ABSTRACT

This paper describes the purification and partial characterization of a putative pancreatic oncofetal antigen. Pancreatic oncofetal antigen is a glycoprotein with a molecular weight between 800,000 and 900,000, which migrates in the \( \alpha \)- to \( \beta \)-region on electrophoresis. It is found in fetal pancreas and pancreatic cancer tissue, but it is not found in normal adult pancreas. Highly purified material migrated as a single band on polyacrylamide gel electrophoresis and immunized rabbits to produce monoclonal antibody without absorption. Pancreatic oncofetal antigen is clearly different from carcinoembryonic antigen, other known tumor-associated antigens, acute-phase reactants, and normal serum proteins. In addition, a quantitative rocket immunoelectrophoresis assay sensitive enough to detect pancreatic oncofetal antigen in the serum of most patients was developed and used to monitor the purification of pancreatic oncofetal antigen and to evaluate its clinical application.

Assays for pancreatic oncofetal antigen were carried out on three panels of sera; one was collected at The University of Chicago, and two were supplied by the Mayo-National Cancer Institute Serum Plasma Bank. The results demonstrate that pancreatic oncofetal antigen is found in the sera of most individuals; however, by far the highest absolute levels and the highest frequency of elevated levels were found in sera of patients with carcinoma of the pancreas. Elevated levels of pancreatic oncofetal antigen were also observed in the serum of some patients with carcinoma of the lung, stomach, colon, biliary tract, and breast as well as in the serum of some pregnant women and other individuals with certain benign conditions. The spectrum of patients who have elevated levels of pancreatic oncofetal antigen in their serum is quite different from that found with carcinoembryonic antigen or other known tumor markers. Serial assays were performed on the sera of several patients with cancer of the pancreas; the results suggest that measurement of pancreatic oncofetal antigen may be valuable in monitoring the course of cancer of the pancreas.

INTRODUCTION

Recently, there has been an increasing interest in the diagnostic and prognostic usefulness of tumor-associated antigens of embryonic and fetal origin. Two oncofetal antigens have been extensively investigated. AFP\(^a\) appears in the serum of patients with hepatoma and embryonal carcinoma (1, 2). CEA is most closely associated with carcinoma of the digestive tract and bronchopulmonary tree (13). In 1974, Banwo et al. (5) reported finding another oncofetal antigen in extracts of fetal pancreas, pancreatic tumor nodules, and sera from patients with carcinoma of the pancreas. They did not detect this antigen in the sera of patients with a variety of other diseases or in extracts of normal adult pancreas. In an attempt to reproduce these results, we have demonstrated an oncofetal antigen that appears to be associated with the pancreas. Since material from Banwo et al. (5) has not been available to us for comparison, we have not been able to determine whether the antigen that we have isolated is identical with that demonstrated previously. However, our antigen is clearly different from CEA and AFP. This report details the isolation and partial characterization of this antigen, which we call "pancreatic oncofetal antigen", and presents the results of assays on over 700 sera from patients with a variety of disorders.

MATERIALS AND METHODS

Sample Collection. Tissues from human fetuses of 18- to 20-week gestation were obtained within 3 hr of prostaglandin-induced abortions. Adult tissues were obtained from surgical specimens or from autopsies within 12 hr of death. All tissues were processed immediately or stored at \(-20^\circ\) until processed. Serum samples from patients with various abdominal diseases were collected over a 2-year period and stored at \(-70^\circ\). One selected patient with pancreatic cancer and elevated pancreatic oncofetal antigen levels underwent plasmaphoresis, and the plasma was stored at \(-20^\circ\). Ascitic fluid from 5 patients with pancreatic cancer was obtained at the time of autopsy. The diagnosis for all patients was confirmed by review of the hospital charts, surgical and pathology reports, slides, and photographs.

The patient sera used in the quantitative assays for pancreatic oncofetal antigen were obtained from 3 sources. First, a panel of 100 coded samples was received from the Mayo-NCI Serum Plasma Bank on September 16, 1975. This panel contained sera from 25 patients with carcinoma.
of the pancreas, 25 with other gastrointestinal cancers, 25 with pancreatitis and other related benign gastrointestinal disorders, and 25 with other benign diseases. The assays on this panel were done by means of a qualitative double immunodiffusion assay, and the results were reported to the NCI, Bethesda, Md., before we obtained the diagnosis. When the present quantitative assay was developed, this panel of sera was reassayed; the results are reported here.

A second panel of 315 sera was obtained from the same Mayo-NCI Serum Plasma Bank on April 13, 1976. These sera were assayed by both the qualitative immunodiffusion assay and the quantitative "rocket" immunoelectrophoresis assay before the results were reported to the NCI and before we obtained the diagnoses. This panel consisted of 50 sera from patients with gastrointestinal cancer, 50 from patients with benign gastrointestinal disease, 25 from patients with breast cancer, 25 from patients with benign breast disease, 30 from patients with lung cancer, 15 from patients with benign lung disease, 40 from smokers, 50 from patients with melanoma, sarcoma, or lymphoma, and 30 from normal individuals.

Sera from pregnant women were collected by Dr. Mel Dodson of Loyola University Medical Center, Chicago, Ill. All remaining sera samples were collected at The University of Chicago Hospitals and Clinics, Chicago, Ill.

Tissue Preparation. Aqueous extracts of fetal and adult tissue were homogenized [200 mg (wet weight) per ml] in PBS (pH 7.3) containing 0.5% e-aminocaproic acid and aprotinin (FBA Pharmaceuticals, Inc., New York, N.Y.) and 20 kallikrein inactivator units/ml, according to the method of Banwo et al. (5). Homogenates were centrifuged for 10 min at 5000 x g, and the pelleted cellular debris and lipid surface layers were discarded.

