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Pancreatic Cholera (W.D.H.A. Syndrome)

Histochemical and Ultrastructural Studies

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Summary. Results of light- and electron-microscopic studies of a primary pancreatic tumor and of metastasis in a new case of *P*ancreatic *C*holera (P.C.) are reported. The primary tumor, but not the metastases, contained unusual, large cystic glandular formations, lined both by pancreatic-duct- and small-intestine-like epithelia and closely connected with the endocrine proliferation. A part from a few D-cells, the endocrine tumoral cells could not be identified by histochemical stainings. Their ultrastructural pattern, with small secretory granules (diameter less than 300 nm) and numerous cytoplasmic bunches of filaments, was very similar to that of gastric and duodenal D1-cells. Normal duodenal D1-cells have been said to produce gastric inhibitory peptide, a substance structurally and biologically similar to the vasoactive intestinal peptide actually secreted by the tumor. The normal histological appearance of gastric, gallbladder, jejunal, ileal, right and left colonic mucosae is consistent with the responsibility of the tumoral secretion in the impairment of gut functions in P.C.

Pancreatic cholera (P.C.) (or Verner-Morrison syndrome) is a well defined clinical entity since its clearcut separation from the Zollinger-Ellison syndrome by Murray *et al.* (1961) on the basis of absent gastric hypersecretion. However, the cellular origin of the pancreatic tumor remains unsettled: histochemical studies, usually limited to a small number of stainings, have led in most cases to the negative conclusion of the "non-B" nature of the tumoral cells (Schoenemann, 1972), and ultrastructural studies have given conflicting results (Elias *et al.*, 1972; Creutzfeld *et al.*, 1970b; Doerr, 1973; Martin *et al.*, 1974; Burkhardt *et al.*, 1974). Similarly, the nature of tumoral secretions has been the matter of various proposals: secretin (Zollinger *et al.*, 1968); prostaglandins (Sandler *et al.*, 1968); "gastrone" (Semb *et al.*, 1970); gastrin plus glucagon (Barbezat and Grossman, 1971); gastric inhibitory peptide (Elias *et al.*, 1972); vasoactive intestinal peptide or V.I.P. (Bloom *et al.*, 1973).

We report here the results of a detailed histochemical and ultrastructural study of the pancreatic tumor and its metastases in a new case of P.C. in which the secretion of V.I.P. has been demonstrated (Rambaud *et al.*, 1974). Histological patterns of gastric, gallblader, small and large intestine walls are also described.

Case Report

A 38-year old housewife, referred in September 1971, suffered since 1964 from bouts of watery diarrhea with periods of nearly complete remission. During the last two years, 5 re-

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gressive attacks of quadriplegia occurred. Finally, the patient was admitted for acute dehydration and anuria, which improved after massive intraveinous water and electrolyte replacement. Klebsiella septicemia and subsequent profuse hematemesis, due to an acute bleeding ulcer evidenced by emergency endoscopy, were cured by medical management. During the following two months, fecal weight varied between 1.7 and 7 kg per day, and several attacks of moderate flushing were noted. Clinical examination was otherwise normal, and past history was not contributive.

Laboratory Investigations

The main findings were hypokalemia (1.8 mEq/l), high fecal-water and potassium losses (3270 ml and 160 mEq/l per day respectively, for a three-day collection period), without evidence of fat, nitrogen, xylose, folic acid and B_{12} malabsorption, and basal gastric achlorhydria in 3 out of 4 studies. Serum calcium level was repeatedly normal, and fasting blood sugar was slightly increased. Fasting plasma levels of gastrin, secretin, glucagon, gastric inhibitory peptide and parathormone were normal on radioimmunoassay, while immunoreactive V.I.P. (1030 pg/ml, $N<60$), prostaglandins E (1316 pg/ml, $N<85$) and Fα (240 pg/ml, $N<22$), and calcitonin (1710 pg/ml, $N<100$) were markedly increased. Selective coeliac angiogram suggested a malignant pancreatic tumor.

