

Gastrointestinal Cancer-associated Antigen in Immunoperoxidase Assay¹

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ABSTRACT

A monosialoganglioside antigen of gastrointestinal adenocarcinomas defined by murine monoclonal antibody was demonstrated by immunoperoxidase (IP) assay in fixed paraffin-embedded tumors in 59% of colonic adenocarcinomas, 86% of pancreatic adenocarcinomas, and 89% of all gastric adenocarcinomas. In all patients with detectable levels of antigen in circulation, the resected tumors also expressed the antigen in IP assay. Six of eight individuals with no detectable levels of antigen in their serum samples expressed the antigen in the tumor tissue. Removal of the sialic acid residue of the antigen abolished the IP reaction. The successful use of the IP assay on fixed tissue to demonstrate the specific sites of gastrointestinal cancer antigen localization in human tumors and normal tissues provides an important tool for the study of developing neoplasia.

INTRODUCTION

Monoclonal antibodies secreted by 2 hybridomas established after immunization of mice with colorectal carcinoma cells detect a GICA⁴ present on the surface of cultured cells of GI adenocarcinomas (6) as defined by RIA or mixed hemadsorption. These antibodies were used to detect an antigen shed into tissue culture (10) and present in the sera of GI tumor patients (2, 5, 9). In order to find out if tumors *in situ* and not normal colonic epithelium are the source of the antigen detected in serum of cancer patients, we have attempted to use freshly resected tumors as target for binding of monoclonal antibodies. Because of the contamination by GI contents and infiltration by inflammatory cells, it was impossible to obtain conclusive results in RIA or mixed hemadsorption binding assays on fresh tumors and adjacent normal tissues. We have therefore adopted the IP assay as an alternate approach to study the distribution of a monoclonal antibody-defined antigen of GI adenocarcinomas in fixed, paraffin-embedded tissues.

MATERIALS AND METHODS

Specimens. Cases were identified from the diagnostic files of Surgical Pathology of either the Hospital of the University of Pennsylvania or American Oncologic Hospital. After slides from each patient were reviewed, tissue blocks which contained both tumor and adjacent normal tissues were selected. Blocks of human fetal tissue of 14 to 17 weeks gestation were also obtained. Blocks were cut into 5- μ m sections, deparaffinized, and used for the studies. Such blocks ranged in age from recent to 10 years old. Most specimens had been fixed in

Bouin's solution, but several were fixed in 10% neutral buffered formalin.

Antibodies. Monoclonal IgG1 antibody 19-9 and IgM antibody 10-17 are secreted by hybridomas which were established after fusion of 653 variant of P3 mouse myeloma cells (3) with splenocytes of mice immunized with colon carcinoma cell line SW 1116 (6). Antibody 19-9 detects GICA, a monosialoganglioside antigen (7) present on the surface of GI adenocarcinomas and absent from other tumors and normal cells in culture (6). Antibody 10-17 defines Le^b blood group antigen (1). As a source of monoclonal antibodies for IP assay, we used tissue culture medium in which hybridomas were maintained. As a control, medium of mouse myeloma P3 of IgG1 isotype was used. Two other IgG1 monoclonal antibodies were included as controls. Antibody H3641-1 was directed against influenza virus, and antibody B₁77-71 has antimelanoma specificity. Hybridoma and myeloma lines were maintained in minimal essential medium supplemented with 2 \times vitamins and 15% fetal calf serum. All antibody supernatants had the same approximate immunoglobulin content.

IP Procedure. The IP assay was performed by a modification of the method of Colcher *et al.* (4). Five- μ m sections were deparaffinized, washed for 5 min in running water, and pretreated with 0.3% H₂O₂ in absolute methanol for 15 min to inhibit endogenous peroxidase, followed by 10% normal swine sera in PBS containing 0.10% BSA for 10 min. The slides were then successively incubated for 30 min at room temperature with approximately 0.2 ml of undiluted tissue culture medium containing mouse monoclonal antibody, goat anti-mouse F(ab')₂ (1/5000), swine anti-goat antibody (1/100), and goat peroxidase antiperoxidase conjugate (1/2000) (Cappel Laboratories, Cochranville, Pa.), with 10-min rinses in PBS/BSA between all steps. The supernatant of P3 or PBS/BSA buffer was used as a control. The slides were then treated with 0.06% diaminobenzidine with 0.01% H₂O₂ in PBS/BSA for 5 min, counterstained with hematoxylin, dehydrated, and mounted.

