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Electron microscope localisation of insulin-like immunoreactivity of conventionally processed human insulinomas

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Summary. Localisation of insulin-like immunoreactivity has been studied using the immunogold staining procedure on thin sections of 6 human insulinomas, conventionally processed for electron microscopy. The labelling was restricted to the secretory granules. Depending on their morphology, these either resembled B-cell granules of human adult pancreas or belonged to the atypical (non-diagnostic) group. Within the former group, those with a crystalloid core or an amorphous dense or moderately dense core were strongly immunoreactive, whereas others, filled with a pale material, were poorly labelled. Most granules of this type were stored together within the heavily granulated cells of 3 insulinomas, presenting the classical features of clinical and biological behaviour and a typical light microscopic staining pattern. In contrast, the non-diagnostic granules, characterized by their smaller size, a very dense core and a thin halo, were mainly found within the poorly granulated cells making up the other tumours, and showed a very uneven labelling. Strongly labelled granules were found in one insulinoma that also belonged to the classical type; these were stored together with a few diagnostic granules within the same cells. Only poorly labelled atypical granules were present in two cases revealing a number of unusual features; these included moderate elevation of insulinaemia, uncertain tumour histology, as well as weak immunostaining for insulin/proinsulin and variable argyrophilia of the tumour in paraffin sections. These findings suggest that human insulinomas differ not only in storage capacity but also in their degree of granule maturation. This may involve some deficiency of either the prohormone conversion or the subsequent processing of the cleavage products.

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Introduction

A number of ultrastructural studies have shown that secretory granules, diagnostic or non-diagnostic, are found within most cells of human insulin-producing tumours (insulinomas) (Creutzfeldt et al. 1973; Creutzfeldt 1980; Capella et al. 1977; Kobayashi et al. 1979; Akaji and Fujii 1981; Liu et al. 1985). However, on immunocytochemical investigation at the light microscope level up to 61.8% of these tumours are multihormonal (Liu et al. 1985). Thus, it is important to determine what peptide is stored within the granules of each morphological type. Varndell et al. (1982), using the immunogold staining procedure at the electron microscope level, detected insulin-like immunoreactivity in a number of granules of 4 insulinomas, but, due to the unosmicated fixation, could not detail their fine morphology. In order to improve ultrastructural preservation, we performed insulin immunolabelling on etched sections of tumour tissue routinely processed for electron microscopy and now report the results obtained from 6 pancreatic insulinomas.

Materials and methods

The 6 tumours were clinically diagnosed as functional (benign or malignant) insulinomas. In each case, tumour resection cured the biological abnormalities. When tested with the peroxidase antiperoxidase procedure on Bouin's fluid fixed paraffin sections, a large number of tumour cells was positively stained for insulin/proinsulin but the staining intensity was variable. No tumour immunoreactivity was found using antisera directed against pancreatic polypeptide (PP), glucagon, somatostatin, gastrin, vaso-active intestinal peptide (VIP), or growth hor-

Table 1. Pathological findings in 6 patients with insulinoma

Case N°	Age	Sex	Tumour size	Histochemistry		Immunohistochemistry		Histology
				Grimelius	Aldehyde-fuchsin	Insulin	Other peptides[a]	
1	75	F	12 mm	−	++	++	some PP cells	benign
2	69	M	12 mm	−	++	++	−	benign
3	34	F	10 mm	−	++	++	−	benign
4	46	F	8 mm	−	++	++	−	benign MEN[b]
5	34	F	17 mm	+	−	++	−	supposed malignant
6	16	M	30 mm	++	−	+(+) low intensity	−	malignant?