Preparation of Antisera to Fetal and Adult Materials. White New Zealand rabbits were immunized with fetal pancreas extract pooled from 4 to 6 specimens. The extracts (0.5 to 1 mg protein per 0.5 ml) were mixed with an equal volume of complete Freund's adjuvant (Difco Labo-

Purification of Pancreatic Oncofetal Antigen. Plasma rich in pancreatic oncofetal antigen was clotted by the addition of 1 mg calcium chloride per ml. One hundred ml of the resulting serum were fractionally precipitated with ammonium sulfate. The material precipitating between 20 and 30% saturation (pancreatic oncofetal antigen-rich frac-

tion) was dissolved in PBS and reprecipitated 3 times at 30% saturated ammonium sulfate. The precipitate was then dissolved in 25 ml PBS and dialyzed extensively against the same buffer. Lipids and lipoproteins were removed by ultracentrifugation as follows (10). The density of the pancreatic oncofetal antigen-rich fraction was adjusted to 1.21 g/ml by the addition of solid NaCl (0.098 g/ml) and NaBr (0.2 g/ml). Ultracentrifugation was carried out at 105,000 x g for 24 hr at 4°. After centrifugation the supernatant, devoid of pancreatic oncofetal antigen, was removed and discarded. The pellet was resuspended in PBS at one-half of the original serum volume and dialyzed against PBS at 4°, and the cryoprecipitate was removed by centrifugation. The supernatant was sterilized through a 0.45-µm Millipore filter and chromatographed on a Sepharose 6B column (2.5 x 91 cm) equilibrated with PBS containing 0.1% sodium azide. Five ml of the sample were chromatographed at a constant flow rate of 2.4 ml/sq cm/hr, and 5.8-ml fractions were collected. Protein peaks were defined by absorption at 280 nm. The pancreatic oncofetal antigen was initially localized in the eluate by double immunodiffusion and subsequently quantitated by rocket immunoelectrophoresis with monospecific antiserum. The eluate fractions containing pancreatic oncofetal antigen were pooled, concentrated by filtration through a Diaflo PM-10 ultrafiltration membrane (Amicon Corp., Lexington, Mass.) to approximately 5 ml, and rechromatographed on the same column. Estimates of molecular weight by gel exclusion chromatography were made by the method of Andrews (3), which was modified by the use of Sepharose 6B as the column matrix. Molecular-weight estimates by this method are dependent on the linear correspondence obtained between the log of the molecular weight of the homologous series of macromolecules and any of the several solute migration indexes. To ensure reproducibility of the results for separate fractionations, we chose the partition coefficient K, to calculate the molecular weight of pancreatic oncofetal antigen. The formula

\[ K_v = \frac{V_v - V_e}{V_v - V_o} \]

uses the values \( V_t \) (total bed volume), \( V_v \) (void volume), and \( V_e \) (sample elution volume).

Immunodiffusion, Immunoelectrophoresis, and Rocket Immunoelectrophoresis. Double immunodiffusion analysis was performed in 0.8% agarose in PBS, pH 7.2, containing 0.1% sodium azide (11). Each plate contained 6 evenly spaced antigen wells, with 1 central well containing antiserum. Diffusion was allowed to occur for 48 hr at room temperature. Immunoelectrophoresis was performed according to the method of Scheidegger (12). Rocket immunoelectrophoresis was carried out with 0.75% agarose in Gelman high-resolution buffer (pH 8.8; ionic strength, 0.03; Gelman Instrument Co., Ann Arbor, Michigan). For analysis of pancreatic oncofetal antigen, the agarose gel was mixed with a predetermined amount of anti-adult pancreatic oncofetal antigen antiserum at 56° and layered onto glass slides held in a Gelman Immunoframe to produce a uniform thickness of 2 mm. The optimal concentration of antiserum required in this assay was determined by evaluating the migration of pancreatic oncofetal antigen standards in gels.
containing varying concentrations of anti-pancreatic oncofetal antigen.

The migration of 5 pancreatic oncofetal antigen standards (100, 50, 25, 12.5, and 6.25 units) was evaluated. These standards were prepared from serum rich in pancreatic oncofetal antigen, from a patient with cancer of the pancreas. The antisera dilution that gave a crisp precipitin reaction with maximum migration was selected for the assay. Aliquots of the undiluted pancreatic oncofetal antigen standard were stored at -20°C. With each quantitative assay a calibration curve was prepared from a freshly thawed standard that was diluted to give 100, 50, 25, 12.5, and 6.25 pancreatic oncofetal antigen units. Holes (3 mm in diameter and 8 mm apart) were cut out 15 mm from the end of each slide. Ten µl of test material and dilutions of the pancreatic oncofetal antigen standard used for calibration were pipetted into the wells. In addition, control sera from patients with low and elevated pancreatic oncofetal antigen values were evaluated. Electrophoresis was performed at 10 ma/frame for 10 hr. Rocket arc lengths were measured to 0.1-mm accuracy with a calibrated digital viewer (Transidyne General Corp., Ann Arbor, Mich.).

The distance traveled by pancreatic oncofetal antigen was proportional to its concentration (Chart 1). Multiple test samples and standards evaluated on the same day and at weekly intervals showed a variation of less than 5% from the observed initial value. Pancreatic oncofetal antigen is stable for several weeks at refrigeration temperature in serum and in concentrated, partially purified preparations. After purification, however, pancreatic oncofetal antigen tends to denature irreversibly, especially in dilute solution. Therefore, we are not yet satisfied with the conversion factor between the quantitative measurements on pancreatic oncofetal antigen and the determination of its protein content. For this reason we chose to report the concentration of pancreatic oncofetal antigen in terms of standard units. If an extinction coefficient of 1 absorbance unit equals to 1 mg protein (E°nm/µg = 10) is assumed, 1 standard unit equals 1.4 µg pancreatic oncofetal antigen per ml. Based on this calculation the 14 standard unit level, which was adopted as a normal range cutoff point, represents approximately 20 µg pancreatic oncofetal antigen per ml.

**Polyacrylamide Disc Gel Electrophoresis.** Samples containing 50 to 200 µg protein were subjected to electrophoresis in 3.75% separating gels (pH 9.5) without a stacking layer. For analysis, 50 µl of the sample were mixed with 3 µl of the tracking dye [bromophenol blue (0.05%) in water] and 50 µl of a 40% sucrose solution. Fifty µl of this mixture were then applied to the separating gel. Electrophoresis was carried out at a constant current of 5 ma/gel. Under these conditions the marker dye moved three-fourths of the way through the gel in approximately 90 min. Gels were removed from the tubes and stained overnight with Coomassie brilliant blue. Polyacrylamide disc gel electrophoresis in the presence of SDS was performed in 4% acrylamide gel according to the method of Weber and Osborn (14).

