Clinical Evaluation of M43: A Novel Cancer-associated Mucin Epitope

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ABSTRACT

A monoclonal antibody to colon carcinoma mucin was found to react with a colon carcinoma-associated carbohydrate epitope. This antibody was used to develop a quantitative solid phase immunoassay, M43. We prospectively and retrospectively evaluated the assay in patients with and without gastrointestinal carcinoma and compared the sensitivity and specificity with that of carcinoembryonic antigen (CEA) and CA 19-9. One hundred ninety-two patients (181 with no evidence of malignancy) referred for upper or lower gastrointestinal endoscopy were prospectively studied. Sera from 172 patients with histologically confirmed gastrointestinal adenocarcinoma were retrospectively studied. Optimal discrimination cutoffs for M43 (5 units/ml), CEA (5 ng/ml), and CA 19-9 (30 units/ml) were determined by receiver operating characteristic curves analysis. M43 was positive in 112 of 151 patients with colorectal carcinoma (sensitivity 74%) and was negative in 167 of 181 patients without carcinoma (specificity 92%). Sensitivity and specificity were 77% and 93% for CEA and 60% and 83% for CA 19-9. Sixty-four percent of 73 patients with colorectal carcinoma limited to the bowel wall had a positive M43 with a mean value of 178 units/ml. Eighty-one percent of 27 patients with nonhepatic metastasis had a positive M43 with a mean value of 223 units/ml. Eighty-four percent of 51 patients with hepatic metastasis had a positive M43 assay with a mean value of 2532 units/ml. Sensitivity in these three groups was 67%, 82%, and 82%, respectively, for CEA and 43%, 68%, and 79%, respectively, for CA 19-9. Of 38 carcinoma patients with a negative CEA, 45% had a positive M43. No correlation between the levels of M43 and CEA in patients with colorectal carcinoma was found. We conclude that M43 is positive in most patients with colorectal carcinoma, even in early stages. As a diagnostic test, its sensitivity and specificity are equivalent to those of CEA. However, the M43 assay is measuring a tumor antigen which is fundamentally different from CEA and which is present in a high percentage of CEA-negative patients.

INTRODUCTION

Many malignancies release a variety of enzymes, hormones, and other tumor-specific products into the circulation. Some of these compounds have been used in patients with cancer as "tumor markers" for determining diagnosis, prognosis, and response to treatment. Cancer of the colon is one of the most frequent causes of cancer among adults in Western countries. Therefore, vigorous attempts have been made to develop sensitive and specific blood tests for colon cancer (1, 2). The most extensively studied colon cancer-associated antigen is CEA (3, 4).

The introduction of monoclonal antibody and enzyme immunoassay technology has resulted in the identification of new tumor markers and the development of new blood tests for monitoring cancer patients (5). Several useful tumor markers are epitopes (antigenic determinants) of mucins which are high-molecular-weight glycoproteins synthesized by most epithelial tissues and released into the circulation by many carcinomas. Antibodies to epitopes of carcinoma-associated mucins, such as CA 19-9, CA 125, and M26, have been used to develop immunoassays to detect mucins in the sera of patients with pancreatic, ovarian, and breast cancer, respectively (6–8). None of these three mucin immunoassays has proved as useful as CEA in the diagnosis of gastric or colonic cancer (9, 10). A new carcinoma-associated mucin epitope, currently detected by an antibody system called M43, is found in increased amounts in the sera of many patients with gastrointestinal malignancies. The purpose of this study was to characterize the mucin epitope of M43, evaluate the M43 assay in patients with and without gastrointestinal malignancies, and compare it with the CA 19-9 and CEA assays.

PATIENTS AND METHODS

Retrospective Group

Serum was obtained from 172 patients seen at the Methodist Hospital and at the Ben Taub General Hospital in Houston, Texas, between 1982 and 1989, and the specimens were frozen at −20°C until assayed. These patients had a variety of gastrointestinal malignancies including pancreatic carcinoma (n = 12), gastric carcinoma (n = 7), rectal cancer (n = 22), limited (T1-3, NO) colon cancer (n = 55), colon cancer with established nonhepatic metastases (n = 26), and colon cancer with documented liver metastases (n = 50). All of these malignancies were established by histopathology, and sera were obtained before definitive therapy (e.g., surgical resection, radiotherapy, or chemotherapy). Many of the patients were identified because of an elevated CEA test, while others were identified without a knowledge of CEA level.

