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Detection of a pancreatic cancer-associated antigen (DU-PAN-2 antigen) in serum and ascites of patients with adenocarcinoma

(monoclonal antibody/radioimmunoassay)

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ABSTRACT **A competition radioimmunoassay was developed, utilizing a murine monoclonal antibody to human pancreatic adenocarcinoma cells. Immunoblotting of a standard antigen preparation from either serum or ascites fluid after electrophoresis in 1% agarose showed that the specific DU-PAN-2 activity resided in two major high molecular weight bands. DU-PAN-2 antigen levels were expressed as arbitrary units based on a standard partially purified antigen preparation. The inhibition curve with standard antigen was reproducible (SD < 10%) and essentially linear from 25 to 200 units/ml. The mean DU-PAN-2 antigen concentration for the sera from 126 normal individuals was 81 units/ml. Sera from pediatric patients with malignancy had a mean of 127 units/ml, while nasopharyngeal, stage III melanoma, and ovarian carcinoma patients had means of 89, 92, and 119 units/ml, respectively. All values in normal subjects as well as the melanoma, nasopharyngeal, ovarian, and pediatric cancer patients were less than 400 units/ml. Intermediate antigen levels were detected in patients with alimentary tract malignancies. Eight of 20 gastric cancer and 8 of 76 colorectal carcinoma patients and 3 patients with benign or nonmalignant gastrointestinal tract disease had DU-PAN-2 values exceeding 400 units/ml. Ascites fluids from 6/6 and pancreatic juice from 2/2 pancreatic cancer patients had values greater than 750 units/ml. Serum from 68% of the 89 pancreatic cancer patients tested had DU-PAN-2 antigen levels greater than 400 units/ml. The mean serum value in this patient population was 4888 units/ml.**

Carcinoma of the pancreas is the fifth leading cause of cancer death in the United States (1) and the incidence of the disease appears to be rising (2). Once discovered, the disease is nearly uniformly fatal. The high mortality relates to the inability to make the diagnosis early, limited successful therapy, and poor capacity for recognizing recurrent or progressive disease. A reliable protocol for detecting and monitoring pancreatic cancer could enhance treatment of the disease. We have produced a monoclonal antibody to pancreatic adenocarcinoma that might provide such a tool.

We have previously reported on the production and partial serological characterization of five monoclonal antibodies elicited to the human pancreatic adenocarcinoma cell line HPAF (3). One of these monoclonal antibodies, DU-PAN-2, detects an antigen present on some cells of fetal pancreas and on ductal epithelial cells of normal adult pancreas, as well as on adenocarcinoma cells of pancreatic and nonpancreatic origin (3, 4). Recent studies of the tissue distribution of this antigen indicated that the antigen is probably a secretory cell product. Although DU-PAN-2 antigen is not a tumor-specific marker, there is a marked difference in the level of expression of the antigen on neoplastic tissues compared to analogous nonneoplastic structures (4). Preliminary studies on the molecular properties of the DU-PAN-2 antigen suggest that the epitope is sensitive to neuraminidase and is expressed on a large, heavily glycosylated molecule (ref. 5 and unpublished data). In this report, we describe the electrophoretic properties of the DU-PAN-2 antigen and its identification by immunoblotting. A competition radioimmunoassay (RIA) for the antigen was also developed, using the DU-PAN-2 IgM monoclonal antibody. This report describes the use of this competition RIA to detect and monitor the levels of DU-PAN-2 antigen in serum and ascites of patients with various malignant and nonmalignant diseases.