To confirm the identity of pancreatic oncofetal antigen within the acrylamide gel, we prepared duplicate gels; one was stained for protein as described, and the other was subjected to immunoanalysis. For the latter the unfixed gels were laid in a trough on an agarose-covered glass plate. Parallel troughs were cut on both sides of the embedded acrylamide gel and were filled with antisera of various specificities. The antigens within the acrylamide gel and the antibodies in the parallel troughs were allowed to diffuse at room temperature for 48 hr, and the resulting immunoprecipitin lines were recorded.

Commercial antisera against α2-macroglobulin, IgM, IgG, human serum, ferritin, β-lipoprotein, α1-acid glycoprotein, haptoglobin, ceruloplasmin, and α1-antitrypsin were purchased from Behring Diagnostics, Woodbury, N.Y. Human proteins used as molecular-weight markers were purified from normal human serum and from sera of selected patients. IgG was purified from normal human serum and from the serum of 1 patient with multiple myeloma. IgM was purified from a patient with Waldenström's macroglobulinemia. For immunoglobulin purification, conventional techniques including ammonium sulfate fractionation, size chromatography on Sephadex G-200 and Sepharose 6B, DEAE-cellulose chromatography, and preparative disc gel electrophoresis were used. Purified preparations were shown, by polyacrylamide disc gel electrophoresis and immunoanalysis with monospecific and polyspecific antisera, to be free of other serum proteins.

For the purification of α2-macroglobulin, we selected 2 normal human sera for type 1-1 haptoglobin to avoid the possibility of haptoglobin-hemoglobin complex contamination in the final preparation. Briefly, serum was diluted with an equal volume of 0.1 M phosphate buffer, pH 7.4, and fractionally precipitated with ammonium sulfate. Material that precipitated between 35 and 40% saturation was collected by centrifugation, dissolved in phosphate buffer, and reprecipitated at 40% ammonium sulfate saturation. The precipitate was dissolved and dialyzed against distilled water at 4°C, clarified, dialyzed against PBS (pH 7.4), and...
RESULTS

Enzyme Susceptibility of Pancreatic Oncofetal Antigen. Isolated preparations of pancreatic oncofetal antigen were reacted for 1 hr at 24 hr at 37°C with and without neuraminidase (250 units/ml in PBS), trypsin (1 mg/ml in PBS), papain (1.5 mg/ml in PBS with 0.01 M cysteine and 0.002 M EDTA), pronase (1 mg/ml in PBS), and pepsin (3 mg/ml in acetic acid buffer, pH 4.0), DNase (1 mg/ml in PBS), and RNase (1 mg/ml in PBS). After incubation each sample was assayed for pancreatic oncofetal antigen reactivity.

Enzyme Susceptibility of Pancreatic Oncofetal Antigen. Isolated preparations of pancreatic oncofetal antigen were reacted for 1 hr and 24 hr at 37°C with and without neuraminidase (250 units/ml in PBS), trypsin (1 mg/ml in PBS), papain (1.5 mg/ml in PBS with 0.01 M cysteine and 0.002 M EDTA), pronase (1 mg/ml in PBS), and pepsin (3 mg/ml in acetic acid buffer, pH 4.0), DNase (1 mg/ml in PBS), and RNase (1 mg/ml in PBS). After incubation each sample was assayed for pancreatic oncofetal antigen reactivity.

Con A Binding. Isolated preparations of pancreatic oncofetal antigen were incubated for 60 min at room temperature with an equal volume of Con A-Sepharose suspension in PBS (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), 1% Sepharose 6B suspension in PBS, and PBS. After incubation, Sepharose gels were removed by centrifugation, and the supernatants were assayed for pancreatic oncofetal antigen reactivity.

Enzyme Susceptibility of Pancreatic Oncofetal Antigen. Isolated preparations of pancreatic oncofetal antigen were reacted for 1 hr and 24 hr at 37°C with and without neuraminidase (250 units/ml in PBS), trypsin (1 mg/ml in PBS), papain (1.5 mg/ml in PBS with 0.01 M cysteine and 0.002 M EDTA), pronase (1 mg/ml in PBS), and pepsin (3 mg/ml in acetic acid buffer, pH 4.0), DNase (1 mg/ml in PBS), and RNase (1 mg/ml in PBS). After incubation each sample was assayed for pancreatic oncofetal antigen reactivity.

RESULTS

Characterization of Antisera to Fetal Pancreas Extracts. Rabbit antisera raised by hyperimmunization with extracts of human fetal pancreas demonstrated several precipitin arcs in double diffusion analysis against fetal and adult pancreas extracts as well as against fetal and adult sera. After absorption for removal of antibodies against normal adult tissues and serum components, the antisera recognized 2 fetal antigens (Table 1). On immunoelectrophoresis, one migrated with α-mobility and was identified as AFP (Fig. 1); the other migrated with β-mobility and showed a clear pattern of immunological nonidentity with CEA (Fig. 1b) and ferritin (Fig. 1c). In addition, purified pancreatic oncofetal antigen did not inhibit the radioimmunoassays for CEA or AFP, and antisera against pancreatic oncofetal antigen had no reactivity with either CEA or AFP in these assays. No immunoprecipitin reaction was detected with extracts of adult pancreas or sera. This suggested the presence of a different fetal antigen, possibly of pancreatic origin. To evaluate this possibility we prepared extracts of multiple fetal and normal adult tissues. As shown in Table 1, the fetal antigen that was subsequently termed pancreatic oncofetal antigen was detected in high concentration in 10 of 13 fetal pancreas extracts. It was not detected in extracts of other fetal tissues, with the exception of a weak reaction observed in 1 of 2 small bowel extracts. It was detected in high concentration, however, in the stomach contents of 1 fetus. Pancreatic oncofetal antigen was not detected in adult tissue, except for trace amounts in 1 of 6 colon extracts and 2 of 6 small bowel extracts. Three different absorbed antisera to fetal pancreas yielded comparable results and were pooled for subsequent use. AFP was detected in high concentration in each fetal liver tested and in lower concentration in extracts of all other fetal tissues. This unexpected result was probably due to contamination of all extracts with fetal serum. AFP was not detected in extracts of any adult tissues. Double immunodiffusion analyses with antisera against fetal pancreas were performed on 110 serum samples from patients with various diseases (Table 2). The presence of pancreatic oncofetal antigen was demonstrated in 20 of 26 patients with carcinoma of the pancreas. Two of 11 sera from patients with colon cancer, 3 of 4 patients with carcinoma of the gallbladder or biliary tree, and 2 of 12 patients with pancreatitis also had detectable concentrations of pancreatic oncofetal antigen in their sera. Antibody analysis of the pancreatic oncofetal antigen in each of these sera with multiple antisera and several standards demonstrated complete immunological identity. No pan-
Pancreatic Oncofetal Antigen

