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Histologic Characteristics of Insulinomas and Gastrinomas

Value of Argyrophilia, Metachromasia, Immunohistology, and Electron Microscopy for the Identification of Gastrointestinal and Pancreatic Endocrine Cells and Their Tumors

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Summary. In a first step of our investigation the staining characteristics, especially the argyrophilia and metachromasia, of immunohistologically identified endocrine cells of the pancreatic islets and of the gastroduodenal mucosa were tested. These staining characteristics were then examined on insulinomas and gastrinomas.

Contrary to normal B cells which generally react positively with aldehyde fuchsin and pseudoisocyanine but not argyrophilic with the Grimelius method, the neoplastic B cells give inconsistent results with conventional staining methods. Yet neoplastic B cells often show argyrophilic structures. Immunohistologically, most benign insulinomas are rich in insulin-containing cells, whereas in malignant types such cells are rare. The carcinomas, however, show a typical and distinct Grimelius argyrophilia. The tumor cells of gastrinomas are Grimelius argyrophilic and slightly metachromatic, as normal G cells, yet, contrary to A_1 cells, they are only exceptionally stainable with the Hellerström method. Despite the great number of Grimelius positive tumor cells, generally only a few reacted with antigastrin serum. Nevertheless, the immunohistology is the most reliable method for the diagnosis of gastrinomas. Electron microscopic results are often difficult to interpret, since gastrinomas, as well as undifferentiated or malignant insulinomas, may predominantly contain atypical secretion granules.

Key words: Islet cell tumors — Insulinomas — Gastrinomas — Staining characteristics — Immunohistology.

Zusammenfassung. In einer ersten Untersuchungsserie werden die Färbeeigenschaften, insbesondere die Argyrophilie und Metachromasie, normaler endokriner, immunologisch identifizierter Zellen der Pankreasinsel und der Gastroduodenalmukosa untersucht. In einem zweiten Schritt werden diese Färbeeigenschaften an Insulinomen und Gastrinomen geprüft.

Während die normalen B-Zellen mit Aldehydfuchsin und Pseudoisozyanin positiv und generell nicht argyrophil nach Grimelius reagieren, lassen sich die neoplastischen B-Zellen nur unregelmäßig mit den konventionellen Methoden anfärben. Sie enthalten aber häufig argyrophile Strukturen. Immunhistologisch kommen praktisch in allen gutartigen Insulinomen reichlich, in den metastasierenden Formen dagegen kaum insulinhaltige Zellen zur Darstellung. Die Malignome weisen jedoch eine besonders ausgeprägte Grimelius-Argyrophilie auf. Die Tumorzellen von Gastrinomen sind wie normale G-Zellen Grimelius-argyrophil, bzw. leicht metachromatisch und lassen sich nur ausnahmsweise wie A_1-Zellen mit der Methode von Hellerström versilbern. Trotz der großen Zahl von Grimelius-positiven Tumorzellen reagieren meist nur wenige mit Antigastrinserum. Dennoch ist die Immunhistologie die zuverlässigste Methode für die Diagnose von Gastrinomen. Die Elektronenmikroskopie liefert dagegen oft schwer interpretierbare Resultate, da in Gastrinomen ähnlich wie in undifferenzierten oder malignen Insulinomen vorwiegend atypische Sekretgranula vorhanden sein können.

Introduction

The accurate identification of the endocrine cells of the gastrointestinal system is growing more important due to the possibility of quantitatively measuring the

corresponding hormone levels in the serum (Grossmann et al., 1974; Raptis, 1974). Thus a differentiated histopathologic diagnosis of endocrine active tumors and hyperplasias is especially important. Various procedures for demonstrating the different endocrine cells of the intestinal tract and their tumors have been developed lately. Among others the argyrophil reactions and the so-called masked metachromasia have been proven as very useful (Solcia et al., 1968; Pearse et al., 1970; Vassallo et al., 1971a). The silver techniques, however, are often very inconsistent, partly contradictory results are therefore not astonishing (Pearse and Bussolati, 1970; McGuigan and Greider, 1971).

Our aim was to examine the significance of the argyrophil and metachromatic reactions. The methods were slightly modified in order to obtain more consistent results. For definite identification of the gastrin and insulin producing cells we also used the corresponding antisera. After having standardized the procedures on fresh autopsy material of normal gastric and intestinal mucosa and of pancreas tissue, the practical value was tested on biopsies of insulinomas and gastrinomas.

Material and Methods

Material

Fresh autopsy tissue of the pancreas and gastroduodenal mucosa was used as test material for the staining methods and immunohistology. The pancreas was cut in blocks of about 7 mm side length, the duodenal and the gastric mucosa in strips of 3–5 mm, and fixed for 12–48 h.

The following appropriate fixative solutions were routinely used: Neutral 4% formalin, Bouin' solution, and the glutaraldehyde-picric acid mixture with sodium acetate (GPA) (Solcia et al., 1968).

There were 39 paraffin embedded biopsies of islet cell tumors examined from the Institute of Pathology of Zürich University, including some from the Institute of Pathology of the cantonal hospital Winterthur. Naturally the material was very heterogeneous with reference to the date when the biopsy was obtained (1957–1975), to preservation and fixation. This may explain the slightly varying results.

According to symptomatology and clinical data, the 39 islet cell tumors could be subdivided in three different groups:

17 insulin producing tumors ("insulinomas")	14 adenomas 3 carcinomas
9 ulcerogenic tumors (Zollinger-Ellison syndrome) ("gastrinomas")	8 carcinomas 1 carcinoid-like tumor
13 endocrine inactive tumors and tumors with atypical symptomatology	

Methods

1. Argentaffinity

Method according to Masson-Hamperl (1932).

2. Other Silver Reactions

a) Silver Reactions in Watery Solution. Method of Grimelius (1968), Holmes (1947) according to the data of Gepts (1957), Sevier and Munger (1965).

The Grimelius silver method was slightly modified: After an impregnation of 3–4 h at 60° C, the slides were reduced at a temperature of 60–70° C instead of 40–45° C, and agitated constantly during the reducing period. Except for excellent material fixed in GPA or Bouin' solution, the slides were reincubated after a short rinse for an additional 1–2 h at 60° C, and reduced as before. In order to get a prominent enough argyrophilic reaction it was generally

necessary to reduce the slides until they appeared light-brown. The silver solution (0.05%) and the reducing solutions were prepared according to the original publication.

b) Silver Reaction in Alcoholic Solution. Hellerström and Hellman (1960).

3. Metachromatic Reactions after Hydrolysis

"Masked metachromasia" according to Manocchio (1960), Solcia et al. (1968), Pearse (1969b).

After a 5–6 h hydrolysis in 0.2 N HCl at 60°C the slides were rinsed in water for 20 min and left overnight at +4°C.

As dyes, toluidine blue (0.05% in 0.02 M phosphate buffer pH 5.5) and pseudoisocyanine (Serva, Heidelberg, W.-Germany) (0.036% watery solution) were used. After a 2–3 min staining time the slides were covered with Sorbit 70%. Examination of the HCl-pseudoisocyanine-colored slides by a Zeiss fluorescence microscope.

4. Methods for B Cells

Aldehyde fuchsin according to Runge (1956), and staining with pseudoisocyanine after oxidation according to Schiebler-Schiessler (1959).