− no or rare cells positively stained; + a moderate number of cells positively stained; ++ most cells positively stained

[a] immunostaining for gastrin, somatostatin, glucagon, PP, VIP, GRF

[b] *MEN* multiple endocrine neoplasia

Table 2. Structural pattern and types of immunoreactive granules[a,b] in 6 insulinomas

Case N°	Structural pattern of cells	Types of immunoreactive granules				
		amorphous pale	amorphous intermediate density	amorphous dense	crystalloid	atypical
1	well granulated	+++ MD: 271±28 nm	+	++ MD: 307±51 nm	++ MD: 257±44 nm	+
2	well granulated	+	+++ MD: 537±57 nm	+	++ MD: 446±53 nm	−
3	mixed	+	+	+	+	+
4	mixed	−	+	−	+	+++ MD: 165±25 nm
5	poorly granulated	−	+	+	−	++ MD: 184±33 nm
6	poorly granulated	−	+	+	−	+++

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

[b] MD: mean diameter

mone-releasing factor (GRF), except for a few PP-immunoreactive cells in case 1 tumour. The data related to each case are summarized in Table 1. Normal pancreas adjacent to the tumours and rat pancreas were similarly processed and used as controls.

The antiserum A564, obtained from Dakopatts, Ltd (Copenhagen, Denmark), had been raised in guinea-pig against porcine pancreatic insulin. Specificity, as measured by radioimmunoassay (RIA), was 100% for porcine insulin and human insulin, and less than 0.05% for glucagon, human growth hormone and PP; the antiserum was shown to cross-react with porcine proinsulin in RIA conditions (data supplied by the manufacturer). Affinity-purified goat antibodies to guinea-pig IgG coating colloidal gold particles of 10 nm or 20 nm mean diameter were purchased from Janssen Pharmaceutica Research Laboratories (Beerse, Belgium).

Tissues were fixed at 4° C for 2 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.30, postfixed for 1 h in 1% buffered osmium tetroxide, dehydrated through a graded ethanol series, transferred into propylene oxide and embedded in Epon 812. Thin sections were placed on uncoated 300-mesh nickel grids, contrasted or used for immunoelectron microscopy.

In order to define technical parameters suitable for the present immunogold procedure, preliminary experiments were performed on both control and tumour tissues, as previously reported for gastrin immunolabelling (Berger et al. 1985a), except that the results were estimated qualitatively. Subsequently, tumour sections were processed as follows: a) they were slightly etched with 10% H202 for 5 min. b) the antiserum, diluted 1:8000 was applied for 24 h at 4° C c) the 10 nm gold reagent was preferred to the 20 nm one and used at a dilution of 1:50. The sections were counterstained with 2% aqueous uranyl acetate for 10 min., then with lead citrate for 10 min. and viewed under a Jeol × 100 electron microscope.

The specificity of the labelling was assessed by incubation of sections with antiserum absorbed with human insulin synthetically derived from porcine insulin (Sigma Chem. Company, Saint-Louis, USA). The 98% pure peptide was added at varying molar concentrations and the results of labelling were compared with those obtained with the non-absorbed antiserum.

Results

Two structural patterns of tumour cells were recognized, with gradations between them. Heavily granulated cells were prominent in cases 1 and 2 as in control B-cells. They were characterized by