Fig. 1. a, double immunodiffusion of pancreatic oncofetal antigen and AFP. Well 1, AFP; Well 4, anti-AFP; Well 2, pancreatic oncofetal antigen; Well 3, antipancreatic oncofetal antigen. The precipitin arcs form a pattern of complete nonidentity. b, double immunodiffusion of pancreatic oncofetal antigen and CEA. Well 1, anti-pancreatic oncofetal antigen; Well 4, pancreatic oncofetal antigen; Well 2, anti-CEA; Well 3, CEA. Pancreatic oncofetal antigen and CEA do not share any antigenic determinants detectable by the antisera used. c, double immunodiffusion of pancreatic oncofetal antigen and ferritin. Well 1, concentrated, partially purified pancreatic oncofetal antigen; Well 4, anti-pancreatic oncofetal antigen; Well 2, ferritin; Well 3, antiferritin. Nonidentity among these antigens is demonstrated by the crossing precipitin reactions. The double precipitin line between Wells 2 and 3 is due to the presence of ferritin and apoferritin in this preparation.

Table 2 Distribution of pancreatic oncofetal antigen in the sera of patients with various diseases

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of patients</th>
<th>No. positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma of pancreas</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>Carcinoma of colon</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Carcinoma of gallbladder-biliary tract</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Carcinoma of duodenum</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Benign biliary stone disease</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Miscellaneous abdominal disease</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Normal</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

Pancreatic oncofetal antigen was detected in sera from normal adults or in sera from patients with hepatoma, carcinoma of the duodenum, benign biliary tract or stone disease, inflammatory bowel disease, or miscellaneous abdominal diseases.

Purification of Pancreatic Oncofetal Antigen. For the initial purification of pancreatic oncofetal antigen, sera containing elevated concentrations of pancreatic oncofetal antigen from 3 separate pancreatic cancer patients were chromatographed on Sepharose 6B. The antiserum prepared to fetal pancreas extracts was used for monitoring of this purification. The typical elution pattern is shown in Chart 2. In each case, all of the antigenic activity of pancreatic oncofetal antigen eluted from the column in the trailing portion of the first protein peak. Major contaminants of pancreatic oncofetal antigen in this fraction were α2-macroglobulin, IgM, and β-lipoprotein. Serum proteins of lower molecular weight eluted from the column after pancreatic oncofetal antigen.

In subsequent purification procedures, pancreatic oncofetal antigen was isolated from plasma obtained from a single patient with adenocarcinoma of the pancreas. Similar results were obtained in 11 separate isolations. The normal serum contaminants that eluted with pancreatic oncofetal antigen during whole-serum fractionation on Sepharose 6B were removed or were greatly reduced in concentration by ammonium sulfate precipitation, cryoprecipitation, and ultracentrifugation, as described in "Materials and Methods." As shown in Chart 3, 90% of the pancreatic oncofetal antigen activity was precipitated by ammonium sulfate in concentrations below 20% saturation. It is almost completely precipitated by 30% saturated ammonium sulfate.

Chart 2. Chromatography on Sepharose 6B of serum rich in pancreatic oncofetal antigen. POA STD, pancreatic oncofetal antigen standard eluted shortly after the void volume (V0). The majority of the serum proteins eluted after pancreatic oncofetal antigen. One small peak eluted just after the total bed volume (Vt). It was identified as nearly pure IgG. The most probable explanation for the elution of this IgG behind the bed volume is that it had weak binding affinity for Sepharose.

Chart 3. Pancreatic oncofetal antigen recovery after ammonium sulfate-fractionated serum rich in pancreatic oncofetal antigen. Pancreatic oncofetal antigen is not precipitated by ammonium sulfate in concentrations below 20% saturation. It is almost completely precipitated by 30% saturated ammonium sulfate.
were separated from pancreatic oncofetal antigen by ultracentrifugation (10). During ultracentrifugation the lipoproteins floated to the surface, whereas pancreatic oncofetal antigen sedimented. When the pancreatic oncofetal antigen-rich pellet was dissolved in and dialyzed against PBS at 4°C, a cloudy cryoprecipitate formed, which was identified as a mixture of IgG and IgM and discarded. In several isolations, 85 to 95% of the pancreatic oncofetal antigen initially present in the serum was recovered after ammonium sulfate precipitation, ultracentrifugation, and cryoprecipitation. Immunoanalysis of this fraction demonstrated several components in addition to pancreatic oncofetal antigen. When subjected to chromatography or Sepharose 6B, pancreatic oncofetal antigen was eluted as before, shortly after the void volume; however, the high-molecular-weight materials that contaminated the fraction containing pancreatic oncofetal antigen were markedly reduced. The pancreatic oncofetal antigen-rich fraction was concentrated and rechromatographed in the same column (Chart 4). More than 80% of the pancreatic oncofetal antigen present in the original serum was recovered after the second chromatography. Immunoanalysis demonstrated that a trace amount of IgM eluted before the pancreatic oncofetal antigen reactivity and that an undefined material eluted slightly after it.

The central portion of the peak containing pancreatic oncofetal antigen from the second Sepharose 6B chromatography was removed, concentrated, and subjected to polyacrylamide gel electrophoresis. After this procedure, 1 major band (96%) and 1 minor band (4%) were demonstrated in polyacrylamide gel electrophoresis. Immunological analysis showed that the major, more anodal band contained almost all of the pancreatic oncofetal antigen activity. In addition, a faint streak of immunoprecipitation extended from the protein band to the origin, and a second small band was seen at the origin; the latter 2 probably represented denatured pancreatic oncofetal antigen. The principal anodal band, which contained most of the pancreatic oncofetal antigen reactivity, was cut out of several gels, concentrated, and subjected to electrophoresis on a similar polyacrylamide gel. A single protein band that contained all of the pancreatic oncofetal antigen reactivity and all protein was produced (Fig. 2).

