Jpn. J. Cancer Res. (Gann), 79, 255–263 ; February, 1988

Immunohistochemistry of Human Gastrointestinal Cancer-associated Antigen Detected by Monoclonal Antibody PA 8-15

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A murine monoclonal antibody (MAb), PA 8-15, was produced against a newly established human pancreatic adenocarcinoma cell line, SUIT-2. With the avidin-biotin immunoperoxidase technique, PA 8-15 MAb reacted strongly with 27 of 28 formalin-fixed paraffin-embedded pancreatic adenocarcinoma tissues. All six gall bladder carcinomas and all nine biliary tract carcinomas also showed positive reactions. In addition, PA 8-15 MAb reacted with gastric carcinomas (6/10), colorectal carcinomas (7/11), and some other primary adenocarcinomas. The distribution of PA 8-15 antigen on the same tissues of pancreatic cancer was different from those of CA 19-9 and DU-PAN-2 antigens and CEA. PA 8-15 MAb stained only the epithelium of the pancreatic duct, gall bladder and bile duct in normal adult tissues, and some normal fetal glandular epithelial cells. However, PA 8-15 MAb was not reactive with inflammatory or benign tumors of the digestive system except for the epithelium, as was seen in normal adults. Reactivity of PA 8-15 MAb with tissue specimens largely disappeared after treatment with neuraminidase, while oxidation with periodate or trypsin digestion did not alter the staining intensity, indicating that antigenic determinants may be at least partly of sialylated carbohydrate nature. These results suggest that PA 8-15 MAb detects a new oncofetal antigen in gastrointestinal cancers, especially of the pancreatico-biliary tract.

Key words: Monoclonal antibody — Gastrointestinal cancer-associated antigen — Human pancreatico-biliary tract cancer — Immunohistochemistry

In recent years, various diagnostic techniques to detect cancers of the digestive system have been developed. Much effort has been made in the search for useful tumor markers. Tumor markers have been effectively employed in diagnosis and monitoring. However, diagnosis of cancers in the early stage is still difficult with conventional diagnostic measures.

In 1979, Koprowski *et al.*[1] reported a new tumor marker, CA 19-9, against a cell line of colorectal cancer. CA 19-9 was found to be highly specific for cancers of the pancreas and biliary tract[2,3] compared with pancreatic cancer-associated antigens, such as pancreatic oncofetal antigen (POA),[4,5] pancreas cancer-associated antigen (PCAA),[6] and carcinoembryonic antigen (CEA).*[2] Recently, tumor-associated antigens of the pancreas and biliary tract characterized by their reactivity with monoclonal antibodies have been reported by several investigators.[7–11]

We produced a monoclonal antibody, PA 8-15, raised against a pancreatic cancer cell line, SUIT-2.[12]

Materials and Methods

Pancreatic Cancer Cell Line A human pancreatic cancer cell line, designated SUIT-2, was a gift from Dr. Iwamura (Department of Surgery I, Miyazaki Medical College, Miyazaki). SUIT-2 was derived from a metastatic liver tumor of human pancreatic carcinoma and classified as moderately differentiated adenocarcinoma. This cell line has been cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FCS (Whittaker Corporation, Walkersville, MD), 100 units penicillin/ml, 100 μg/ml streptomycin (Meiji Medical, Inc., Tokyo).

Production of Monoclonal Antibodies A hybridoma was produced according to the method of Galfre *et al.*[13] with some modification. Briefly,

*[1] To whom correspondence should be addressed.
*[2] Abbreviations: MAb, monoclonal antibody; CEA, carcinoembryonic antigen; FCS, fetal calf serum; PEG, polyethylene glycol; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; ABC, avidin-biotin-peroxidase complex; BSA, bovine serum albumin.

three BALB/c female mice (2 to 3 months old) were used for each set of fusions and were immunized by ip injection (days 1, 14, 21, and 28) of SUIT-2 cells (approximately 10^7 cells). For each fusion, 5×10^7 spleen cells were fused with 10^7 mouse myeloma cells, P3×63-AG-8-U1,[14] by addition of 0.5 ml of polyethylene glycol (PEG 4000; Sigma Chemical Co., St. Louis, MO). Cloning was carried out by limiting dilution. The hybridoma supernatant was assayed by ELISA after the appearance of macroscopically visible colonies.