Laparotomy Findings and Postoperative Course

Laparotomy showed a tumor of the body of pancreas, adhesive to the posterior wall of the stomach, and responsible for a splenic vein thrombosis; multiple small metastases were found on liver, peritoneum, and ovaries. Tumoral resection being hazardous, several biopsies of metastases, proximal jejunum, distal ileum, right and left colon were taken, and a double-barrel ileostomy was performed.

During the postoperative period, ileostomy output varied between 1.5 and 7.1 kg per day, while no losses via the anus were observed. After ineffective courses of steroids, cyclophosphamide, and cobalt teletherapy, resection of the pancreatic tumor, which necessited a total gastrectomy and a splenectomy, was eventually performed. 15 days later, the ileostomy was closed and a cholecystectomy was performed, the gallbladder containing biliary mud. Radioimmunoassay and bioassay of pancreatic tumor extracts revealed high amounts of V.I.P.; other hormones were absent, except for a small amount of glucagon. Immunofluorescence study with antisera against calcitonin, gastrin, secretin, insulin, and glucagon was negative. After a 15 months remission, there was a relapse of diarrhea and the patient died. Post-mortem examination was refused.

Material and Methods

Light-Microscopic Studies

2-mm sections of the resected pancreas and of lymph-node, liver-, and peritoneum metastases were fixed in 10% neutral buffered formaldehyde, in Bouin's fluid and in 6% glutaraldehyde in a saline phosphate buffer 0.01 mol (pH 7.1). Sections were stained with hematoxylin-eosin-saffron (H.E.S.), periodic acid-Schiff (P.A.S.), Masson's trichrome and Thioflavine T. Special stains for polypeptide secretory granules of pancreatic and gastro-intestinal endocrine cells were performed according to the methods and classification used since the Wiesbaden International Symposium (Creutzfeld *et al.*, 1970a; Pearse *et al.*, 1970; Polak *et al.*, 1973; Pearse, 1973). The same fixation and staining techniques were applied to fundic and antral fragments of the stomach; jejunal, ileal and colonic biopsies, and a gallbladder fragment were fixed in neutral formaldehyde and stained with H.E.S.

Electron-Microscopic Studies

Pancreatic tumor and metastatic tissues were either fixed by 3% glutaraldehyde and post-fixed by osmium tetroxide, or fixed by osmium tetroxide alone. Thin sections were