Characterization of Antisera against Adult Pancreatic Oncofetal Antigens. Antisera produced by injection of rabbits with isolated adult pancreatic oncofetal antigen had a much higher titer than that produced by injection of rabbits with extracts of fetal pancreas. The material used for immunization was obtained from the second Sepharose 6B chromatography; it had not been subjected to acrylamide gel electrophoresis. This antisera contained weak reactivity to IgM and strong reactivity against pancreatic oncofetal antigen. The reactivity to IgM could not be detected by gel diffusion after a 1:4 dilution of this antisera; however, strong reactivity to pancreatic oncofetal antigen was present after a 1:128 dilution. This antisera was rendered specific for pancreatic oncofetal antigen by a single absorption with glutaraldehyde-insolubilized normal human serum. That we might ensure complete removal of contaminating specificities, this antisera was absorbed 3 times.

Additional rabbits were immunized with the material recovered from the final acrylamide gel electrophoresis. These animals produced high-titer antisera specific for pancreatic oncofetal antigen without absorption. Antisera were considered specific for pancreatic oncofetal antigen when they failed to form any visible precipitation in double-immunodiffusion, immunoelectrophoresis, or rocket immunoelectrophoresis analyses with any material in serum except pancreatic oncofetal antigen. Each antiserum was tested with anti-fetal pancreatic oncofetal antigen against at least 20 sera rich in pancreatic oncofetal antigen from patients with a variety of conditions. Each precipitin reaction was studied by double immunodiffusion with known fetal and adult pancreatic oncofetal antigen standards. Lines of complete immunological identity were formed in each sample.
case (Fig. 3). Consequently, our antisera against both fetal and adult pancreatic oncofetal antigen appear to recognize identical specificities and to be incapable of detecting any heterogeneity in pancreatic oncofetal antigen derived from various sources.

**Characterization of Pancreatic Oncofetal Antigen.** The molecular weight of pancreatic oncofetal antigen was estimated by molecular-sieve chromatography on Sepharose 6B. The elution pattern of pancreatic oncofetal antigen was compared with that of highly purified preparations of IgG, ferritin, α₂-macroglobulin, and IgM. As shown in Chart 5, this procedure gives a molecular size for pancreatic oncofetal antigen of between 800,000 and 900,000. Similar but less precise estimates were obtained from SDS-acrylamide gel electrophoresis and ultrafiltration on Amicon filters of varying pore sizes. Pancreatic oncofetal antigen barely entered the gel in SDS-polyacrylamide gel electrophoresis (4% gel with bovine serum albumin standards), indicating that its molecular weight is far in excess of that of the bovine serum albumin tetramers (270,000 daltons). In addition, no detectable dissociation of pancreatic oncofetal antigen was observed; this suggested that pancreatic oncofetal antigen is a single homogeneous molecule rather than a loose aggregation of subunits. No detectable pancreatic oncofetal antigens passed through Amicon filters that had an exclusion limit of 500,000. The pancreatic oncofetal antigen moved as a single discrete entity in all fractionation procedures. We carried out partial characterization of pancreatic oncofetal antigen derived from several other sources, in an effort to detect heterogeneity. Multiple serum samples from patients with various tumors, extracts of 3 pancreas cancers, and 2 pools of 6 fetal pancreas were subjected to immuno-electrophoresis. In each case the pancreatic oncofetal antigen migrated to the β-region in a single compact arc. The quantities of pancreatic oncofetal antigen present in tissue extracts were not sufficient for further analysis. Serum samples from 4 patients with pancreas cancer and ascitic fluid from 2 other patients were chromatographed on Sepharose 6B. In each case the elution pattern of pancreatic oncofetal antigen was indistinguishable from that described above.

Pancreatic oncofetal antigen appears to be a glycoprotein. Its immunoreactivity was destroyed by exposure to trypsin, papain, pronase, and pepsin, at 37° for 1 hr. It was completely unaffected by DNase or RNase. When pancreatic oncofetal antigen was reacted with neuramindase, its immunoreactivity was reduced; however, it could not be completely destroyed. Incubation with a 1% solution of Con A-Sepharose completely removed all pancreatic oncofetal antigen immunoreactivity, indicating that this protein has a carbohydrate component. No detectable loss in pancreatic oncofetal antigen was observed when it was reacted with a 1% suspension of Sepharose alone. Pancreatic oncofetal antigen was irreversibly precipitated with perchloric acid at several concentrations. When equal volumes of serum rich in pancreatic oncofetal antigen were mixed with perchloric acid (2.0, 1.0, 0.5, and 0.125 M), no detectable immunoreactivity was recovered from either the supernatant or the solubilized precipitate. The majority of the protein precipitated with perchloric acid could not be solubilized; therefore irreversible denaturation of pancreatic oncofetal antigen seems to have occurred during this procedure. Pancreatic oncofetal antigen did not stain with Oil Red O, suggesting the absence of lipid component; it stained with the periodic acid-Schiff reaction, however, providing additional evidence for a carbohydrate component.

**Studies with Patient Sera.** The results of tests for pancreatic oncofetal antigen in the 2 sets of sera obtained from the Mayo-NCI Serum Plasma Bank were in general agreement with each other and with those obtained for sera collected at The University of Chicago, Chicago, Ill. Consequently, most of the results were pooled for ease of presentation.

The results of testing for pancreatic oncofetal antigen in the sera of patients with cancers of the pancreas, pancreatitis, islet cell tumors, and carcinoids and in sera of normal individuals are presented in Chart 6. The source of the sera from patients with cancer of the pancreas is indicated to show that the values for the 3 panels are indeed comparable. Sera from patients with pancreatic cancer had elevated levels of pancreatic oncofetal antigen more frequently than
had those of any other group (Table 3). Twenty-six of 48 (54%) of the sera collected at The University of Chicago had levels above 14 standard units. Twelve of 32 (37%) of sera obtained from the NCI were also above this level. The patients with serum pancreatic oncofetal antigen levels higher than 25 standard units had moderate to well-differentiated adenocarcinomas; most of those with levels below 10 units had poorly differentiated tumors. All of the sera collected at The University of Chicago from patients with pancreatic cancer were obtained before surgery or before any therapy had begun. Those obtained from the NCI were drawn at various times with respect to therapy. Two of the 3 patients with pancreatitis who had pancreatic oncofetal antigen levels above 14 standard units had a chronic form.