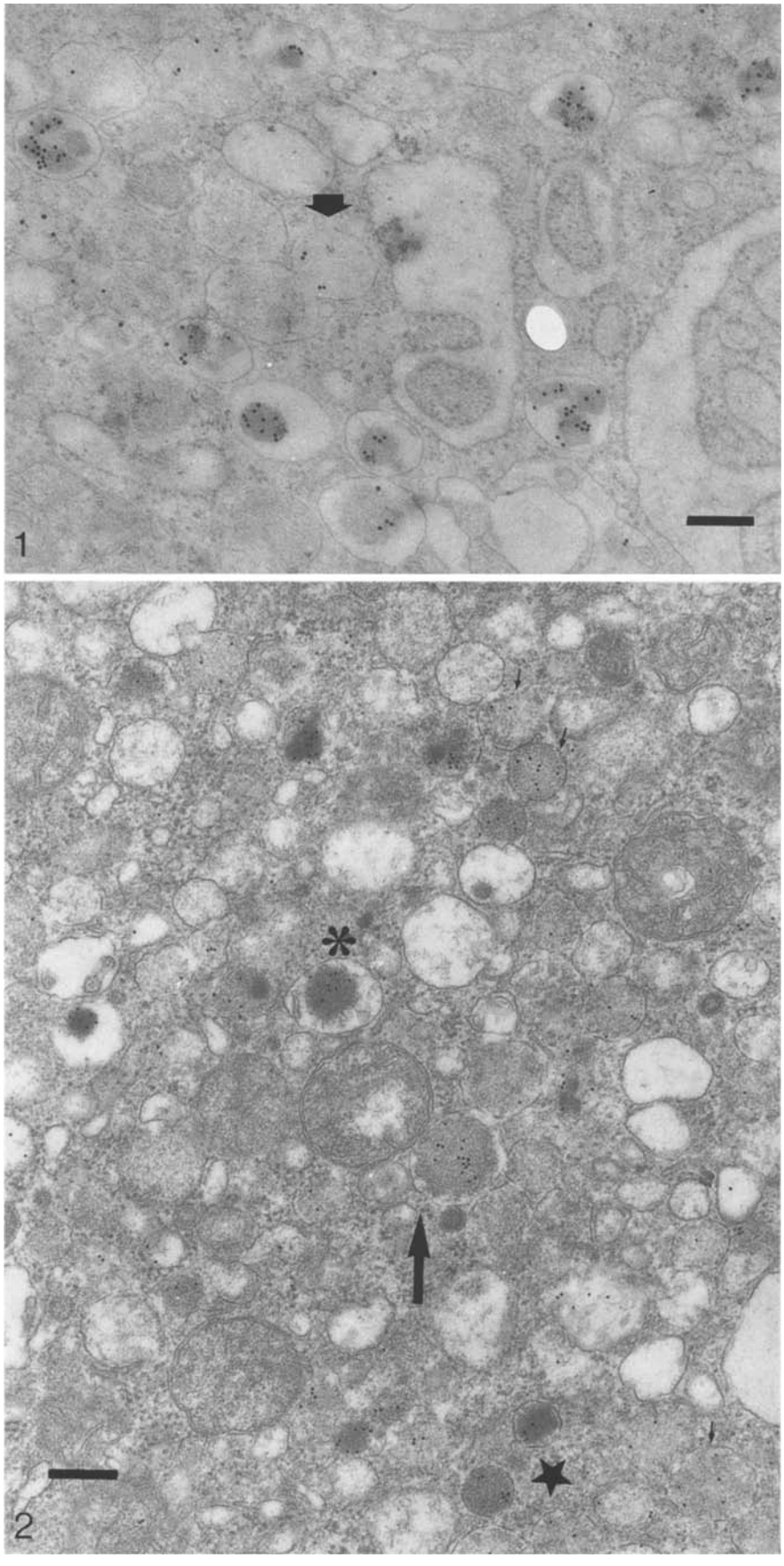
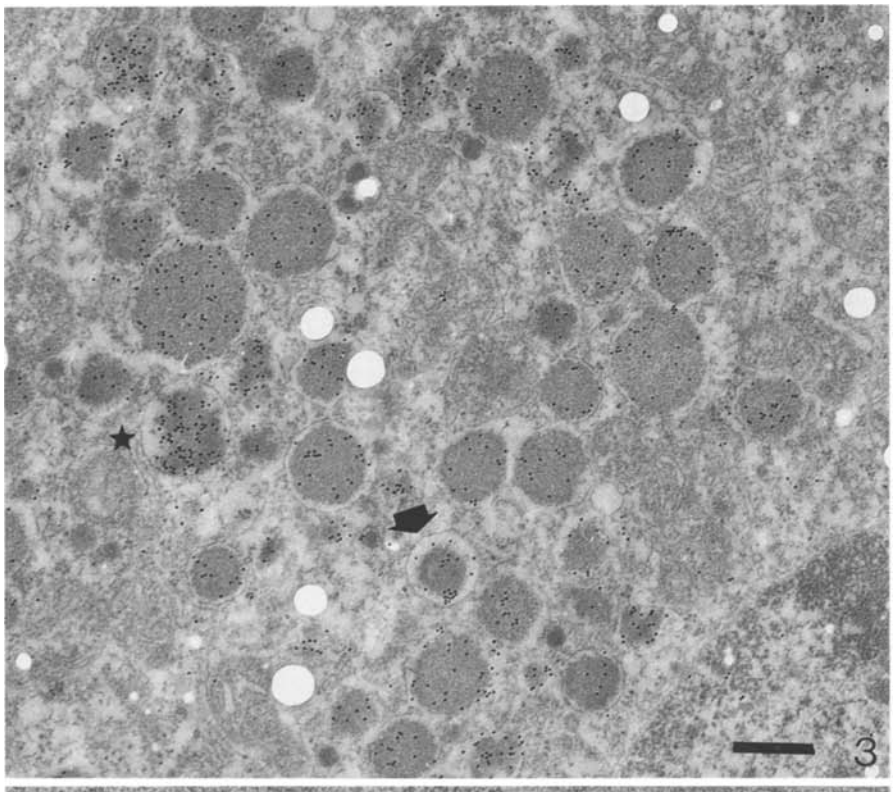