Prospective Group

Between December 1989 and May 1990, 847 patients were seen in the Gastrointestinal Endoscopy Laboratory at Ben Taub General Hospital in Houston, Texas. Of these, 192 had blood drawn for tumor antigen assays. Patients were selected on the basis of their need for an i.v. line placed for the administration of sedative drugs for endoscopy. After informed consent, 10 ml of blood were drawn through the i.v. line before any drugs were administered. The patients were selected for blood drawing only on the basis of informed consent and requirement for placement of the line, not on the basis of their presumed diagnosis. Serum was separated and stored at −20°C for up to 2 weeks before the assays were performed. Results were obtained and recorded without knowledge of the patients' diagnoses. Serum was drawn from 124 patients undergoing upper endoscopy. The diagnoses based on endoscopy and/or biopsy (without knowledge of assay results) were normal endoscopy (n = 33), normal endoscopy with gastric inflammation on biopsy (n = 15), esophagitis (n = 14), duodenal ulcer (n = 13), esophageal varices (n = 13), hiatal hernia (n = 9), benign gastric ulcer (n = 9), squamous carcinoma of the esophagus (n = 5), benign esophageal stricture (n = 3), Mallory-Weiss tear (n = 2), benign gastric polyp (n = 2), carcinoma of the stomach (n = 2), bezoar (n = 2), Crohn's disease (n = 1), and postoperative gastric carcinoma (n = 1). Sixty-eight patients undergoing colonoscopy had blood drawn. The diagnoses in this group were found to be adenomatous polyps (n = 25), normal colonoscopy (n = 14), ulcerative colitis (n = 6), hemorrhoids (n = 5), diverticulosis (n = 5), hyperplastic polyp (n = 4), adenocarcinoma (n = 3), Crohn's disease (n = 2), lymphoma (n = 1), benign colonic ulcer (n = 1), benign colonic stricture (n = 1), and proctitis (n = 1).

Immunooassays for Tumor Antigens

CA M43: Preparation of Colon Adenocarcinoma-associated Mucin.

Four products were used as sources of mucin with which to immunize mice. The first were samples of malignant pleural effusions from patients with gastrointestinal cancers seen at the Virginia Mason Clinic in Seattle, Washington.
ton. These effusion fluids contained predominantly soluble mucin and were stored at -20°C until used. The second mucin-containing product was from colon adenocarcinoma surgical specimens which were pooled and stored at -70°C. Mucin was extracted with 95% ethanol, 100 mM HCl, phenylmethylsulfonyl fluoride (32 μg/ml), and aprotinin (2 mg/ml), and the mixture was stirred for 16 h at 4°C. Insoluble material was collected by sedimentation at 10,000 × g for 30 min at 4°C and resuspended in H₂O at a concentration of approximately 4 g/ml (w/v), and guanidine-HCl was immediately added to a final concentration of 6 M. The mixture was vortexed vigorously, allowed to stand overnight at 4°C, and then sedimented at 1000 × g for 10 min, and the supernatant was then collected, following removal of the lipid phase. The third mucin product was a more purified form of the second mucin extract mentioned above. This product was further purified with an equilibrium sedimentation in a cesium chloride gradient density gradient. CsCl (source) was added at 0.6 g/ml (original volume), and the density was adjusted to 1.33–1.35 g/ml and centrifuged in a Beckman 50.2 rotor at 40,000 rpm (145,000 × g average) for 60 to 65 h at 21°C. Fractions were dialyzed against H₂O and then assayed by polyacrylamide gel electrophoresis. The fourth mucin product was a further purification of the third product by column chromatography using either a PSK-63000SW or a TSK-6400SW column. Fractions containing molecules larger than M, 200,000 were collected as determined by protein standards under similar conditions.

**Generation and Screening of Monoclonal Antibodies CT43 and CT66.**
Hybridomas producing monoclonal antibodies CT43 and CT66 were developed by immunizing BALB/c mice i.p. with an injection of 200 μl of cesium chloride gradient-purified colorectal adenocarcinoma mucin with 1 mg of silica as adjuvant. Fourteen days later, 140 μl of column chromatography-purified mucin were injected s.c. with 1 mg of silica as adjuvant. Column chromatography-purified mucin was used as the immunogen for subsequent immunizations on days 28 (500 μl), 35 (200 μl), and 84 (200 μl). On day 171 pleural effusion mucin was produced by column chromatography using either a PSK-63000SW or a TSK-6400SW column. Fractions containing molecules larger than M, 200,000 were collected as determined by protein standards under similar conditions. Mucin was run with the glycolipids, and a duplicate chromatogram treated under hexasaccharide ceramide. A positive control of colorectal carcinoma mucin was run with the glycolipids, and a duplicate chromatogram treated under identical conditions but without monoclonal antibody CT43 was run as a negative control.