Scoring of Slides. Monoclonal antibody-incubated slides were compared to control P3-incubated slides. A very strong brown-black color was scored as ++, definite brown granular pattern was +, and a brownish color darker than in the control was graded as \pm but scored as negative (-) in the final tabulation. If cells showing strong staining were present in clusters on the background of negative cells, the reaction was described as focal.

Neuraminidase Treatment. The IP procedure was begun as described previously, but after pretreatment with normal swine sera and before incubation with the monoclonal antibody, the slides were incubated at 35° for 2 hr with various concentrations of type VI clostridial neuraminidase (Sigma Chemical Co., St. Louis, Mo.) diluted in 0.2 ml of PBS, pH 6, ranging from 0.5 to 0.01 units per slide. After neuraminidase treatment, the slides were washed, exposed to monoclonal antibodies, and processed as described before. As a control, slides were exposed to PBS at pH 6.0 without neuraminidase and then stained in the usual IP procedure. The antibody 10-17 was used for positive control purposes, since the hapten of Le^b antigen detected by this antibody is free of sialic acid.

Correlation of GICA Presence in Tissue and Serum. Sera from 13 patients with colon carcinoma were collected prior to surgical resection and afterwards at regular intervals. Tissues from both tumor and resection margins of these cases were fixed in Bouin's solution or formalin and stained by IP assay for GICA. The results established in IP assay were correlated with the presence of the same antigen in the patient's serum (2, 5, 9).

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⁴ The abbreviations used are: GICA, gastrointestinal cancer antigen (a monosialoganglioside); GI, gastrointestinal; RIA, radioimmunoassay; IP, immunoperoxidase; 19-9, 1116 NS 19-9; 10-17, 1116 NS 10-17; P3, P3 \times 63Ag8; Le^b, Lewis b; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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RESULTS

Expression of GICA in Tumor Specimens. A variety of tumors were tested by IP staining with the 19-9 antibody (Table 1). Forty of 68 cases (59%) of colonic adenocarcinomas demonstrated antigen in the primary tumor (Fig. 1). In 6 of the tumors, a focal staining pattern was observed, while in others, most tumor cells were stained. Of 23 positive cases having adjacent normal mucosa, 5 had focal staining of these areas. In 15 colon specimens obtained from patients with diverticulitis but without cancer, IP reaction was negative (Fig. 2). The control IgG-1 antibodies either did not react with these tissues (H3641-1) or reacted with melanomas but not with GI tumors or normal cells (B₁77-71).

Nineteen of 22 pancreatic carcinoma specimens expressed the GICA (Table 1). The staining of tumor cells was always diffuse and strong (Fig. 3). Of 10 pancreatic tissue specimens available from patients without tumor, 7 showed staining of columnar epithelium of large and small ducts (Fig. 4) but not of acini and islets.