Fig. 1. Part of a control B-cell of adult human pancreas immunolabelled for insulin/proinsulin using 20 nm gold. The particles are seen over the crystalloid or amorphous dense cores of the secretory granules. In contrast, granules with amorphous pale content (*arrow*) are inconstantly and poorly labelled. Antiserum A 564. × 35000. *Scale bar*: 0.30 μm

Fig. 2. Immunogold staining of case 1 tumour for insulin/proinsulin using 10 nm gold. Many of the secretory granules belong to the pale variety and show poor labelling. The large arrow points to a granule of intermediate density, which presents moderate labelling. Large granules with amorphous dense (*asterisk*) or crystalloid cores are strongly labelled. Note two poorly labelled granules of the small dense core type (*star*). Antiserum A 564. × 35000. *Scale bar*: 0.30 μm



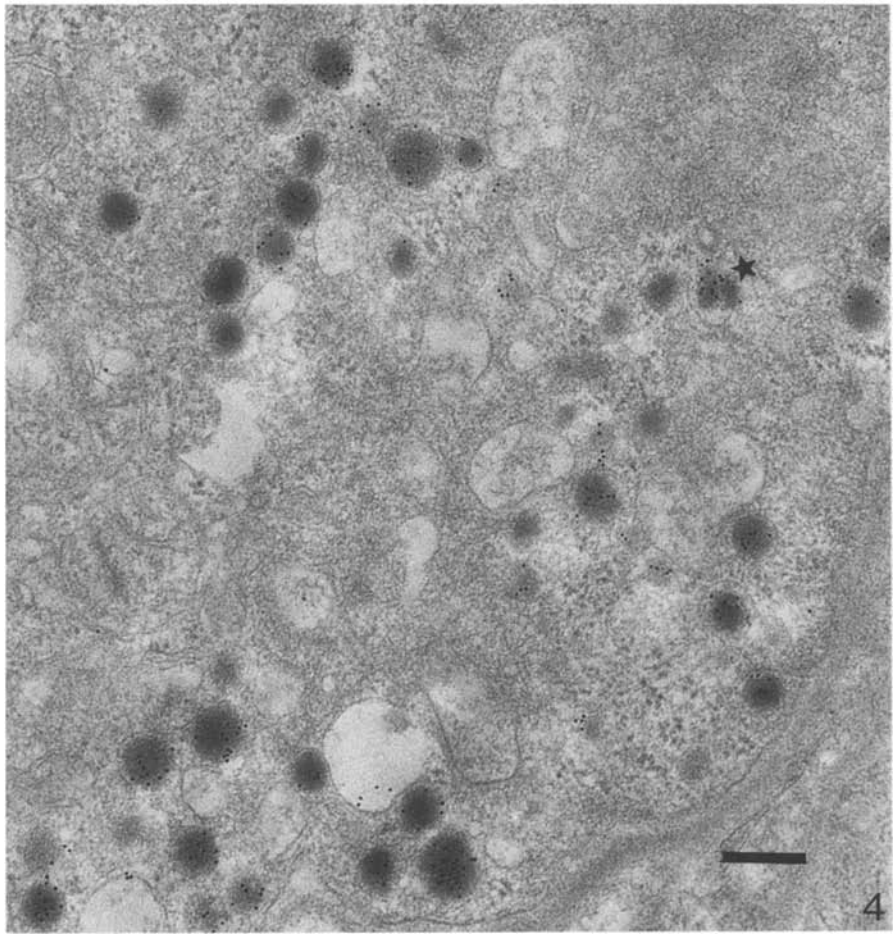


Fig. 3. Part of a well granulated cell from case 2 tumour immunolabelled for insulin/proinsulin with 10 nm gold. The secretory granules are strongly immunoreactive. Most of them belong to the amorphous moderately dense type and some (*short arrow*) to the amorphous dense type. The strongest labelling is present over the few crystalloid granules (*star*). Antiserum A 564. ×24000. *Scale bar*: 0.43 μm

