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Localisation of C-terminal gastrin immunoreactivity in gastrinoma cells

An immunoelectron microscopy study on conventionally processed tissue

Gérard Berger[1], Françoise Berger[1], Françoise Boman, Jean-Alain Chayvialle[2], and Jacques Féroldi[1]

[1] Laboratoire d'Anatomie Pathologique; [2] INSERM, Unité 45. Hôpital Edouard-Herriot, Place d'Arsonval, F-69374-Lyon Cédex 2-France

Summary. Localisation of C-terminal gastrin immunoreactivity has been studied, using the immunogold staining procedure, on ultrathin sections of 6 human gastrinomas conventionally processed for electron microscopy. The specific labelling, whose density depended on the mean diameter of the gold marker, was restricted to endocrine secretory granules. However, in poorly differentiated cells from malignant tumours, a number of granules remained unreactive. The labelling pattern depended also on the functional state of each cell. The immunoreactive granules showed various morphological features. A moderate number of gold particles was demonstrated over the floccular content of the infrequent diagnostic G-type granules. Non-diagnostic round granules of varying size and electron density were prevalent in most cells; their usually strong immunostaining allowed immediate recognition of cell specificity. Dense granules which were large in size and angular in shape and present in one case, were also intensely labelled. In the same tumour, unequal labelling occurred over polymorphous, often elongated granules, of varying size. Granules of different types, including intermediate forms, could be found in the same cell, indicating a spectrum of granule maturation towards well-defined types of the fetal or adult normal tissues.

The present methodology would help to identify gastrin-producing cells in prospective or retrospective electron microscopy studies of multihormonal endocrine tumours.

Key words: Gastrin, Gastrinomas, Ultrastructure, Immunogold technique

Introduction

The ultrastructural features of gastrin-producing tumour cells have been extensively investigated (Vassallo et al. 1982; Creutzfeldt et al. 1974; Capella

Offprint requests to: G. Berger at the above address

et al. 1977). Both diagnostic and/or non-diagnostic (atypical) endocrine secretory granules have been demonstrated. The former resemble those of human pyloric G-cells that mostly store little gastrin (G-17), whereas some of the latter resemble gastrin-like peptide-storing granules reported in a few cells of the adult or fetal human intestine (Larsson and Jorgensen 1978; Buchan et al. 1979).

Because of the functional heterogeneity of endocrine peptide-secreting tumours, direct localisation of gastrin-immunoreactivity using ultrathin sections is essential but has not yet been successfully achieved. This may be due to technical shortcomings associated with immunoelectron postembedding methods, which are time-consuming (serial semithin-thin technique) or which mask the fine morphology of granules (immunoenzyme technique). However, the recently introduced colloidal gold methods have proved helpful in investigating gastrin-immunoreactive cells of normal pyloric mucosa (Garaud et al. 1982; Varndell et al. 1983). This prompted us to use the immunogold technique in order to define the labelling pattern of gastrin-producing tumour cells. For this particular study, an appropriate procedure was selected so as to preserve the fine morphology of the cells and, at the same time, to detect most of the gastrin-like peptides. This consisted of a) a conventional processing of the tissues for electron microscopy, and b) the use of C-terminal directed anti-gastrin sera.

Materials and methods

Tissues. Six endocrine tumours, including four pancreatic or duodenal tumours and two metastatic tissues, from patients with Zollinger-Ellison syndrome, were collected during the 1980–1982 period. In all cases, hypergastrinemia had been proven by radioimmunoassay. Immunocytochemistry, performed on deparaffinized sections of Bouin's fluid fixed tissues, showed that a majority of tumour cells stored gastrin-like peptides. The main clinical and pathological features are presented in Table 1.

Table 1. Clinical and pathological findings of 6 patients with gastrinoma

Case	Sexe/ age	Clinical data	Tumour			Metastases	Immunochemistry	
			location	size	microscopy		gastrin	others
1	F, 46	ZE	duodenum	2 cm	CT	−	+++	−
2	F, 30	ZE	amp. of Vater	1.5 cm	CT	−	+++	+SOM
3	F, 58	ZE	amp. of Vater	1 cm	CT	+	+++	+HPP
4	F, 38	ZE CUSH	pancreas	large	MEPT	+	+++	+ACTH +INS
5	F, 43	ZE	pancreas	3 cm	MEPT	+	+++	−
6	M, 20	ZE	pancreas	large	MEPT	+	++	+SOM

Abbreviations. ZE = Zollinger-Ellison syndrom; CUSH = Cushing syndrome amp. of Vater = ampulla of Vater; CT = carcinoid tumour; MEPT = malignant endocrine pancreatic tumour; SOM = somatostatin; INS = insulin

In addition, samples of pyloric mucosa from adult patients with unaltered gastro-intestinal function were prepared in the same way as the tumour tissues for both immunooptical and immunoelectron microscopy. This material was used as control to test anti-gastrin sera and to define technical parameters suitable for the immunogold staining procedure.