Synthetic glycopeptides used to screen for CT43 binding contained 10–20 mol of purified oligosaccharide covalently coupled per mol of either human or bovine serum albumin. Three different chemical spacer arms were used to couple the oligosaccharides to proteins (a) p-aminoethyl; (b) aminophenyl-ethyl; and (c) acetyl phenylene diamine. Both p-aminophenyl and aminophenyl-ethyl attach the oligosaccharide to the protein glycosidically, retaining the anionic configuration of the reducing sugar. Acetyl phenylene diamine, however, attaches the oligosaccharides to protein by reduction amination, thereby converting the sugar to an aminoalcohol.

Binding of CT43 and CT66 to immobilized glycolipids and the synthetic glycopeptides was also examined by ELISA. Glycolipids were dried from methanol in microtiter wells by incubation of glycoprotein diluted to 200 mg/ml in PBS (pH 7.4). Purified CT43 was assayed at a concentration of 10 μg/ml in 0.01 M Tris-HCl (pH 7.4) containing 1% BSA and antibodies of 1:100 in the same buffer.

**Isotype Determination**
Nine-six-well plates were coated with column chromatography-purified mucin as described above. Fifty μl of supernatant cultures of the CT43 cell line were added and incubated at room temperature for 1 h. Wells were again washed, and monospecific goat anti-mouse immunoglobulin horseradish peroxidase conjugate (1:2000 in 10% FCS/PBS; Cappel Laboratories) was added to the wells for 30 min at room temperature. Wells were washed. The second monoclonal antibody, CT66, was conjugated with horseradish peroxidase. Conjugate was diluted 1:2 in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M NaCl, and 20% FCS, then 50 μl were added to each well, and the wells were washed. The second monoclonal antibody, CT66, was conjugated with horseradish peroxidase. Conjugate was diluted 1:2 in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M NaCl, and 20% FCS, and 50 μl were added to each well and allowed to incubate for 60 min at room temperature. 3.3'-5.5' Tetramethylbenzidine in citrate/phosphate buffer (pH 6.0) with 0.0015% hydrogen peroxide was added, and after 15 min 1 N sulfuric acid was added.
added to stop the reaction. The absorbance values for positive control, standards, and test samples were determined spectrophotometrically at 450 nm with a microplate reader.

Standard curves were constructed for each test by plotting absorbance value versus concentration for each standard. The antigen concentrations of diluted samples and diluted positive controls were then read from the standard curves.

The assay coefficients of variation at intermediate and high values determined from 10 duplicates were 2.7% and 1.9%, respectively. Between-assay variation determined in 16 separate assays on a specimen with a mean concentration of 10.5 units/ml was 5.0%.

CEA values were determined using Abbott CEA-EIA reagents. Retrospective specimens were assayed with both the EIA polyvalent and the one-step EIA kit. Specimens were assayed for CA 19-9 antigen with the kit developed by Centocor, Inc. (Malvern, PA) and marketed by Amersham.

### Statistics

Sensitivities and specificities for the three assays were calculated using standard definitions: the sensitivity of an assay is the proportion of patients with gastrointestinal malignancy having a positive assay, whereas specificity is the proportion of patients without malignancy (benign conditions) having a negative assay. Optimal cutoffs to separate positive from negative assay results were obtained by ROC analysis (11). Thus, cutoffs obtained from ROC analysis were used to dichotomize CEA, M43, and CA 19-9 test results into positive versus negative. Furthermore, it is both clinically and mathematically reasonable to consider more than two categories of test results: we used the concept of likelihood ratios (12) to define assay test characteristics when three categories of test results are considered: positive, intermediate ("gray zone"), and negative.

Proportions were compared using the χ² test (with Yates’ continuity correction when indicated) or McNemar’s test for matched-pair analysis (13). Student’s t test, paired when indicated, was used to compare means of continuous variables. One-way analysis of variance or the Kruskal-Wallis test was used to compare the distribution of a continuous variable in more than two samples. Pearson correlation coefficients were used to investigate the relationship between two continuous variables (14).