MATERIALS AND METHODS

DU-PAN-2 Antigen. The DU-PAN-2 antigen used as target antigen for the RIA and as a standard antigen for the inhibition curves was prepared from ascites of a patient with pancreatic adenocarcinoma. An immunoaffinity column could not be used for antigen purification because the DU-PAN-2 IgM antibody shows poor antigen binding when coupled to an insoluble matrix. Therefore, the antigen was partially purified by other techniques. A 50–75% saturated ammonium sulfate fraction of the ascitic fluid contained >95% of the DU-PAN-2 antigen. Since human serum albumin in the ascites fluid precipitated with DU-PAN-2 antigen in the 50–75% saturated ammonium sulfate fraction, material from that fraction was dialyzed against 20 mM sodium phosphate, pH 7.1, and chromatographed on a Cibacron blue F3G-A-Sepharose CL-6B column to remove albumin. More than 90% of the DU-PAN-2 antigen was eluted within one bed volume, while 90% or more of the albumin was removed. The antigen preparation recovered from the Cibacron blue F3G-A-Sepharose CL-6B column was then treated with 1.0 M perchloric acid for 15 min in an ice bath, centrifuged at 15,000 × *g* for 5 min, decanted, neutralized, and dialyzed overnight against phosphate-buffered saline.

For electrophoretic and immunoblot analysis, DU-PAN-2 antigen was also purified by cesium bromide density gradient centrifugation (6) from serum of a pancreatic cancer patient. This serum had 1000 units of DU-PAN-2 antigen per ml by competition RIA. The serum was dialyzed against deionized water. Cesium bromide (42%, wt/wt) was then added to achieve a loading density of 1.43 g/cm^3. The samples were centrifuged for 72 hr at 10°C and 30,000 rpm in a Beckman SW 41 Ti rotor. The high-density fractions were collected, dialyzed against water, and lyophilized. A different approach

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was taken to purify the antigen from serum due to the high concentration of other serum proteins, which interfered with the standard ascites protocol.

Competition RIA. Flexible polyvinyl chloride 96-well plates (Dynatech, Alexandria, VA) were prepared for antigen coating by adding 40 μl of 50 μg/ml poly(L-lysine) (Sigma, P1274) per well. After a 30-min incubation at room temperature, the plates were washed once with phosphate-buffered saline and were incubated overnight at 4°C with 50 μl of phosphate-buffered saline containing 1000 units (defined below) of partially purified DU-PAN-2 antigen (see above). Meanwhile, 50 μl of a test sample or control material was incubated overnight at 4°C with 50 μl of DU-PAN-2 IgM antibody from tissue culture supernatant (0.2–0.3 μg of IgM per ml) in RIA buffer (1% bovine serum albumin/1% normal goat serum in phosphate-buffered saline). After the overnight incubation, the antigen-coated plates were washed once with phosphate-buffered saline and incubated 30 min at room temperature with 50 μl of RIA buffer. RIA buffer was then removed from the wells and 40 μl of the test sample/DU-PAN-2 antibody mixture was added to duplicate wells. The plates were incubated for 1 hr at room temperature and washed four times with phosphate-buffered saline. RIA buffer (100 μl) containing approximately 8×10^5 cpm of ^{125}I-labeled F(ab')$_2$ fragments of goat antiserum to mouse Ig was then added and the mixture was incubated for 2 hr at room temperature. The plates were then washed seven times in phosphate-buffered saline and dried, and radioactivities in individual wells were determined in a gamma spectrometer. The conditions and materials described above were judged to be optimal for DU-PAN-2 antigen detection by varying the concentrations of the first and second antibody and the times and temperatures of the incubations. The overnight incubation of the test sample and DU-PAN-2 antibody at 4°C could be changed to 2 hr at room temperature. However, since the antigen coating of the plates required an overnight incubation we preferred to use the overnight incubation at 4°C for the antibody inhibition phase as well. DU-PAN monoclonal antibodies 1, 3, 4, and 5 (3) failed to show significant binding with the DU-PAN-2 antigen in this RIA.

Agarose Gel Electrophoresis and Immunoblotting. Lyophilized DU-PAN-2 antigen from both serum and ascites was dissolved in sodium dodecyl sulfate/polyacrylamide gel electrophoresis sample buffer (7) without 2-mercaptoethanol and then subjected to horizontal slab gel electrophoresis in 1.0% agarose in Tris/boric acid/EDTA buffer containing 0.1% sodium dodecyl sulfate, at 100 V, for 4 hr (8). Transfer to nitrocellulose paper was performed by electroblotting at 30 V, overnight in the cold. The sheet was then incubated with 5% bovine serum albumin at 40°C followed by DU-PAN-2 antibody or a control antibody at room temperature for 1 hr with continuous rocking. After washing, the paper was incubated for 1 hr with rabbit antibody to mouse IgM, followed by 1-hr incubation with ^{125}I-labeled staphyloccal protein A at 10^6 cpm/ml. The blots were again extensively washed, blotted dry, and exposed to Kodak X AR-5 x-ray film with an intensifying screen at −70°C for 1 hr.

