

Ultrastructural immunogold study on the various cell types of cultured pancreatic islets of adult rats

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*Whereas several reports describing the ultrastructure of the intact pancreatic islets have been recorded, published experience with the ultrastructural integrity of the cultured pancreatic islets is limited. The present study was, therefore, undertaken to provide an ultrastructure identification of the different cells in the cultured islets of the adult rat pancreas, after marking their secretory granules with gold particles. Pancreatic islets were isolated from adult male Wistar rats by the intraductal perfusion of collagenase technique. The islets were cultured in RPMI-1640 medium for 3 days and processed for preparation of ultrathin sections. The sections were stained with the indirect immunogold technique for insulin, glucagon, somatostatin, and pancreatic polypeptide. Ultrastructural examination of the cultured islets clearly identified the presence of B, A, D and PP-cells, as indicated by the numerous gold particles concentrated predominantly over the secretory granules. The secretory granules of the various cell types of the cultured islets demonstrated several similarities as well as differences from the recorded results of the corresponding secretory granules of the intact islets. The differences probably reflect a deviation in the underlying mechanisms of synthesis, maturation and secretion of the different secretory products of the cells in the cultured islets as they adapt to the *in vitro* environment.*

key words: culture, electron microscope, immunogold, islets of langerhans, pancreas, rat, ultrastructure

INTRODUCTION

With the increased demand of the isolated and cultured pancreatic islets as an important preparation for the biological and transplantation studies, comprehensive morphological evaluation seems feasible. Few reports have been recorded about the ultrastructure of the isolated [12,19,30,32] and cultured islets [6,10,24]. These studies have focused on the identification of B-cells, whereas identification of the non-B cells of the cultured islets has not been recorded. Furthermore, identification of the B-cells of the islets in these studies relied on the morphology of their

secretory granules. Although this method may clearly identify well-granulated cells of intact islets, difficulties are encountered when the cells are scarcely granulated or contain secretory granules with various forms of pleomorphism [32]. These difficulties are especially expected in cultured islets, where the cells are adapting to an *in vitro* environment. Therefore, specific marking of the secretory granules by the immunogold technique would provide accurate identification of the different cells in the cultured islets, prior to their ultrastructural examination.

The aim of the present study is to provide a precise ultrastructural identification of the various cells in the cultured islets of the adult rat pancreas, after marking their secretory granules with the immunogold technique. Since previous ultrastructural identification of islet cells relied on the criteria of their secretory granules, it may suffice to focus essentially on the description of the marked secretory granules. Cultured rat islets were selected, since there is no definite information about the rat, which is the most extensively used animal for experimental purposes.

MATERIAL AND METHODS

Animals

Adult white Wistar male rats aged 60 days and weighing 230–280 g, were used. Nonfasting blood glucose concentrations were measured to check that the animals were normoglycaemic.

Isolation of the pancreatic islets

Pancreatic islets were isolated from five rats using the intraductal perfusion method of Sutton et al. [31], which is based on the original collagenase digestion technique of Lacy and Kostianovsky [15]. The rats were anaesthetised with ether inhalation, and the common bile duct was cannulated and infused with 7 ml of cold, freshly prepared, collagenase Sigma type V (2 mg/ml) dissolved in medium 199 (M 199). The pancreas was quickly excised and incubated at 37°C for 22 min. The action of collagenase was stopped by adding cold M 199. The islets were separated by discontinuous Ficoll gradient (density = 1.095) and further purified by hand picking of the non-islet tissue with the help of a binocular dissecting microscope. The whole process was carried out under aseptic conditions using a laminar-flow hood to avoid contamination of the isolated islets.

Culture of the pancreatic islets

The isolated islets were cultured in 94 mm sterile polystyrene culture dishes, in groups of approximately 100 islets per dish. The culture medium consisted of RPMI-1640 medium supplemented with L-glutamine (3%), rat serum (5%), penicillin-streptomycin (50 IU/ml and 50 µg/ml, respectively), gentamycin (10 µg/ml) and nystatin (100 IU/ml). Glucose concentration of the culture medium was 2 g/litre and the osmolality between 310 and 340 mOsmol/kg. The cultured islets were maintained at 37°C in a humidified atmosphere of 5% (v/v) carbon dioxide in air and the culture medium was changed daily.