Pancreatic oncofetal antigen levels in sera of normal individuals ranged from 0 to 4 standard units. The source of sera from these individuals is shown because the mean level of pancreatic oncofetal antigen is higher for the normal individuals in the Mayo-NCI Serum Plasma Bank panel than for those at The University of Chicago. The reasons for these differences in pancreatic oncofetal antigen values are not clear. Most of the sera from normal individuals in The University of Chicago panel were obtained through the blood bank when people were donating blood. The normal sera from the plasma bank were obtained at the time of yearly physical examinations of employees. We have tested the samples obtained by treating them with a variety of anticoagulants and storing them under various conditions, in an effort to identify factors that might explain these differences. Pancreatic oncofetal antigen levels were not detectably affected by anticoagulants, freezing, thawing, or the refrigerator storage of sera for a period of at least several weeks. It is likely, therefore, that there is some systematic difference between the blood donors at The University of Chicago and the employees of the Mayo Clinic who reported for yearly physical examinations.

The second highest proportion of patients with pancreatic oncofetal antigen levels greater than 14% of standard had malignant biliary tract disease. Five of 14 (36%) patients with a diagnosis of gallbladder or biliary tract cancer had pancreatic oncofetal antigen levels above 20% of the standard. Three of 11 patients in the group with benign biliary tract disease had pancreatic oncofetal antigen levels above 14% of standard. One had alcoholic cirrhosis (26.5%), 1 had biliary cirrhosis (18%), and 1 had cholecystitis (16.5%). None of the patients with hepatoma had elevated levels of pancreatic oncofetal antigen. The elevation of pancreatic oncofetal antigen in sera of patients with liver disease did not correlate with degree of jaundice or other abnormal liver function tests.

### Table 3

Quantitative serum pancreatic oncofetal antigen levels in individuals with various conditions

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of individuals studied</th>
<th>No. of individuals with POA% values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0-14%</td>
</tr>
<tr>
<td>Pancreatic disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinoma of the pancreas</td>
<td>80</td>
<td>42</td>
</tr>
<tr>
<td>Pancreatitis</td>
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<tr>
<td>Islet cell or carcinoid tumor</td>
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<tr>
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<td>Hepatoma</td>
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<tr>
<td>Benign biliary stone disease or cirrhosis</td>
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<td>8</td>
</tr>
<tr>
<td>Bronchopulmonary disease</td>
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<tr>
<td>Bronchogenic carcinoma</td>
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<td>Small cell carcinoma of the lung</td>
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<td>Smokers</td>
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<td>Gastrointestinal disease</td>
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<td>Carcinoma of the colon or rectum</td>
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<td>Inflammatory bowel disease</td>
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<td>Carcinoma of the duodenum and small bowel</td>
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<td>Sarcoma</td>
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<td>Miscellaneous benign abdominal diseases</td>
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<td>Normal</td>
<td>102</td>
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</table>

*POA, pancreatic oncofetal antigen.*

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**Note:**

- Table 3 provides quantitative serum pancreatic oncofetal antigen levels in individuals with various conditions.
- The results for all other groups were pooled from the 3 panels. Values are expressed as standard units pancreatic oncofetal antigen, pending more accurate determination of the conversion factor to mg/ml.
- The line was drawn at 14 standard units; this corresponds approximately to 20 µg pancreatic oncofetal antigen per ml serum. The 3 points above 40 units were measured at 60, 65, and 100 units.
- The second highest proportion of patients with pancreatic oncofetal antigen levels greater than 14% of standard had malignant biliary tract disease. Five of 14 (36%) patients with a diagnosis of gallbladder or biliary tract cancer had pancreatic oncofetal antigen levels above 20% of the standard. Three of 11 patients in the group with benign biliary tract disease had pancreatic oncofetal antigen levels above 14% of standard. One had alcoholic cirrhosis (26.5%), 1 had biliary cirrhosis (18%), and 1 had cholecystitis (16.5%). None of the patients with hepatoma had elevated levels of pancreatic oncofetal antigen. The elevation of pancreatic oncofetal antigen in sera of patients with liver disease did not correlate with degree of jaundice or other abnormal liver function tests.
Thirteen of 53 (24.5%) patients with bronchogenic carcinoma had pancreatic oncofetal antigen levels greater than 14%; 8 patients had pancreatic oncofetal antigen levels between 23 and 79%. These concentrations of pancreatic oncofetal antigen were comparable to those found in patients with pancreatic cancer. Several patients were studied serially and, as happened to patients with pancreatic cancer, the levels of pancreatic oncofetal antigen fell with therapy and increased with expanding tumor mass. Sera from patients with small cell carcinomas and adenocarcinomas of the lung had much lower levels of pancreatic oncofetal antigen. One patient with small cell carcinoma had a pancreatic oncofetal antigen level of 16.5%, and 4 patients with adenocarcinoma of the lung had pancreatic oncofetal antigen levels of 14, 16, 18, and 34%. All had metastatic disease at the time of diagnosis. The patient with benign lung disease and an elevated pancreatic oncofetal antigen level had a lung abscess and bronchiectasis.

Six of 42 (14%) patients with carcinoma of the colon or rectum had pancreatic oncofetal antigen levels in excess of 14%, and 1 of the 6 had a pancreatic oncofetal antigen level greater than 20%. All of these individuals had metastatic adenocarcinoma. Patients with ulcerative colitis or chronic bowel disease had comparable serum pancreatic oncofetal antigen levels and were pooled under inflammatory bowel disease; all except 1 of these patients (pancreatic oncofetal antigen level, 18%) were found to have normal pancreatic oncofetal antigen levels.

Four of 16 patients with carcinoma of the stomach had pancreatic oncofetal antigen levels greater than 14%; one of the 4 had a pancreatic oncofetal antigen level of 23%. Neither the location of the carcinomas in the stomach nor the extent of their invasion of peripancreatic tissues or lymph nodes is known.