Subcloning and Growth of Hybridoma in Mice Hybridomas of interest were expanded and subcloned by limiting dilution. These hybridomas were propagated as ip tumors in mice. Ascitic fluid was centrifuged to remove debris and stored at −20° until use.

Immunoglobulin Subclass Determination The immunoglobulin subclass was determined by double-immunodiffusion analysis (Ouchterlony) by means of isotype-specific anti-mouse antibody (Miles Laboratories, Indiana, IN) and ELISA with horseradish peroxidase-conjugated anti-mouse Ig Kit (MMT 03K, Serotec Ltd., Blackthorn, Bicester, England).

Hemagglutination Assay Blood group antigens were tested with erythrocytes from healthy individuals, whose major and minor blood types were confirmed by the indirect Coombs test (Lewis a+, b−: 2 subjects, Lewis a−,b+: 6 subjects, ABO: 4 subjects each). Agglutination was achieved by addition of 50 μl of 1:10 diluted $F(ab')_2$ fragment of anit-mouse IgM (Cooper Biomedical Inc., Malvern, PA).

Tissue Samples All human specimens were obtained from Kumamoto University Medical School and University Hospital, Department of Surgery II, and Surgical Pathology. There were 98 specimens of various tumors, 102 of normal adult and inflammatory tissues, and 18 of normal fetal tissues. All tumors were primary, obtained at surgery or autopsy. Normal tissues adjacent to malignant tumors and inflammatory tissues were obtained at surgery. Human fetal tissues (12W-33W) were collected from the Department of Pathology II of our Medical School at autopsy. All materials were obtained incidental to diagnostic or therapeutic procedures not related to this study.

All specimens were routinely fixed in neutral buffered formalin within 3 weeks and embedded in paraffin. The period of preservation in paraffin-embedded blocks was less than 6 years. Representative sections of the tumors were stained with hematoxylin and eosin for light microscopic examination. Diagnosis was confirmed by at least two independent pathologists. Pancreatic cancers were diagnosed according to the WHO classification.

Immunohistochemistry Immunoperoxidase staining was performed by the method of Hsu *et al.*[15] with some modification. Briefly, 4-μm cryostat tissue sections on slides were deparaffinized in three changes of xylene (5 min each) and rehydrated through successive dilution of ethanol (5 min each change): absolute ethanol and 80% ethanol. After washing of the sections in distilled water for 5 min, endogenous peroxidase activity was blocked by preincubation in methanol containing 0.3% H_2O_2 for 30 min. All procedures were performed at room temperature. The sections were washed in PBS and then placed in a solution of 2% normal goat, horse or rabbit serum (Vector Laboratories, Burlingame, CA) in 0.05*M* Tris buffer (pH 7.6) with 3% BSA (Sigma Chemical Co.). The primary antibody was added and the sections were incubated overnight at 4°, then washed with PBS. An aliquot of biotinylated goat anti-mouse IgM antibody (Vector Laboratories), biotinylated horse anti-mouse IgG (Vector Laboratories) or biotinylated rabbit anti-goat IgG (MBL Co. Ltd., Nagoya) was added and the sections were incubated for 30 min. After being washed with PBS, the slides were treated similarly with the avidin DH-biotinylated horseradish peroxidase H complex (Vector Laboratories). The slides were washed again with PBS, and an aliquot of a 0.05% diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka) and 0.01% H_2O_2 solution in 0.05*M* Tris buffer (pH 7.2) was applied. The slides were incubated for 1 min and then rinsed in distilled water. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted under a cover slip. For comparison, monoclonal antibodies to CA 19-9 (a gift from Centocor Inc., Malvern, PA), and DU-PAN-2 (a gift from Kyowa Medix Co., Ltd., Tokyo), and the antibody to CEA (MBL Co., Ltd.) were used. Hybridoma supernatants containing non-specific immunoglobulin were used as a negative control. Staining intensity was determined as follows. Strong brown staining was scored as ++, moderate staining as +, faint staining ±, and no staining −. Faintly stained (±) specimens were not included in the positive group.