Clinical Samples. Serum, plasma, and ascites samples were obtained from normal volunteers and patients with malignant and nonmalignant disease. Some of the samples were obtained directly from the patient and then tested before freezing, while other samples, often from the serum banks of collaborating investigators, were tested after freezing. The samples from pediatric patients with lymphomas or solid tumors were obtained from Dr. Barry Dowell, Texas Childrens Hospital, Houston; the sera from nasopharyngeal carcinoma patients were from Dr. T. C. Lynn, National Taiwan University Hospital, Taipei; and the sera from ovarian cancer patients was obtained from Dr. Stanley Gall, Department of Obstetrics–Gynecology, Duke University Medical Center, Durham, NC.

RESULTS

Immunoblot of DU-PAN-2 Antigen. We have analyzed the binding reactivity of DU-PAN-2 antibody with soluble antigen preparations from ascitic fluid and serum of pancreatic adenocarcinoma patients. Both preparations were partially purified, subjected to electrophoresis in 1% agarose, and immunoblotted as described in *Materials and Methods*.

The DU-PAN-2 antibody gave similar patterns of reactivity with the antigen preparations from both serum (Fig. 1, lane B) and ascites fluid (Fig. 1, lane C). Two prominent bands were seen with each antigen and the apparently higher molecular weight bands from both sources migrated to the same positions on the gels. The lower molecular weight band from serum migrated slower and was less intense than that from ascites. An IgM monoclonal antibody to a human myeloid cell antigen failed to detect bands in these regions (Fig. 1, lane A) in either preparation.

RIA. Rather than express the results as percent inhibition and titer, we express the quantity of DU-PAN-2 antigen in a sample as arbitrary units/ml based on reference to the partially purified standard antigen sample (see *Materials and Methods*). The amount of DU-PAN-2 antigen in 20 μl of a 1:500 dilution of the standard antigen preparation was designated as 100 units/ml. As shown in Fig. 2, a semilogarithmic plot of the percent inhibition vs. DU-PAN-2 antigen concentration is essentially linear over the range of 20–70% inhibition and could therefore be used as a standard curve for calculating antigen concentration in an unknown sample. The standard deviations ranged from 9.8% at 25 units/ml to 6.5% at 200 units/ml and were calculated from the mean of 13–21 determinations for each point. Each of these determinations was done on different testing dates over a 2- to 3-month period. Thus, the between-assay variability for the standard antigen preparation was 10% or less. Purified carcinoembryonic antigen, ABO, and Lewis blood group antigens gave less than 5% inhibition in the RIA. Body fluid samples being tested for DU-PAN-2 antigen were then titered by 1:1 dilutions and units/ml were calculated from the dilution giving between 30% and 70% inhibition.

Partially purified DU-PAN-2 antigen was stable at 100°C for more than 15 min and there has been no loss of DU-PAN-2 antigen activity in the standard antigen preparation stored at −20°C for over 1 year. Sera from patients with high levels of DU-PAN-2 antigen and sera from normal donors have not

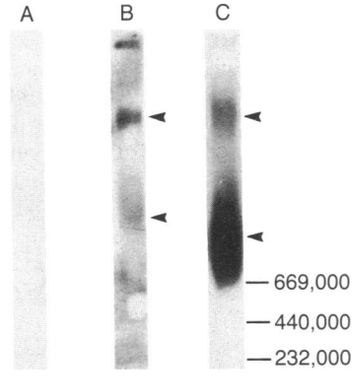

FIG. 1. Analysis of DU-PAN-2 antigens by 1% agarose gel electrophoresis and immunoblotting. Lane A, immunoblot of either antigen with DU-HL60-3, an IgM antibody to a myeloid cell antigen. DU-PAN-2 antibody was used in immunoblots of serum antigen (lane B) and ascites antigen (lane C). Thyroglobulin (M_r, 669,000), ferritin (M_r, 440,000), and catalase (M_r, 232,000) were used as molecular weight standards.

