Immunohistochemical Analysis of Antiserum from Rhesus Monkeys Immunized with Human Colon Carcinoma

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ABSTRACT

In an attempt to generate antibodies which recognize novel tumor-associated antigens we have immunized Rhesus monkeys (Macaca mulatta) with human colon carcinoma cells prepared from freshly excised tumors. Immunohistochemical characterization of polyclonal antisera from one monkey (DF6) revealed preferential reactivity with primary and metastatic colon carcinoma tissue, and a general lack of recognition of nonneoplastic mucosa. Immunoreactivity was localized to the luminal contents of glandular structures and to the apical surfaces of cells lining these glands. Immunoreactivity was not observed with any normal tissue examined. Examination of neoplastic tissues revealed reactivity with two gastric carcinoma specimens (n = 2) and one breast carcinoma (n = 7). In reactive colon carcinoma tissues, the pattern of staining with DF6 was similar to that of several other antibodies including anti-carcinoembryonic antigen, B72.3, anti-Lea and anti-Leb. However, the panel of tissues recognized by these antibodies and DF6 differed significantly, suggesting that the DF6-reactive epitopes are unique. Human colon carcinoma cell lines maintained in vitro also expressed antigens recognized by DF6 in a pattern similar to that of surgically excised tissue. This preliminary characterization of DF6 antiserum suggests that immunization of Rhesus monkeys is a potentially useful protocol for identifying antigens preferentially expressed by human colon carcinoma.

INTRODUCTION

Attempts at generating immunological reagents that distinguish neoplastic from normal cells have involved immunization of heterologous species with cultured tumor cells or freshly excised tumor tissues. While partially successful, this approach has one serious limitation in that the generated antibodies, whether monoclonal or polyclonal, have generally displayed significant cross-reactivity with antigens on normal cell populations (1-3). This promiscuous reactivity has restricted the diagnostic and therapeutic use of these immunoreagents since both applications rely upon selective recognition of tumor-associated antigens (4, 5).

Most currently available immunoreagents have been produced by the immunization of rabbits and rodents which, because of their phylogenetic separation from humans, may predispose to the generation of antibodies cross-reactive with normal cell constituents. However, there is evidence that the immune system of nonhuman primates may preferentially react with tumor-associated antigens on human tumor cells with significantly less "background" recognition of epitopes common to both normal and neoplastic tissue (6-8). To maximize the possibility of generating antibodies that distinguish between malignant and normal colon we have immunized Rhesus monkeys (Macaca mulatta) with human colon carcinoma cells isolated from freshly resected surgical specimens.

Characterization of antisera from one monkey (DF6) revealed preferential reactivity with primary and metastatic colon carcinoma, and minimal reactivity with nonneoplastic tissue. Immunoreactivity was restricted to the luminal contents and the apical membrane of cells lining glandular structures. Colon carcinoma cell lines maintained in vitro also displayed DF6-reactive antigens with a pattern of staining similar to that of freshly excised tumors. DF6 antiserum failed to recognize a variety of normal tissues. Examination of neoplastic specimens revealed immunoreactivity with gastric carcinoma (2 of 2) and a single breast carcinoma (1 of 7), but not with other specimens. A comparison of DF6 reactivity with several antibodies previously reported to recognize colon carcinoma-associated antigens suggests that the epitopes recognized by DF6 are unique and are preferentially expressed by primary and disseminated colorectal neoplasms.

MATERIALS AND METHODS

Monkeys. Mature female Rhesus monkeys (Macaca mulatta) were obtained from Hazelton Domestic Bred Primates (Alice, TX). The animal studies described in this report adhere to the standards established by the NIH publication "Guide for the Care and Use of Laboratory Animals," (9). Preimmune sera, collected from two monkeys, were examined for immunoreactivity. Both monkeys then received either six (monkey DF6) or five (monkey 345N) immunizations. Each monkey was immunized with colon carcinoma cells isolated from a single tumor specimen. DF6 was immunized with a Dukes' stage B2 moderately differentiated tumor (proximal colon) from a 75-year-old male patient with O-type blood. Histological analysis revealed the presence of stroma, but the majority of cells for immunization were neoplastic epithelium.