Fig. 4. Case 4 tumour cell storing small dense-cored (atypical) granules strongly labelled for insulin/proinsulin with 10 nm gold. Note one granule with a crystalloid core (*star*). Antiserum A 564. ×35000. *Scale bar*: 0.30 μm

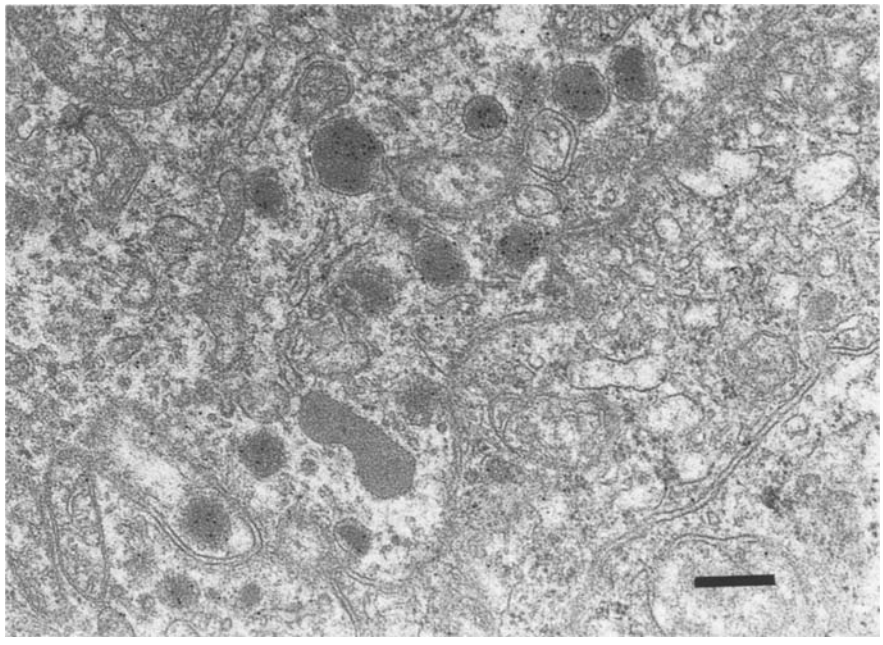


Fig. 5. A group of atypical granules in the vicinity of the membrane within a poorly granulated cell from case 5. Note their moderate to strong immunoreactivity to insulin/proinsulin with 10 nm gold. Antiserum A 564. ×28000. *Scale bar*: 0.37 μm

a high number of secretory granules, usually scattered over the whole cytoplasmic area, with a moderate to strong labelling. These cells exhibited only a few profiles of rough endoplasmic reticulum (RER) and rare mitochondria. Poorly granulated cells were present in case 3 and prominent in cases 4–6. The granules were lined along the cell membrane or gathered within cell processes and showed an unequal labelling. A large amount of RER, prominent Golgi complex and numerous mitochondria were other striking features. No significant labelling was detectable over the Golgi complexes. Lysosome-like bodies, including residual bodies, were abundant in all tumours. In contrast to multigranular bodies found in control rat B-cells, the tumour bodies were unreactive. In all cases, the addition of insulin to the antiserum, at a concentration as low as 0.35 nmol/ml, inhibited the labelling.