As shown in Table 1, 16 of 18 gastric carcinomas were positive for GICA in IP assay. The staining was strong and diffuse in all tumor cells (Fig. 5). In all these specimens, we have also observed single positive cells scattered throughout mucosa adjacent to the tumor. Seven of 19 normal samples of gastric epithelium showed the presence of antigen in single cells in the midzone of the glands (Fig. 6). In one of 11 specimens of hepatocellular carcinoma (Table 1), the antigen was detected as a focal staining of tumor cell nests. The antigen was also detected in epithelium of small biliary ducts of normal liver (Table 1). Two of 5 gallbladder adenocarcinomas showed presence of antigen with focal distribution (Table 1). Small foci of columnar cells stained in IP assay were found in 6 of 11 gallbladders removed because of mild cholecystitis. The only other normal epithelium binding 19-9 antibody in IP assay were bronchial glands in normal lung specimens, large ducts of salivary glands, and the prostate. Large breast ducts were very focally positive in one case but totally negative in 10 other cases. Fetal bowel from 5 specimens of 14 to 17 weeks gestation demonstrated GICA focally within the surface cells. A large number of other tissues found to be negative in all specimens tested by IP assay for GICA included esophagus, small intestine, pleura, urinary bladder, kidney, testis, ovary, uterine cervix and endometrium, Fallopian tube, skin, thyroid, adrenal gland, skeletal muscle, myocardium, and umbilical cord. The glandular epithelium of nasal sinus was also negative. None of 4 samples of normal bone marrow, of normal spleen, and of normal lymph node and one of thymus showed any binding of 19-9 antibody.

A variety of tumors of other origins have also been tested for the presence of the GICA. It was present overall in 28 of 66 lung carcinomas including 15 of 29 adenocarcinomas, 6 of 21 bronchioloalveolar cell carcinomas, 3 of 4 squamous carcinomas, 4 of 9 small-cell anaplastic carcinomas, and 0 of 3 large-cell undifferentiated tumors. It was present in only one of 18 breast carcinomas and one of 12 malignant mesotheliomas. Other tumors that demonstrated GICA include: papillary carcinoma of the thyroid (4 of 8 cases, very focally positive); papillary transitional cell carcinoma of urinary bladder (2 of 3 cases); papillary cystadenocarcinoma of the ovary (2 of 3 cases); and one prostate and renal cell carcinoma. All sarcomas tested were negative. All blocks with negative staining by

19-9 antibody have demonstrated positive staining with other mouse monoclonal antibodies.

Neuraminidase Sensitivity of GICA. The antigen detected by monoclonal antibody 19-9 was found to be sensitive to treatment with neuraminidase in both tumor cells of gastric adenocarcinoma and normal bronchial glands (Table 2). While antigen detected by 19-9 antibody is neuraminidase sensitive, another glycolipid antigen (Le^b) detected by monoclonal antibody 10-17, included for control purposes, since it contains no sialic acid residue, was not affected by the concentration of 0.5 to 0.01 units of neuraminidase (Table 2).

Presence of 19-9 Antigen in Tumor and Serum of the Same Patient. Results shown in Table 3 indicate that all patients with detectable levels of 19-9 antigen in their serum showed presence of this antigen in their primary colon tumor specimens. Of the 8 patients with no detectable levels of the antigen in circulation (2), 6 showed presence of the antigen in their tumor tissue.

DISCUSSION

These studies demonstrate that IP procedure with 19-9 monoclonal antibody used on paraffin-embedded fixed tissue showed expression of a GICA in 59% of colonic adenocarcinomas, 86% of pancreatic adenocarcinomas, and 89% of gastric carcinomas tested. Since the binding site for the 19-9 antibody is the sialic acid-containing residue of the monosialoganglioside of the antigen (7), the staining reaction in IP is abolished by treatment with neuraminidase. The specificity of the neuraminidase sensitivity of the 19-9 antigen was confirmed by the fact that this treatment did not affect the staining after incubation with 10-17 monoclonal antibody, which recognizes on Le^b antigen, a glycolipid but not a sialoganglioside (1). Further evidence of specificity of this IP procedure is the comparison of the reactivity of the 19-9 antibody to the other

Table 1
GICA presence in GI tissues determined by IP assay

Tissue origin	Ratio of GICA positive to total tissue samples of			
	Tumor	%	Normal	%
Colon	40/68	59	0/15	0
Pancreas	19/22	86	7/10 ^a	70
Stomach	16/18	89	7/19 ^a	37
Liver	1/11	9	7/11 ^a	64
Gall bladder	2/5	40	6/11 ^a	54

^a In normal tissues, antigen was found only in some cells of the columnar epithelium.

