Immunological Heterogeneity of Carcinoembryonic Antigen: Immunohistochemical Detection of Carcinoembryonic Antigen Determinants in Colonic Tumors with Monoclonal Antibodies

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ABSTRACT

The immunoperoxidase localization of carcinoembryonic antigen (CEA) determinants was studied in colon adenocarcinomas using four murine monoclonal antibodies to CEA in a bridged avidin:biotin technique. One of the monoclonal antibodies, NP-1, recognizes a CEA epitope shared with the non-specific cross-reacting antigen and meconium antigen. Two others, NP-2 and NP-3, discriminate two separate CEA epitopes shared with meconium antigen only, whereas NP-4 reacts with a unique determinant expressed on a subpopulation of CEA molecules. The monoclonal antibodies and polyclonal goat antisera against CEA and nonspecific cross-reacting antigen stained columnar epithelial cells in morphologically normal mucosa. Neutrophils were stained by only the NP-1 monoclonal antibody and goat anti-nonspecific cross-reacting antigen antisera. All moderately differentiated colorectal adenocarcinomas and most of their nodal and liver metastases reacted with the goat antiserum and cross-reactive monoclonal antibodies. Thirty percent of these primary tumors and most of the nodal and/or liver metastases from six patients with NP-4-positive primary tumors failed to stain with NP-4. These results suggest heterogeneity in the expression of a CEA variant and/or determinant recognized by the NP-4 monoclonal antibody that perhaps identifies a subgroup of colonic cancers which differ in their functional differentiation.

INTRODUCTION

The immunohistochemical localization of CEA has been widely studied in a variety of epithelial cancers (7, 8, 15, 17). Since it is present to varying degrees in normal and noncancerous diseased tissue, benign tumors, and atypical epithelium of different organs, the detection of this antigen in tissue sections cannot be used to distinguish normal or benign cells from malignant ones. Most studies have not found a correlation between CEA staining of primary tumors and disease stage or prognosis, although measurement of blood antigen level can aid in prognosis and monitoring of therapy (17). Shousha and Lysiosiotis (23) and Shousha et al. (24) found a relationship between positive CEA staining of malignant breast tumors and regional lymph node involvement. It was also observed that patients with CEA-negative primary breast tumors appeared to have a more favorable prognosis. In colon tumors, CEA content increases in the more differentiated types as contrasted to a weak or negative staining of poorly differentiated carcinomas (15, 17). Wiley et al. (26) reported recently an increase in the incidence of metastases from tumors of the transverse and distal colon that were negative for both CEA and blood group antigens, irrespective of tumor histology.

Several studies have described the development of monoclonal antibodies to CEA (1, 11, 12, 21, 22, 25), but their use for the immunohistochemical detection of this antigen in tissue sections has not been explored extensively (1). We characterized recently the specificity of several monoclonal antibodies derived against colon tumor CEA (20). This paper reports the staining of colorectal adenocarcinomas with 4 of these monoclonal antibodies in comparison to conventional goat antisera.

MATERIALS AND METHODS

Antibodies. The preparation of goat anti-CEA and -NCA antisera and the NP-1, 2, -3, and -4 murine monoclonal antibodies to CEA are described in detail elsewhere (17, 18, 20). All antibodies were derived against antigen purified from liver metastases of a colon adenocarcinoma. Before use, the goat anti-CEA antiserum was cross-absorbed with NCA and meconium to render it CEA specific, whereas the goat anti-NCA antisera was absorbed with CEA. The Class I antibody, NP-1, recognizes a CEA determinant shared with the CEA-related antigens, NCA and MA (20). The 2 Class II antibodies, NP-2 and NP-3, distinguish 2 separate epitopes on CEA that are also shared with MA but not NCA. Although it is not possible to determine the relative contribution of CEA and the related antigens to the total staining reaction produced by NP-1, -2, and -3, particularly that due to MA since specific antibodies against this antigen have not been developed, these antibodies were included for comparison to the Class III antibody, NP-4, which reacts with a unique determinant expressed on a subpopulation of CEA molecules. NP-4 binds about 50% of the radiolabeled CEA molecules recognized by the goat anti-CEA antiserum and the NP-1, -2, and -3 monoclonal antibodies (20). The NP-1, -2, and -3 monoclonal antibodies, all of IgG1 (κ) isotype, were purified from tissue culture medium by ion-exchange chromatography (20), whereas ascites fluid was used for NP-4, which has an IgG1 (κ) isotype. Average antibody affinity constants determined in competitive CEA radioimmunoassay by the method of Muller (13) were as follows: goat anti-CEA antiserum, 6.3