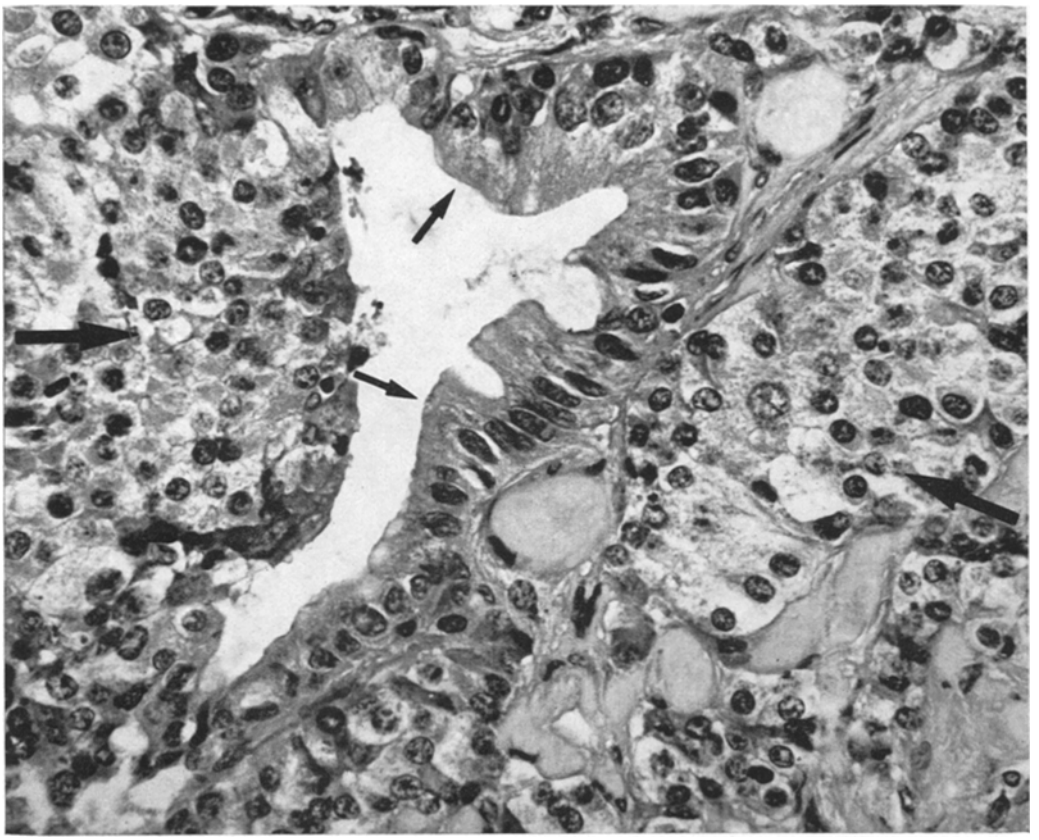

Fig. 1. Pancreatic endocrine tumor (big arrows) and intestinal-like structures (small arrows) with goblet cells. (Hematoxylin-Eosin-Saffron. ×450)

stained with uranyl acetate and lead citrate. Thiery's method (Thiery, 1967) for polysaccharide characterization was also used.

Results

Light-Microscopic Studies

Pancreas. The resected pancreas contained a 5×3 cm hard and whitish tumor, consisting of a trabecular arrangement of regular polyhedric cells with eosinophilic cytoplasm and round or oval nuclei without atypia. The cellular strands were either separated by a hyaline fibrosis or juxtaposed with intervening narrow connective tissue septae containing numerous capillaries; no amyloid deposits were observed. In some areas, the endocrine structure was mixed with glandular forms, either small or cystic and filled with mucus. These cysts were lined in some places by PAS-positive goblet cells and by columnar cells resembling normal enterocytes, with a well-differenciated brush border, and in other areas by cuboidal cells with a poorly visible brush border, similar to pancreatic duct cells.

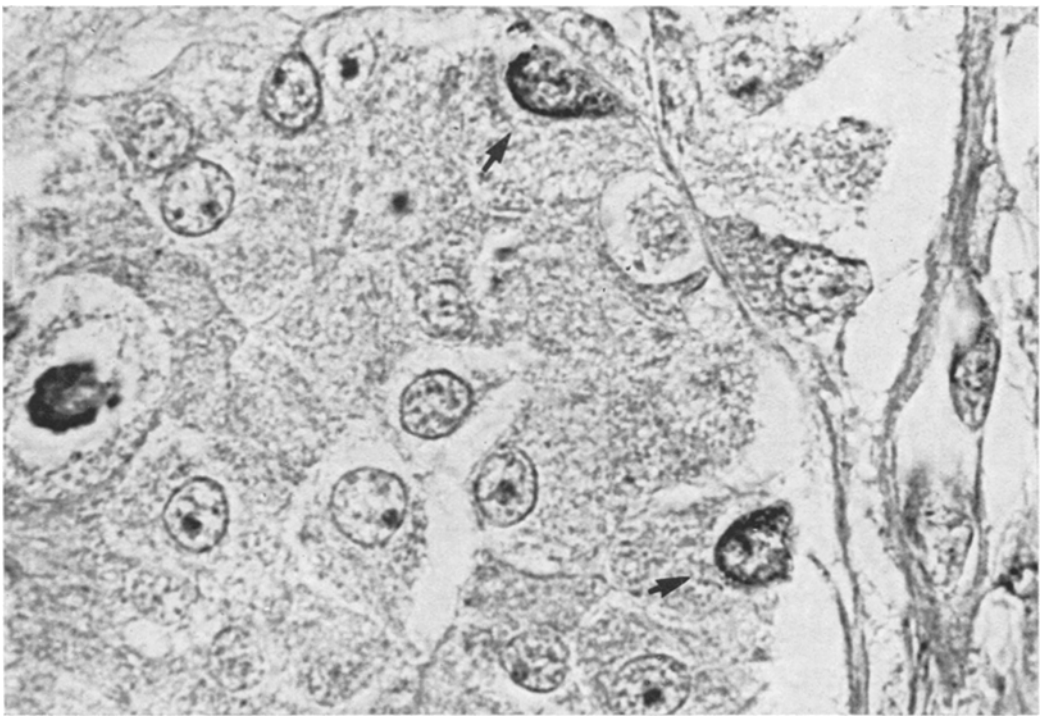

Fig. 2. Pancreatic endocrine tumor. Hellman-Hellerström's staining showing rare D-cells (arrows). ×1650