Most of the granules stored within the well granulated cells of cases 1–3 tumours resembled those seen in control human B-cells. They were classified into four main types according to the morphological features and immunostaining pattern (see Table 2).

The most characteristic displayed a single or multiform dense crystalloid body, surrounded by a wide electron lucent halo. Although the diameters of such granules varied greatly, the cores were always strongly labelled in both control and tumour tissues (Fig. 1 and 2). Granules of unequal size with an homogeneous dense core surrounded by a wide translucid halo were also easily recognized. Again, the often eccentric cores were strongly labelled (Fig. 1 and 2). Other granules of larger size (537 nm mean diameter) stored an amorphous material of intermediate density, encompassed by a thin regular halo. They were abundant in tumour 2 but scarcely represented in human control B-cells. The labelling was moderate to strong (Fig. 3). Medium-sized granules (271 nm mean diameter, estimated in the tumour 1) were found in the cells of tumours 1–3, filled with a low electron dense content of floccular appearance. They were also present in human control B-cells. In contrast to the previous varieties, they could not be labelled by 20 nm gold and the labelling obtained using 10 nm particles was rather weak (Fig. 2). A moderate labelling was also present over similar granules, apparently uncoated, in the close proximity of the Golgi complex in rare cells from case 3.

The poorly or moderately granulated cells that composed tumours 4–6 contained a predominant population of small granules (165 nm and 184 nm mean diameter, as estimated in tumours 4 and 5 respectively), characterized by a central core of high electron density separated from the limiting membrane by a very narrow halo. Using 10 nm gold, the labelling over the granules was rather strong in case 4 (Fig. 4), moderate in case 5 (Fig. 5) but very weak in case 6. Indeed, in the latter tumour, only a few were labelled, since the labelling was mainly restricted to larger-sized granules that displayed transitional features towards the amorphous dense or moderately dense varieties (Fig. 6).