5. Immunohistology (Indirect Immunofluorescence)

a) Anti-Insulin Serum. The fixed and deparaffinized slides were incubated for 30 min at 37°C in antibovine insulin serum of the guinea pig (Nutritional Biochemical Corp., Cleveland, Ohio, USA), diluted 1:40 with NaCl, 0.01 M phosphate buffer pH 7.4. After rinsing for 30 min at room temperature the slides were covered for 30 min at 37°C with fluorescein-isothiocyanate (FITC)-labeled rabbit antiguinea pig gamma globulin (Behring, Marburg, W.-Germany).

Controls: 1. Anti-insulin serum, preincubated with an excess of pig insulin (Actrapid-MC, Hoechst, W.-Germany).
2. Serum of not immunized guinea pig.
3. FITC-antiguinea pig gamma globulin serum alone.
4. Anticalcitonin and antigastrin serum (see below) with FITC-antiguinea pig gamma globulin.

b) Antigastrin Serum. The serum was kindly supplied by Prof. W. Gepts, Director of the Institute of Pathology of the Vrije Universiteit, Brussels, Belgium. The serum was produced by immunization of rabbits with synthetic human gastrin (SHG 2–17). For the characteristics of the serum see Lotstra et al. (1974). We diluted the serum 1:10. Reincubation in FITC-labeled goat antirabbit gamma globulin (For procedure see 5. a).

Controls: 1. Serum of not immunized rabbit.
2. FITC-labeled goat antirabbit gamma globulin alone (Behring).
3. Anti-insulin serum and anticalcitonin serum.

c) Anticalcitonin Serum. The antiserum to synthetic human calcitonin was obtained in a goat (goat 6A, Day 143) (Dietrich et al., 1975). It was kindly supplied by PD Dr. J. A. Fischer (Orthopedic Clinic of Zürich University) and Prof. F. M. Dietrich (Ciba-Geigy, Basle). Dilution 1:40. Procedure according to 5. a, b. We looked for a possible calcitonin content of the islet cell tumors of the pancreas, especially the ones rich in amyloid; on the other hand, the serum was needed as an additional control for nonspecific fluorescence.

6. Re-Staining and Double-Staining

a) Re-Staining. Characteristic areas of the slides were examined immunohistologically and photographed. The same slides were then stained according to Grimelius, HCl-toluidine blue, and HCl-pseudoisocyanine respectively, and identical areas photographed.

b) Double-Staining. Amazingly, in most normal cells and sometimes also in tumor cells, specific immunofluorescence persisted after Grimelius silver impregnation. This method permitted the simultaneous examination of argyrophilia and hormone content of the endocrine cells.

7. Electron Microscopy

Six recent biopsies were fixed in 2% glutaraldehyde buffered at pH 7.4 with 0.2 M cacodylate, embedded in epon, refixed in osmiumtetroxide, contrasted with lead- and uranyl acetate, and examined with a Philips EM 201.

Results

1. Pancreatic Islets

The cells of the pancreatic islets demonstrated by staining with aldehyde fuchsin (Runge) or pseudoisocyanine after oxidation correlated well with the immunohistologically characterized B cells. The immunologic method seemed to be more sensitive by making most of the B cells visible. As expected, the B cells in general showed no argyrophilia, neither with the method of Grimelius nor with the ones of Sevier-Munger and Holmes. Occasionally, however, a slight but distinct argyrophilia could be demonstrated in some cells which had to be classified as B cells according to their central location in the islets. Examinations on re-stained slides, immunohistology first, silver impregnation thereafter, seemed to confirm this observation (Fig. 1). In double-stained slides, however, no overlapping of argyrophilia and fluorescence could be shown.

The Grimelius, Sevier-Munger and Holmes argyrophilic cells generally corresponded with the HCl-toluidine blue and HCl-pseudoisocyanine *slightly* metachromatic cells.

Moreover, in the HCl-toluidine blue staining a small cell fraction constantly became visible which reacted intensively red-violet, i.e., *strongly* metachromatic. On the contrary, with HCl-pseudoisocyanine, strongly metachromatic islet cells were very rare. The large pointed or round HCl-toluidine blue metachromatic cells could also partly be identified in slides colored with the Grimelius method. They correlated in form and location with the Hellerström argyrophilic cells. The latter, however, seemed to be more numerous than the HCl-toluidine blue strongly metachromatic cells in our test slides. Immunohistologically, none of these argyrophilic and slightly or strongly metachromatic cells gave a positive reaction with antigastrin serum.

2. B cell Tumors, Insulinomas

Of the 14 *benign insulinomas,* B cells could be identified only in 6 cases by aldehyde fuchsin staining, yet in 8 cases by pseudoisocyanine staining after oxidation (Table 1). In contrast, the very high sensitivity of immunohistologic procedures proved to be extremely valuable in investigating these tumors. In 11 out of the 14 insulinomas there were cells reacting with anti-insulin serum. The oldest preparation still showing a distinct immunofluorescence was from 1960, i.e., 15 years old. In the 3 negative cases the islets in the bordering pancreatic tissue also showed practically no reaction, which was probably due to autolysis or inappropriate fixation. Generally the tumor cells reacted immunohistologically weaker than the B cells of the islets; this discrepancy was especially striking in older preparations.

Morphologically two tumor types could be distinguished (Table 1):

1. Adenomas of trabecular pattern with equal fluorescence in all the tumor cells. The capillary cell pole was always brighter.

Table 1. Histologic findings of 17 insulinomas: 14 adenomas (1–14), 3 carcinomas (15–17)

Case	Year of biopsy	Type[a]	Immunohistology			Pseudoisocyanine after oxidation	Aldehyde fuchsin	Grimelius argyrophilia
			Number of pos. cells	Intensity of fluorescence	Islets			
1	1959	1	−	−	−	−	(+)	?
2	1960	1+2	+++	+	++	?	+	?
3	1962	1	+++	++	0	++	++	+/r
4	1966	2	++	++	++	−	−	+
5	1966	1	+	+	0	(+)	0	−
6	1967	2+1	+	++	++	++	−	+/r
7	1968	2	+	+	++	+	+	++/r
8	1968	1	+++	+++	+++	+	+	+/r
9	1968	2+1	++	++	0	+	−	(+)
10	1968	2	?	?	+	−	−	+/r
11	1969	2	?	?	?	−	−	+/r
12	1969	2	+++	++	++	++	−	+++/r HCl-Tol +
13	1973	2	+	+++	+++	?	?	++/r
14	1974	1	+++	+++	+++	+++	++	+/d
15	1957	2	−	−	−	−	−	+++/d
16	1968	2	+	+	0	−	−	+++/d
17	1973	2	−	−	−	−	−	+++/d

+++ = many/intensive; ++ = medium; + = few/weak; (+) = borderline; − = negative; 0 = not present/not investigated; r = reticular Grimelius argyrophilia; d = dispersed Grimelius argyrophilia; ? = questionable

[a] Histologic type: 1 = trabecular, 2 = medullary

2. Medullary adenomas with irregularly positive cells scattered between slightly or nonreacting cells.

In several tumors these two patterns merge into each other.

Surprisingly most of the insulinomas showed a slight Grimelius argyrophilia (Table 1). The silver granules were often cloddy, reticular, and deposited irregularly on one cell pole or along the cell membrane. In one case with particularly marked argyrophilia, the same structures showed a metachromasia with HCl-toluidine blue.