Processing for electron microscopic examination

Specimens of islets were processed for electron microscopy after 3 days of culture. The culture medium was removed and the islets were rinsed three times in M 199. They were fixed in Trump's fixative (2% glutaraldehyde and 1% formaldehyde in 0.2 M cacodylate buffer, pH 7.2) for 30 min, at 4°C and then dehydrated in graded ethanol series. The islets were exposed to two changes of propylene oxide-absolute ethanol mixture and two changes of pure propylene oxide and then embedded in epoxy resin at beam capsules and cured at 60°C for 24 hr. At each step, the islets were pelleted at 1,000 rpm (200 g) for 1 min to facilitate its processing. Ultrathin sections were stained with immunogold technique and examined with Philips 300 Transmission Electron Microscope at 60 KV.

Immunogold staining

The indirect immunogold technique of DeMey et al. [3] was used to localise insulin, glucagon, somatostatin, and pancreatic polypeptide-producing cells at the ultrastructural level. The procedure consisted of exposing the non-osmicated sections, on nickel grids to a solution of 2% normal goat serum (NGS) and 2% bovine serum albumen (BSA) in Tris-buffered saline (TBS) at pH 7.6 for 30 min. This was followed by incubation of the grids with the primary antisera for 1 hour, at room temperature. The primary antisera were guinea pig anti-pig insulin serum at a concentration of 1:50 (for B-cells), rabbit anti-porcine glucagon serum at a concentration of 1:100 (for A-cells), rabbit anti-human somatostatin serum at a concentration of 1:50 (for D-cells), rabbit antihuman pancreatic polypeptide serum at a concentration of 1:100 (for PP-cells). The grids were then rinsed in a solution formed of 2% NGS and 2% BSA in TBS at pH 7.6.

The secondary antibody for B-cells was goat anti-guinea pig IgG conjugated with 15 nm gold particles. The secondary antibody for A, D, and PP-cells was goat anti-rabbit Ig conjugated with 10 nm gold particles. The secondary antibodies were purified by centrifugation at 9,000 x g for 30 sec and used at concentrations of 1:20 for 1 h. Subsequent steps included rinsing in a solution of 2% NGS-2% BSA in TBS at pH 7.6, and then with distilled water. The sections were counterstained for 15 min in 2% aqueous uranyl acetate [34]. All the staining steps were carried out at room temperature and the grids were stained by immersing them in 60 µl drops of the staining solutions. The drops were put on a parafilm paper and the grids were kept gently agitated inside

the drops by putting the parafilm on top of a magnetic stirrer.

Controls for the specificity of the immunocytochemical method consisted of replacing the primary antisera with normal (non-immunised) serum or included an excess of the immunogold solution. Controls for the antibody specificity consisted of absorption of the primary antisera with an excess of the antigen. The controls gave the appropriate negative results.

Morphometric analysis

Secretory granules in about 10 cells, randomly chosen, were used for this analysis. For each islet cell type, the diameters of 400 secretory granules were measured on micrographs at magnification of about $\times 50,000$.

The profile diameters (d) of the secretory granules were calculated from the equation $d=2\sqrt{ab}$, where a and b are the major and minor at right angle semi-axis, respectively. The mean axial ratio of the profiles was calculated. Assuming that the secretory granules are spheroid structures, the formula of Fullman [35], for the ungrouped profile range of sizes, was used to calculate the mean true diameter (\bar{D}) for the secretory granules.

$$\bar{D} = \frac{\pi}{2} \times \frac{N}{1/d_1 + 1/d_2 \dots 1/d_N}$$

where N represents the total profiles measured and d_1, d_2, \dots, d_N represent the profile diameters.

Gold particles were counted over each secretory granule's profile. The immunogold labelling density was calculated as the number of gold particles per unit surface area of the secretory granules.