Antisera. Two experimental antisera (code c7d and 91a) were applied to each section and compared. Both were obtained from rabbits after multiple injections of synthetic 1–17 human gastrin I (ICI, Macclesfield, England) conjugated with bovine serum albumin (BSA) by carbodiimide. They had been tested by radioimmunoassay. Antiserum 91a was initially selected because of a rather strong cross-reaction with the NH_2 – terminal portion of human gastrin – 17. However, in inactivation tests performed on deparaffinized sections of Bouin's fluid fixed pyloric mucosa the staining was totally abolished by prior incubation of each antiserum with excess pentagastrin (Peptavlon, ICI). Thus both antisera were considered to be solely directed against the COOH – terminal sequence of gastrin/cholecystokinin (CCK) at the optical immunocytochemical level.

Affinity purified goat antibodies to rabbit immunoglobulins G coating colloidal gold particles of 10 nm, 20 nm or 40 nm mean diameter were obtained commercially from Janssens Pharmaceutica Research Laboratories (Beerse, Belgium).

Electron microscopy. All tissues were fixed at 4° C for 1 h in 2,5% glutaraldehyde in 0,1 M phosphate buffer pH 7,30, postfixed for 1 h in 1% buffered osmium tetroxide, dehydrated in graded ethanol, transferred to propylene oxide and embedded in epon 812.

Ultramicrotome sections were stained with uranyl acetate and lead citrate and examined under a Zeiss EM 9 electron microscope to select at least three specimens representative of granule diversity for each case. Additional ultrathin sections were serially cut from the corresponding blocks and mounted on uncoated 300 mesh nickel grids for immunoelectron microscopy.

Immunogold staining procedure. The procedure was adapted from Garaud et al. (1982) and Varndell et al. (1982).

Each section was slightly etched (10% H_2O_2 for 2 mn), and successively floated on drops of the following reagents: 1) normal goat serum diluted 1:30 2) anti-gastrin serum diluted in 0,05 M Tris-buffered saline (TBS) pH 7,2 containing 1% BSA (for 1 h) 3) colloidal gold-antibodies diluted in TBS pH 8,2 with 1% BSA (for 1 h). Optimal dilutions of antisera/antibodies were determined in quantitative preliminary experiments performed on similarly processed pyloric mucosa and found to correspond to 1:10,000 (c 7d), 1:5,000 (91a), and 1:25 to 1:100 (gold reagent, depending on the size). Steps 2 and 3 were followed by jet-washing with buffer. The sections were finally rinsed in distilled water and stained with 2% aqueous uranyl acetate (5–20 mn) and lead citrate (5–10 mn).

Specificity tests. The specificity of immunolabelling for C-terminal gastrin was assessed by the following controls: 1) incubation of the ultrathin sections with each primary antiserum previously inactivated for 1 h at 37° C with excess gastrin sequences, followed by the gold reagent. Pentagastrin (Peptavlon, ICI), human gastrin – 17 (ICI) and the sequence 1–20 of human big gastrin (Fluka AG, Buchs, Switzerland) were used at the respective concentrations of 10, 10 and 100 ®g per ml of diluted antisera 2) replacement of the antigastrin serum by normal rabbit serum 3) additional labelling experiments using rabbit anti-somatostatin (code 56 K) and rabbit anti-human pancreatic polypeptide (HPP) (code 54 E) sera were comparatively performed on serial sections.

Results

General observations

In both tumour and control cells the gold particles were restricted to endocrine secretory granules, whereas the rough endoplasmic reticulum (RER)

and the Golgi apparatus were not labelled by the highly diluted antisera. Even in the nuclear area, the background level was negligible. Although no quantification was attempted on tumour sections, the labelling pattern appeared unmodified whatever primary anti-gastrin serum was applied at the optimal dilution. In contrast, the labelling density depended on the size of the marker. The number of 40 nm particles bound to each granule area was low (0–2 per granule). The number increased to 1–40 and 1–80 respectively when 20 nm and 10 nm gold-tagged antibodies were used.

When the primary incubation was carried out using normal rabbit serum, or anti-gastrin sera inactivated with pentagastrin or gastrin −17, no aggregation of gold particles occurred, except for a very low non-specific binding over the nuclear and cytoplasmic area. In contrast, the 1–20 sequence of big gastrin did not inactivate the anti-gastrin sera. No labelling was obtained when the anti-somatostatin and the anti-HPP sera were applied (except for a few cell processes from case 2 tumour).

Cell labelling pattern

Case 1, a well-differentiated duodenal carcinoid tumour, was composed of two cell varieties, which reacted unequally with anti-gastrin sera. Heavily granulated cells displayed a very dense labelling over each granule. The other cells harbored a large amount of dilated RER and only a small number of granules, whose reactivity was similar.

Case 2 was a vaterian poorly aggressive carcinoid tumour with abundant cytofilaments. In a majority of cells, numerous granules, scattered over the whole cytoplasmic area, showed a moderate unequal labelling. The other cells were unreactive.

Cases 3 and 4 were malignant endocrine tumours originating in the ampulla of Vater and the pancreas respectively. Their polyhedral well-differentiated cells contained abundant granules scattered throughout the cytoplasmic area or lined along the membrane. Most of them bound a number of gold particles.