Connections between endocrine and exocrine-like structures were tight (Fig. 1): the exocrine epithelium often laid directly upon endocrine strands, or exocrine and endocrine cells succeeded to each other without any transition. Further, the cysts were rarely found isolated in the tumoral stroma and never within the normal surrounding pancreas tissue.

In tumoral endocrine tissue, paramethylaminobenzaldehyde (DMAB) (Ganter and Jolles, 1969) and phosphotungstic hematoxylin (PTAH) (Terner *et al.*, 1964) stains for pancreatic A-cells, and aldehyde-fuschin stain (Ganter and Jolles, 1969) for pancreatic B-cells, were negative. A few cells, stained by Hellerstrom and Hellman (1960) and Grimelius (1968) methods, and by lead hematoxylin without hydrolysis (Solcia *et al.*, 1969), but not by the Fontana-Masson argentaffin staining (Ganter and Jolles, 1969), could be classified as D-cells (Fig. 2). In the intestinal-type epithelium lining the "exocrine" cysts, some E.C. cells (stained by the Fontana-Masson method) and cells stained by the Hellerstrom-Hellman technique were observed. In the pancreatic tissue surrounding the tumor, the number and size of Langerhans islets were normal, as well as the proportion of B to A and D cells.

Metastases. Lymph-node, liver and peritoneum metastases were composed exclusively of an endocrine-type tissue, without any exocrine figure. All methods of staining used for the pancreatic tumor studies were negative.

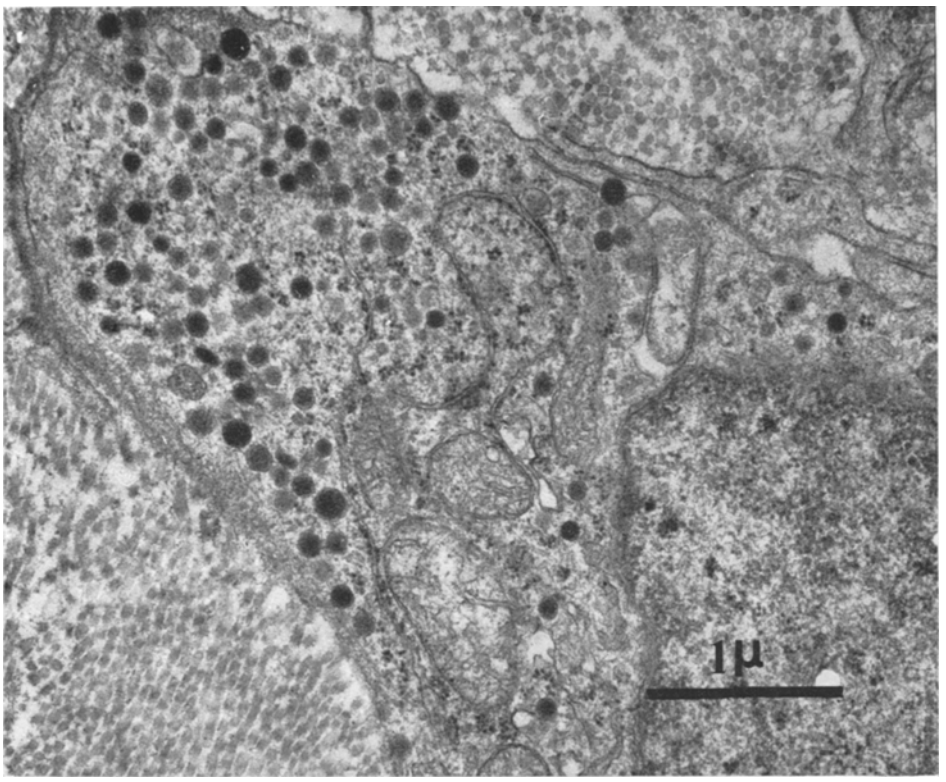

Fig. 3. Electron micrograph of pancreatic tumor (×25000). Secretory granules are limited by a thin membrane. They have a dense content without peripheral clear space or crystals