In order to compare immunoreactive cells and argyrophilia, double-staining on appropriate slides was undertaken. It appeared that the neoplastic cell could not stand this procedure as well as normal B cells. The fluorescence was scarcely visible, whereby only a few nonargyrophilic cells reacted.

More information was supplied by two recent cases of insulinomas having been investigated electron-microscopically as well. One of them, a medullary type, displayed reticular argyrophilia (case 13, Fig. 2e). Only few scattered immunofluorescent cells could be found (Fig. 2 d). Likewise, with the electron microscope a small number of cells containing polygonal and spheric granules

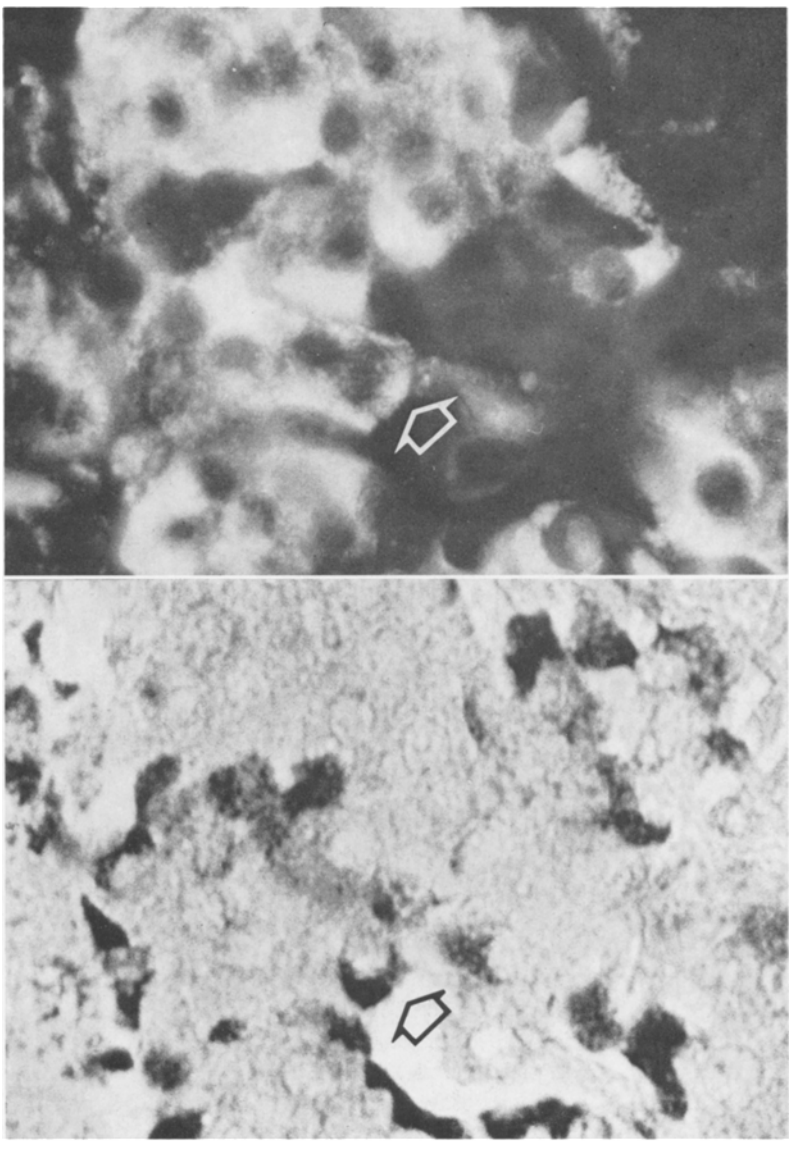
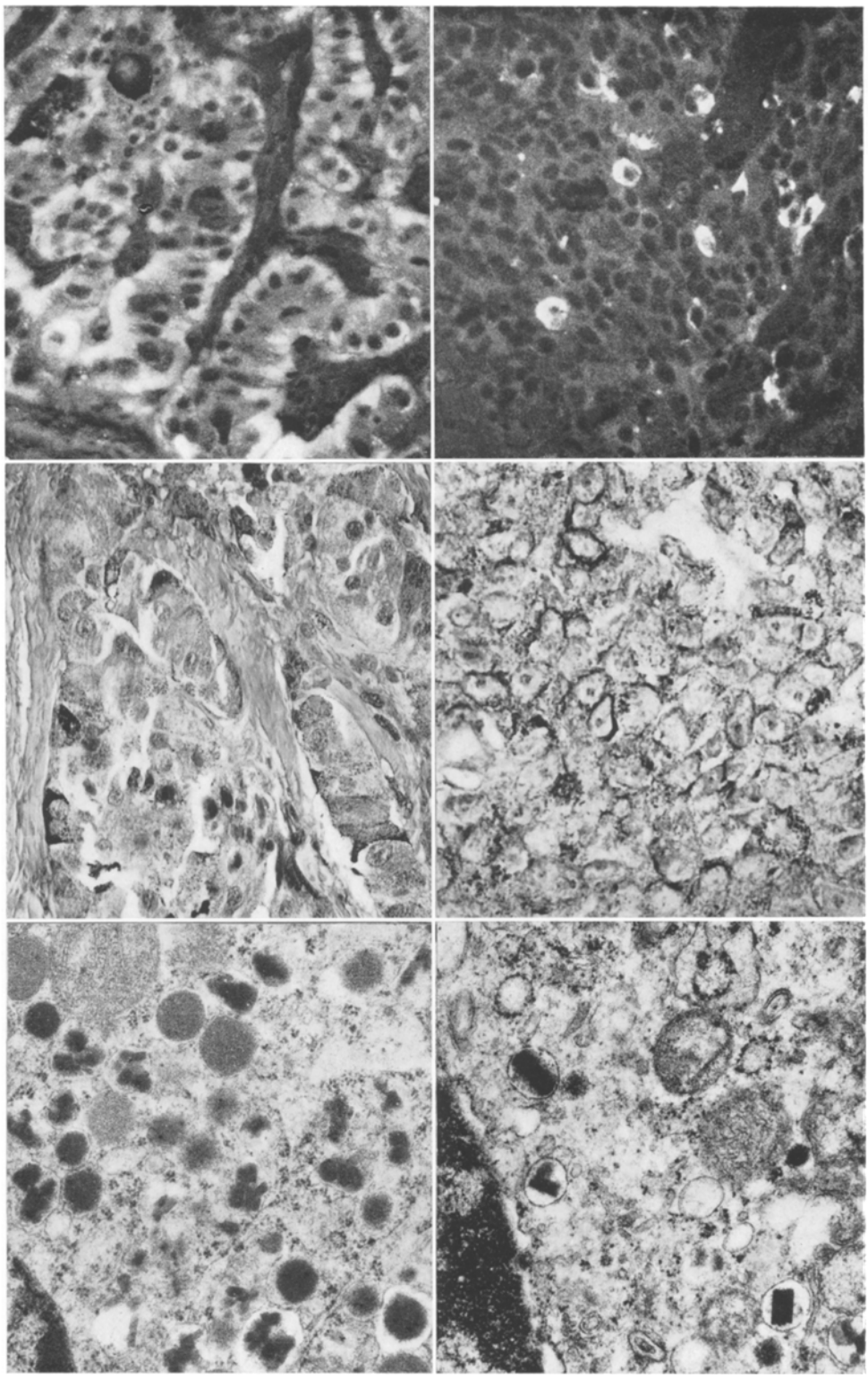