**RESULTS**

**Monoclonal Antibody Isotypes and Epitope Characterization.**

Isotype determination showed that CT43 was of the IgG2b isotype and CT66 was of the IgG1 isotype. The CT43 epitope was determined to be resistant to neuraminidase and protease K and sensitive to periodate. Therefore, the antigenic determinant is unlikely to involve sialic acid residues and does not appear to be protein in nature. Sensitivity to treatment with periodate indicates the antigenic determinant to be carbohydrate in nature. The CT66 epitope was found to be sensitive to neuraminidase and periodate and thus appears to contain sialic acid.

Table 1 lists the glycolipids and synthetic glycoproteins tested. CT43 was not found to bind strongly to any of the glycoconjugates tested. Very weak reactivity was detected to H-type oligosaccharide. CT66 bound strongly to glycoconjugates containing Lea and sialyl Lea.

**M43, CEA, and CA 19-9 Performance.** In the retrospective study of 172 cancer patients, 164 had sufficient sera to measure M43, 172 to measure CEA, and 171 to measure CA 19-9. In the prospective study of 192 patients undergoing gastrointestinal endoscopy, all had assays for M43, CEA, and CA 19-9. Since the prospective study was directed toward the usefulness of the assays to discriminate between adenocarcinoma and benign disease, the five patients with squamous carcinoma of the esophagus and the patient with lymphoma of the colon were excluded. However, all but one of these patients had all three of the assay values within the normal range. Therefore, in the prospective study there were three patients with adenocarcinoma of the colon, two patients with adenocarcinoma of the stomach, and 181 patients with benign disease.

Fig. 1 displays the ROC curves for the three assays. The ROC curves for CEA and M43 are fairly close, covering approximately the same area, whereas the curve for CA 19-9 is substantially lower, covering less area. This indicates that CEA and M43 are comparable in their accuracy as diagnostic tests at comparable levels, whereas CA 19-9 performs at a lower level. By ROC analysis of our data, optimal cutoffs were 5 ng/ml for CEA, 5 units/ml for M43, and 30 units/ml for CA 19-9. Hence, in the computation of sensitivity and specificity of the three assays, we took for upper limits of normal 5 ng/ml for CEA, 5 units/ml for M43, and 30 units/ml for CA 19-9.

Table 2 summarizes the sensitivity and specificity for M43, CEA, and CA 19-9 in various disease categories. The sensitivity of M43 for gastrointestinal adenocarcinoma was 73%, with a specificity of 92%. Sensitivity and specificity for CEA were 77% and 93%, respectively, whereas CA 19-9 performed more poorly, with a sensitivity of 60% and a specificity of 83%. McNemar’s test shows that differences in sensitivity and specificity between CEA and M43 are not statistically significant (P values of 0.28 and 0.66 for sensitivity and specificity, respectively). On the other hand, the superiority of both CEA and M43 over CA 19-9 was highly significant statistically (P = 0.004 and 0.0006 for differences in sensitivity and specificity, respectively, between CEA and CA 19-9; P = 0.007 and 0.001 for differences in sensitivity and specificity, respectively, between M43 and CA 19-9). Clearly, sensitivities for malignancy of all tests increase markedly when only metastatic disease is being considered. CEA and M43 appear to perform at similar levels, and CA 19-9 is 2 less sensitive than either of the two other assays, even in metastatic disease.