Preparation of Human Colon Carcinoma Cells. Primary colon tumors were placed in chilled Dulbecco's modified Eagle's medium immediately upon surgical excision and were rinsed several times with sterile PBS. Tumor cells were dissociated by gently teasing through stainless steel screens and then cryopreserved in aliquots of 5-7.5 x 10⁶ cells in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum and 10% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO).

Immunizations and Serum Collection. After rapid thawing and washing with chilled PBS, cells were suspended in 0.5 ml PBS and vortex mixed with an equal volume of the RIBI adjuvant system, containing 125 µg monophosphoryl lipid A, 125 µg trehalose dimycolate, 10 µl squalane, and 1 µl Tween 80 (RIBI Laboratories, Rochester, MN). This suspension was injected s.c. into the right forearm of individual Rhesus monkeys that had been lightly sedated by i.m. administration of Ketaset (Bristol Laboratories, Syracuse, NY). Immunizations were repeated at 4-week intervals, and on each occasion 20 ml of blood were collected from the femoral vein into Vacutainer tubes (Becton Dickinson, Rutherford, NJ). Serum was separated by centrifugation and stored in 0.5-ml aliquots at -70°C.

Normal and Neoplastic Human Tissues. Tissue blocks prepared for routine histopathological evaluation were retrieved from hospital archives. Colonic tissues included seven cases of paired primary and metastatic carcinomas with corresponding histologically normal adjacent mucosa, as well as two blocks of normal colonic mucosa from patients with diverticular disease. Noncolonic tissues included a variety of neoplastic surgical specimens and normal tissues collected at necropsy.

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The abbreviations used are: PBS, phosphate-buffered saline; CEA, carcinoembryonic antigen.
Human Colonic Cell Lines. The human colon carcinoma cell lines LS174T, SKC01, SW403, SW480, SW620, COLO 320DM, COLO 320HSR, DLD-1, the nonneoplastic human colonic epithelial cell line, HCMC, and the human fetal colonic fibroblast line, CCD 12, were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described (10). For immunocytochemical analysis, cells were harvested by gentle scraping with a rubber policeman, washed with PBS, fixed for 30 min in 10% neutral buffered formalin, and pelleted by centrifugation. Pellets were wrapped in tea paper and processed for paraffin sectioning with routine procedures.

Monoclonal and Polyclonal Antibodies. Antibodies were diluted in PBS containing 3% bovine serum albumin (Sigma Chemical Co.). Appropriate working concentrations were determined by evaluating serial dilutions on positive tissue sections. Monkey antiserum was diluted 1:100. Tissue reactivities of the following antibodies were also examined: rabbit anti-CEA antiserum (Accurate Scientific, Westbury, NY); anti-CEA monoclonal antibody AA115/MCE (Accurate Scientific); B72.3, provided by Dr. Jeffrey Schlom, NIH, Bethesda, MD (11, 12); CaCo 4/23, obtained from Dr. Andrea Quaroni, Cornell University, Ithaca, NY (13); monoclonal anti-Lea antibody 55-2 (14), obtained from Dr. Zenon Steplewski, The Wistar Institute, Philadelphia, PA; and monoclonal anti-SSEA-1 (Lea), provided by Dr. Barbara Knowles of The Wistar Institute, Philadelphia, PA (15, 16). Secondary antibodies included goat anti-mouse IgG, heavy and light chain specific; goat anti-mouse IgG heavy and light chain specific; and goat anti-mouse IgM, ß chain specific. Secondary antibodies were fluorescein conjugated (Cappel Laboratories, Malvern, PA).

Immunochemistry. Five-μm sections were cut from blocks of paraffin-embedded tissues or cultured cells with an American Optical Model 820 microtome. After drying at 37°C, sections were deparaffinized in xylene and rehydrated through graded ethanol and distilled water. Following three changes of PBS, sections were incubated with primary antibodies at 4°C overnight in a humidity chamber. Primary antibodies were diluted with PBS containing 3% bovine serum albumin and 0.01% sodium azide. Following extensive washing, the sections were incubated at room temperature for 30 min with secondary antibody, rinsed in several changes of PBS, mounted by using a PBS-glycerol mountant, and examined on an Olympus BH-2 microscope equipped with UV epiillumination.