Sections from cases 5 and 6 tumours showed a majority of poorly granulated, usually mitochondria-rich, cells. The granules were mainly stored in cell processes. The labelling appeared heterogeneous since a number of granules of each cell failed to react with the antisera.

Morphology of the immunoreactive granules

G-type vesicular granules were rather abundant in case 6, scarcely represented in cases 3 and 5 and absent in the other tumours (Table 2). However, the diameter, as estimated in case 6 tumour, was smaller than that of pyloric G-cells vacuolar granules (mean diameter (MD) 209 and 329 nm, maximal profile diameter (MPD) 250 and 415 nm, respectively). In both cases, a specific labelling of moderate density was obtained over the often eccentric floccular content (Figs. 1 and 2). In addition, a few particles were frequently

Table 2. Types of C-terminal gastrin-immunoreactive granules in 6 gastrinomas

Case	Round	Granules[a]		Polymorphous granules[a]		
N°	Vacuolar	Pale	Dense	Angular	Elongated (large)	Elongated (small)
1	+ (few)	+ + +	+ + +	−	−	−
2	−	−	+ + +	−	−	−
3	+ (few)	+ +	+ + +	−	−	−
4	−	−	+ + +	−	−	−
5	+ (few)	−	+ +	+	+ +	+ +
6	+ +	+	+	−	−	−

[a] The number of granules of each type has been roughly estimated from + to + + +

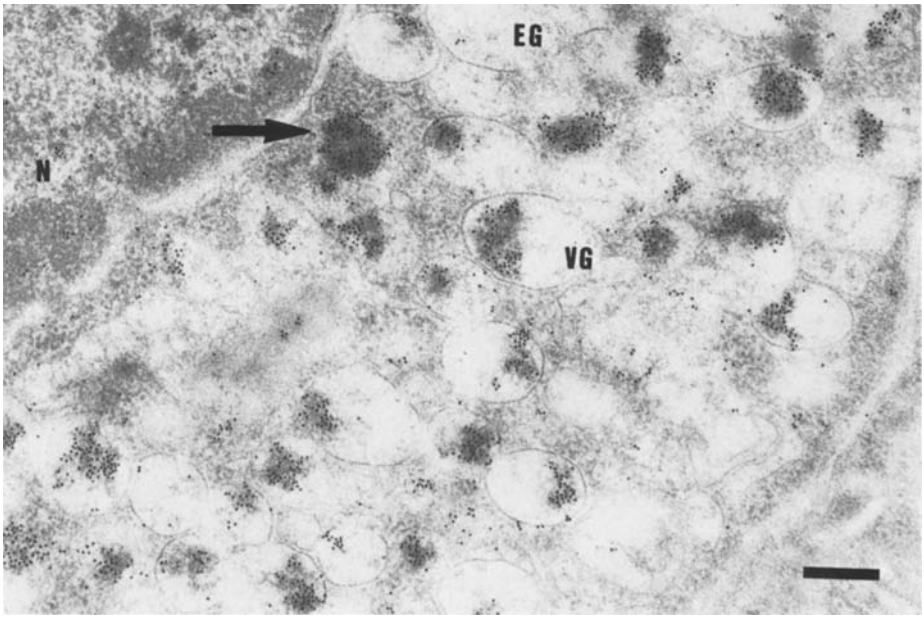

Fig. 1. Part of a control pyloric G-cell immunolabelled for C-terminal gastrin with 10 nm gold. The particles are seen over the dense granule (arrow) and the vacuolar granules (*VG*). Emptylooking granules (*EG*) are poorly labelled. Nucleus: *N*. Antiserum 91a. ×25,700. Scale bar = 0.39 μm

seen close to the limiting membrane. Empty-looking granules, as found in control G-cells, were not found in tumour cells.

Most of the immunoreactive granules were of the solid round type. They formed the whole granule population (case 4) or were admixed with gastrin-storing granules of other types. Moreover, they varied in size, electron density and labelling density from cell to cell and from case to case. However, two subtypes could be defined (Figs. 3 and 4), which resembled dense granules found in control pyloric G-cells. Subtype 1 was composed