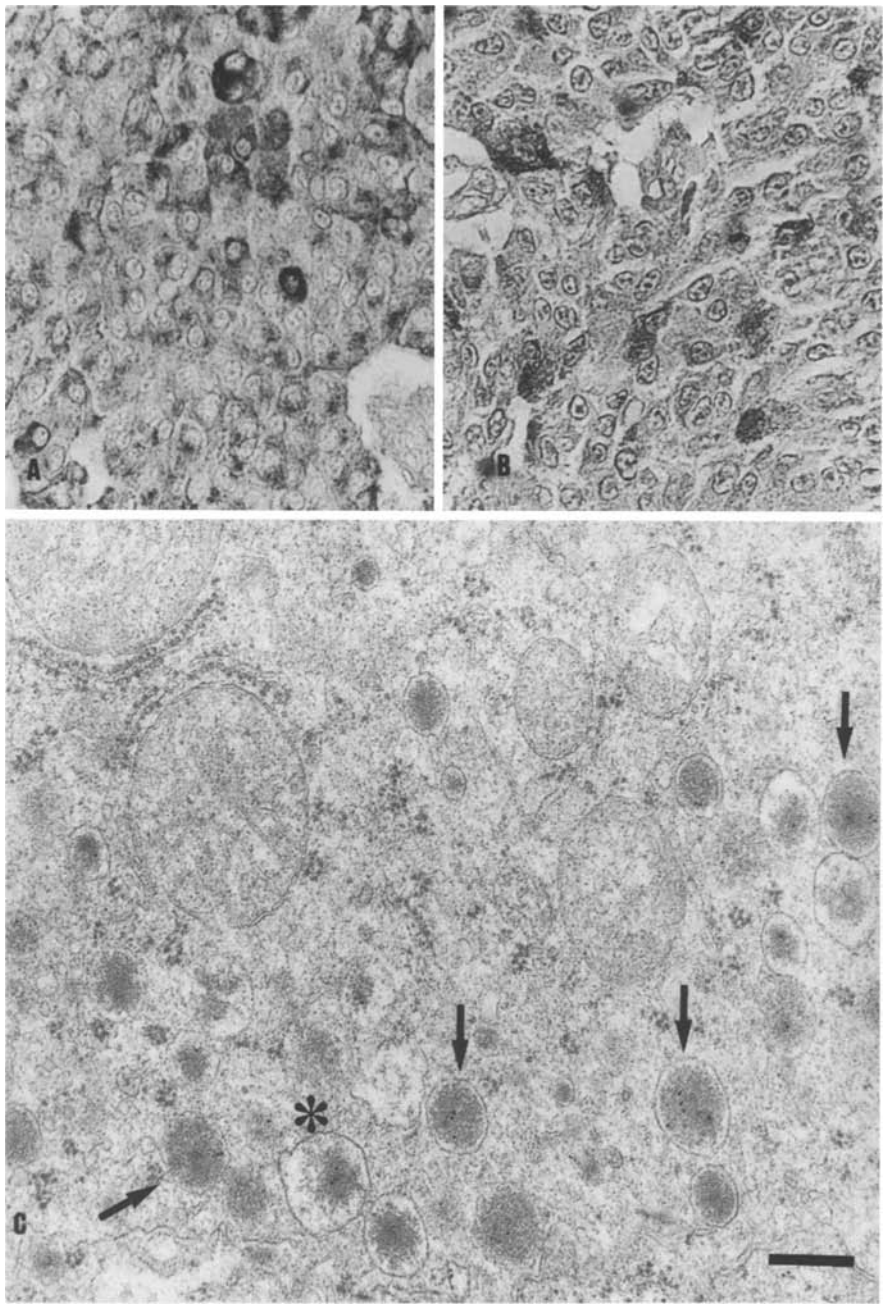


Fig. 6A–C. Case 6 insulinoma. **A** Marked argyrophilia of the tumour cells (light microscope Grimelius staining. ×1200) **B** Weak immunostaining of most tumour cells for insulin/proinsulin (light microscope PAP procedure. ×1200) **C** Only a few granules are (poorly) labelled with the anti-insulin/proinsulin serum using 10 nm gold (*arrows*). Note that the labelling is present over granules that show transitional features towards larger forms of the maturing type (*asterisk*). Antiserum A 564. ×35000. *Scale bar*: 0.30 μm

It is noteworthy that in these cells a number of small (areactive) granules showed degenerative features, such as an irregular shape, a wrinkled membrane or a partial vacuolisation. Some harbored a curious double contour characterized by a core of moderate electron density and a peripheral rim of higher density.

Discussion

The present localisation of insulin-like immunoreactivity was achieved on slightly etched sections of double-fixed conventionally embedded tumour tissues, using gold labelling. This method, introduced by Bendayan and Zollinger (1983) and Roth et al. (1985), does not impair the precise interpretation of B-cell morphology and results in a traditional electron microscopy picture. As we noted previously (Berger et al. 1985a) the use of small (10 nm) gold particles is decisive when poorly immunoreactive tumour material is investigated. Since polyclonal antisera raised against human, bovine or porcine insulin or human C-peptide cross-react with human proinsulin (Rubenstein

et al. 1970) no attempt was made to localise the precursor molecules and the cleavage products separately in the present material.