Stomach. Serosa of the fundic posterior wall was invaded by endocrine tumoral strands, in front of which a small fibrous area was observed in the submucosa, covered by a dystrophic mucosa. The thickness of gastric mucosa was normal, but the height of the infundibulum area was slightly increased at the expense of fundic and pyloric glands. In fundic glands, where the parietal to chief cell ratio was normal, histochemical studies showed some E.C.-like cells as characterized by Vassalo *et al.* (1971), while cells stained by Hellerstrom-Hellman, lead-hematoxylin and Grimelius techniques, but not by the Fontana-Masson procedure, were numerous in the middle third part of the antral mucosa. Whether these cells are D or G cells is undetermined.

Gallbladder, Small and Large Intestine. The histological pattern of gallbladder, jejunum, right and left colon walls was normal. In ileum, the number of goblet cells, equal to that of enterocytes, was increased.

Electron-Microscopic Studies

The same type of cell composed the primary pancreatic tumor and the hepatic and lymph-node metastases. They were packed together and connected by cell

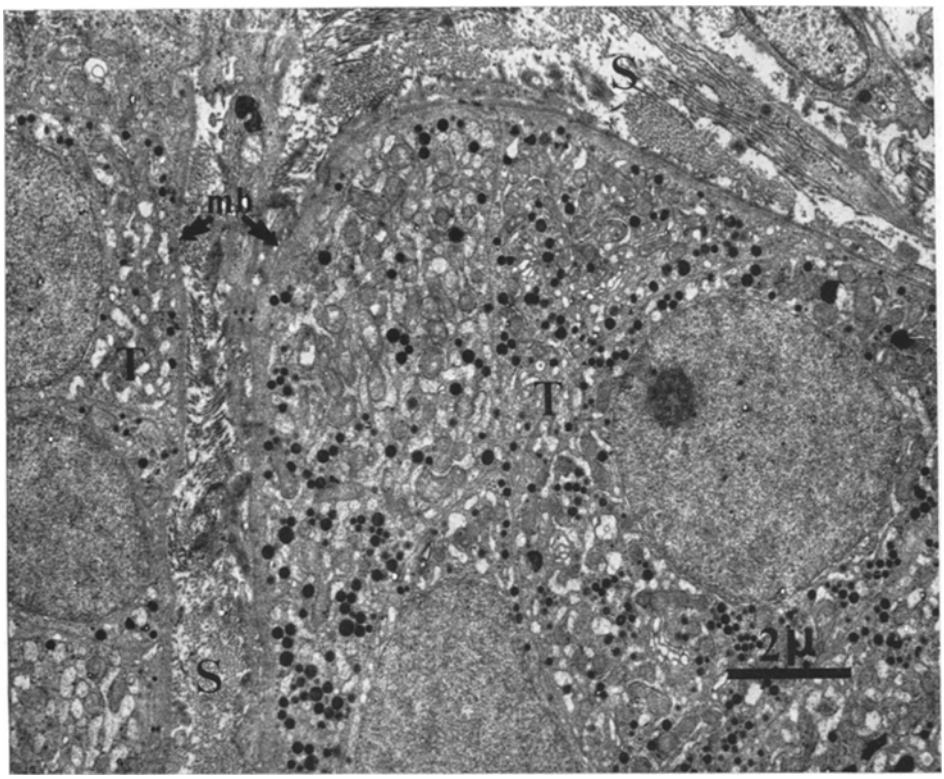

Fig. 4. Electron micrograph of hepatic metastasis (×8000). The tumor cells (*T*) are separated from the stroma by a basement membrane (*mb*). The size of the round secretory granules ranges up to 300 nm but is sometimes less than 100 nm

junctions, tight junctions, and desmosomes (Fig. 3). Some sets of cells were separated from the stroma by a thin basement membrane (Fig. 4). In metastasis, tumor-cell-lined true canaliculi with microvilli were a remarkable, although rare, finding. The tumoral cells had a light electron density, were middle-sized and had a large, round to oval nucleus. The nucleoli were small. The rough endoplasmic reticulum was sometimes vesicular. The Golgi apparatus was never large, centrioles were rare, and lipid droplets exceptional. Mitochondria often appeared altered; their matrix was clarified and their crystae rarefied. In the hyaloplasm, polysomes, monoparticulate glycogen and, in the primary tumor cells only, numerous filamentous bunches were observed (Fig. 3).