Four patients with carcinoma of the duodenum or small bowel were studied. All 4 patients had pancreatic oncofetal antigen levels within our normal limits.

Three of 10 patients with gastric ulcers had slightly elevated levels of pancreatic oncofetal antigen (14 to 19%), and 1 of 30 patients with duodenal ulcers had an elevated level of 26%. This latter patient had a chronic ulcer and esophagitis. In all cases neither the depth of erosion nor the positions of the gastric and duodenal ulcers in relation to the pancreas are known.

Twenty-six of 28 breast cancer patients had normal pancreatic oncofetal antigen levels. The 2 patients with elevated levels had metastatic disease. Twenty-five of 27 patients with breast disease had normal serum pancreatic oncofetal antigen levels. The 2 with elevated levels were from the Mayo-NCI Serum Plasma Bank, and additional information about their diagnoses was not available to us.

Fifty-three of 54 patients with melanoma, sarcoma, and lymphoma had normal serum pancreatic oncofetal antigen levels. The 1 patient with an elevated pancreatic oncofetal antigen level was a 22-year-old with metastatic synovial sarcoma. A patient with duodenal diverticulum had the single elevated serum pancreatic oncofetal antigen level in the "miscellaneous abdominal disease" category.

The sera from pregnant women were obtained at random from patients visiting the clinics for prenatal care. Follow-up data were available on 5 of the 11 patients who had elevated pancreatic oncofetal antigen levels. All 5 patients were in the second trimester of pregnancy when the samples were drawn. One pregnant woman, who works in our laboratories, had a serum level of 8 standard units during the first trimester, which rose to 22 units during the middle of the second trimester and fell to 14 units in the early third trimester of pregnancy.

CEA levels were determined for many of our serum samples from patients with pancreatic cancer. As shown in Chart 7, samples were found with high pancreatic oncofetal antigen levels but without a detectable increase in CEA. The reciprocal to this was also observed in some samples, as well as an increase in both CEA and pancreatic oncofetal antigen in others. These findings complement the immunochemical information also presented in this paper and demonstrate that pancreatic oncofetal antigen is distinct from CEA. Similar results were obtained in tests for AFP.

Serial blood samples were tested on 9 patients with pancreatic cancer (Chart 8a). Three of the patients had high serum levels of pancreatic oncofetal antigen which fell sharply when the tumors were resected; the pancreatic oncofetal antigen levels rose again in 1 of the 3 at the time of recurrence. In 4 other patients who had initial levels below 14 units, no significant change was found after resection (Chart 8b). Pancreatic oncofetal antigen levels rose in parallel with recurrence in 2 other patients from whom no sera were obtained prior to surgery.

**DISCUSSION**

The general characteristics of pancreatic oncofetal antigen suggest that it is a distinct oncofetal antigen quite different from any other tumor-associated or oncofetal antigen or serum component studied previously. We believe
that studies with pancreatic oncofetal antigen have the potential for leading to the development of a clinically useful assay. However, many questions about the immunological and physicochemical characteristics of pancreatic oncofetal antigen must be resolved before clinical testing can be considered seriously.

Pancreatic oncofetal antigen is highly sensitive to a variety of proteolytic enzymes, but it is resistant to DNase and RNase and has no detectable lipid. Although studies with binding to Con A-Sepharose, periodic acid-Schiff staining, and neuraminidase sensitivity demonstrated that it is a glycoprotein, pancreatic oncofetal antigen is highly sensitive to perchloric acid. This suggests that it has much less carbohydrate than CEA.

The molecular weight of pancreatic oncofetal antigen is approximately 800,000 to 900,000, as determined by gel filtration chromatography on a calibrated Sepharose 6B column. Gel filtration chromatography for molecular weight determination yields only approximate values because the partitioning differences between molecules depend on their shapes and interactions in addition to their size. Nevertheless, we believe that 800,000 to 900,000 is a reasonable value because independent measurements made with SDS-polyacrylamide gels and Amicon ultrafiltration indicate that pancreatic oncofetal antigen has a molecular weight considerably in excess of 500,000. No evidence for noncovalently bound subunits has been found.

Early attempts to isolate pancreatic oncofetal antigen from fetal pancreas were unsuccessful because of the small quantities of material available. When a large volume of plasma rich in pancreatic oncofetal antigen had been obtained from a patient with pancreatic cancer, isolation of the antigen became a fairly straightforward procedure. The best evidence for the high purity of the final product is that it migrated as a single band on polyacrylamide gel electrophoresis and that it stimulated monospecific antibody in hyperimmunized rabbits.

Several other tumor-associated antigens including CEA, AFP, and ferritin exist in multiple isomeric forms. Such isomers of pancreatic oncofetal antigen were sought, but they were not found. Samples of each of the sera and tissues described in Tables 1 and 2 were subjected to double immunodiffusion against antiserum to adult pancreatic oncofetal antigen and fetal pancreatic oncofetal antigen. In each instance a line of complete immunological identity was produced, indicating that the antiserum used are incapable of detecting immunological heterogeneity among pancreatic oncofetal antigen samples from various sources. Limited physicochemical characterization of pancreatic oncofetal antigen derived from adult and fetal serum and from pancreatic cancer tissue confirmed this homogeneity. Material from each source behaved identically in immunoelectrophoresis and Sepharose 6B chromatography, indicating that all of the preparations had similar size and charge properties. Antiserum against glycoproteins frequently contain antibodies directed to carbohydrate determinants, which are present in a wide variety of tissues. If any such antibodies had been produced by immunization with pancreatic oncofetal antigen, they may have been removed by the absorption with human AB RBC and agar used to remove antibodies to Forssman antigens, which are naturally present in rabbit serum.

It would be expected that a molecule present in concentrations higher than 100 µg/ml in the sera of some individuals would have been described previously. We have evaluated many proteins but have not found 1 with the immunological and physicochemical properties of pancreatic oncofetal antigen. However, CEA, AFP, ferritin, acute-phase reactants, and all normal serum proteins to which we were able to obtain antisera showed complete immunological nonidentity with pancreatic oncofetal antigen. Recently, Chu et al. (6) reported on the isolation of a glycoprotein by perchloric acid extraction from ascitic fluid of patients with pancreatic carcinoma. This antigen is not the same as pancreatic oncofetal antigen in molecular weight, perchloric acid sensitivity, and cross-reactivity with anti-CEA antibody.