Periodic Acid and Enzyme Treatment of Tissue Sections Periodate (10m*M* in sodium acetate buffer, pH 4.5; Wako Industries) oxidation was carried out for 10 min or 1 hr at room temperature. The slides were incubated at 37° for 2 hr with various concentrations of clostridial neuraminidase (type VI; Sigma Chemical Co.) diluted in 0.2 ml of PBS (pH 6), ranging from 1.0 to 0.02 unit per slides. The DU-PAN-2 and CA 19-9 antibodies were used as positive controls. Similarly, 0.1% trypsin (DIFCO Laboratories, Detroit, MI) digestion was done for 30 min at 37°. After treatment, the slides were washed, exposed to monoclonal antibodies, and processed as described before.

RESULTS

Production of Monoclonal Antibody PA 8-15 A fusion of spleen cells from a mouse immunized with SUIT-2 cells with P3×63-AG-8-U1 myeloma cells resulted in the production of 215 hybrid cultures. Among them, antibodies produced by 26 hybridomas reacted with SUIT-2 cells by whole-cell-preparation ELISA. These hybrid cultures were then screened with formalin-fixed paraffin-embedded sections of SUIT-2 tumor transplanted in nude mice, and three of 26 hybridoma supernatants reacted with the tissue sections by the ABC staining technique. One of the three hybridomas that reacted strongly with pancreatic cancer tissues was selected and recloned three times by limiting dilution. Finally, one hybridoma, PA 8-15, which continuously released monoclonal antibody in the supernatant, was produced.

The monoclonal antibody PA 8-15 was identified to be of the IgM (lambda) subclass, as determined by double immunodiffusion analysis and ELISA using a peroxidase-conjugated immunoglobulin kit (data not shown).

Preliminary Antigen Characterization The binding of PA 8-15 MAb to pancreatic cancer tissues was markedly reduced after treatment with neuraminidase. However, oxidation with periodate and trypsin treatment did not affect the reactivity of PA 8-15 MAb, whereas bindings of both DU-PAN-2 and CA 19-9 MAb to the same tissue sections, as positive controls, were drastically reduced by both periodate and neuraminidase treatments. These results suggest that the antigenic determinants of PA 8-15 are of sialylated carbohydrate nature. PA 8-15 MAb did not react with various blood cells in hemagglutination assays.

Immunohistochemistry The ABC immunoperoxidase technique was used to define the reactivity of PA 8-15 MAb. First, the staining titers of PA 8-15 MAb in both hybridoma supernatants and ascitic fluids of the hybridoma produced in mice were determined by using strongly stained pancreatic cancer sections. Staining of culture supernatants was pronounced at a dilution of 1:20, but disappeared at 1:30. In contrast, staining of ascitic fluids was pronounced up to a dilution of 1:5000.

Table I shows the reactivity of PA 8-15 MAb with neoplastic and non-neoplastic

Table I. Immunohistochemical Reactivity of PA 8-15 Antibody with Various Tumors

Tumor tested	No. tested	No. of tissue specimens			
		++	+	±	−
Cancer					
Pancreas	28	12	15	1	
Gall bladder	6	3	3		
Bile duct	8	3	5		
Liver	4			4	
Esophagus	6			2	4
Stomach	10	2	4	4	
Colorectum	11	2	5	4	
Thyroid	4				4
Lung	4				4
Breast	6				6
Ovarium	3		1		2
Other malignancy					
Sarcoma of the colon	1				1
Malignant thymoma	1				1
Benign					
Insulinoma	1				1
Adenoma of the stomach	3				3
Adenoma of the colon	3				3

Staining intensity: ++, strongly positive; +, positive; ±, faintly positive; −, negative.