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Tissue Processing and Fixation. Tissues were obtained from 23 individuals with colorectal cancer. Of these adenocarcinomas, 3 were in the cecum, 6 in the ascending colon, and 14 in the rectosigmoid. One was poorly differentiated, whereas the remaining were moderately well differentiated. Morphologically normal mucosa adjacent to the tumor and/or at resection margins was available in 21 of these cases. The Dukes' classification (5) was used for clinical staging.

Surgical specimens were routinely fixed in 10% buffered formalin, pH 7.2, while in 6 cases, they were also fixed in EA as described previously (16). Tissues were embedded in paraffin and serially sectioned 5 μm thick. The sections were mounted on gelatinized slides, deparaffinized with xylene, and rehydrated with decreasing ethanol concentrations as finally, PBS.

Immunohistochemical Procedures. A bridged avidin:biotin immunoperoxidase method was used for most of the staining reactions with goat and monoclonal antibodies (9). The basic procedure with goat antibodies consisted of the sequential application of primary goat antibody, biotinylated rabbit anti-goat IgG (50 μg/ml), free avidin (100 μg/ml), and biotinylated horseradish peroxidase (50 μg/ml). Biotinylated reagents and avidin were obtained from Vector Laboratories (Burlingame, Calif.). All reagents were diluted in PBS except avidin, which was diluted in 0.05 M Tris (pH 8.6):0.15 M NaCl. The basic procedure was modified for the detection of antigen with murine monoclonal antibodies by the introduction of an affinity-purified goat anti-mouse IgG (30 μg/ml) step after the initial application of the monoclonal antibody. All immune and biotin:avidin reactions were carried out in a humidified atmosphere for 20 min at 37°C and 30 min at room temperature, respectively. Each step was followed by two 5-min washes in PBS. One section on each slide received the test antibody, while an adjacent section was exposed to a control preparation. The goat anti-CEA and -NCA antisera were used at a 1:600 and 1:100 dilution, respectively. Controls for these antisera consisted of similarly diluted antigen-neutralized CEA antiserum and/or normal goat serum. The monoclonal antibodies were used at 5 μg/ml, and normal mouse IgG (Pel-Freeze, Rogers, Ark.), 50 μg/ml, as well as antigen-neutralized purified monoclonal antibodies and ascites fluid were used for control purposes. Before application of the primary antibody, hydrated sections were incubated in 3% H2O2 in methanol, washed in PBS, and then incubated for 10 min at 37°C with diluted normal rabbit serum. After the biotinylated enzyme step, the histochemical reaction was developed with 0.01% diaminobenzidine and 0.003% H2O2 in 0.05 M Tris-HCl, pH 7.6, at room temperature for 15 min. Immunoperoxidase-stained slides were counterstained lightly with Harris's hematoxylin.

An unlabeled antibody method using glucose oxidase:antiglucose oxidase complexes (4) was used for the detection of NCA in neutrophils. The basic procedure was similar to the bridged avidin:biotin technique, except donkey anti-goat IgG and goat antiglucose oxidase:glucose oxidase complexes were substituted for biotinylated rabbit anti-goat IgG and biotinylated horseradish peroxidase, respectively. The avidin and H2O2 steps were also omitted, and preincubation with undiluted normal horse serum was used instead of normal rabbit serum. The enzymatic disclosing reaction consisted of the following: 6.7 mg β-D-glucose per ml (Calbiochem-Behring Corp., LaJolla, Calif.); 0.67 mg nitroblue tetrazolium per ml (Research Organics, Inc., Cleveland, Ohio); and 0.0167 mg phenazine methosulfate per ml (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M Tris, pH 8.3. The glucose and nitroblue tetrazolium were preheated to 37°C for 1 hr, at which point the phenazine methosulfate and the tissue sections were added. After further incubation for 45 min at 37°C, the slides were washed and counterstained with nuclear Fast red.