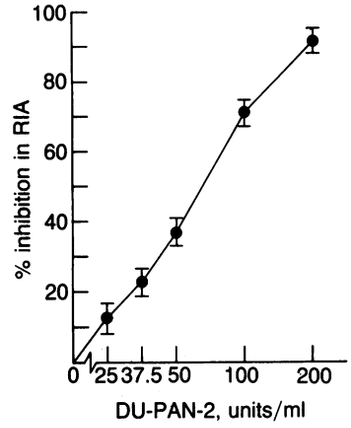

FIG. 2. DU-PAN-2 standard antigen titration. Partially purified DU-PAN-2 antigen was titrated in the competition RIA and the values are plotted as arbitrary units vs. percent inhibition. Bars indicate the standard deviation of at least 13 different determinations over a 3-month period.

shown any marked changes in DU-PAN-2 units after storage at −20°C for 6 months. Patients' sera stored longer than 2 years at −20°C still had high levels of DU-PAN-2 antigen when tested.

Detection of DU-PAN-2 Antigen in Ascites and Cyst Fluids. Ascites fluid from the patient from whom the HPAF cell line was derived was used as the source of the standard antigen. The untreated and perchloric acid-extracted ascites fluid from this patient contained more than 50,000 units/ml, whereas ascites fluid from a patient with chronic renal failure had less than 10 units/ml. Ascites or cyst fluid samples were not tested at less than a 1:4 dilution. All of the ascites fluids from patients with pancreatic adenocarcinoma had 750 or more units/ml, whereas ascites fluid from one patient with chronic renal failure and three patients with leukemia or lymphoma had <10 units/ml (Table 1). Patients with other types of solid tumors gave intermediate levels ranging from <100 units/ml to 1000 units/ml. Fluid aspirated from pseudocysts from two patients with pancreatitis had <50 units/ml, whereas fluid from a patient with a mucinous cyst adenocarcinoma of the pancreas had >100,000 units/ml. Fluid from the pancreatic duct of two pancreatic adenocarcinoma patients was taken at the time of surgical resection and was found to have 2400 and 5200 units/ml. Pancreatic duct fluid from a patient with a nongastrointestinal tract malignancy had only 83 DU-PAN-2 units/ml.

Detection of DU-PAN-2 Antigen in Serum. Sera from normal volunteers, from patients with a variety of malignant diseases, and from patients with pancreatitis were titered in the competition RIA; the DU-PAN-2 units/ml were calculated and expressed in a dot chart (Fig. 3). Data on the sera from patients with pancreatitis and pancreatic adenocarcinoma were plotted irrespective of their clinical status at the time the serum sample was obtained. The patients with other malignancies had progressive disease at the time the sample was taken but some were receiving therapy. The mean DU-PAN-2 concentration for the sera from 126 normal individuals was 81 units/ml. Sera from pediatric patients with solid tumors or lymphomas had a mean of 127 units/ml, while nasopharyngeal carcinoma, stage III melanoma, and ovarian cancer patients with a heavy tumor burden had means of 89, 92, and 119 units/ml, respectively. All values in normal subjects as well as the melanoma and pediatric cancer patients were less than 300 units/ml and only infrequently were values above 200 units/ml in these groups. Of the 55 ovarian cancer patients tested in this study, none had values >400 units/ml. Intermediate antigen levels were detected in patients with alimentary tract malignancies. Eight of 20 gastric cancer and 8 of 76 colorectal carcinoma patients had values greater than 400 units/ml. Only 3 of 48 patients with benign gastrointestinal tract disease, including pancreatitis, had values exceeding 400 units/ml. Of 89 tested patients with pancreatic carcinoma, 77% had DU-PAN-2 antigen levels greater than 300 and 68% had values greater than 400 units/ml. The mean DU-PAN-2 value in this patient population was 4888 units/ml. Serum from 14 of the pancreatic cancer patients had levels between 10,000 and 100,000 units/ml (shown as >7000 in Fig. 3).