RESULTS

Immunoreactivity of Monkey Serum with Human Colonic Tissues. Preliminary evaluation of antiserum collected from both monkeys (DF6 and 345N) after the third immunization revealed reactivity with the immunizing colon carcinoma tissues, but not with adjacent normal mucosa. The humoral response of monkey DF6 was chosen for detailed characterization and 20 ml of serum were collected following the sixth immunization.

Table 1 Immunoreactivity of DF6 antiserum and selected monoclonal and polyclonal antibodies on normal, neoplastic, and metastatic human colonic tissues

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Anti-CEA</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF6 Rabbit MAB*</td>
<td>B72.3 CaCo 4/23 Anti-Lea Anti-Lea</td>
</tr>
<tr>
<td>Nonneoplastic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mucosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Adjacent normal</td>
<td>1/7</td>
<td>5/5</td>
</tr>
<tr>
<td>Total</td>
<td>1/9</td>
<td>7/7</td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>7/7</td>
<td>7/7</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>7/7</td>
<td>7/7</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>2/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* Results of immunohistochemistry are expressed as number reactive/number examined. Reactivity is not classified by extent or intensity, a tissue is considered reactive if fluorescence is detected within the epithelial component.
* MAB, monoclonal antibody.
* Diverticular disease.

Fig. 1. Immunoreactivity of DF6 antiserum with normal and neoplastic colon (a, b, c, d); immunofluorescence (a, c, d); phase contrast (b). Normal adjacent mucosa (a, b); primary colon carcinoma (c); colon carcinoma liver metastasis (d). Immunoreactivity of DF6 preimmune serum with primary colon carcinoma (e, f); immunofluorescence (e); phase contrast (f).

nization. Immunohistochemical analysis of DF6 antiserum revealed negligible background staining, and preferential reactivity with colon carcinoma at a dilution of 1:100. All subsequent stainings were performed at this dilution.

DF6 antiserum was incubated with paraffin-embedded tissue sections from seven cases of primary colon carcinoma, and paired liver and lymph node metastases (removed during primary tumor resection), from 7 and 3 of these cases, respectively. Lumen contents and apical membranes of cells within glandular structures were immunoreactive in all primary adenocarcinomas examined (Table 1; Fig. 1). The fraction of immunoreactive glands varied among individual specimens, with essentially all glands reacting in the majority of tumors and in only isolated glands in others. All seven liver metastases and two of three lymph node metastases displayed immunofluorescence in a pattern identical to the primary lesions (Table 1; Fig. 1).
Fig. 2. Immunoreactivity of rabbit anti-CEA antiserum with normal and neoplastic colon (a, b, c, d); immunofluorescence (a, c, d); phase contrast (b). Normal adjacent mucosa (a, b); primary colon carcinoma (c); colon carcinoma liver metastasis (d). Immunoreactivity of an anti-Le\(^+\) monoclonal antibody with normal and neoplastic colon (e, f, g, h); immunofluorescence (e, g, h); phase contrast (f). Normal adjacent mucosa (e, f); primary colon carcinoma (g); colon carcinoma liver metastasis (h).

Samples of adjacent histologically normal colonic mucosa from each colon carcinoma patient were also examined, in addition to mucosal specimens from two patients without evidence of neoplasia. Immunoreactivity was observed only in a single mucosal specimen (from a patient with colon carcinoma) and was limited to the apical membrane of cells and luminal contents in the lower portion of isolated crypts (Table 1).

Comparison with Previously Described Immunoreagents. The immunoreactivity of several antibodies previously reported to recognize colon carcinoma was compared with DF6 antiserum by examining reactivity on the same set of colonic tissues (Table 1; Fig. 2). Like DF6, reactivity with colon carcinoma specimens was predominantly focused on luminal contents and apical cell membranes, but there were significant differences between this panel of antibodies and DF6. The most striking difference involved cross-reactivity with histologically normal adjacent mucosa. In contrast to DF6, which displayed a virtual absence of reactivity with normal mucosa, several of the other antibodies (including anti-CEA, B72.3, CaCo 4/23, anti-Le\(^+\), and anti-Le\(^-\)) showed readily detectable staining, although the level of immunofluorescence was significantly less compared to that of neoplastic tissue. In addition (but with the exceptions of DF6 and B72.3), the remaining antibodies cross-reacted with at least one colon specimen from patients with no evidence of neoplastic disease.