Fig. 1a and b. Re-staining technique of the same slide of normal islet. (a) Grimelius argyrophilia. (b) Immunohistology with anti-insulin serum. *Arrow*: argyrophilic, insulin containing cell. (×1,000)

Fig. 2a—f. Insulinoma type 1, on the left side (case 14). (a) Immunohistology: trabecular pattern with equal insulin content in almost all cells (×320). (b) Slight Grimelius argyrophilia of some cells (×320). (c) Electron microscopy: cells are rich in polygonal and spheric secretory granules (×20,000). Insulinoma type 2, on the right side (case 13). (d) Immunohistology: insulin is demonstrated only in some scattered cells (×320). (e) Cells show partly reticular Grimelius argyrophilia (×500). (f) Electron microscopy: only few cells contain secretory granules, and even in these cells granules are sparse (×24,500)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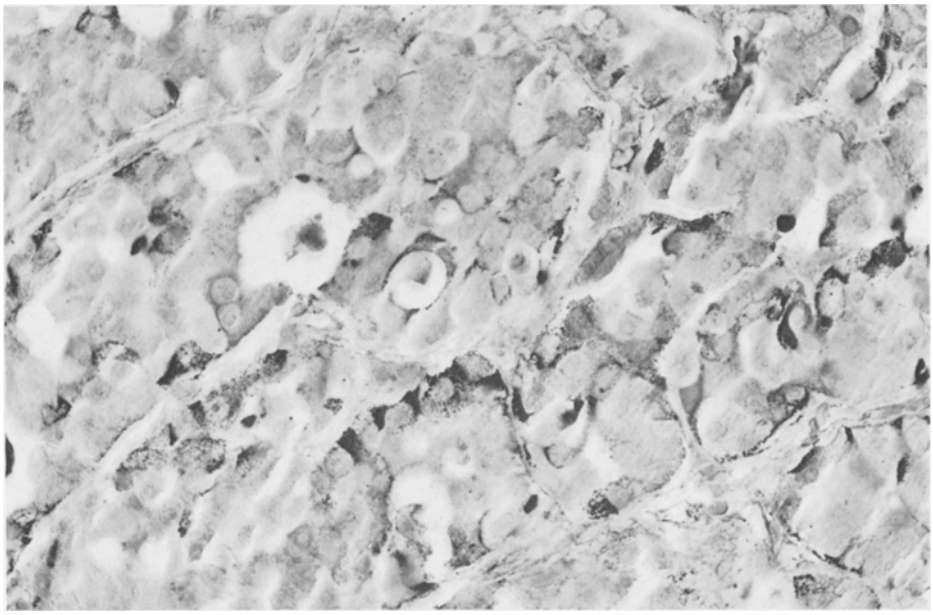


Fig. 3. Typical Grimelius argyrophilic cells in an insulin-producing carcinoma (case 15). Polyendocine acrtivity is probable (×400)

could be demonstrated (Fig. 2f). The second case displayed a hardly visible dispersed argyrophilia (case 14, Fig. 2b). All the cells of this trabecular adenoma reacted intensively with anti-insulin serum and pseudoisocyanine after oxidation (Fig. 2a). Electron-microscopically the cells were filled with polygonal and noncristalline granules (Fig. 2c).

The three *islet cell carcinomas* producing hypoglycemia but possibly combined with other endocrine activities displayed a strong, finely dispersed argyrophilia in many cells (Fig. 3). The HCl-toluidine blue metachromasia was difficult to assess (Table 1). One tumor also contained reticular argyrophilic structures in its cells and showed a weak fluorescence with anti-insulin serum. In the two other carcinomas there was no insulin demonstrable, neither by staining nor by immunohistology. In all three cases no positive reaction with antigastrin serum was seen. Electron-microscopically one of these tumors contained in most cells granules resembling the G cell granules (Fig. 4). Only a few cells with B cell granules could be found.

3. Pyloric and Duodenal Mucosa

Two cell types could be distinguished with the argyrophilic methods of Grimelius, Holmes, and Sevier-Munger after GPA- and Bouin's fixation. One cell type with long, slender processes located on the basis of the crypts was characterized by strong argyrophilia. By their argentaffinity and their morphology these cells correspond to the enterochromaffine cells. They were also Hellerström positive. It could not be decided whether enterochromaffine and Hellerström positive cells

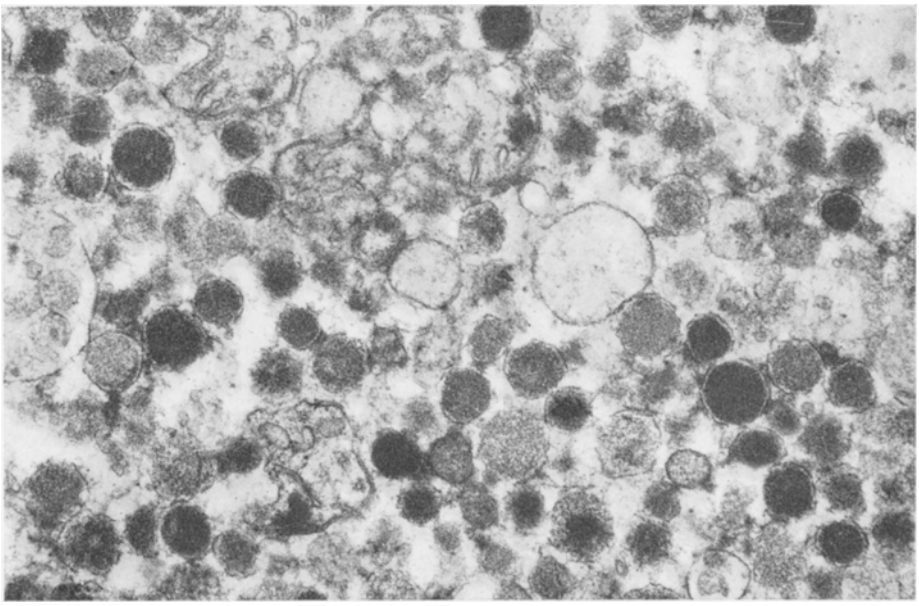


Fig. 4. Insulin-producing carcinoma (case 17). Granules remind one of G cell granules (EM $\times$ 22,000)

were always identical. However, enterochromaffine and Hellerström positive cells seemed to correlate quantitatively in the gastric and duodenal mucosa and in the carcinoid-like tumor of the pancreas (see below). On the other hand, we were unable to demonstrate Hellerström argyrophilia in three argentaffine carcinoids of the small intestine not included in the present study.

The second cell type was characterized by larger, mostly round, and slightly argyrophilic cells located in middle parts of the glands. This argyrophilia was missing after formalin fixation. These cells were Masson-Hamperl- and Hellerström negative. They were very clearly demonstrated by HCl-toluidine blue and HCl-pseudoisocyanine by reacting *slightly* metachromatic in contrast to the *strongly* metachromatic enterochromaffine cells. Most of the argyrophilic, slightly metachromatic cells of the antrum, and part of them in the duodenum and the Brunner's glands displayed a fluorescence with antigastrin serum. The identity of these argyrophilic, metachromatic cells with the gastrin containing cells was confirmed by re-staining and especially by the double-staining method (Fig. 5). Using double-staining, however, it was shown that the intensity of the argyrophilia did not always correlate with the immunofluorescence. On the one hand, some weak or nonargyrophilic cells were intensively fluorescent, on the other hand, argyrophilic cells not reacting with antigastrin were found in the antrum.