The staining intensity of specimens was evaluated at ×125 magnification and was graded as negative, +/−, or +. The +/− designation was used for specimens showing a weak but definite staining, and all tumor specimens were considered positive when greater than 10% of the tumor tissue had a + or + staining intensity.
classified as NP-4 negative gave a weak focal reaction in less than 10% of the tumor glands.

For 6 specimens of primary tumor, sufficient material was available for alternative fixation in EA. The intensity of staining by all the antibodies was enhanced in EA-fixed specimens over that observed following formalin fixation for each case. Cytoplasmic staining of tumor cells was very evident in EA-fixed specimens, whereas following formalin fixation, it was either absent or tended to be weak. This enhancement of staining intensity and cytoplasmic localization is depicted in Fig. 2, A, B, D, and E, which compares staining by the goat anti-CEA antiserum and NP-4 monoclonal antibody of a tumor specimen fixed in formalin or EA. Although the increase in cytoplasmic staining in EA-fixed tissue was not as great with the NP-4 antibody compared to that of the goat antiserum, the overall staining reaction obtained with the former antibody was appreciably greater in the EA-fixed tissue as compared to the formalin-fixed specimen.

Only one case of anaplastic carcinoma was studied, and this was completely negative with all the goat and monoclonal antibodies.

NP-4 Staining of Metastases. Of the 22 cases of moderately differentiated adenocarcinomas examined, 1 of 1, 5 of 7, 3 of 4, and 6 of 10 of the primary tumors from Dukes' Stages A to D, respectively, reacted with NP-4. Although the number of patients is too few to arrive at a precise correlation between NP-4 reactivity and clinical staging, these preliminary results suggest that an absolute relationship will not be established. A relationship was also not apparent between NP-4 reactivity and the location of the tumor in the colon or rectum.

Regional lymph node and/or liver metastases were available for study as formalin-fixed specimens from 8 of the above patients, 2 and 6 cases from Dukes' Stages C and D, respectively. These specimens were stained with the goat anti-CEA and -NCA antibodies and the NP-3 and NP-4 monoclonal antibodies (Table 1). The NP-4 antibody gave only a few positive staining reactions in metastatic tumors even in patients who had over 80% of their primary tumor tissue, as visualized in single sections, staining positive with this antibody. The majority of these metastases did retain reactivity with the goat antibodies and the NP-3 monoclonal antibody. Fig. 3 illustrates the staining characteristics of liver and lymph node metastases from Patient D-6 when reacted with the goat anti-CEA antiserum and the NP-3 and NP-4 monoclonal antibodies. The reaction of this patient's primary tumor with these antibodies is shown in Fig. 2. For the goat anti-CEA antiserum and NP-3 monoclonal antibody, the staining features of both the primary and metastatic tumors were essentially the same, although the NP-3 antibody stained metastases somewhat stronger. The NP-4 monoclonal antibody stained about 50% of the primary tumor tissue from this case, but the metastases were unreactive. Fig. 4 shows a similar relationship in the staining pattern obtained with these antibodies between the primary tumor and a lymph node metastasis from Patient D-7. The remaining 4 nodes that were infiltrated with tumor from the latter patient demonstrated the same staining pattern as the one depicted.

The regional lymph node and/or liver metastases of 2 patients, C-2 and D-3, showed further differences in their staining phenotype (Table 1). Two of the nodes from Patient C-2 were unreactive with all the antibodies, while the third node gave a positive staining reaction with the goat and NP-3 antibodies. Table 1.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>NP-4 positivity of primary tumor (%)</th>
<th>Metastatic site</th>
<th>Antigen positivity in metastases</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Goat antisera</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CEA</td>
</tr>
<tr>
<td>C-2</td>
<td>90</td>
<td>3 nodes</td>
<td>a</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c</td>
</tr>
<tr>
<td>C-3</td>
<td>90</td>
<td>1 node</td>
<td>+</td>
</tr>
<tr>
<td>D-1</td>
<td>90</td>
<td>3 nodes</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>D-3</td>
<td>80</td>
<td>3 nodes</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 nodes</td>
<td>a</td>
</tr>
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<td></td>
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<td></td>
<td>b</td>
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<td>1 node</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Liver</td>
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<tr>
<td>D-7</td>
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<tr>
<td>D-9</td>
<td>Negative</td>
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<td>+</td>
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<tr>
<td>Total positivity of metastases</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>4:5</td>
</tr>
</tbody>
</table>