After application of the anti-insulin/proinsulin serum, most granules found in well granulated tumour cells resembled those observed in normal human B-cells in both morphology and immunoreactivity. Our findings are consistent with those of Varndell et al. (1982) who used unosmicated tumour material, in that we found a strong labelling over the crystalloid cores that characterize diagnostic B-cell tumour granules. The halos did not show any labelling. It has been suggested that they could store C-peptide (Steiner et al. 1971) but direct cytochemical evidence for this is lacking. The large granules with a dense or moderately dense core were also strongly immunoreactive. In contrast, those with a pale content were poorly labelled. The latter are also considered to be diagnostic but this is mainly due to the usual costorage in the same cell of more characteristic varieties. Whereas the degree of granule density may partially depend on artefacts of fixation and dehydration, the labelling pattern is more likely related to the biochemical maturation of the peptide content. Recent investigations by Orci et al. (1985; 1987) of prohormone conversion in rat and human B-cells support this hypothesis; using monospecific monoclonal antibodies, they demonstrated that the precursor predominates in Golgi-derived clathrin-coated vesicles whereas insulin is mainly stored in mature granules. Thus, the weak labelling that we noticed over the pale tumour granules, considered to be early granules (Steiner et al. 1974), may suggest that they store predominantly cross-reacting proinsulin. The abundance of such granules in insulinoma cells corroborates the increased proinsulin percentage reported in insulinoma extracts when compared with pancreatic tissue (Creutzfeldt et al. 1973).

We also observed a specific insulin-like labelling of very unequal intensity over small dense-cored granules that were prominent in poorly granulated cells of 3 other tumours. They were similar in size and shape to the "atypical granules" described in insulinomas by Creutzfeldt et al. (1973), who used them to categorize such tumours as follows: tumours with cells storing only typical beta granules (type 1), both typical and atypical granules (type 2), only atypical ones (type 3), or virtually agranular (type 4). However, the significance of these non-diagnostic granules remained controversial. First, as noticed by Creutzfeldt et al. (1973), types 3 and 4 insulinomas may present morphological, biochemical or clinical particularities including a) an unexpected light microscope staining pattern b) high proinsulin and rather low insulin concentrations of tumour content and c) a higher rate of malignant behaviour. Secondly, atypical granules found in insulinomas cannot be distinguished on morphology alone from those that store immunoreactive gastrin and progastrin (Berger et al. 1985b, c), VIP (Capella et al. 1983) or PP (Bordi et al. 1977; Tomita et al. 1983) in a number of non-B-cell endocrine tumours of pancreas. Finally, in immunoelectron microscopy investigation of human adult pancreas, similar granules cannot be found within B-cells but within PP-cells. For these reasons, it has been suggested that no insulin is present in type 3 insulinoma cells, which might represent an immature cell type (Creutzfeldt et al. 1973). In fact, the present procedure helps to identify those non-diagnostic granules that store insulin or proinsulin and to distinguish the corresponding cells from others that might contain non-insulin-related peptides such as PP.

In addition, our results revealed heterogeneity of the insulin-related atypical granules. Those found in the tumours with classical light microscope staining pattern were strongly immunoreactive. In contrast, the labelling was weak and unconstant over those found in the two tumours with unusual staining pattern, including variable argyrophilia, aldehyde-fuchsin negativity and faint staining with the anti-insulin serum. In these cases, the moderate elevation of the patient's insulinemia in spite of the rather large tumour size was also remarkable. Moreover, both tumours showed a number of emboli in the vascular channels, suggesting malignancy. The atypical granules with a strong labelling may store an appreciable amount of non-crystallised insulin, whereas the poorly labelled ones may contain a lower amount and/or a cross-reacting peptide such as proinsulin. The costorage in the same cells of rare typical mature granules together with the former variety and of only subtypical maturing granules together with the second could indicate that both varieties represent different degrees of granule maturation. The problem needs to be further investigated using monospecific monoclonal antibodies. It is of interest that morphologically similar granules have been described in small numbers within B-cells of human embryos (Like and Orci 1972). The reasons that govern the incomplete granule maturation observed in some insulinomas with reduced storage capacity are still speculative. A deficiency of specific (converting) enzyme activities (or a defect of the clathrin-coated compartment?) might be involved,

or alternatively abnormalities might occur within the maturing granules and concern the processing of the cleavage products.

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