Table 2
Effect of different concentrations of neuraminidase on the expression of GICA and Le^b antigen (10-17) in the cells of gastric carcinoma and bronchial glands as detected by IP assay

Deparaffinized sections were treated for 2 hr at 35° with 0.5, 0.1, and 0.01 units of type VI clostridial neuraminidase before monoclonal antibodies were applied.

Tissue tested	Mono-clonal antibody	Neuraminidase treatment (units)			
		0.5	0.1	0.01	PBS
Gastric adenocarcinoma	19-9	-	± ^a	±	++
	10-17	++	++	++	++
	P3	-	-	-	-
Bronchial glands	19-9	-	-	±	++
	10-17	++	++	++	++
	P3	-	-	-	-

^a For scoring of slides, see "Materials and Methods."

Table 3
Comparison of the expression of GICA in the tumor specimens with its presence in the serum

Patient's code	Staining of the tumor cells in IP assay	Presence of GICA in serum
242	++ ^a	++
349	++	++
485	++	++
600	++	++
459	++	+
14	++	-
261	++	-
271	++	-
273	++	-
16	+	-
V. P.	+	-
237	-	-
90	-	-

^a For scoring of slides, see "Materials and Methods."

IgG-1 antibodies. The anti-influenza antibody did not react with tumors or normal epithelium, and the anti-melanoma antibody reacts with melanoma but not GI tumors.

This IP procedure proved to be very sensitive for GICA, since the 19-9 monoclonal antibody tissue supernatant could be diluted to 2000 times, and a positive staining reaction could still be obtained. Neither the type of tissue fixative nor the age of the paraffin blocks appeared to influence the presence of antigen, since the relative percentage of positive specimens was always maintained. The length of antibody incubation was also not a critical factor with 19-9 antibody, since strong reactivity could be demonstrated with less than 1 min of primary incubation.

In analysis of correlation of presence of GICA in serum of patients with presence of antigen in resected colonic carcinoma, all of the specimens resected from patients with circulating antigen were shown by IP to express GICA. Previous studies (2, 9) have found detectable GICA in serum of pancreatic, gastric, and colonic carcinoma patients. Six of the 8 individuals, however, with no detectable 19-9 antigen in their serum samples showed presence of this antigen in IP assay of their tumor specimen (Table 3). This indicates that the absence of 19-9 antigen in the serum does not preclude its presence in the tumor. The possible reasons for this difference are that the concentration of the antigen in circulation was too low to be detectable or that the tumor cells produce this antigen but do not secrete it. Some of the tissue culture cell lines that have membrane and cytoplasmic antigen detectable in RIA do not shed the antigen into tissue culture medium in which the cells were maintained (10).

The histogenesis of the pancreatic adenocarcinoma from the secreting epithelium of the pancreatic ducts may account for the presence of this antigen in both types of tissue. The same may be true for the origin of some gastric and gallbladder carcinomas and for those hepatomas which demonstrate this antigen; perhaps, they do not originate from hepatocytes but from small biliary ducts within the liver. On the other hand, presence of the IP-detected GICA in cells of colorectal adenocarcinomas cannot be accounted for by the expression of the antigen in normal histogenetically related adult cells, since normal epithelial cells were unstained in most cases. The GICA, however, was detected in both fetal bowel and meconium (7); thus, it is possible that it belongs to a class of oncodevelopmental antigens, expression of which is shut off during differentiation and is reexpressed in malignant tissues.

In this study, we have concentrated our observations on primary tumors of GI tract. In one case of primary gastric adenocarcinoma, however, we have detected the GICA in IP assay on primary tumor and liver metastasis but not in 2 different specimens of the metastases to the bone (8). Thus, the IP reaction with 19-9 antibody may be useful in the study of antigenic modulation of tumor cells metastasized to different anatomical sites.