Cross-Reactivity with Noncolonic Tissues. To characterize further the specificity of DF6 antiserum, cross-reactivity with a variety of human neoplastic surgical specimens and normal necropsy tissues was examined (Table 2). Immunoreactivity was detected in each of two gastric adenocarcinoma specimens examined and, like the colonic tumors, this reactivity was restricted to luminal contents and apical surfaces of cells lining glandular structures. Normal adjacent gastric mucosa failed to react. One breast carcinoma specimen also demonstrated immunoreactivity. Weak membranous fluorescence was observed in the single infiltrating lobular carcinoma examined. Breast

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Positive/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neoplasms(^a)</td>
<td></td>
</tr>
<tr>
<td>Prostatic</td>
<td>0/6</td>
</tr>
<tr>
<td>Lung</td>
<td>0/4</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>0/6</td>
</tr>
<tr>
<td>Breast</td>
<td>1/7</td>
</tr>
<tr>
<td>Gastric</td>
<td>2/2</td>
</tr>
<tr>
<td>Renal cell</td>
<td>0/3</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>0/3</td>
</tr>
<tr>
<td>Fibrous histiocytoma</td>
<td>0/2</td>
</tr>
<tr>
<td>Normal(^b)</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0/5</td>
</tr>
<tr>
<td>Liver</td>
<td>0/3</td>
</tr>
<tr>
<td>Prostate</td>
<td>0/2</td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td>0/3</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/5</td>
</tr>
<tr>
<td>Small Intestine(^c)</td>
<td>0/4</td>
</tr>
</tbody>
</table>

\(^a\) Surgical specimens.  
\(^b\) Necropsy specimens.
cancers of the infiltrating ductal variety were not immunoreactive. Immunostaining was not detected in other human neoplastic or normal tissues (Fig. 3).

Immunoreactivity with Colon Carcinoma Cell Lines. The expression of DF6-reactive antigens was examined in colon carcinoma cell lines established in culture (Table 3). Extracellular amorphous material, often plasma membrane associated, was strongly immunoreactive in all lines examined (Fig. 4). In most cases, including COLO 320HSR and LS174T, this reactivity was evenly distributed throughout the section; however, in others (including SW620, SW480, and DLD-1), only isolated positivity was observed. Occasional cytoplasmic reactivity was also detected. HCMC and the fibroblast cell line CCD 112 (Table 3) displayed sporadic immunostaining but the intensity was significantly diminished compared to the colon carcinoma cultures.

CEA expression was also examined in these cell populations (Table 3; Fig. 4). The majority of cell lines displayed immunofluorescence, but in a pattern clearly distinct from that of DF6 antisera. In contrast to the amorphous material immunoreactive with DF6, extracellular CEA staining was primarily granular. Membranous and cytoplasmic reactivity was also observed in all reactive cultures. Some lines (e.g., SKCO1) displayed uniform cytoplasmic immunofluorescence. CEA was undetectable in two colon lines, COLO 320HSR and COLO 320DM, which were strongly recognized by DF6. Anti-CEA antisera did not recognize nonneoplastic cultures (HCMC and CCD 112).

**DISCUSSION**

Polyclonal and monoclonal antibodies generated against tumor-associated antigens through immunization of rabbits and rodents have proven useful as research reagents and increasingly as tools for both *in vitro* and *in vivo* diagnostics. However, they also have important limitations in that they cross-react with epitopes present on normal tissue (1–3). Thus there is a continuing need for antibodies that are more efficient in recognizing antigens expressed specifically by neoplastic cell populations (4, 5). One alternate approach is the immunization of nonhuman primates with tumor material since there is evidence that the immune system of these animals recognizes epitopes distinct from those seen by standard species such as rabbits, goats,
and rodents (6-8). For example, Seigler et al. (17) and Metzgar et al. (18) have demonstrated that serum from monkeys immunized with either human lymphocytic or myeloid leukemia cells discriminated among individual classes of hematopoietic neoplasms, while serum from rabbits similarly immunized failed to do so. In addition, Rouslahti et al. (7) have shown that while immunization of rabbits with a human tumor-associated antigen frequently generated antibodies which cross-react with antigens present on normal cells, the immunization of monkeys eliminated this cross-reactivity.