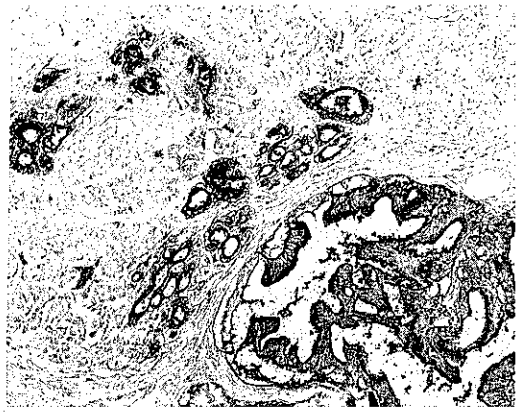

Fig. 1. Section of moderately differentiated adenocarcinoma of the pancreas stained by immunoperoxidase with PA 8-15 MAb, showing strong staining of tumor cells and adjacent non-staining stroma and normal pancreatic tissue. Hematoxylin counterstain (×40).

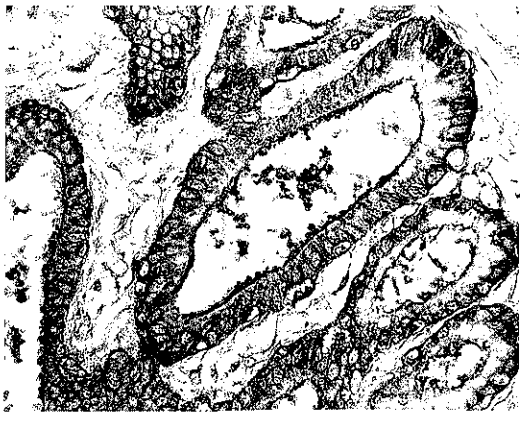

Fig. 2. Section of moderately differentiated adenocarcinoma of the pancreas stained by immunoperoxidase with PA 8-15 MAb, showing prominent staining of the cancer cell surface of the luminal side compared to the cytoplasm and surrounding non-staining stroma. Hematoxylin counterstain (×200).

Table II. Immunohistochemical Reactivity of PA 8-15 Antibody with Normal and Inflammatory Tissues in Adults

Tissue tested	No. tested	No. of tissue specimens			
		++	+	±	−
Normal					
Pancreatic ducts	14		14		
Acini and islets	14				14
Gall bladder	6		3	3	
Bile duct	9		9		
Liver	6				6
Esophagus	4				4
Stomach	7				7
Small intestine	6				6
Colorectal	9				9
Brain	1				1
Thyroid	3				3
Lung	3				3
Breast	7				7
Kidney	3				3
Spleen	6				6
Inflammatory					
Chronic pancreatitis[a)]	9				9
Cholecystitis[a)]	2				2
Chronic hepatitis	2				2
Mastopathy	3				3

Staining intensity: ++, strongly positive; +, positive; ±, faintly positive; −, negative.
a) Except for the epithelium, as seen in normal adult.

Table III. Immunohistochemical Reactivity of PA 8-15 Antibody with Normal Fetal Tissues

Tissue tested	No. tested	No. of tissue specimens			
		##	+	±	−
Esophagus	2		1	1	
Stomach	2			2	
Small intestine	2	1	1		
Colon	3	2	1		
Pancreatic ducts	3		3		
Acini and islets	3			3	
Liver	3				3
Lung	3		1	1	1

Staining intensity: ##, strongly positive; +, positive; ±, faintly positive; −, negative.