The results of the morphometric study were presented as the arithmetic mean \pm standard deviation (SD). Data obtained from the various islet cells were compared statistically by one way analysis of variance (ANOVA) followed by Scheffe pairwise comparisons using a current SPSS statistical package. Significant results were determined at $P < 0.05$ throughout the study.

RESULTS

Ultrastructural examination of the immunogold stained cultured islets clearly identified four distinct types of islet cells, as indicated by the presence of numerous gold particles concentrated predominantly over the secretory granules and occasionally over the rough endoplasmic reticulum (RER) (Fig. 1–4).

B-cells

They were the most numerous cells of the cultured islets and were located in the central region of the islets. They were almost polyhedral in shape and had a rounded or slightly oval nucleus with irregular contour and narrow perinuclear cisterns. The majority of the B-cells were either moderately or sparsely granulated, whereas few of them appeared well-granulated (Fig. 1). The secretory granules were mostly distributed in the marginal cytoplasm and abutting the plasma membrane. Mature and immature forms of secretory granules were observed. The mature forms represented almost 44% of the total secretory granules. This type of secretory granules showed a mean true diameter of 367.6 nm. They showed an inner core of moderate to dark electron density, of variable shape (although usually rounded), surrounded by a rather peripheral zone of electron lucency between the core and the limiting membrane (Fig. 1). The immature secretory granules, however, represented about 56% of the total secretory granules. They had a mean true diameter of 423 nm and showed a fluffy core with moderate electron density, consisting of a porous substance with ill-defined contour surrounded with either a narrow lucent zone or a fitting membrane (Fig. 1). Secretory granules with a homogeneous core of moderate electron density, surrounded with an outer zone of light density, were rarely seen. The profile diameter of the B-secretory granules was found to be 272 ± 58 nm.

A-cells

They were less numerous and located at the peripheral part of the islet. They were almost polyhedral in shape and their nuclei were rounded with an undulating contour. Their secretory granules showed a homogeneous large rounded core of moderate to high electron density and a narrow lucent-halo or well-fitting limiting membrane (Fig. 2). The profile diameter of the A-secretory granules was 155 ± 38 nm. The cytoplasm also showed vacuolar spaces of variable sizes.

D-cells

They were located at the peripheral part of the islet. Their cytoplasm was moderately granulated with immature small secretory granules, having moderate electron density and well-fitting limiting membrane (Fig. 3). Mature secretory granules were few and were surrounded with a narrow electron-lucent halo. The profile diameter of the D-secretory granules was 104 ± 30 nm.