### Table 1 Glycoconjugates tested for binding of monoclonal antibody CT43

<table>
<thead>
<tr>
<th>Glycolipids</th>
<th>Glycoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM1</td>
<td>Lacto-N-fucopentose-I-HSA</td>
</tr>
<tr>
<td>GM2</td>
<td>Lacto-N-fucopentose-II-HSA</td>
</tr>
<tr>
<td>GM3</td>
<td>Lacto-N-fucopentose-III-HSA</td>
</tr>
<tr>
<td>GD1b</td>
<td>Lacto-N-difucohexose-I-HSA</td>
</tr>
<tr>
<td>GD3</td>
<td>Maltose-HSA</td>
</tr>
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<td>G</td>
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<td>GT16</td>
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<tr>
<td>QT1b</td>
<td>Lacto-N-neohexose-HSA</td>
</tr>
<tr>
<td>H type 1</td>
<td>Melibiose-HSA</td>
</tr>
<tr>
<td>H type 2</td>
<td>Cellbiose-HSA</td>
</tr>
<tr>
<td>A type 1</td>
<td>A-trisaccharide-HSA</td>
</tr>
<tr>
<td>CDH</td>
<td>B-tetrasaccharide-HSA</td>
</tr>
<tr>
<td>CTH</td>
<td>A-tetrasaccharide-HSA</td>
</tr>
<tr>
<td>Globoside</td>
<td>A-heptasaccharide-HSA</td>
</tr>
<tr>
<td>Forsmann glycolipid</td>
<td>H-type 2-HSA</td>
</tr>
<tr>
<td>Lea-active hexaasaccharide ceramide</td>
<td>A-heptasaccharide-HSA</td>
</tr>
<tr>
<td>Leb-active hexaasaccharide ceramide</td>
<td>Gangliotetraose-HSA</td>
</tr>
<tr>
<td>Sulated Lea-active hexaasaccharide ceramide</td>
<td>T-antigen</td>
</tr>
<tr>
<td>B-active hexaasaccharide</td>
<td>3'-Sialyllactose</td>
</tr>
<tr>
<td>Asialo GM1</td>
<td>6'-Sialyllactose</td>
</tr>
<tr>
<td>Asialo GM2</td>
<td>Sialylacto-N-tetraose</td>
</tr>
<tr>
<td>GD2</td>
<td>Sialylacto-N-tetraose b</td>
</tr>
<tr>
<td>GT7a</td>
<td>Sialylated lacto-N-tetraose</td>
</tr>
<tr>
<td>Man,GlCNac</td>
<td>Chitotriose</td>
</tr>
<tr>
<td>Man,GlCNac</td>
<td>Man,GlcNAc</td>
</tr>
<tr>
<td>Biantennary-ocactasaccharide</td>
<td>Globotriose</td>
</tr>
<tr>
<td>Globotriose</td>
<td>Globotetraose</td>
</tr>
<tr>
<td>Lea-active hexaasaccharide</td>
<td>Sialylated lacto-N-fucopentose</td>
</tr>
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</table>

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In addition to the above, we considered a fourth test category combining CEA with M43. Patients were considered positive if either CEA or M43 was above normal. This combination enhanced sensitivity (CEA alone, 77%; M43 alone, 73%; either CEA or M43, 87%) but reduced specificity (CEA alone, 93%; M43 alone, 92%; either, 87%).

Fig. 2 displays scatterplots of the three assays with juxtaposed box-and-whisker plots. Patients with cancer had higher levels of all assays than patients with benign disease. Because a log scale is used in Fig. 2, the magnitude of the differences between benign and malignant disease is less apparent than it is when comparing the mean values in Table 2. Patients with benign disease had mean values of 3 units/ml, 2 ng/ml, and 23 units/ml for M43, CEA, and CA 19-9, respectively, compared with 1973 units/ml, 758 ng/ml, and 2390 units/ml in patients with malignancy.

Another way of assessing diagnostic test performance is to define three levels of test results for each assay, rather than just dichotomizing into positive versus negative. We sought two cutoff values that would allow for three possible test results for each assay: positive, negative; and intermediate ("gray zone"). We determined these cutoffs so that they would yield a likelihood ratio for the "gray zone" approximately equal to 1, with a small likelihood ratio for a negative test result and a large likelihood ratio for a positive test result. That is, we sought cutoffs such that a "gray zone" assay result would not appreciably change the likelihood of disease once the assay result was known, while maximizing discriminatory power for negative and positive test results. In this fashion, the cutoffs which were most favorable for each assay were 3 and 7 units/ml for M43, 3 and 7 ng/ml for CEA, and 10 and 100 units/ml for CA 19-9. Table 3 summarizes characteristics for the three tests with these cutoffs. It is apparent from the likelihood ratios displayed in Table 3 that the discriminatory capacities for CEA and M43 are similar, whereas CA 19-9, even when cutoffs are chosen to optimize its discriminatory capacity outside the gray zone, does not perform as well.