All tumoral cells contained rather numerous secretory granules. The latter were sometimes elongated, but in most cases spherical. Their diameter varied, but never to more than 300 nm and sometimes to less than 100 nm (Fig. 4). Their matrix was homogenous, of variable density, most often osmiophile. A thin, limiting membrane was frequently seen and was sometimes detached from the dense core but never separated by a true clear halo (Fig. 3). The granules never contained crystals, but had a clearer vacuolar constituant of probable lipid nature.

The Thiery's method was negative. No intermediate cells, containing both zymogen and endocrine-like secretory granules, were found in the tumor or its metastases. The sections studied did not contain the exocrine structures revealed by light-microscopic studies.

Discussion

Tumoral Endocrine Structures

The primary tumor, but not the metastases, contained a few cells with histochemical characteristics of D-pancreatic cells, as in two previously published cases of P.C. (Lopes *et al.*, 1970; Greider *et al.*, 1974). Although these cells are supposed to secrete gastrin, the immunoreactive gastrin content of the tumor was negligible, and immunofluorescent study with antiserum against gastrin was negative. In two cases studied by Burkhardt *et al.* (1974), a weak and diffuse argyrophilic reaction with the Grimelius method and a slight and patchy staining by lead-hematoxylin were merely considered to be a general feature of APUD-cell tumors.

Most of the cells in the primary tumor, and all of them in the secondary deposits, could not be identified by the current methods of staining of pancreatic and gastrointestinal endocrine cells. Referring to the normal cells of these two endocrine systems, the secretory granules observed by electron microscopy seemed to be distinct of that of B-cells (because of the constant absence of crystals), A-cells (absence of a clear halo), D-cells (a smaller size and less prononced density), of L and I-cells (the smaller size), of EC and EC-like cells (a less marked polymorphism), and of G-cells (a smaller size and less variable density). Although their diameter was slightly greater, the granules resembled that of duodenal S-cells. Above all, considering the morphology of the secretory granules and the presence of numerous bunches of filaments, the ultrastructure of the tumoral cells appeared very similar to that of D1-cells of Vassalo *et al.* (1971); the ultrastructure of these D1-cells appears morphologically identical to the human antral gastrin cells of Greider *et al.* (1972). A cell type with identical granules has been described among the normal non-A, non-B-pancreatic islet cell population (Munger, 1972; Jirasek and Kubes, 1972). Cells with similar granules have been found in other cases of P.C. (Elias *et al.*, 1972; Greider *et al.*, 1974; Burkhardt *et al.*, 1974), but also in the pancreatic tumor of patients with the Zollinger-Ellison syndrome (Vassalo *et al.*, 1972; Greider *et al.*, 1974), in insulinomas (Creutzfeld *et al.*, 1973), and carcinoid syndrome (Black and Haffner, 1968). Conversely, different larger granules have been described in other cases of P.C. (Creutzfeld *et al.*, 1970b; Martin *et al.*, 1974). These discrepancies could be due to pathological changes of the endocrine tumoral cells, or might be relevant to the hypothesis that these tumors are derived from a common precursor cell with variable morphological differenciation (Vassalo *et al.*, 1972).

Whatever the interpretation of these conflicting data, they make it very difficult to establish a correspondance between morphology, as given by ultrastructural studies, and the secretory activity, as obtained from histochemical and immunofluorescence studies or tumoral extractions (Burkhardt *et al.*, 1974). This task is difficult even in normal cells (Frexinos *et al.*, 1973) and

presented a problem in the present case, in which tumoral cells resembled gastric and duodenal D1-cells: production of V.I.P. by the tumor has been demonstrated, whereas normal D1-cells have been tentatively identified as G.I.P.-secreting cells (Polak *et al.*, 1973). It is possible that these two peptides, the structure and physiological activities of which are closely related (Bodanszky *et al.*, 1973) are secreted by cells of similar morphological appearance; however, V.I.P.-secreting cells have recently been suggested to be D-cells of the mammalian gut (Polak *et al.*, 1974).