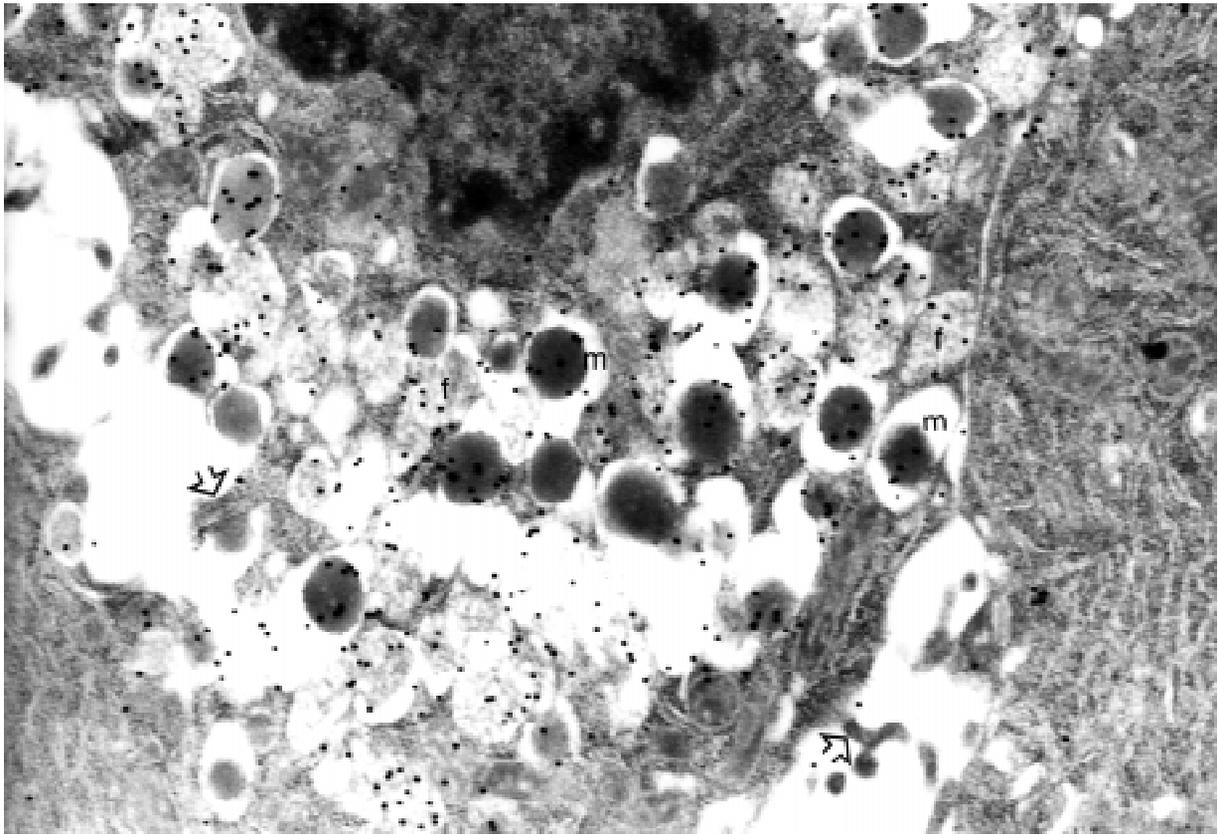


Figure 1. Electron micrograph of a B-cell of an islet cultured for 3 days. The insulin secretory granules are positively marked with 15 nm gold particles. Mature secretory granules (m) have a central or eccentric electron-dense core surrounded with a wide electron-lucent halo. Immature secretory granules have a fluffy central core (f) surrounded with either a narrow lucent-halo or a well-fitting limiting membrane. Several secretory granules could be seen adjacent to the plasmalemma in various phases of exocytosis. Microvillous processes could be seen extending from the surface of the cell (open arrows). The cell at the right side of the micrograph appeared to have shut-off its secretory activity. Immunogold for insulin. x 40,000.

PP-cells

They were located at the peripheral part of the islet. The cells were almost polyhedral in shape and showed oval or rounded nuclei with slightly undulating contours. The cytoplasm was moderately granulated, showing immature secretory granules with rounded or oval homogeneous cores of variable electron density. Mature secretory granules having a core with high electron density separated from the limiting membrane by a narrow electron-lucent halo were also seen (Fig. 4). The profile diameter of the PP-secretory granules was 110 ± 25 nm.

For the most part, cells located at the periphery of the islets displayed numerous microvillous projections extending from the cell surfaces into the adjacent unoccupied space (Fig. 1–4). A similar but less marked phenomenon was apparent within the interior of clusters, whenever cells were grouped so as to delimit an unoccupied space.

The results of the morphometric study are presented in Table 1. It showed that the mean axial ra-

tio of the secretory granules in the different islet cells ranged from 1.1 to 1.3, which means that the islets could be treated as spheroids. Applying the formula of Fullman showed that the true diameters of B, A, D, and PP-secretory granules were 410, 228, 149 and 165 nm, respectively. The results of ANOVA showed that the diameters of the secretory granules in the different cells were significantly different from each other, except for D and PP-secretory granules. Whereas B and A-secretory granules showed moderate immunogold labelling density, D and PP-secretory granules showed high labelling density (Table 1). Significant differences in immunogold labelling densities were detected between the secretory granules of the different cell types.