In order to assess the correlations between the three assays, we computed the Pearson correlation coefficients between M43, CEA, and CA 19-9. CA 19-9 levels correlate with both CEA ($r = 0.52, P < 0.001$) and M43 ($r = 0.15, P = 0.023$). However, there is no statis-

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### Table 2. Comparison of sensitivity and specificity for M43, CEA, CA 19-9, and the combination of M43 or CEA

<table>
<thead>
<tr>
<th></th>
<th>M43</th>
<th>CEA</th>
<th>CA 19-9</th>
<th>M43 or CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTn</td>
<td>%</td>
<td>Mean u/ml</td>
<td>PTn</td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All malignancy</td>
<td>123/169</td>
<td>73%</td>
<td>1973</td>
<td>136/177</td>
</tr>
<tr>
<td>All colorec</td>
<td>112/151</td>
<td>74%</td>
<td>981</td>
<td>120/156</td>
</tr>
<tr>
<td>Colorec</td>
<td>47/73</td>
<td>64%</td>
<td>178</td>
<td>50/75</td>
</tr>
<tr>
<td>Colorec-met</td>
<td>22/37</td>
<td>81%</td>
<td>223</td>
<td>23/28</td>
</tr>
<tr>
<td>Coloreciv met</td>
<td>43/51</td>
<td>84%</td>
<td>2532</td>
<td>47/53</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>7/10</td>
<td>70%</td>
<td>18513</td>
<td>11/12</td>
</tr>
<tr>
<td>Gastric</td>
<td>4/8</td>
<td>50%</td>
<td>24</td>
<td>5/9</td>
</tr>
<tr>
<td>Adenomatous polyps</td>
<td>167/181</td>
<td>92%</td>
<td>3</td>
<td>169/181</td>
</tr>
</tbody>
</table>

* PTn = positive/total.

* Colorec, adenocarcinoma of the colon and/or limited to the intestine; Colorec-met, adenocarcinoma of the colon or rectum with known metastases to lymph nodes and other organs but not the liver; Colorec-liv-met, adenocarcinoma of the colon or rectum with known metastases to the liver; Pancreatic, adenocarcinoma of the pancreas; Gastric, adenocarcinoma of the stomach; All colorec, combined results of Colorec, Colorec-liv-met, and Colorec-met; All malignancy, combined results of all patients with cancer.

* Mean elevated because of one value of 183,250 units/ml.
restricting the analysis to exclusively benign conditions demonstrated a statistically significant correlation with correlation coefficients ranging from 0.26 (CEA with CA 19-9) through 0.89 (M43 with CA 19-9) for the three pairs. This analysis suggests that while all three parameters are correlated on benign conditions, the tumor-related biological products which are being measured may be quite different in the case of CEA and M43, whereas a strong association exists between what is measured by CEA and CA 19-9.

Table 2 also gives mean values for all three assays in cancer patients analyzed according to type of cancer and stage of disease. In all three assays, levels increase with increasing extent of disease. This is shown graphically in Fig. 3 for M43. Mean values of M43 for localized colorectal, nonhepatic, and hepatic metastases were 178, 223, and 2532 units/ml, respectively. The median values of M43 for these three diagnostic categories were 17, 74, and 242 units/ml, respectively. The Kruskal-Wallis test reveals statistical significance at the P = 0.0001 level in the difference between these three distributions.

Within the group of 181 patients with benign disease, there were 25 who had known adenomatous polyps of the colon. The mean values of those with benign polyps compared to those with other benign disease were not statistically different: for M43, 1.7 versus 3.2 (t test: P = 0.4); for CEA, 2.1 versus 2.3 (P = 0.7); for CA 19-9, 14.5 versus 22.8 (P = 0.4). The specificity for each assay among patients with adenomatous polyps indicates that patients with benign colon polyps are no more likely to have positive assays than patients with other benign diseases.

Fourteen of 181 patients with benign disease in the prospective group were found to have an elevated M43 value. A review of the clinical records for up to 1 year after the assays were performed showed no evidence of undiagnosed malignancy. A variety of medical disorders were present in this group, but no obvious common features were found to account for a false positivity of M43 or CEA. Repeat assays were not performed.

One hundred sixty-nine patients had a malignancy with both CEA and M43 positivity recorded. Of these, 63 patients had either CEA < 5 ng/ml and M43 ≥ 5 units/ml (17 patients) or M43 < 5 and CEA ≥ 5 (25 patients) or both assays < 5 (21 patients), i.e., 63 patients had at least one false negative test result. Of the 38 patients with malignancy who had a false negative CEA, 17 (45%) had a positive M43 assay. It is of particular interest to note that of the 23 patients with potentially resectable colorectal cancer and negative CEA, 11 (48%) had a positive M43 level.

Of the 181 patients with benign conditions, 23 had at least CEA, M43, or both falsely elevated; 3 were false positive by both tests, 9 were false positive by CEA but had normal M43, and 11 were false positive by M43 but had normal CEA.