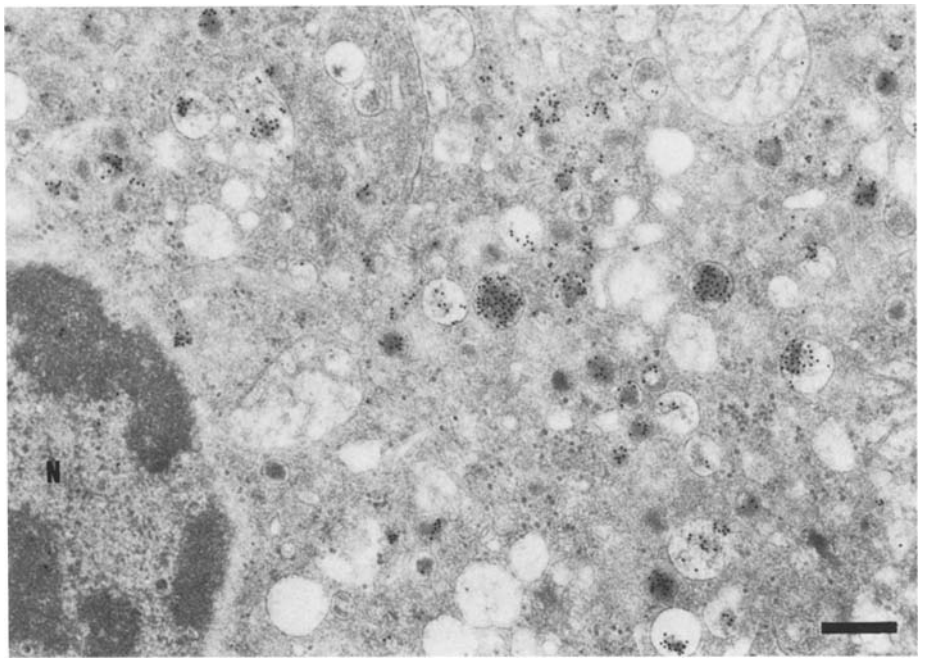

Fig. 2. Part of two cells from case 6 tumour immunolabelled for C-terminal gastrin with 10 nm gold. Both G-type vacuolar granules and small dense granules are labelled. Nucleus: *N*. Antiserum 91 a. × 30,000. Scale bar: 0.33 μm

of small to medium-sized granules (MD 260 or 174 nm, MPD 314 or 249 nm as estimated in cases 1 and 3 tumours respectively), with a moderately dense core surrounded by a tightly fitting membrane. Subtype 2 consisted of medium-sized to large granules (MD 331 or 232 nm, MPD 405 to 280 nm in cases 1 and 3 tumours respectively) filled with a pale content without a distinct halo. These granules were easily characterized in cases 1, 4 and 6 tumours, since they could be found together in the same cell in addition to a few intermediate forms. Moreover, transitional features between subtype 2 and vacuolar G-type granules were occasionally present. Further, when labelled with small particles, both subtypes were strongly immunoreactive. The labelling density was usually more intense in subtype 1 granules (Fig. 3).

In cases 2 and 4 the majority of granules also resembled subtype 1. However, their nature was more difficult to define due to the lack of vacuolar/floccular forms. Moreover, the granule size varied greatly in the same cell and the halo was unequally distinct. Using anti-gastrin sera, strong labelling occurred over most granules of all cells in case 4 (Fig. 5). In case 2, moderate immunoreactivity was localised on a number of granules of unequal size (MD 180 nm, MPD 235 nm) in most cells (Fig. 6a). A few cells stored larger granules (MD 258 nm, MPD 470 nm) of similar or lower electron density, which were unreactive. In contrast with the smaller gran-

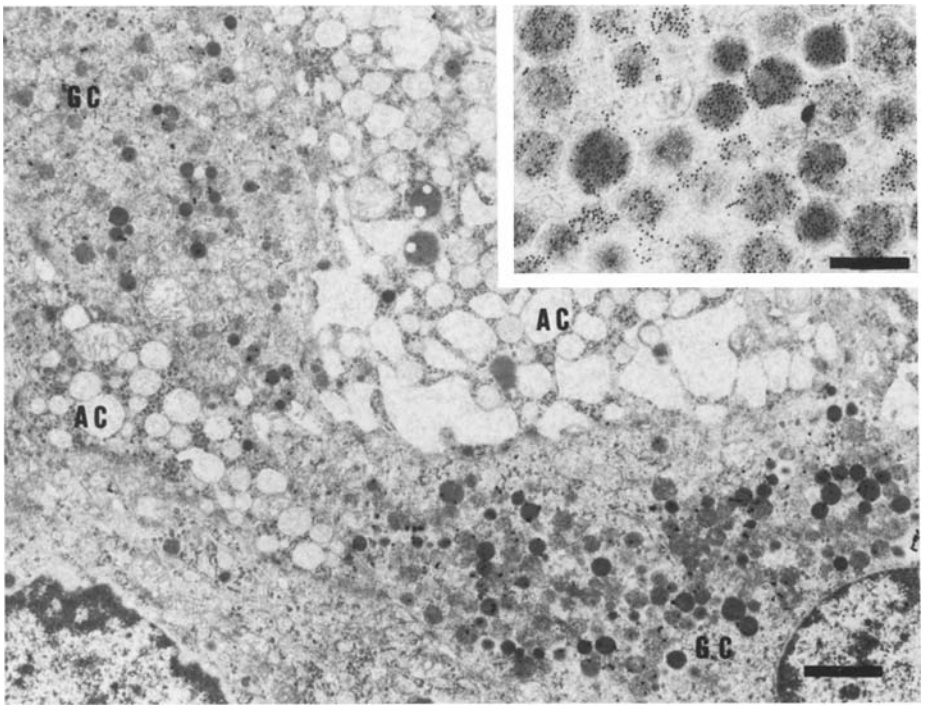

Fig. 3. Immunogold labelling of case 1 tumour for C-terminal gastrin using 40 nm gold. Note two virtually agranular cells (*AC*) and two well-granulated cells (*GC*). The labelling is restricted to some granules of the latter cells (belonging to subtypes 1 and 2). Antiserum c7d × 7,500. Scale bar: 1.33 μm. *Inset:* immunolabelling with 10 nm gold. The labelling is seen over all the granules, although it is stronger over the small dense ones. Antiserum c7d. × 27,500. Scale bar: 0.39 μm

ules, many of them could be labelled using the antisomatostatin serum (Fig. 6b).