a Percentage of primary tumor glands staining positive with NP-4.
b Letter designates Dukes' stage; the number specifically identifies individual patients.
c Ratio of number positive to total number tested.
negative reaction with only the NP-4 antibody. One node from Patient D-3 was not stained by any of the antibodies, and, in addition, another node and the liver metastasis failed to stain with the goat anti-CEA antisera and the NP-3 and NP-4 monoclonal antibodies. By contrast, the remaining 4 nodes and the liver metastasis were NCA positive. When these specimens from Patient D-3 were tested against the NCA cross-reactive monoclonal antibody, NP-1, the staining pattern was identical to that obtained with the goat anti-NCA antibody (data not shown).

DISCUSSION

The possibility that the eventual aggressive behavior of morphologically similar tumors can be ascertained by the detection of certain antigenic markers they elaborate has emerged from immunohistochemical studies of some cancer types (3, 19). With other markers, the discrimination of molecular or subtle determinant changes of an antigen might be missed due to the diversity of epitopes inherently recognized by polyclonal antibodies (6). The identification of these molecular changes would be significant if they are indeed linked to a concomitant alteration in the biological expression of the cancer cell. The precise immunospecificity of monoclonal antibodies provides a tool by which one can probe for these antigenic modifications, and, in this study, we have compared the staining characteristics of colonic adenocarcinomas using a polyclonal goat antibody and 4 monoclonal antibodies derived against CEA.

As expected from our previous studies (16), which demonstrated by immunohistochemistry with conventional antisera the presence of CEA in normal colon, this tissue was stained by the 3 cross-reactive monoclonal antibodies. This was also true for the NP-4 antibody which lacks cross-reactivity with NCA and MA but appears to recognize a subpopulation of CEA molecules based on labeled antigen binding (20). The staining by these antibodies appeared to be localized within the same cell as visualized in separate tissue sections reacted with individual antibodies. Like the conventional antisera used in this study but unlike those of some other investigators (10), the monoclonal antibodies failed to stain the mucus of colonic goblet cells. Of the monoclonal antibodies, it was also found that only NP-1 stained neutrophils which likewise reacted with goat anti-NCA antibody, as predicted by the known synthesis of NCA by these cells (2). Thus, immunohistochemical procedures can both confirm the specificity of monoclonal antibodies to CEA determined by other methods (20) and be useful in identifying their NCA cross-reactivity when other methods are not readily available.

With the exception of the NP-4 antibody, the other monoclonal antibodies and the conventional goat antisera stained all of the primary moderately differentiated colonic tumors and most of the regional lymph node and liver metastases examined in this study. A different staining pattern emerged with NP-4 in that about 30% of the primary tumors were unreactive, and most of the metastases arising from NP-4-positive primary tumors did not stain with this antibody. This does not mean that the molecule or determinant recognized by NP-4 is universally deleted from metastatic tumor cells, since this antibody was developed against CEA purified from liver metastases. Conversely, the lower affinity of NP-4 for CEA compared to that of the other antibodies, possibly in conjunction with lower tissue levels of NP-4-reactive CEA determinants, could account for the lack of staining. Increased sensitivity of the NP-4-reactive determinant to alteration or masking as a result of tissue fixation, compared to that of other CEA epitopes, may further contribute to the reduced staining observed with NP-4. Nakayama and Taylor (14) reported recently that 6 of 9 monoclonal antibodies to prostate acid phosphatase stained only frozen, unfixed tissue specimens. We found that the staining intensity obtained with the NP-4 monoclonal antibody was considerably improved in primary tumor specimens fixed in EA rather than formalin. Alternatively, the almost virtual absence of NP-4 staining of metastases in patients with positive primary tumors may suggest a quantitative, if not qualitative, deletion in the expression of its recognized determinant or antigen from metastatic tumor cells or the cloning of antigen-negative tumor cells.

