminished with the remaining case. That one case remained positive may be a consequence of the particular cyst fluid used in the absorption procedure. We have noted that not all ovarian tumor fluids and/or extracts were capable of removing the observed reactivity, and it would have been ideal to attempt absorption of the antiserum with cyst fluid obtained from the remaining positive case. Unfortunately, this cyst fluid was not available to us, and we must await further study of this aspect before reaching a conclusion regarding CMA and ovarian tumor cross-reactivity.

Several investigators have described antigens associated with normal colonic tissue. With immunohistochemical techniques, Nairn et al. (10) reported a gastrointestinal-specific antigen with properties similar to CMA. The antigen, an acid mucopolysaccharide with a low isoelectric point, was present in goblet cells and as a secreted product in the stomach and intestinal tissue as well as one of the 2 mucoid gastric carcinomas were positive in the immunofluorescent reaction. That these results contrast with those reported in the present study may be due to differences in the preparation of antiserum. It is of interest that this group has also noted a cross-reactivity between colon and ovarian tumors (12). Their antiserum was reactive with ovarian mucinous cystadenoma and cystadenocarcinoma. After absorption of the antiserum with a cystadenoma tissue extract, the staining reaction with the ovarian tumors was abolished, whereas the reaction with the colon although diminished was still positive.

Tappeiner et al. (21) have described also an antigen associated with normal colonic goblet cells and secreted mucin. This antigen was found in both normal and neoplastic colon; however, no attempt was made to characterize the organ specificity of the antigen. Broberger and Perllman (1) suggested that a colon-specific antigen, present in a crude phenol-water extract of normal colon, was responsible for the autoimmune phenomena associated with inflammatory bowel disease. Lagercrantz et al. (8) examined the incidence of antibodies to this antigen and found high-titered sera in individuals with inflammatory bowel disease, whereas individuals with colon cancer had antibody titers similar to healthy adults. Zweibaum et al. (22) have identified a polymorphic determinant system in colonic secretions (WZ) having no correlation with blood group ABH and Lewis determinants. In a study of colon carcinomas, the WZ determinants were found only in those tumors secreting mucin (17). Finally, Pant et al. (15) have recently described a colon-specific antigen (CSA) which by hemagglutination inhibition analyses was present in 18 of 20 colon carcinomas as well as 2 of 2 gastric carcinomas and 3 of 5 mucinous cystadeno- carcinomas of the ovary.

The evidence suggests that CMA may prove useful as an organ-specific marker of neoplasia. The presence of CMA, as detected by immunohistochemical methods, could be of value in the diagnosis of metastases of unknown origin. Additionally, the potential for detection of antigen in the serum of patients with colon cancer and/or colon disease in general deserves attention. Studies in our laboratory are aimed at the development of a radioimmunoassay, which we hope will enable us to define more clearly the tissue specificity and role the antigen might have in cancer medicine. Finally, it should be noted that in the current study the antiserum was prepared with CMA derived from normal colonic tissue. The potential identification of a tumor-specific determinant by an antiserum prepared with tumor-derived CMA, as has been described in a rat colon carcinoma model, is intriguing.

ACKNOWLEDGMENTS

The excellent technical assistance of T. Kreader, D. Nelson, and M. Southgate in certain aspects of this study is gratefully acknowledged.

REFERENCES

Fig. 2. Normal colon showing immunoperoxidase staining of CMA within goblet cells. × 150.

Fig. 3. Moderately differentiated colon carcinoma showing absence of staining when normal rabbit serum absorbed with ovarian cystadenocarcinoma fluid was utilized as the primary antiserum. The section was counterstained lightly with hematoxylin. No goblet cells are discernible. × 150.

Fig. 4. Same tumor as that shown in Fig. 3, stained with ovarian cyst fluid-absorbed anti-CMA antiserum as the primary antiserum and counterstained lightly with hematoxylin. Pleomorphic mucin granules are evident in the apical region of the cells as well as mucin extruded into the lumen of the gland. × 150.

Fig. 5. Moderately differentiated colon carcinoma stained with ovarian cyst fluid anti-CMA antiserum and lightly counterstained. Individual mucin granules are evident within the apical region of the cytoplasm of the glandular cells. × 300.

Fig. 6. Moderately differentiated colon carcinoma stained with ovarian cyst fluid-absorbed anti-CMA antiserum (uncounterstained). Pools of cytoplasmic mucin are stained. × 300.

Fig. 7. Same tumor as in Fig. 6, stained with ovarian cyst fluid-absorbed anti-CMA and lightly counterstained to provide cellular orientation. Pools of cytoplasmic mucin staining are evident. × 300.

Fig. 8. Moderately differentiated colon carcinoma stained with ovarian cyst fluid-absorbed anti-CMA and lightly counterstained. Scattered cytoplasmic pools of mucin are stained by the antiserum. × 150.

Fig. 9. Poorly differentiated colon carcinoma, stained with ovarian cyst fluid-absorbed anti-CMA and lightly counterstained, shows granular cytoplasmic staining of pleomorphic cells. × 300.
Immunoperoxidase Localization of Colonic Mucoprotein Antigen in Neoplastic Tissues

David V. Gold

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