4. Ulcerogenic Tumors, Gastrinomas

Eight of the 9 endocrine active tumors combined with recurrent ulcers consisted mostly in cells of finely dispersed argyrophilia (Grimelius, Sevier-Munger, Holmes) (Fig. 6). Only one case additionally displayed a Hellerström

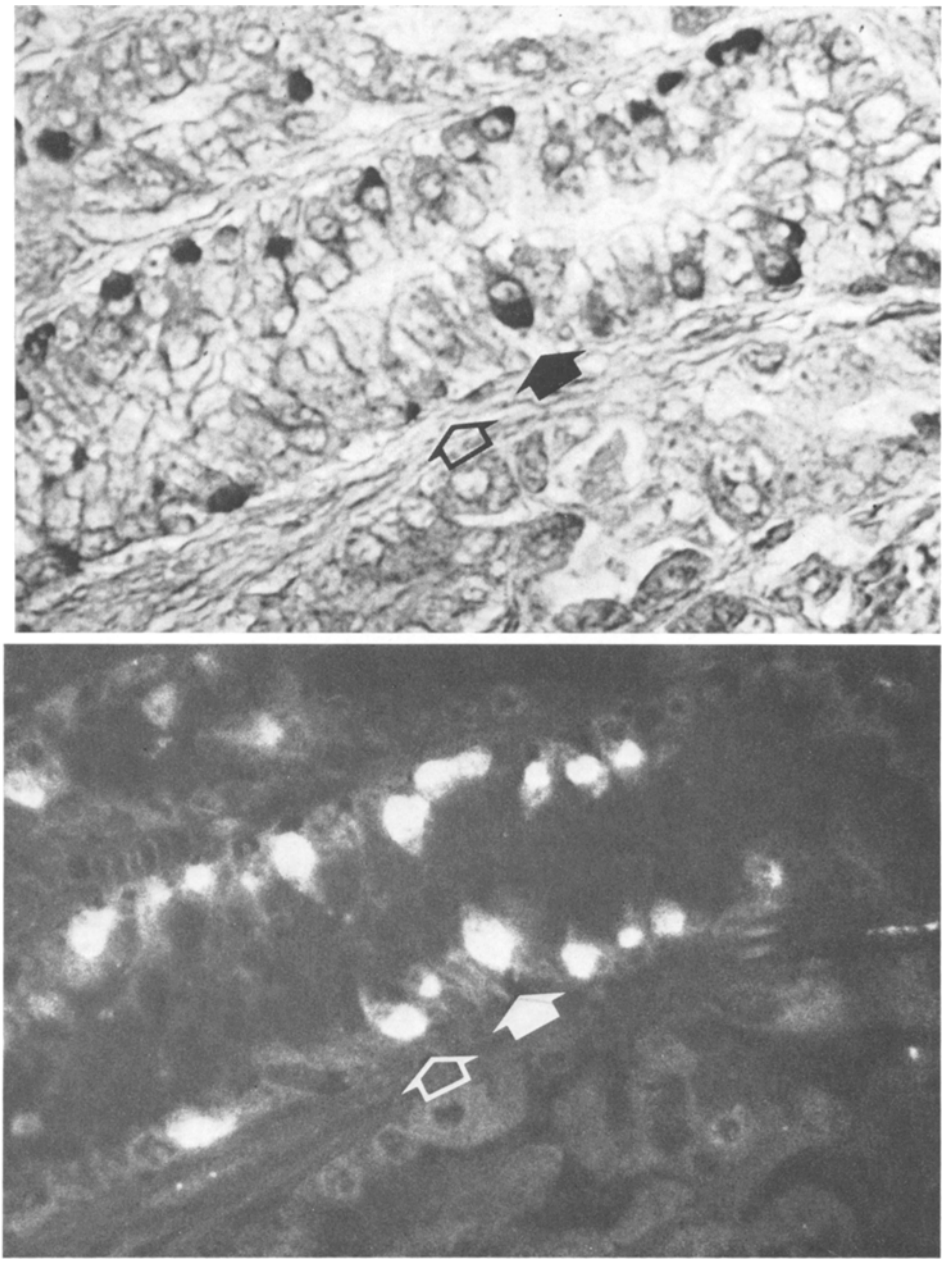


Fig. 5a and b. Double-staining technique of antral mucosa. (a) Grimelius argyrophilic cells. Argyrophilia is varying from one cell to another. (b) Immunohistology with antigastrin serum shows identical cells, but immunofluorescence does not always correspond to intensity of argyrophilia (*arrows*). (×500)

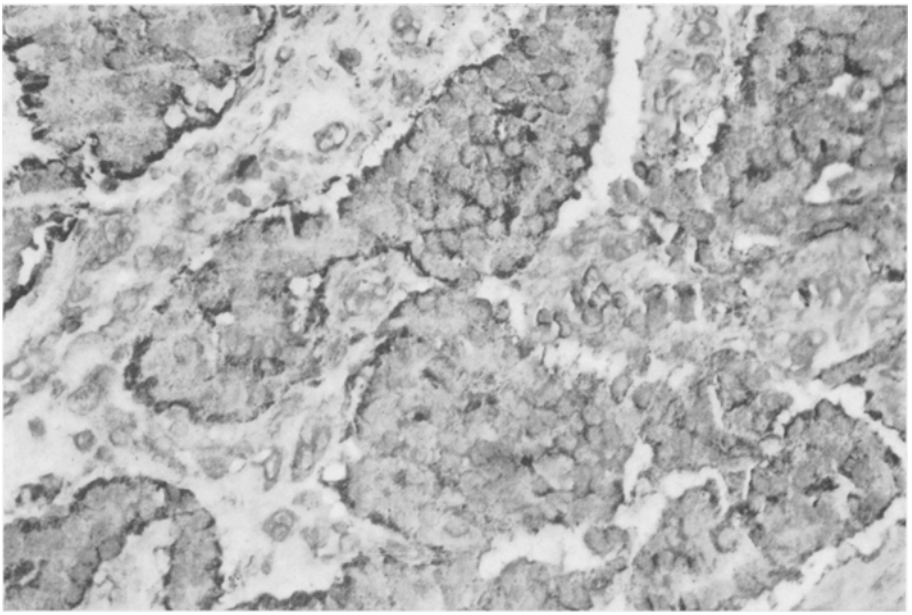


Fig. 6. Typical Grimelius argyrophilia of gastrinoma (case 6). (×400)

argyrophilia. In old, formalin-fixated preparations it was sometimes laborious to make the argyrophilic granules visible (Table 2). The results with toluidine metachromasia were less constant but correlated in the more recent biopsies well with the argyrophilia. The weak HCl-pseudoisocyanine metachromasia was difficult to judge in these tumors because of its little contrast. Moreover, in most cases some strong metachromatic cells of slender and pointed shape were found. These cells which were demonstrable in insulinomas as well were stained strongly eosinophilic in routine preparations and appeared homogenous brown-black with the Hellerström method. In our opinion, they are not identical with the pancreatic Hellerström positive cells by lacking the typical granules. Possibly their staining characteristic reflects a special state of cellular cytoplasm.