Although we have not as yet compared determinant expression in tissues with that in the circulation, the existence of tissue CEA determinant heterogeneity indicates that potential problems will arise in the correlation between blood levels of this antigen, measured with monoclonal antibodies, and disease activity. Rogers et al. (22) have already reported several instances of discordance in blood CEA levels in their comparison of conventional assays with one using a monoclonal antibody to CEA, providing additional support for the existence of different immunological forms of CEA. We appreciate that several other factors, unrelated to the immunospecificity of the assay used, can contribute to the level of a marker in the blood. On the basis of the immunohistochemical studies presented here, however, we would anticipate divergent assay results and a poor correlation with disease activity, depending upon the relative contribution to circulating antigen by tumor in different locations and the specificity of the monoclonal antibody used in the immunoassay.

While fundamental questions remain to be answered with regard to antibody specificity and the presence of reactive antigen in the tissue and blood, it is clear that immunohistochemistry, which can be conveniently applied to conventional histopathology specimens, will have a major role in distinguishing monoclonal antibodies reactive with CEA variants or determinants that have prognostic and diagnostic value. This also applies to the appropriate selection of monoclonal antibodies for radioimmunoassay and antibody-directed therapy of colonic tumors (1).

REFERENCES


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Fig. 1. Immunohistochemical detection of CEA or related antigens in morphologically normal colonic mucosa (A through F) and neutrophils (G and H). The immunoperoxidase technique was used to test the reactivity of goat anti-CEA antiserum (A), goat anti-NCA antiserum (B), NP-1 antibody (C), NP-3 antibody (D), NP-4 antibody (E), and normal mouse IgG control (F) with normal mucosa. Only the normal mouse IgG control is shown, since the goat antisera controls were identical to it. All sections in this group were counterstained with hematoxylin. The immunoglucose oxidase procedure was used to demonstrate the reactivity of the NP-1 antibody (G) and NP-3 antibody (H) with neutrophils. Only the NP-1 antibody and goat anti-NCA antiserum (not shown) stained neutrophils. Immunoglucose oxidase-stained sections were counterstained with nuclear Fast red. All specimens were fixed in EA. ×130.
Fig. 2. Immunoperoxidase detection of CEA or related antigens in a colonic adenocarcinoma. The localization with goat anti-CEA antiseraum (A and D) and the NP-4 antibody (B and E) is compared between the specimen fixed in formalin (A and B) and EA (D and E). The normal mouse IgG controls for the EA- and formalin-fixed specimens are shown in G and H, respectively. The goat antiseraum controls were identical to those of the normal mouse IgG controls. Localization with the NP-1 (C) and NP-3 (F) monoclonal antibodies reacted against the formalin-fixed specimen is shown for comparison to that of the goat anti-CEA antiseraum (A) and NP-4 antibody (B) on the same specimen. Hematoxylin counterstain, ×130.
Fig. 3. Immunoperoxidase detection of CEA or related antigens in formalin-fixed specimens of liver and regional lymph node colonic tumor metastases. The staining characteristics of the primary tumor from this patient are depicted in Fig. 2. The reactivity of liver (A, B, C, and G) and lymph node (D, E, F, and H) metastases with goat anti-CEA antiserum (A and D), NP-3 antibody (B and E), NP-4 antibody (C and F), and normal mouse IgG control (G and H) is shown. The goat antiserum control was identical to that of the normal mouse IgG control. Hematoxylin counterstain, x130.
Fig. 4. Immunoperoxidase detection of CEA or related antigens in formalin-fixed specimens of a primary colonic adenocarcinoma (A, B, C, and G) and its lymph node metastases (D, E, F, and H). The staining by goat anti-CEA antiserum (A and D), NP-3 antibody (B and E), NP-4 antibody (C and F), and normal mouse IgG control (G and H) is shown. The goat antiserum control was identical to that of the normal mouse IgG control. Hematoxylin counterstain, ×130.
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