Table 1. Detection of DU-PAN-2 antigen in ascites and cyst fluids from patients with malignant and nonmalignant disease

Patient source (diagnosis)	Type of fluid	DU-PAN-2, units/ml
1. Pancreatic carcinoma	Ascites	>50,000
2. Pancreatic carcinoma	Ascites	>20,000
3. Pancreatic carcinoma	Ascites	>20,000
4. Pancreatic carcinoma	Ascites	2,960
5. Pancreatic carcinoma	Ascites	750
6. Pancreatic carcinoma	Ascites	800
7. Gastric carcinoma	Ascites	750
8. Metastatic carcinoma (primary unknown)	Ascites	1,000
9. Metastatic carcinoma (primary unknown)	Ascites	350
10. Metastatic carcinoma (primary unknown)	Ascites	300
11. Colon carcinoma	Ascites	150
12. Colon carcinoma	Ascites	360
13. Lung carcinoma	Ascites	<100
14. Chronic renal failure	Ascites	<10
15. Leukemia	Ascites	<10
16. Lymphoma	Ascites	<10
17. Lymphoma	Ascites	<10
18. Cystadenocarcinoma of the pancreas	Cyst	>100,000
19. Pancreatitis with pseudocyst	Cyst	<50
20. Pancreatic carcinoma	Pancreatic duct	5,200
21. Pancreatic carcinoma	Pancreatic duct	2,400
22. Nongastrointestinal tract malignancy	Pancreatic duct	83

Some of the pancreatic adenocarcinoma patients reported in Fig. 3 to have low DU-PAN-2 antigen values were receiving therapy and were classified as having stable or minimal diseases. We were, therefore, interested in doing serum antigen determinations on different dates to investigate possible correlations between antigen levels and disease status. Serial serum samples from most pancreatic cancer patients were not readily available for this type of retrospective study. Samples from two patients were available, however, and data from these patients are indicative of the problems and questions that need to be addressed by future prospective studies (Table 2). Serum DU-PAN-2 antigen levels in patient A were mildly elevated (380 units/ml) when the patient was first seen for obstructive jaundice. The antigen levels declined after a bypass operation. The patient's bilirubin was also elevated initially due to the common bile duct obstruction. When the hepatic duct was later obstructed, both the DU-PAN-2 antigen and bilirubin levels were at their highest, 1400 units/ml and 18.3 mg/dl, respectively. When the hepatic duct obstruction was relieved, liver function improved and the DU-PAN-2 antigen concentration became normal. Throughout this period, patient A was not receiving therapy and her tumor burden remained stable as well as could be clinically documented.