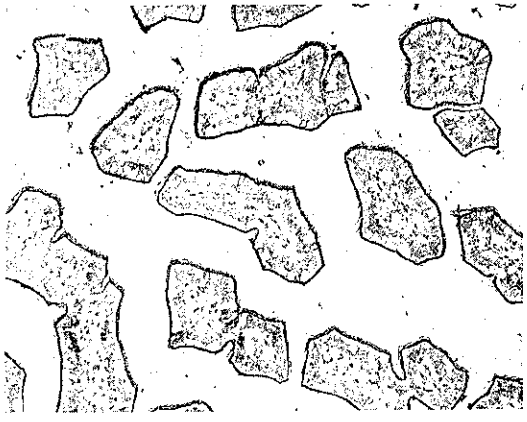

Fig. 3. Section of normal fetal small intestine (13 W) stained by immunoperoxidase with PA 8-15 antibody, showing reactivity of the apical free cell border of cells. Hematoxylin counterstain (×100).

Table IV. Immunoperoxidase Reactivity of Antibodies with the Same Tissues of 28 Pancreatic Cancers

Antibody	No. of tissue specimens				Positivity (%)
	##	+	±	−	
PA 8-15	13	14	1		96
CA 19-9	11	5	3	9	57
DU-PAN-2	4	16	3	5	71
CEA	3	9	3	13	43

Staining intensity: ##, strongly positive; +, positive; ±, faintly positive; −, negative.

tissues from the pancreas and common tumors from various sites in adults. PA 8-15 MAb reacted with 27 of the 28 pancreatic carcinomas examined. All gall bladder and choledochal carcinomas also showed pronounced diffuse staining of all the tumor cells, as seen in pancreatic cancer. Six of the 10 stomach carcinomas and seven of the 11 colorectum carcinomas showed a positive reaction with both the cell surface and cytoplasm of all cancer cells. However, intratumor difference of staining intensity was observed in most of the cancer tissues of the stomach and colorectum. None of the other tumors, including benign tumors, showed reactivity except for one ovarian adenocarcinoma in which the staining pattern was similar to that of the stomach and colorectum cancers. Well and moderately differentiated adenocarcinomas of the pancreas showed a pronounced staining on the cell membrane and in the cytoplasm, whereas the fibroblasts and connective tissue surrounding the tumor cells were not stained (Figs. 1 and 2).

Reactivity of PA 8-15 MAb with normal and inflammatory tissues is shown in Table II. Among the normal tissues, the apical free cell border of cells of the pancreatic duct was stained positive. A similar positive staining pattern was observed only in the apical surfaces of epithelial cells of both the gall bladder and bile duct. However, the acinar and islet cells of the pancreas and epithelium of the stomach and colorectum were not reactive with PA 8-15 MAb. The antigens recognized by PA 8-15 MAb were not expressed on other

Table V. Correlation between Immunoperoxidase Reactivity with Antibodies and Histological Types and Degrees of Differentiation of 28 Pancreatic Cancers

Classification	No. tested	No. positive (%)		
		PA 8-15	CA 19-9	DU-PAN-2
Adenocarcinoma	25			
Well[a]	11	11 (100)	8 (73)	8 (73)
Moderate[b]	12	11 (92)	6 (50)	9 (75)
Poor[c]	2	2 (100)	0 (0)	2 (100)
Cystadenocarcinoma	1	1 (100)	1 (100)	1 (100)
Undifferentiated	2	2 (100)	0 (0)	1 (50)

a) Well differentiated type.
b) Moderately differentiated type.
c) Poorly differentiated type.

normal tissues of the gastrointestinal or non-gastrointestinal system in adults. Interestingly, no staining was observed in nine tissues of chronic pancreatitis, two of cholecystitis, two of chronic hepatitis except for the apical cells, which were positive in normal adult tissues, and three of mastopathy.

Importantly, positive staining of normal fetal tissues (12W-33W) was noted in all of the pancreatic duct samples and six of the nine gastrointestinal mucosa, and the apical free cell border of cells was stained as seen in normal adult tissues (Table III, Fig. 3). Bronchial epithelial cells of one case were also positive, whereas normal pancreatic acinar and endocrine cells were negative. The fetal lung and liver parenchyma were negative but the antigen was barely detectable in fetal gastric mucosa. Thus, some differences in positively stained organs were observed between adults and normal fetuses. However, only the apical surface was positive, as was seen in adults.