Immunohistologically, gastrin-containing cells have been demonstrated in 7 of the 9 ulcerogenic tumors (Table 2). In all tumors these cells were much less numerous than the argyrophilic cells (Figs. 6, 7). By double-staining of appropriate biopsies the argyrophilia of the tumor cells and the well-preserved immunofluorescence did not correlate; frequently, cells lacking silver deposits showed an immunofluorescence and vice versa (Fig. 8). In two cases insulin-containing cells were also demonstrated.

The carcinoid-like tumor, causing mainly diarrhea and steatorrhea, consisted of argentaffine, Hellerström positive cells in addition to Grimelius argyrophilic cells. Most of the Grimelius argyrophilic cells were intensely fluorescent with antigastrin serum (case 9).

In the three electron-microscopically investigated tumors, some cells showed relatively abundant round granules of different size and density, separated by a

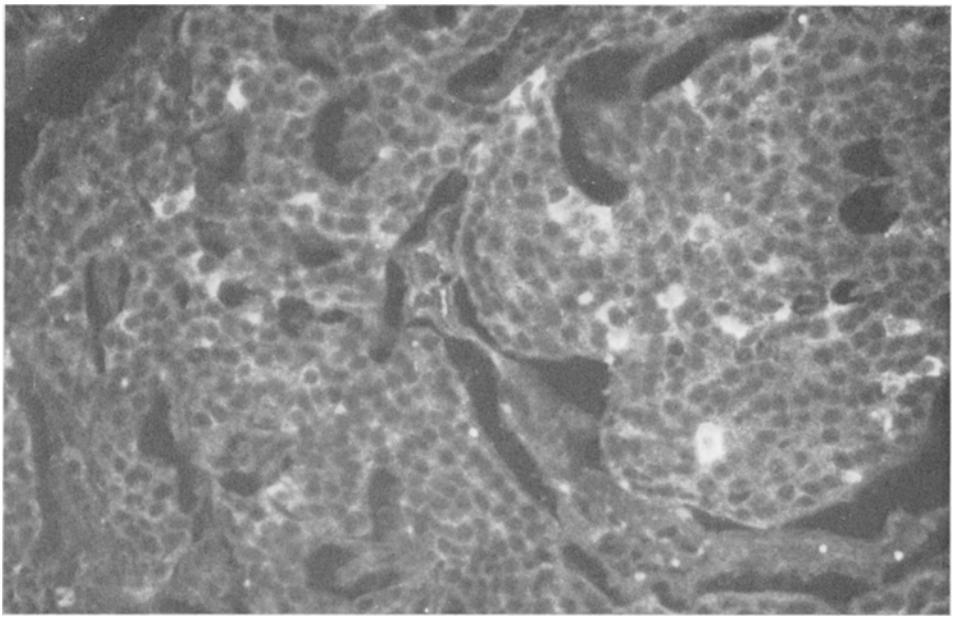


Fig. 7. Immunohistology of gastrinoma. Only few scattered cells show immunofluorescence with antigastrin serum (case 9). (×310)

Table 2. Histologic findings of 9 gastrinomas

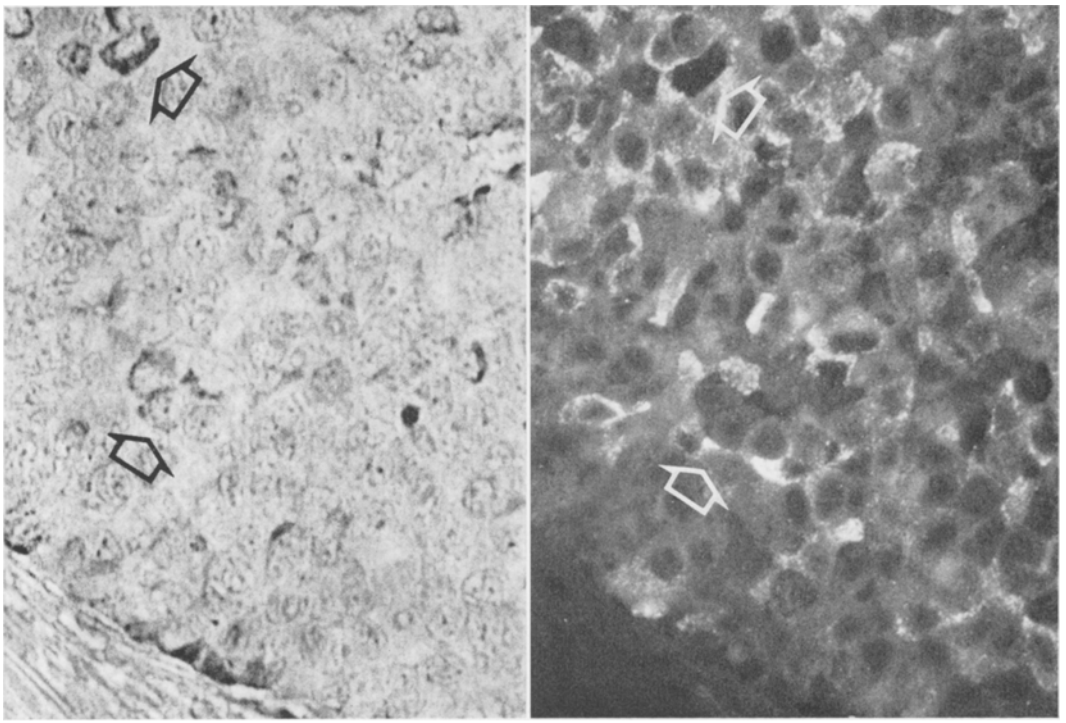
Case	Year of biopsy	Type[b]	Immunohistology		Grimelius argyrophilia	Sevier-Munger	HCl-tol. blue	Hellerström argyrophilia	Argentaffinity	Anti-insulin serum
			Number of cells	Intensity of fluorescence						
1	1965	2+1	+	++	+	−	++	−	−	−
2	1965	2	−	−	++	++	(+)	−	−	−
3	1966	2	+	(+)	+	−	−	+	−	−
4	1968	1	+	(+)	−	0	−	−	−	−
5	1971	2	−	−	++	0	+	−	−	−
6	1972	2	+++	+	++	+	+	−	−	+
7	1973	2	++	+++	+++	++	+++	−	−	−
8	1975	2	+	++	++	+	++	−	−	+
9[a]	1972	2	++	+++	++	+	+	++	++	−

+++ = many/intensive; ++ = medium; + = few/weak; (+) = borderline; − = negative;
0 = not present/not investigated

[a] Carcinoid-like tumor

[b] Histologic type: 1 = trabecular, 2 = medullary

narrow gap from the membrane (case 7, Fig. 9). They resembled G cell granules. The carcinoid-like tumor consisted mostly of cells with small granules of low density.



a b

Fig. 8. Double-staining technique of a gastrinoma (case 6). Argyrophilia (a) and immunofluorescence (b) do not correspond. Argyrophilic cells are immunohistologically negative (*arrows*). (×500)

5. Inactive Tumors and Tumors with Atypical Symptomatology

Out of the 13 pancreatic islet cell tumors with vague or no endocrine activity, 5 cases displayed numerous and 3 only a few Grimelius argyrophilic cells. Some tumors reacted metachromatically, none were Hellerström positive. All cases were gastrin negative, but in 2 cases there was evidence of insulin positive cells. Rests of tumor-invaded pancreatic islets could not be excluded.

There was no tumor in our whole material which exhibited a fluorescence with anticalcitonin serum.