In the prospective group serum was obtained from only three patients with adenocarcinoma of the colon and two patients with adenocarcinoma of the stomach. This is a small number of patients, which makes the conclusions unreliable. However, it does approximate the prevalence of cancer in a routine endoscopy practice. Sensitivity, specificity, and positive predictive values of all three assays using only the patients obtained in the prospective group were: M43, 80%, 92%, and 22%; CEA, 40%, 93%, and 14%; and CA 19-9, 60%, 83%, and 9%, respectively.

DISCUSSION

The development and evaluation of a new tumor marker should be contingent upon the potential uses of tumor markers and the performance of currently available tumor markers. The enthusiasm for studying tumor-associated antigen-antibody systems is based, in part, on the hope that such systems will be useful in the diagnosis and management of cancer. Diagnosis would be enhanced by the development of sensitive and specific blood tests as well as labeled antibody imaging tests. Management would be enhanced if new blood tests or imaging techniques were proved to be of use in staging, prognosis, monitoring response to treatment, and early detection of relapse. In addition, chemotherapeutic agents could be linked to tumor-specific antibody to enhance efficacy and/or reduce systemic toxicity.

CEA is a family of related glycoproteins produced in larger-than-normal quantities by many colon cancers. As a diagnostic blood test, CEA has a published sensitivity of 30–85% (9, 16) and a specificity of 84–98% (3, 4, 16, 17) (normal, <5 ng/ml). In limited disease CEA has been found to be positive in 18–47%, but in metastatic disease sensitivity is in the range of 65–85% (9, 10, 16, 18, 19). The test is negative in most patients with early curable disease. Therefore, CEA is not very useful in the screening of asymptomatic individuals or in the evaluation of patients with suspected colonic malignancy (1–4). In large groups of patients with documented colon cancer, the CEA level correlates with stage of disease and prognosis (9, 16, 20). However, in individual patients the CEA is not helpful in staging or prognosis. The most common clinical use of CEA is in follow-up, after attempted curative resection or chemotherapy to assess efficacy and to monitor for early recurrence (9, 16, 21, 22). However, the cost of such monitoring in conjunction with early second-look operations is high compared to the questionable increase in quality or quantity of life attained (23, 24). Since CEA performs poorly in purely local disease, attempts have been made to image colon cancer with isotope-labeled anti-CEA (25). Whether this technology will overcome some of the deficiencies of the serum CEA assay is not known. Targeting treatment of colon cancer by linking radiotherapeutic (26) and chemotherapeutic (27) compounds to anti-CEA is a new treatment modality approach in the very early stages of development.

In view of the lack of proven impact of the CEA test on colon cancer diagnosis and management, similar evaluations were directed toward CA 19-9 when it was discovered to be “overproduced” in some patients with colon cancers (28, 29). CA 19-9 is an antigen-antibody
Clinical series have confirmed that the CA 19-9 test is rarely elevated, detects 20-53% of patients with recurrence, whereas CEA detects but advanced stages are positive in 29-68% (3, 9, 10, 18, 19). CA 19-9 has been used to monitor recurrence after surgery (17). CA 19-9 is highly sensitive to CA 19-9 as it has been shown to do for CEA (16, 30). The sensitivity of any blood test for pancreatic adenocarcinoma (75%-93%) (22). However, it has consistently been shown to have the highest sensitivity of any blood test for pancreatic adenocarcinoma (75%-93%) (10, 17, 22, 23, 30, 31). A level above 120 units/ml is highly suggestive of pancreatic carcinoma. Like CEA, sensitivity of CA 19-9 in the diagnosis of colorectal cancer is related to clinical stage (19, 30). Patients with Duke’s stage A and B show a positive test in 6-16%, but advanced stages are positive in 29-68% (3, 9, 10, 18, 19). CA 19-9 has been used to monitor recurrence after surgery (17). CA 19-9 detects 20-53% of patients with recurrence, whereas CEA detects 78-90%. With only a few exceptions, CEA has consistently been shown to be more sensitive than CA 19-9 in all aspects of colorectal cancer (9, 10, 18).

The results reported here represent the first clinical evaluation of a new antibody system currently called M43, specific for a colon cancer-associated mucin epitope. The monoclonal antibody providing the specificity is an IgG2b directed against a carbohydrate epitope chemically and immunologically distinct from CEA, CA 19-9, or any previously described mucin epitope. We have demonstrated that patients with gastrointestinal malignancies have much higher levels of M43 than a group of patients without known malignancies (Fig. 2). From ROC analysis of our data, the value of 5 units/ml is the best cutoff for “normal” versus “elevated” M43 values.