In the present study we have immunized Rhesus monkeys with human colon carcinoma cells with the goal of identifying polyclonal antibodies that recognize epitopes preferentially expressed by this neoplasm. Characterization of the antiserum was performed on paraffin-embedded tissue which, for the most part, preserves antigen integrity, and permits retrospective analysis of hospital-archived specimens. Using this protocol we established that antibodies from monkey DF6 reacted with essentially all primary and metastatic colon carcinoma specimens tested, but recognized only a single (histologically) normal section of colonic mucosa, which was from a patient with frank colon carcinoma. Reactivity in this specimen was weak and restricted to the base of isolated colonic crypts. In general, DF6 reactivity with colon primary tumors, and lymph node and hepatic metastases consistently displayed a uniform pattern of reactivity recognizing both luminal contents and apical surfaces of cells within glandular structures. A limited survey of normal and neoplastic tissue from other organs revealed DF6 immunoreactivity in two gastric carcinoma specimens, with a pattern of fluorescence identical to that of colon tumors. Additionally, one breast carcinoma of the infiltrating lobular variety demonstrated membrane-associated immunoreactivity, although the intensity of this fluorescence was much lower than that observed in the gastrointestinal malignancies.

This selectivity of DF6 toward neoplastic tissue compares favorably with several other antibodies commonly used in the immunoanalysis of colorectal tumors, including anti-CEA, B72.3, anti-Le', and anti-Le'. In addition, the panel of tissue specimens recognized by these antibodies differed significantly from that of DF6, suggesting that DF6 reacts with previously unrecognized tumor-associated antigens. However, further (biochemical) studies are required to support this conclusion.

The antigenic determinants recognized by DF6 antiserum were also expressed by human colon carcinoma cells propagated in vitro, with a pattern of staining similar to that of the surgical samples. DF6 immunoreactivity did not correlate with any other property of these cell lines, including population doubling time, tumorigenicity, oncogene expression, and CA19-9 display (10).

According to previous studies, nonhuman primates are capable of mounting a humoral response against epitopes on the CEA molecule (7, 8). However, three pieces of evidence suggest that the predominant reactivity of DF6 is not directed toward this antigen. First, DF6 and the anti-CEA antibodies differ in

Fig. 4. Immunoreactivity of DF6 antiserum (a, b, e, f, k, l), rabbit anti-CEA antiserum (c, d, g, h), or DF6 preimmune serum (i, j) with colon carcinoma cell lines; immunofluorescence (a, c, e, g, i, k); phase contrast (b, d, f, h, j, l). The following cell lines were reacted: COLO 320 HSR (a, b, c, d); LS174T (e, f, g, h, i, j); DLD 1 (k, l).
their recognition of colon carcinoma cell lines maintained in vitro. For example, rabbit polyclonal anti-CEA antiserum failed to recognize two carcinoma cultures, Colo 320HSR and Colo 320DM, although both were strongly reactive with DF6. Second, the single nonneoplastic colon specimen recognized by DF6 was not reactive with anti-CEA monoclonal antibody, although this latter reagent did recognize occasional normal and “normal adjacent” colon mucosa (Table 1). Third, while DF6 recognized cells at the base of the colonic crypt, both monoclonal and polyclonal anti-CEA reacted predominantly with cells at the crypt neck and along the superficial surface of histologically normal mucosa (Fig. 2a). These results do not rule out the possibility that CEA and DF6-reactive antigens share common epitopes, but they do indicate that the epitopes recognized by the monoclonal and polyclonal CEA antibodies are distinct from those recognized by DF6.

This preliminary characterization suggests that DF6 antiserum recognizes antigens preferentially expressed on colon carcinoma tissue, but evaluation of a much larger tissue panel is required before the distribution of DF6-reactive antigens can be documented with certainty. These studies are in progress. One approach currently under way is the generation of monkey-mouse hybridomas by using cells harvested by partial splenectomy of monkey DF6. Monoclonal antibodies produced by these hybridomas will allow a more detailed analysis of antigen distribution, and aid in their isolation and identification. The high level of antigen expression noted in certain colon carcinoma cell lines, including LS174T and COLO 320HSR, indicate that a convenient source of antigen is available for future study.

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