The staining pattern and positivity were compared with those of CA 19-9, DU-PAN-2 MAbs and CEA antibody by using the same specimens of 28 pancreatic carcinomas (Table IV). Positivity was 96% for PA 8-15, 57% for CA 19-9, 71% for DU-PAN-2, and 43% for CEA. Thus, PA 8-15, CA 19-9, and DU-PAN-2 MAbs had high reactivity with pancreatic adenocarcinoma. PA 8-15 and DU-PAN-2 MAbs strongly stained both the cell surface and the cytoplasm of undifferentiated carcinoma, whereas PA 8-15 and CA 19-9 MAbs prominently stained the cell surface of the luminal side compared to the cytoplasm of poorly, moderately and well differentiated carcinomas. However, CA 19-9 MAb was not reactive in two cases of poorly differentiated adenocarcinoma and two of undifferentiated carcinoma. Poorly differentiated adenocarcinoma and undifferentiated carcinoma of the pancreas showed strong staining but intratumor heterogeneity was scarcely seen against PA 8-15 MAb. Interestingly, the staining of PA 8-15 and CA 19-9 MAbs among positive cases showed little intratumor heterogeneity, whereas DU-PAN-2 MAb exhibited it in several cases. Furthermore, connective tissue was sometimes positive for DU-PAN-2 MAb (Fig. 4).

Although PA 8-15 MAb did not show intratumor heterogeneity in cancers of the pancreas and biliary tract, heterogeneous stainings were observed in cancers of the stomach, colorectum and ovarium.

Discussion

The PA 8-15 monoclonal antibody was prepared against human pancreatic cancer. PA 8-15 MAb detected most of the pancreaticobiliary tract cancers but did not react with chronic pancreatitis, other inflammatory tissues or benign tumors.

The results of enzyme treatment suggested the involvement of sialylated carbohydrate in the epitope recognized by PA 8-15 MAb. In this respect, the epitope recognized by PA 8-15 MAb resembles those of CA 19-9, DU-PAN-2 and CA-50.[7, 16–18] Histochemical studies, in which the determinant of PA 8-15

antigen was detected in the bowel and some other tissues of the fetus, indicated that it belongs to a class of oncodevelopmental antigens. Expression of the PA 8-15 antigen may be shut off during cell differentiation and re-expression may occur during malignant changes because only cancer showed this antigen among adult tissues except for the normal epithelium of the pancreas and biliary tract.

PA 8-15 antigens were distributed in normal adult and fetal tissues of humans as was reported for CA 19-9 and DU-PAN-2 by other investigators,[8, 19] and were localized in apical free cell border of cells of the pancreatic duct, gall bladder and bile duct epithelia in normal adult and several fetal tissues. In fact, in our study, DU-PAN-2 MAb often reacted with connective tissues, and acinar and islet cells of the normal pancreas in adults, while CA 19-9 MAb revealed a reactive pattern similar to that of PA 8-15 MAb in normal tissues. On the other hand, PA 8-15 antigens, as well as CA 19-9 and DU-PAN-2, were localized in both the cell surface and cytoplasm of various paraffin-embedded specimens of carcinomas. In normal and non-neoplastic tissues, ductular structures composed of cells displaying the apical-basal orientation are intact. However, lack of this orientation in malignant change would result in a change of the antigen localization.