Polymorphous dense granules of varying immunoreactivity were found in some cells from case 5 tumour. A large number of gold particles was present over irregularly shaped granules, often angular or oblong, with a very dense content and a thin halo. Although most of them were large, their size varied greatly (MD 268 nm, MPD 387 nm) (Fig. 7). Other cells from the same tumour stored elongated haloed granules of large size (maximal length (ML) 491 nm). Their moderately dense content was usually strongly labelled (Fig. 8). An irregular and rather low labelling pattern was observed over small elongated granules (ML 185 nm), either scattered over the whole cytoplasmic area or more usually stored in cell processes. Round haloed granules of very small size (about 50 to 100 nm in diameter), which could represent transverse sections of the elongated forms, were also labelled. Although granules of each subtype were mainly stored in distinct cell processes, mixed cells were also seen, including either large and small elongated granules, or angular and large elongated granules together with few vacuolar forms.

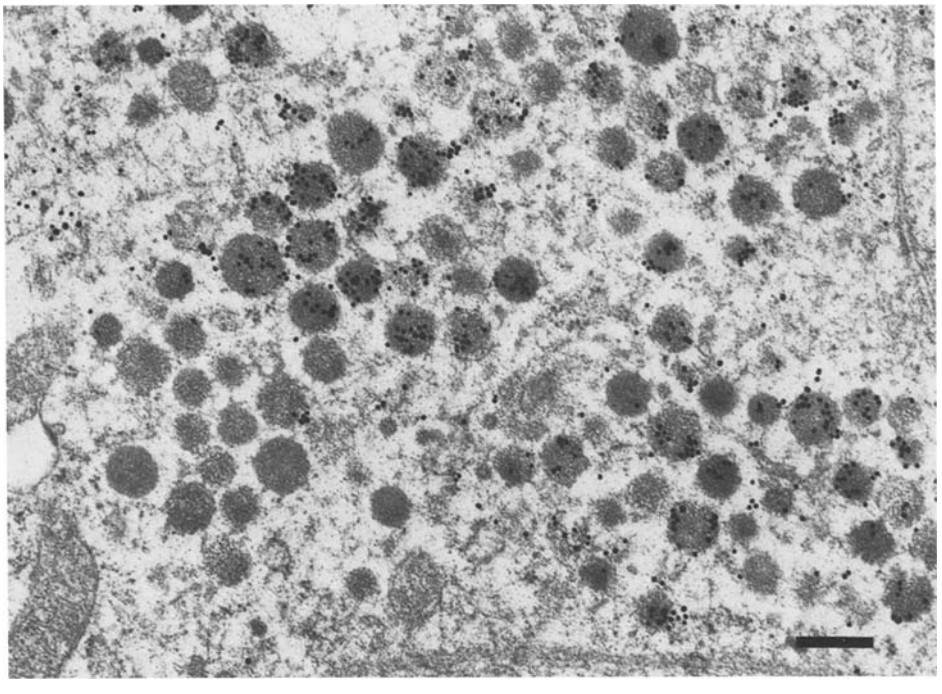

Fig. 4. Part of a cell from case 3 tumour labelled for C-terminal gastrin with 20 nm gold. A varying number of particles are found over subtype 1, round, dense granules, which lack a halo. Antiserum c7d. ×25,700. Scale bar: 0.39 μm