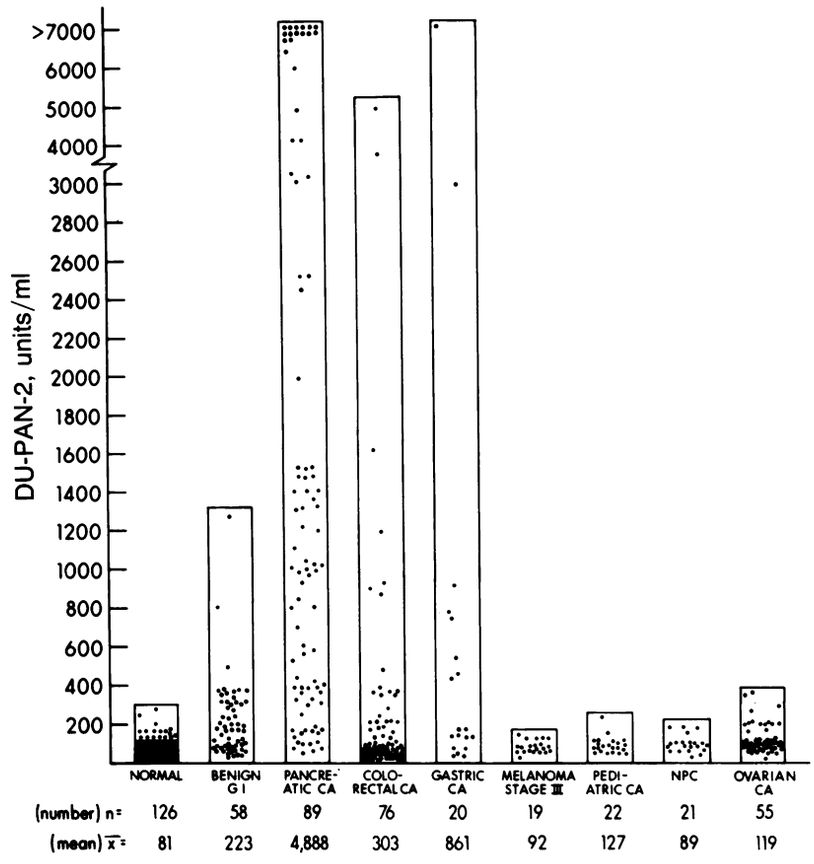

FIG. 3. DU-PAN-2 antigen levels of sera from normal volunteers and patients with pancreatitis or cancer. Each dot represents the mean of triplicate determinations from a single testing date. Samples from normal donors having 100 units/ml or less were too numerous to depict as dots and are shown as a solid rectangle. CA, carcinoma; NPC, nasopharyngeal carcinoma. The benign GI category includes pancreatitis and benign or nonmalignant gastrointestinal tract disease.

Patient B (Table 2) showed a drop in DU-PAN-2 antigen from 992 to 190 units/ml within 3 days of resection of his primary pancreatic tumor. The patient remained clinically stable for over 1 year with normal DU-PAN-2 levels and then was found to have recurrent and progressive disease with elevated DU-PAN-2 antigen in June 1982. The patient expired in July 1982, when DU-PAN-2 antigen was 1216 units/ml. There was a poor correlation in this patient between bilirubin/alkaline phosphatase levels and DU-PAN-2 antigen levels.

Table 2. Serial data on pancreatic cancer patients

Sample date	Clinical summary	AP	Bilirubin	DU-PAN-2, units/ml
	Patient A			
10/06/82	Obstructive jaundice due to tumor	199	8.6	380
11/16/82	No clinical jaundice	ND	ND	126
12/23/82	Extreme clinical jaundice	260	3.6	ND
1/25/83	Deeply jaundiced; obstructed hepatic duct	ND	18.3	1400
3/03/83	Subclinical jaundice	164	2.7	ND
4/27/83	No clinical jaundice; obstructed duct relieved	ND	ND	166
	Patient B			
12/10/81	Preoperative sample; extensive primary disease; no evidence of metastases	135	2.4	992
12/14/81	Three days postoperative	103	1.0	190
1/04/82	Stable clinically; no evidence of disease	98	0.4	176
6/18/82	Progressive disease	576	2.2	992
7/12/82	Progressive disease	295	1.0	1216

AP, alkaline phosphatase; ND, not done. Alkaline phosphatase is expressed as international units/ml; bilirubin is mg/dl.

DISCUSSION

Agarose gel electrophoresis followed by immunoblotting (Fig. 1) provides a means of assessing the molecular properties of the DU-PAN-2 antigen, the nature of the antigenic determinant, and a method for determining the relationship of DU-PAN-2 to other tumor-associated antigens with similar properties. The highly glycosylated nature of DU-PAN-2 molecules and their behavior on gels and density gradients allows for their separation from many proteins and glycoproteins. More detailed studies on the molecular properties of the DU-PAN-2 antigen will be reported separately. However, we did want to provide the reader of this report with some visual data on the DU-PAN-2 antigen.

The competition RIA, as described in this report, appears to be a rapid and sensitive assay for fluids containing molecules with the DU-PAN-2 epitope. The quantitation of DU-PAN-2 antigen in terms of protein concentration is not justified because our most purified antigen preparations to date had little protein and the epitope is carbohydrate. When sufficient quantities of highly purified DU-PAN-2 antigen are available we will be able to determine the weight of a standard antigen and express the results in weight units. For the present purposes, however, quantitation of antigen concentration by comparison to a standard reference has been shown to be reliable and useful. We were initially concerned that undiluted serum from normal donors showed some

inhibition in the competition RIA. Although the reason for this inhibition is still unknown, there are striking quantitative differences in the levels of inhibition by normal donor sera and those from pancreatic cancer patients. This difference became apparent when the concentration of DU-PAN-2 antigen was expressed in arbitrary units.