Using 5 units/ml as the upper limit of normal, we found that the M43 serum assay was abnormal in 74% of all patients with colorectal malignancies. Even in patients with tumor limited to the bowel wall the assay was elevated in 64% (Table 2). This is higher than the published positivity rate of 18-47% for CEA in patients with Dukes’ A and B colorectal cancer (9, 10, 18, 19). Although the patients with gastrointestinal cancer in our prospective study were few in number, two were positive by M43, while only one was positive by CEA. In our patients with lymph node or hepatic metastases, 81% and 84%, respectively, were positive for M43. This is comparable to the published positivity rate for CEA in similar patients (9, 10, 18, 19). The positivity rate for patients in all stages of colorectal cancer in our series far exceeds that found with CA 19-9 or any other marker for colorectal carcinoma.

Because we performed CEA and CA 19-9 assays as well as the M43 assay in most patients in the retrospective group, we do not have to rely on comparisons with previously published data. However, the conclusions are the same: M43 shows equivalent positivity to CEA and positivity rates superior to those of CA 19-9 (Table 2). However, since many of the patients in the retrospective study were identified by virtue of an elevated CEA, this comparison may make CEA look falsely favorable compared to M43. Further prospective studies should be done to assess the M43 positivity rates, especially in early, potentially resectable colorectal carcinoma. Our data indicate that most patients with colorectal carcinoma, even in early stages, have elevated values of M43.

Our study shows that some tumors “overproduce” M43 and not CEA or CA 19-9. Fifteen of 151 colorectal cancer patients were positive for M43 and negative for CEA. The main clinical use of CEA now is in the clinical follow-up of patients with resected tumors. We found that 11 of 73 patients with potentially resectable colorectal cancer were negative for CEA and positive for M43. These figures alone may be a justification for the new tumor marker, especially when many of our patients were identified based on a positive CEA test. It is known that at least 15% of colon cancers do not produce CEA at all (32).

Only 14 of 146 patients with benign disease had an elevated M43 assay. This is similar to the published specificity of CEA and CA 19-9 (3, 4). The data presented here suggest that the tumor specificity of M43 is equivalent to that of currently available markers. We reviewed the charts of the patients who had an elevated M43 but no obvious malignancy. There were no features common to these patients, but occult carcinoma could not be ruled out.

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**Table 3: Likelihood ratios for M43, CEA, and CA 19-9 when they are trichotomized**

<table>
<thead>
<tr>
<th></th>
<th>Low</th>
<th>High</th>
<th>( LR (+test)^a )</th>
<th>( LR (gray zone)^b )</th>
<th>( LR (-test)^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>M43</td>
<td>3 units/ml</td>
<td>7 units/ml</td>
<td>13.1</td>
<td>1.00</td>
<td>0.15</td>
</tr>
<tr>
<td>CEA</td>
<td>3 units/ml</td>
<td>7 units/ml</td>
<td>13.4</td>
<td>1.06</td>
<td>0.18</td>
</tr>
<tr>
<td>CA 19-9</td>
<td>10 ng/ml</td>
<td>100 ng/ml</td>
<td>12.6</td>
<td>0.71</td>
<td>0.50</td>
</tr>
</tbody>
</table>

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\( LR (+test) = likelihood ratio of positive test result, i.e., M43 \geq 7, CEA \geq 7, CA 19-9 \geq 100.\)

\( LR (gray zone) = likelihood ratio of intermediate test result, i.e., 3 \leq M43 < 7, 3 \leq CEA < 7, 10 \leq CA 19-9 < 100.\)

\( LR (-test) = likelihood ratio of negative test result, i.e., M43 < 3, CEA < 3, CA 19-9 < 10.\)
In conclusion, M43 is a colorectal carcinoma-associated mucin epitope which can be identified in the serum of most patients with colorectal cancer. It is immunologically and biochemically distinct from CEA and is present in some cancer patients in whom CEA is absent. The high prevalence of M43 in colon cancer patients and its specificity justify further prospective studies to determine its usefulness in detecting recurrence after surgery, in follow-up of therapy, and potentially in targeting diagnostic and therapeutic substances.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of John Pang, B.S., and Marie Kolp, P.A.C., and wish to thank Ms. Lina Ricci for her assistance with the preparation of the manuscript.

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