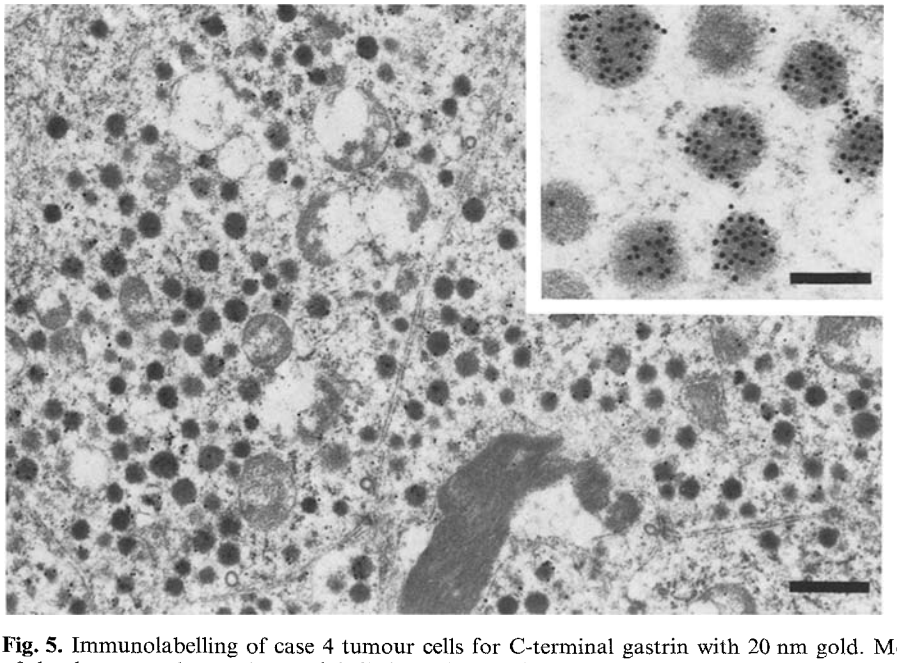

Fig. 5. Immunolabelling of case 4 tumour cells for C-terminal gastrin with 20 nm gold. Most of the dense-cored granules are labelled. Antiserum 91a. ×15,000. Scale bar: 0.66 μm. *Inset:* higher magnification. The granules lack a halo. ×60,000. Scale bar: 0.17 μm

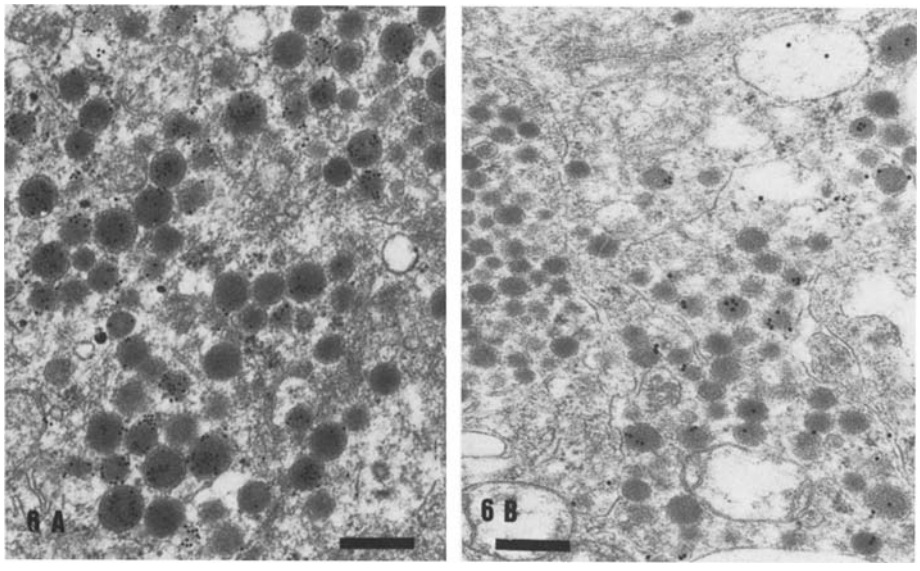

Fig. 6 A, B. Case 2 tumour cells storing either small dense-cored granules or larger ones with a pale content. **A** Immunolabelling of the small haloed granules for C-terminal gastrin (using 10 nm gold). Antiserum c7d. × 30,000. Scale bar 0.33 μm. **B** Immunolabelling for somatostatin with 20 nm gold. The small granules (*left*) are unreactive. Larger granules stored in adjacent cell processes (*bottom right*) are labelled. Antiserum 56K × 15,000. Scale bar: 0,66 μm

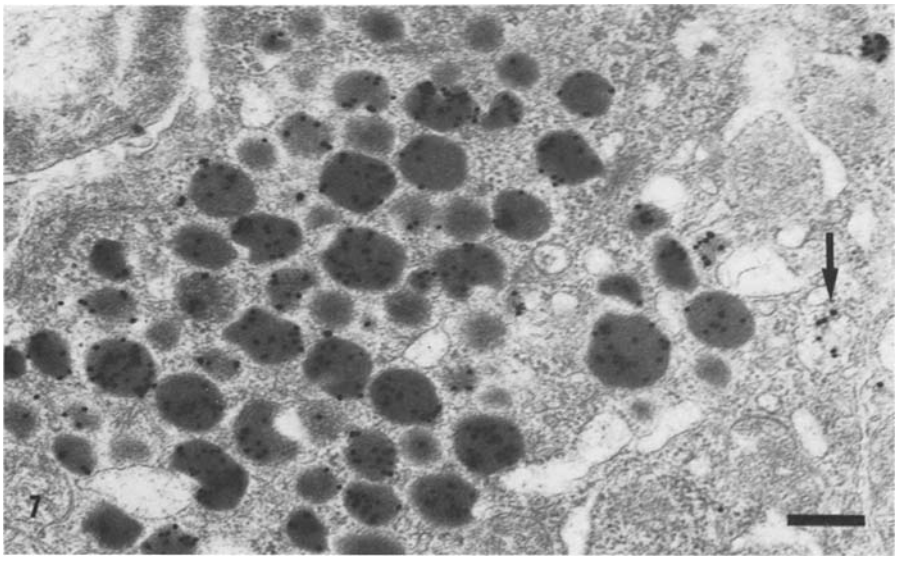

Fig. 7. Part of a cell from case 5 tumour labelled for C-terminal gastrin with 20 nm gold. A high number of gold particles are seen over large dense granules of angular shape. Note one vacuolar granule (*arrow*). Antiserum 91a × 25,700. Scale bar: 0.39 μm