We were unable to adapt the DU-PAN-2 monoclonal antibody to a radioimmunometric type assay because this IgM antibody lost much of its ability to bind antigen when coupled to an insoluble matrix. "Second generation" murine monoclonal antibodies and polyclonal rabbit antibodies to purified DU-PAN-2 antigen preparations could result in production of IgG antibodies or antibodies with greater binding affinities to this antigen.

The detection of soluble tumor antigen in ascites and cyst fluids may offer an approach to diagnosis of carcinoma independent of cytologic examination. The number of patients with ascites or cyst fluid samples tested in this report for DU-PAN-2 antigen is small, but impressive differences were noted between the antigen activity in ascites from patients with chronic renal failure or nonepithelial tumors and ascites due to pancreatic carcinoma (Table 1). There were also quantitative differences between the ascitic fluids from pancreatic tumor patients and some of those from patients with other solid tumors. This suggests that assay of DU-PAN-2 antigen in ascitic fluids of patients with adenocarcinoma may help in determining the primary site of a tumor. We were also able to detect high levels of antigen in pancreatic duct fluids (Table 1). Consequently, it is possible to evaluate DU-PAN-2 antigen levels in pancreatic secretion samples taken from a variety of patients during endoscopic cannulation of the pancreatic duct. Since many pancreatic adenocarcinomas are located in the head of the pancreas and often obstruct the pancreatic duct, these secretions may be a good source of material for assays designed to facilitate early detection of a primary tumor. The cyst fluid from the patient with mucinous cystadenocarcinoma of the pancreas and some of the ascites from pancreatic cancer patients also have provided enriched sources of antigen for purification and characterization studies.

It is the detection of DU-PAN-2 antigen in serum, however, that is of the greatest potential diagnostic value. Sera from 77% of patients with pancreatic adenocarcinoma, irrespective of therapy or disease status, had increased levels (>300 units/ml) of DU-PAN-2 antigen. Another antigen (CA 19-9), detected by a monoclonal antibody in a radioimmunometric assay, has also been reported to be increased in serum from patients with pancreatic adenocarcinoma (9, 10). This antigen was shown to be a sialylated lacto-*N*-fucopentose II epitope that on cell lines and tumor cells is associated with a monosialoganglioside. In serum this antigen occurs as a mucin (9, 10). Reagent exchanges, specificity studies, and the inability of the 19-9 antibody to immunoblot the DU-PAN-2 antigen indicate that the epitopes and molecules detected by the 19-9 and DU-PAN-2 monoclonal antibodies are different (unpublished data).

The presence of very high levels of antigen (10,000 to 100,000 units/ml) in sera from some pancreatic cancer patients may be a reflection of liver disease, since all of the patients with these high levels had nonresectable liver metastases. Thus, as with carcinoembryonic antigen, the serum levels of the antigen may reflect not only its shedding or release from a given tumor but also the ability of the patient to catabolize the antigen (11). Elevation of serum DU-PAN-2 antigen is not strictly a reflection of liver disease *per se*, since some patients with colorectal carcinomas in this study had impaired liver function with normal antigen levels, presumably because their tumors did not express DU-PAN-2 antigen (4).

This initial retrospective serum study does not allow us to correlate DU-PAN-2 antigen levels with the status of disease or liver function since it was not possible to determine the exact tumor burden or have available the bilirubin levels on many of the patients studied. This type of clinical correlation can best be accomplished in a prospective study in which the appropriate clinical and clinical laboratory data can be obtained on the dates when body fluid samples are taken for DU-PAN-2 antigen testing. However, the data from patient B (Table 2) suggest that the assay may provide a sensitive indicator for recurrence in a disease that has traditionally been difficult to monitor. The test could be helpful for identifying the origin of tumors with metastases, particularly to the liver. We feel that the clinical applications of the determination of DU-PAN-2 antigen in body fluids is encouraging but that a more comprehensive testing program is warranted.

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