An important potential of this IP technique is that if other monoclonal antibodies are found which recognize either different determinants or other antigens of tumors not expressing GICA, then it will be possible to separate GI adenocarcinomas into categories based on antigenic content which up to now could not be done by histological examination alone. It may also be possible to determine similarities in behavior of tumors based on their antigenic characteristics instead of site of origin or histological classification.

GICA was detected focally in colonic mucosa adjacent to GICA-positive tumors in 5 of 23 cases. When GICA appears in such histopathologically "normal" mucosa, it may mean only that GICA is secreted by the tumor and then absorbed onto surrounding tissue. GICA has also been identified, however, in some colonic polyps including both tubular and villous adenomas, and this will be the subject of a further report. This seems to indicate that the presence of the oncodevelopmental antigen might be a useful tool to study developing neoplasia within the GI tract in such premalignant conditions as ulcerative colitis and colonic polyps.

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Fig. 1. Section of villous adenocarcinoma of the colon showing strongly stained cells with 19-9 monoclonal antibody and adjacent nonstaining areas. IP counterstained with hematoxylin, $\times 400$.

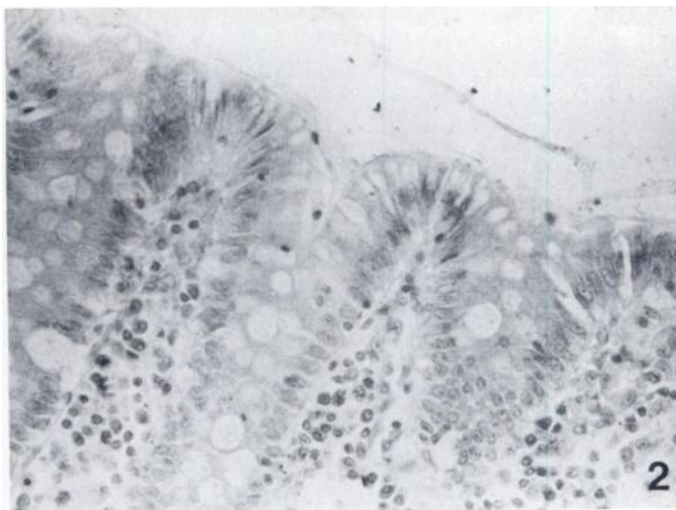


Fig. 2. Section of normal colon from patient with diverticulitis demonstrating no detectable GICA. IP counterstained with hematoxylin, $\times 640$.

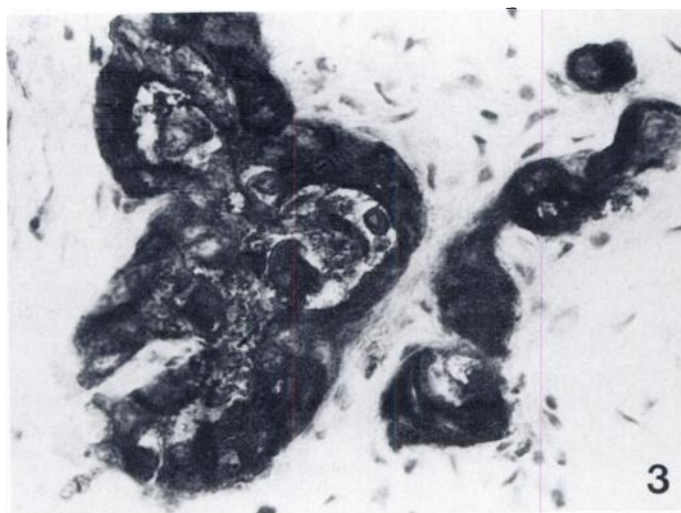


Fig. 3. Poorly differentiated adenocarcinoma of pancreatic origin showing diffuse, strong staining of malignant cells by 19-9 monoclonal antibody. IP counterstained with hematoxylin, $\times 640$.