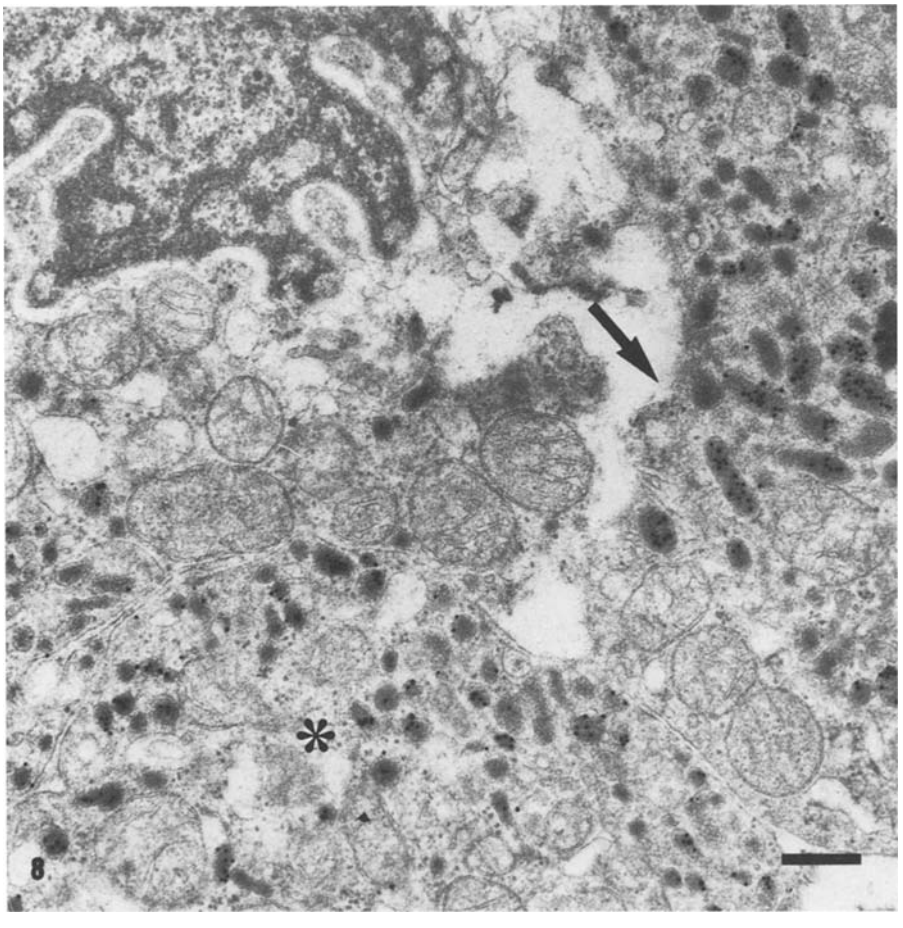

Fig. 8. Adjacent cell processes from case 5 tumour labelled for C-terminal gastrin using 20 nm gold. The particles are seen over elongated haloed granules of large (*arrow*) or small (*asterisk*) size and over small to medium-sized round haloed granules. Antiserum 91**a**. ×25,700. *Scale bar:* 0.39 μm

Discussion

In contrast with the current practice, which recommends etched sections of unosmicated tissue for postembedding electron immunocytochemistry, the present localisation of C-terminal gastrin immunoreactivity was achieved on briefly etched sections of double-fixed tissue. This procedure, adapted from Garaud et al. (1982), allows a satisfactory preservation of the membranes, presents artifactual bleaching of endocrine secretory granules and permits retrospective investigations. Moreover, when combined with the use of colloidal gold as tracer, it allows conventional counterstaining of the ultrathin sections and, thus, results in a traditional electron microscopy picture.

When the labelling density was quantitatively evaluated on similarly processed pyloric G-cells, the diameter of the gold marker was found to be an important determinant. (Berger et al. in preparation). Its influence proved to be decisive when tumour cells were investigated. At optimal dilution, antibodies tagged with small-sized (10 nm or 20 nm) gold resulted in dense labelling of large numbers of granules, whereas the 40 nm gold reagent bound only to a few granules from strongly immunoreactive tumour cells. The phenomenon, initially reported in labelling experiments of surface antigens (Horisberger 1981), would appear to reflect a still poorly understood sterical hindrance.

When applied in accordance with the present procedure, both antisera used in our study were specific for the bioactive C-terminal sequence of gastrin-17. This was demonstrated by inactivation tests performed at both optical and ultrastructural levels, with excess pentagastrin. Such antisera would react with a number of gastrin-CCK related peptides; they were selected because of the storage in gastrinoma cells of several gastrin molecular forms, most of them including the C-terminal sequence, as demonstrated by gel permeation chromatography of tumour extracts (Creutzfeldt et al. 1975; Dockray et al. 1975).