Immunohistochemical reactivity of PA 8-15 MAb in the same pancreatic cancers differed from those of CA 19-9, DU-PAN-2, and CEA antibody. Higher positivity (96%) of PA 8-15 MAb was achieved as compared with those of CA 19-9 (57%), DU-PAN-2 (71%) and CEA (43%). One tissue not reactive with PA 8-15 MAb was histologically moderately differentiated adenocarcinoma. This should not be due to antigen degeneration by prolonged fixation, because all specimens were routinely processed for examination. Furthermore, the markers showed different patterns for pancreatic cancer. These results suggest that PA 8-15 MAb recognizes a

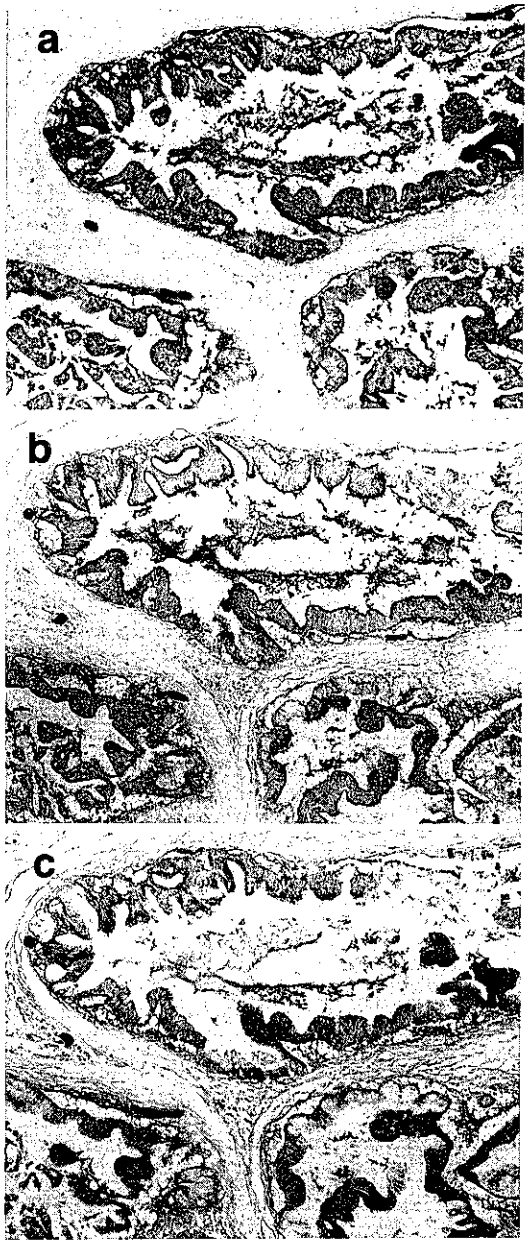

Fig. 4. Comparison of stainability by immunoperoxidase between (a) PA 8-15, (b) CA 19-9 and (c) DU-PAN-2 MAbs in the identical area of pancreatic carcinoma. (a) Strong staining both of the cell surface and cytoplasm of cancer cells. (b) Strong staining of the cancer cell surface, but weak staining of the cytoplasm of cancer cells compared to PA 8-15 MAb. (c) Marked intratumor heterogeneity and granular strong staining in the cytoplasm of cancer cells, with weak staining of the cancer cell surface and normal connective tissue. Hematoxylin counterstain (×100).

different epitope from those of CA 19-9 and DU-PAN-2. Nonspecific staining was found in the cases of DU-PAN-2 and CEA. However, with each antibody, no apparent correlation was observed between positivity or intensity of staining and level of differentiation in pancreatic carcinomas. The staining intensity of CA 19-9 MAb correlated well with the serum values of CA 19-9 (data not shown). A similar tendency was observed for CEA (data not shown), suggesting that PA 8-15 antigen is a useful tumor marker for cancers of the digestive system.

Furthermore, since this monoclonal antibody retains its reactivity with formalin-fixed paraffin-embedded material, it could become a useful reagent for retrospective studies of histological subclassification and differentiation of gastrointestinal cancers. It could also be useful for diagnostic purposes such as serum diagnostics and tumor imaging of pancreatico-biliary tract carcinomas.

However, we must test more cases for the presence of the antigen recognized by PA 8-15 MAb to further clarify its distribution and applicability.

(Received Aug. 26, 1987/Accepted Dec. 9, 1987)

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