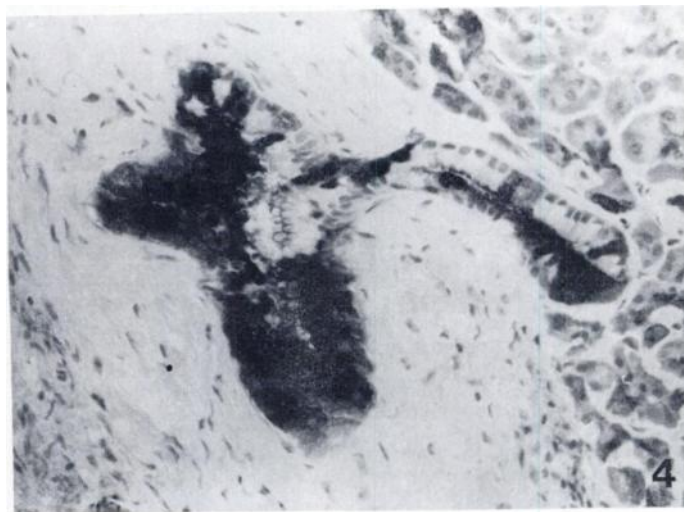


Fig. 4. Medium-sized pancreatic duct adjacent to a pancreatic adenocarcinoma, demonstrating many ductal cells with strong staining by 19-9 monoclonal antibody and no staining of acinar cells. IP counterstained with hematoxylin, $\times 400$.

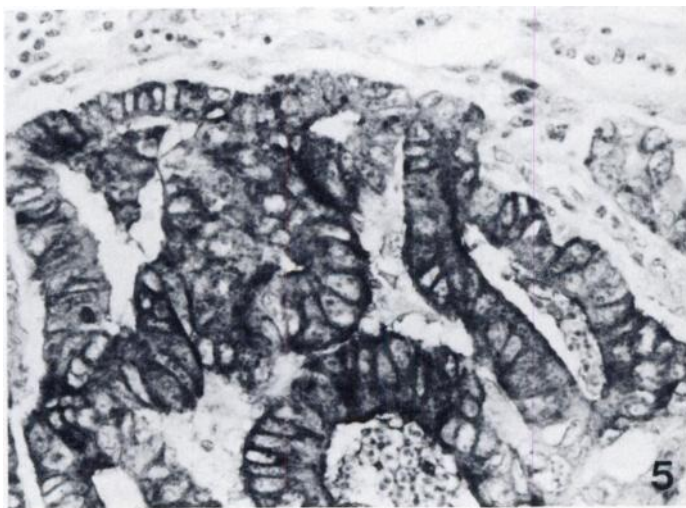


Fig. 5. Gastric adenocarcinoma showing strong staining with 19-9 monoclonal antibody. IP counterstained with hematoxylin, $\times 640$.

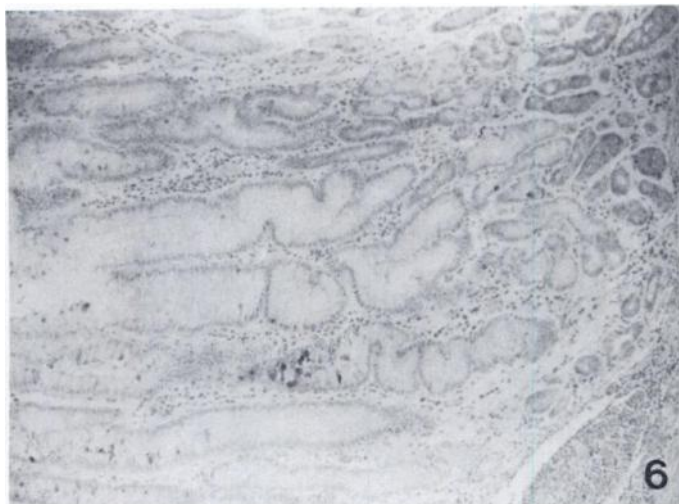


Fig. 6. Section of apparently normal stomach mucosa adjacent to gastric adenocarcinoma showing a few scattered cells that are stained with 19-9 monoclonal antibody. IP counterstained with hematoxylin, $\times 125$.

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