Discussion

1. Pancreatic Islets and Insulinomas

B cells of *normal pancreatic islets* are histochemically and immunohistologically a well-defined population. The argyrophilic and the slightly or strongly metachromatic cells, on the other hand, form a heterogeneous group. There may also be an overlap of the different cell types in between this group of non-B cells. Thus some Hellerström argyrophilic cells seem to be identical with Grimelius argyrophilic cells (Grimelius, 1968; Greider et al., 1970), and even single B cells

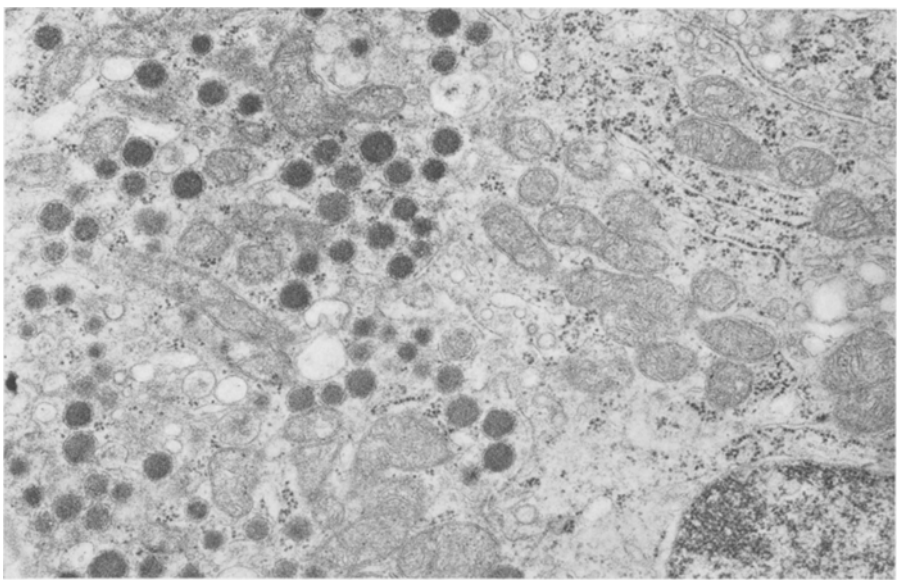


Fig. 9. Electron microscopy of a gastrinoma (case 7). Secretory granules resembling granules of inactive gastroduodenal G cells. (×13,000)

reacted slightly argyrophilic (Fig. 1). Furthermore, there is a quantitative difference between the Hellerström positive (A_1 cells), the HCl-pseudoisocyanine strongly metachromatic, and the HCl-toluidine blue intensively metachromatic cells (D cells). Either these techniques are relatively unspecific or the non-B cell group consists of more than two cell types. The latter possibility is favored by recent electron microscopic studies demonstrating up to 5 different cell types in the normal pancreatic islets (Björkman et al., 1966; Deconinck et al., 1971; Munger, 1973). Most of the Grimelius argyrophilic cells might, however, correspond to the glucagon-producing A_2 cells (Bussolati et al., 1971). As the three methods with watery silver solutions (Grimelius, Sevier-Munger, Holmes) yield comparable results in islets and intestinal mucosa, we confined ourselves to the Grimelius method which, in our experience, was most satisfactory.

The high sensitivity of the immunohistologic technique is of great value for the examination of routinely fixed biopsy material of normal pancreatic tissue and insulinomas (Table 1) (Bürkle et al., 1971; Thomsen, 1971; Creutzfeldt et al., 1973). Since the tumor content of insulin and its precursors is known to diminish with its progressing dedifferentiation, in some cases only the immunohistology yields positive results. Thus 11 of our 14 *benign insulinomas* could be characterized with anti-insulin serum, compared with only 8 by pseudoisocyanine and 6 by aldehyde fuchsin staining. With regard to the degree of dedifferentiation we believe to distinguish two histologic tumor patterns: a well-differentiated trabecular adenoma with a regular fluorescence in all the tumor cells, and a medullary, less differentiated type with scattered, irregularly fluorescing cells (Fig. 2 a–f). In

older preparations the cells of the undifferentiated medullary tumors show a fainter fluorescence than cells of the differentiated adenomas (Table 1, cases 3, 8), or the normal B cells of the same age. Arnold et al. (1972) also found that the immunoreactivity of adenoma cells is more rapidly fainting than that of the normal B cells. They assume an atypical tumor-insulin. We rather believe that insulin and its precursors in undifferentiated cells are less stable than in the normal B cells. This view is also supported by the fact that it was impossible to show a fluorescence in tumor cells after double-staining procedures, in contrast to normal B cells.

The rather reticular or clotted argyrophilia found in the cells of our insulinomas differed in most tumors from the dispersed silver deposits of other polypeptide-producing cells (Pearse, 1969b; Pearse et al., 1974) (Fig. 2e). As we used partly old and not appropriately fixed material, an artifact cannot be completely excluded. Nevertheless, we believe that the substrate of this argyrophilia is related to that of other hormone-producing cells of the APUD system. This view is supported by the observation that in one case the same structures reacted slightly metachromatic with HCl-toluidine blue. Pearse et al. (1974) and Creutzfeldt et al. (1975) also described argyrophilia in insulinoma cells. On the basis of two recently investigated adenomas we presume that the silver affinity depends on the degree of differentiation or the endocrine activity of the tumor cells. Thus, a reticular argyrophilia was shown in the first tumor, an undifferentiated adenoma with only a few immunoreactive cells and electronmicroscopically rare granules (Fig. 2, right side, d–f). The second tumor, a highly differentiated trabecular adenoma with plenty of granules, displayed a very weak silver reaction (Fig. 2, left side, a–c). However, in our heterogeneous material it was not possible to demonstrate a strict correlation between the histologic pattern and the degree of immunofluorescence or argyrophilia (Table 1). Furthermore, the question arises whether the argyrophilia is a sign of polyendocrine activity, as we can suppose in our cases of insulin-producing carcinomas.

All three *insulin-producing carcinomas* showed the typical dispersed silver granulation of APUD cells and an inconsistent metachromasia with HCl-toluidine blue (Table 1). By these features they could not be distinguished from non-B cell tumors, i.e., gastrinomas (Fig. 3). This may be even more confusing for diagnosis, as conventional staining methods, immunofluorescence, and electron microscopy often allow no further differentiation. Only one of our cases exhibited a slight fluorescence with anti-insulin serum. In addition, the symptomatology of all three cases is reminiscent of the syndromes of polyendocrine active tumors (Woodtli and Hedinger, in prep.). In one case the disease began with recurrent ulcers, later on severe attacks of hypoglycemia developed. Gastrin could not be demonstrated in the serum, but there were large amounts of proinsulin-like components (case 17). Staining methods and immunofluorescence did not allow characterization of the tumor. By the electron microscope, however, a few polygonal B cell granules in addition to G cell-like granules could be demonstrated (Fig. 4).

On the basis of these cases the question cannot be answered whether the argyrophilia represents a sign of dedifferentiation or of a multihormonal activity.