A rocket immunoelectrophoresis assay for pancreatic oncofetal antigen was developed because it is accurate, reproducible, and sufficiently sensitive to detect pancreatic oncofetal antigen in the sera of the majority of individuals. It has advantages over a radioimmunoassay in that it facilitates, in much the same way as Ouchterlony double immunodiffusion, the detection of contaminating antigens and antibodies. The assay is reproducible within about 1 standard unit when tests are run either simultaneously or at intervals of up to 6 months. The results are reported as standard units, pending more accurate determination of the correlation between protein content and assay results on purified preparations.

The specificity of the quantitative assay was evaluated in extensive studies. We were especially anxious to determine whether the pancreatic oncofetal antigen found in sera of patients with various diseases was the same as that found in our purified adult standard and fetal pancreas sera. All of the sera from both Mayo-NCI Serum Plasma Bank panels and many collected at The University of Chicago were...
tested in double immunodiffusion assays with antisera against both fetal and adult pancreatic oncofetal antigen and antigens extracted from fetal pancreas and from our adult standard sera. Whenever pancreatic oncofetal antigen was found in a serum sample, it formed a line of complete immunological identity with our standards. We have seen no evidence of isomers or other variants.

The line drawn at 14 standard units was chosen arbitrarily and serves to emphasize the relative levels of serum pancreatic oncofetal antigen; the highest proportion of samples with levels over 14 standard units was found in patients with carcinoma of the pancreas. This observation together with the increased levels of pancreatic oncofetal antigen in cancerous pancreatic tissue and in fetal pancreas supports the notion that pancreatic oncofetal antigen is associated more closely with the pancreas than with other organs.

The documentation of the diagnoses of patients from whom sera were drawn proved to be an unexpectedly difficult problem. The diagnoses for the patients at The University of Chicago was confirmed by review of the hospital charts, surgical and pathology reports, slides, and photographs. Many sera had to be discarded because of improper and incomplete clinical documentation; this was done without knowledge of the results of the assays for pancreatic oncofetal antigen. Since the clinical management of patients with different types of widespread abdominal carcinomas is similar, there is frequently little incentive for a vigorous attempt to establish the correct diagnoses. In addition, some patients have more than 1 disease, which might cause elevated serum levels of pancreatic oncofetal antigen. Relatively small numbers of inaccurate diagnoses on patients with multiple diseases can introduce significant errors into a study such as this. Consequently, the present results relating the occurrence and levels of pancreatic oncofetal antigen in the sera of patients with various diseases must be considered as preliminary. They show trends which undoubtedly exist, but much additional, careful work needs to be done.

The usefulness of determinations of serum pancreatic oncofetal antigen in the diagnosis of pancreatic cancer is not yet known. The high proportion of false-negative results makes this an unlikely candidate for a "stand-alone" screening test. Nevertheless, since the diagnosis of pancreatic cancer, even in advanced cases, is frequently difficult, our experience suggests that measurements of serum pancreatic oncofetal antigen may be of significant value in the work-up of patients with suspected abdominal cancer. The common initial approach to the investigation of these patients could have multiple diseases which might cause elevated serum levels of pancreatic oncofetal antigen. Relatively small numbers of inaccurate diagnoses on patients with multiple diseases can introduce significant errors into a study such as this. Consequently, the present results relating the occurrence and levels of pancreatic oncofetal antigen in the sera of patients with various diseases must be considered as preliminary. They show trends which undoubtedly exist, but much additional, careful work needs to be done.

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The designation of pancreatic oncofetal antigen as an oncofetal antigen requires discussion. The term pancreatic oncofetal antigen was adopted because the material was found in elevated concentration in fetal pancreas, in pancreatic cancer tissue, and in the serum of the majority of patients with pancreatic cancer. It was subsequently found in elevated quantities in the serum of a lesser percentage of patients with other diseases. By means of the rocket assay, it was also found in low concentrations in the serum of nearly all normal individuals. All of these observations are consistent with the designation of pancreatic oncofetal antigen as an oncofetal antigen. Available data are not sufficient, however, to provide a precise determination of its origin and significance. The low concentrations in normal adult pancreas and serum could reflect increased rates to degradation rather than decreased synthesis. Likewise, the finding of elevated levels of pancreatic oncofetal antigen in the serum of some patients with diseases unrelated to the pancreas does not imply that the material is not synthesized by the pancreas. These patients could have occult pancreatic disease. The definitive classification of pancreatic oncofetal antigen as a tumor antigen will not be possible until much more information is available. However, present evidence seems sufficient to justify the operational designation of pancreatic oncofetal antigen as an oncofetal antigen that is associated in some way with the pancreas.

REFERENCES


2. Abelev, G. J. α-Fetoprotein as a Marker of Embryo-Specific Differentiations... 9, 16. If any of these examinations give positive results, the investigation continues with more invasive procedures such as endoscopy, arteriography and, finally, surgery. These procedures are expensive and require highly skilled personnel. In an effort to find more effective diagnostic procedures, we have studied a variety of polypeptide hormones and tumor-associated antigens, including CEA, AFP, human chorionic gonadotrophin, parathyroid hormone, C-peptide, gastrin, glucagon, calcitonin, and proinsulin (15). Only CEA and pancreatic oncofetal antigen were found to be of diagnostic value in pancreatic cancer; 80% of such patients had elevations of 1 or both of these markers. Thus, simultaneous assays of pancreatic oncofetal antigen and CEA appear to be quite useful in the work-up of patients with suspected cancer of the pancreas.

Another possible application of assays for pancreatic oncofetal antigen is the prediction of recurrence during therapy of pancreatic cancer. In each of the patients who had elevated pancreatic oncofetal antigen values and resectable tumors, the serum levels of pancreatic oncofetal antigen fell to within the normal range after surgery. In severalf cases, the levels of pancreatic oncofetal antigen rose again in parallel with recurrence. The full development of indications for an interpretation of the assays still requires an enormous amount of work.

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Purification, Partial Characterization, and Clinical Evaluation of a Pancreatic Oncofetal Antigen