Even in active cells of case 1 tumour, the labelling was restricted to secretory granules. A similar finding has been obtained in pyloric G-cells of controls by Garaud et al. (1982), Varndell et al. (1983) and ourselves. In contrast, some authors reported successful localisation of endocrine peptides or glycoproteins in RER or Golgi cisternae, applying immunoenzyme or protein A – gold methods on normal tissues similarly processed (Ravazzola et al. 1981; Bendayan and Zollinger 1983; Childs 1983). The conflicting results may be due to methodological differences and/or may reflect different properties of precursors.

Except for well-differentiated cells of case 1 tumour, the labelling pattern was heterogeneous. Unstained granules were found mainly in poorly granulated cells of malignant tumours. This suggests a low storage of gastrin-like peptides in such granules, although a partial loss of immunoreactivity during processing cannot be excluded. Whether the immunoreactive granules were scattered throughout the whole cytoplasm, marginated in cell processes or along the membrane or scarcely represented, probably reflects different functional states of the cells.

Our observations revealed a variety of gastrin-immunoreactive granules, including vacuolar, round compact and polymorphous types. The former, known as diagnostic G-type granules, were abundant in only 1 out of 6 tumours. The gold particles were mainly scattered over the floccular content. These features resembled those of vacuolar granules found in control pyloric G-cells, except for the smaller size and the absence of empty-looking varieties.

Round small to medium-sized granules, with either a dense or a pale content, were prevalent in most cells, whose morphology and labelling pattern often resembled those of the dense granules stored in small numbers

in the control pyloric G-cells. Indeed, the co-storage in some cells of a few labelled granules belonging to the large floccular/vacuolar types or intermediate types, lends support to this interpretation. However, in the absence of vacuolar forms, the population of round granules appeared heterogeneous with regard to the size, electron density or presence of a halo. Moreover, the labelling density was unequal, including a number of unreactive granules. Thus, the relationship with the previous granules appears uncertain. Similar granules, already reported in a variety of peptide-secreting tumours, such as gastrinomas (Vassallo et al. 1972; Creutzfeldt et al. 1975; Capella et al. 1977) or vipomas (Capella et al. 1983), were said to resemble those of D_1 cells of gut and pancreas. In addition, distinguishing them from PP cell, P cell or D cell granules can be difficult. Moreover, the functional heterogeneity of gut D_1 cells has now been demonstrated, including motilin cell and intestinal gastrin (IG) cell (Polak and Buchan 1981). This might be true also for tumour cells. Thus, the immunoelectron localisation of C-terminal gastrin in tumour cells may be of diagnostic value when G-type granules are few or absent. As suggested by Solcia et al. (1980), the analogy of the round compact granules of gastrinoma cells with those of IG cells, known to store mainly big gastrin (Buchan et al. 1979), represents indeed an attractive hypothesis. The prevalence of such granules in those gastrinomas that store and secrete predominantly G 34 does favour it (Varndell et al. 1982). Our preliminary results (data not shown) are confirmative since a strong labelling for the sequence 1 – 20 of gastrin – 34 occurred in most granules from cases 1, 3 and 4.

In one tumour, we were able to localise C-terminal gastrin-immunoreactivity on large angular granules, resembling those previously reported by Solcia et al. (1980) in some cells from 3 gastrinomas. They considered these cells as equivalent of the VL cells described in the human small intestine (Solcia et al. 1978), or of the type 3 cells described in the fetal duodenum and already shown to store C-terminal gastrin (Larsson and Jorgensen 1978). Additional studies using other region-specific antibodies would be necessary to substantiate such a relationship.

The unequal, often dense labelling that occurred over polymorphous elongated granules of case 5 tumour was an unexpected finding. In spite of abnormally great variations in size, their morphology could suggest EC cell granules. It must be stressed that EC cells from similarly processed adult pyloric mucosa were unreactive. Moreover, anti-gastrin sera inactivated with pentagastrin failed to stain the polymorphous tumour granules. Although EC-type cells have been occasionally reported in ultrastructural studies of gastrinomas (see Solcia et al. 1981), their possible immunoreactivity for gastrin has not yet been investigated. On the other hand, Larsson and Jorgensen (1978), using the semithin-thin procedure, demonstrated both polymorphous EC-type granules and smaller round granules in C-terminal gastrin – immunoreactive cells from the fetal human duodenum mucosa. In addition, they observed at the optical microscope level that, during the fetal life, a number of gut endocrine cells store both immunoreactive gastrin

(or CCK or secretin) and cytochemically defined serotonin. In the present case, however, no argentaffin cell was found on deparaffinized sections from the tumour.

Finally, our immunoelectron microscopy findings confirmed that a spectrum of granule maturation occurred in gastrinoma cells towards gastrin-storing granules, as defined in normal human tissues during adult or fetal life.

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