2. Antral and Duodenal Mucosa and Gastrinomas

By immunofluorescence the G cells were demonstrated as round cells in circumscribed areas of the middle part of the antropylcric mucosa. Regularly isolated cells were found in the duodenal mucosa and in the Brunner's glands. They are slightly metachromatic with HCl-toluidine blue and HCl-pseudoisocyanine. In agreement with Pearse and Bussolati (1970), Vassallo et al. (1971a, b), and contrary to McGuigan and Greider (1971), Mitschke (1971), and Lotstra et al. (1974), they displayed a weak but distinct silver granulation with the methods of Grimelius, Sevier-Munger and Holmes after fixation in Bouin and GPA solutions. These contradictory results are probably due to the fact that G cells—unlike pancreatic A_2 cells which react argyrophilic also after formalin fixation—show their argyrophilia only after Bouin and GPA fixation and intensive reduction. The identity of the Grimelius positive and gastrin-containing cells could most impressively be demonstrated with the double-staining method (Fig. 5). However, the fluorescence did not always correlate with the intensity of the argyrophilia. A small fraction of the immunohistologically definite G cells displayed no or a very weak silver affinity. This suggests that the argyrophilia is not correlated directly with the hormone content of the cell. On the other hand, some argyrophilic cells did not react with antigastrin serum. In these cells the production of another hormone cannot be excluded.

By the method of Hellerström, only the enterochromaffine cells gave a positive reaction, the G cells were negative. Thus the G cells can be clearly distinguished from the Hellerström positive and mostly Grimelius negative A_1 cells in the pancreatic islet. This characterization, based on conventional staining methods, is in contrast to the immunohistologic findings of Lomsky et al. (1969), Greider and McGuigan (1971), and Vassallo et al. (1972) who believe that the A_1 cells are involved in the gastrin production as well. Recent studies of Hökfelt et al. (1975) show the presence of somatostatin-like immunoreactivity in the A_1 cells.

Eight of the nine ulcer-producing tumors showed a sometimes weak, dispersed silver granulation with the Grimelius technique, and partly a slight metachromasia with HCl-toluidine blue (Table 2, Fig. 6). In seven of these gastrinomas some cells reacted with antigastrin serum. We therefore believe that the gastrinoma, after proper fixation and preparation, is characterized as a Grimelius argyrophilic tumor, but it is sometimes difficult to visualize the few gastrin-containing cells by immunohistology (Fig. 7). This may explain that Greider et al. (1974) found a positive reaction in only one out of four ulcerogenic tumors. The fact that gastrin is demonstrable in a limited number of cells may be due to the occurrence of molecular species of gastrin, which are not necessarily immunoreactive with the particular antiserum used (Yalow and Berson, 1971; Rehfeld and Stadil, 1973). Or it may be that not all the gastrin species found in the Zollinger-Ellison syndrome preserve their immunoreactivity after the employed fixations. Polak et al. (1971) demonstrated that the cells in the duodenum which contain heterogeneous enteroglucagon-like hormones only reacted after carbodiimide fixation, while the immunoreactivity of the homogeneous glucagon of the A_2 cells was not altered by the usual fixation. The sparsity of immunoreactive cells in gastrinomas may also be caused by an increased hormone secretion, as it is seen in undifferentiated insulinomas. This interpretation would correlate well with the fact that

all of our gastrinomas were malignant, and that only one of them displayed a trabecular pattern. In addition, one tumor contained argentaffine cells and two others B cells, suggesting that the pancreatic gastrinomas may possibly arise from endocrine cells of the pancreas by progressive metaplasia.

Tumors with Hellerström argyrophilic cells, as in one of our cases, have to be regarded as an exception. Vassallo et al. (1972) found 1 Hellerström positive tumor among 3 gastrinomas, Greider et al. (1974) only 1 among 16 non-B cell tumors, and Creutzfeldt et al. (1975) none in their set of 10 Grimelius positive carcinomas. These results are in contradiction to the findings of Cavallero et al. (1967) and Bretholz and Steiner (1973) who classified the gastrinoma simply as a A_1 cell tumor of the pancreas. Thus, the gastrinoma cells seem to be related to the G cells rather than to any pancreatic islet cells. This view is also supported by electron microscopic studies in three cases of our series. The cells of all three tumors contained round granules of different size and density without a halo, as described in G cells (Orci et al., 1968; Pearse et al., 1970; Greider et al., 1972; Lechago and Bencosme, 1973) (Fig. 9). It must be mentioned, however, that often atypical granules were found in ulcer producing tumors by different authors (Vassallo et al., 1972; Mitschke, 1973; Greider et al., 1974; Creutzfeldt et al., 1975) (gastrinoma case 9), and that in normal islets and nongastrin-producing tumors similar granules can be identified (Lotstra et al., 1974) (insulinoma case 17).

3. Argyrophilia and Metachromasia

Our study showed that argyrophilia and metachromasia are not hormone-specific reactions. Both features belong to the standard characteristics of the APUD cells (Pearse, 1969b; Pearse, 1974). As routine screening methods they only allow the diagnosis of APUD cell or APUD tumor. In spite of this nonspecificity we believe that the quantitative estimation of the argyrophilia and metachromasia give some additional information. The A_2 cells and the enterochromaffine cells generally display a stronger coloration—also demonstrable after formalin fixation—than G cells and other gastrointestinal endocrine cells. This would suggest, for instance, the diagnosis of a glucagonoma if a carcinoid can be excluded by negative argentaffine reaction (Woodtli et al., 1976). The significance of Hellerström positive or intensively metachromatic cells is still controversial (Somatostatin: Hökfelt et al., 1975; Gastrin: McGuigan and Greider, 1971; No gastrin: Lotstra et al., 1974). These features are of little help for the differential diagnosis of the APUD cell tumors at this time.

On the other hand, the argyrophilia seems to depend on the state of activity of the APUD cells. Virtually all gastrinoma cells show an argyrophilia while only single, often not argyrophilic cells seem to store the hormone (Figs. 6, 7). Tateishi et al. (1972) found some correlations between argyrophilia, ultrastructural granule content, and amyloid formation in C cell carcinomas of the thyroid. Additionally, silver precipitating structures were found in several B cell adenomas and in all our carcinomas. This observation suggests that the argyrophilia may indicate an increased hormone synthesis or secretion of the APUD cells. However, in insulinomas a multiendocrine activity of the dedifferentiating tumor cells cannot be excluded.

The substrate of the argyrophilia still remains unknown (Vassallo et al., 1971b; Bussolati et al., 1971). From our experience we believe that the argyrophilia is based on structures related to the metachromasia. Solcia et al. (1968, 1969), and Pearse (1969a) suggest that the metachromasia is caused by the acid side chain groups of the hormones and their precursors which are "demasked" by hydrolysis.

Conclusion

1) Insulinomas are generally not difficult to characterize by staining, immunofluorescence, and electron microscopy if they are not too undifferentiated. The argyrophilia we described should not influence the diagnosis. The histologic diagnosis of insulin-producing carcinomas, however, might be troublesome because these tumors often seem to display the APUD argyrophilia alone, without definite cell specific features. Additionally, the characterization can be complicated by the frequent incidence of polyendocrine activity (Broder and Carter, 1973).

2) It is not possible to define the gastrinomas by staining methods alone, since their argyrophilia and metachromasia are nonspecific. The rarely seen Hellerström argyrophilia does not allow the diagnosis either. In this tumor the immunohistology is of high diagnostic value. The results of immunohistology and electron microscopy demonstrate the relationship of the tumor cells with the gastroduodenal G cells if the dedifferentiation is not too pronounced.

3) The argyrophilia and metachromasia may give some indication as a routine screening method for characterizing the normal or tumoral APUD cells, and to some extent their activity. We assume that the identification of the substrate of argyrophilia and masked metachromasia will bring an important diagnostic significance to these techniques.

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