Tumoral Exocrine Structures

Some islet-cell tumors may contain small canalicular formations, lined by exocrine-type pancreatic cells (Frantz, 1959; Floquet, 1967), which should not be confused with the tubular or acinar pattern frequently observed within the endocrine proliferation of pancreatic cholera (Murray *et al.*, 1961; Deleu *et al.*, 1964; Greider *et al.*, 1974) and Zollinger-Ellison syndromes (Greider *et al.*, 1974). But the presence within such tumors of large cysts, lined in some places by a small intestine-like epithelium, as observed here, is a previously undescribed feature. One cannot completely exclude that such endocrine structures may have proceeded from invasion and inclusion of normal pancreatic ducts within the endocrine tumoral tissue since (a) a small number of goblet, enterocyte-like and argentaffin cells has been observed in normal pancreatic ducts (Glenner and Mallory, 1956); (b) in our patient, metastases exclusively contained endocrine cells. However, in view of the very peculiar topographical relationships between exocrine and endocrine structures within the primary tumor, the existence of a mixed tumoral proliferation appears more likely. Two hypothesis could account for such a mixed proliferation in view of the current theories concerning the origin of pancreatic islet cells.

According to the first hypothesis, which relies on morphological data observed in numerous animal species, the origin of islet cells has been ascribed to the pancreatic ducts (Pearse *et al.*, 1973), or to the "acinar-insular transformation", i.e. the occurrence in animal- (Leduc and Jones, 1968; Melmed *et al.*, 1972) and human (Zagury *et al.*, 1961) pancreas of mixed or intermediate cells containing both zymogen and endocrine granules. Such a unifying theory suggesting that Langerhans islets are derived from exocrine pancreas would fit with the previous report, in P.C. and Zollinger-Ellison syndrome patients, of pancreatic areas with "tubulo-islet neoformation" (Stoker and Wynn, 1970; Bloodworth Jr. and Elliott, 1963) or with an embryonal pancreas appearance (Deleu *et al.*, 1964). However, numerous arguments support the second hypothesis, according to which islet cells belonging to the A.P.U.D. system would migrate from the neural crest and colonize the primary exocrine pancreas derived from the foregut (Pearse, 1973; Pearse *et al.*, 1973). In this dualist concept, one may speculate that an abnormal intrication of exocrine and endocrine tissues could result in a hamartoma, the endocrine component of which would only later be submitted to malignant transformation. Thus, the origin of our patient's tumor could be compared to that of "mixt insular-duct adenomas" (Glenner and Mallory, 1956), a variety of pancreatic cystadenoma closely associating normal Langerhans islets to cystic cavities covered with a small

intestine or pancreatic duct-like epithelium, and to that of a carcinoid tumor found within the wall of a pancreatic cystadenoma (Persaud and Walrond, 1971).

Gastric, Gallbladder, Small and Large Intestine Morphology

Investigations performed in the present patient (Rambaud *et al.*, 1974) and in previously published cases of P.C. (Pessayre, 1973) have revealed several abnormalities in gastric juice and gallbladder bile composition, and in small and large intestine functions. The normal histological pattern of stomach, gallbladder, jejunum, ileum, and right and left colon observed here confirms the results of numerous but fragmentary studies (Chears *et al.*, 1960; Parkins, 1961; Marks *et al.*, 1967; Zollinger *et al.*, 1968; Stoker and Wynn, 1970; Gjone *et al.*, 1970; Lopes *et al.*, 1970; Jacobs *et al.*, 1972; Andersson *et al.*, 1972), and strongly suggests the responsibility of a humoral agent, secreted by the pancreatic tumor, in the pathogenesis of these abnormalities. There is now evidence that V.I.P. is this agent (Bloom *et al.*, 1973; Rambaud *et al.*, 1974), but further work is necessary before all P.C. tumors can be considered "vipomas".

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