DISCUSSION

Since the pancreatic islets form 1–2% of the mass of the pancreas, procedures are available, using collagenase enzyme, to isolate them from the surrounding tissues for purposes of obtaining purified endo-

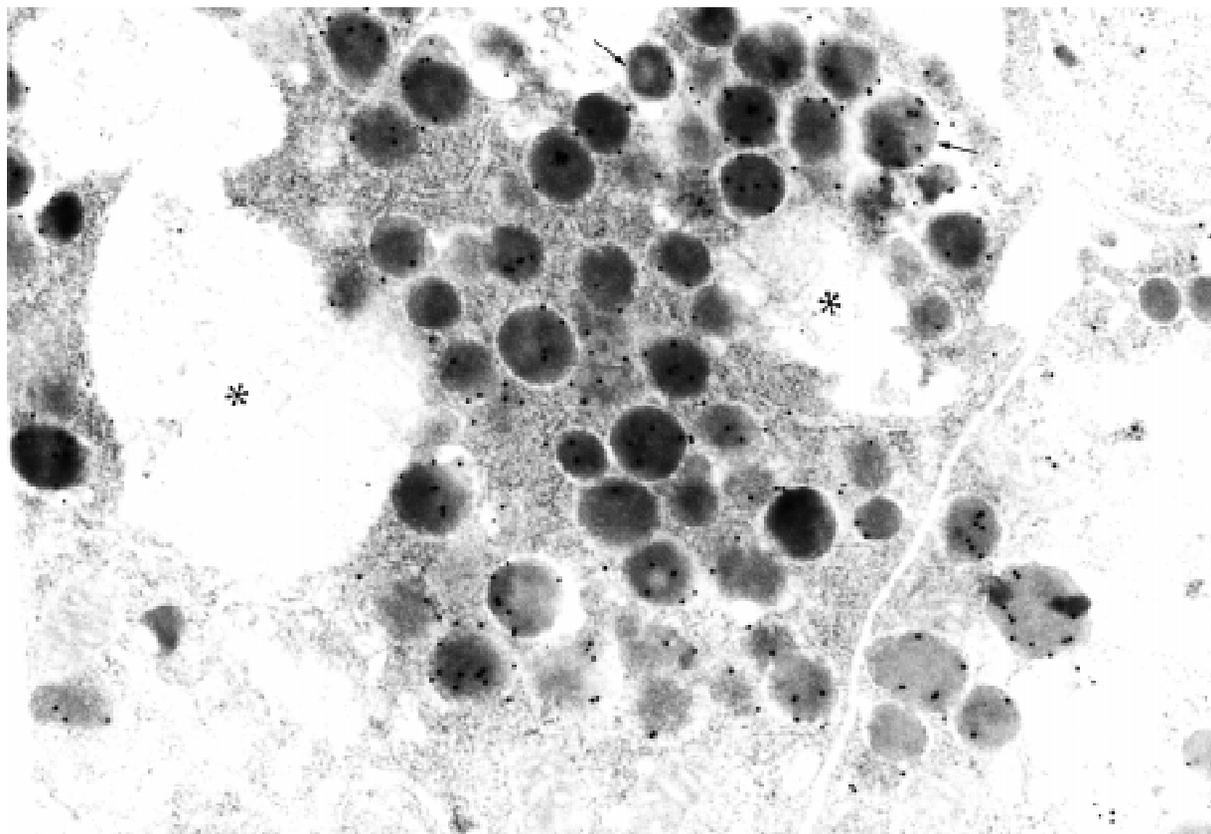


Figure 2. Electron micrograph of two A-cells of an islet cultured for 3 days. The glucagon secretory granules are positively marked with 10 nm gold particles. They have a homogeneous large electron dense core and a narrow lucent-halo or a well-fitting limiting membrane. Several secretory granules could be seen adjacent to the plasmalemma in various phases of exocytosis (arrows). Vacuolar spaces of variable sizes appear in the cytoplasm (stars). Notice the moderately granulated cell at the right lower angle and a non-A-cell at the upper right angle. Immunogold for glucagon. x 51,500.

ocrine preparation for culture experiments. It is evident that the use of collagenase enzyme to isolate the islets inevitably leads to injury of the peripherally situated non-B cells [1,21,30,32,33]. The significance of the non-B cells was evidenced by reporting that they may interact with B-cells for the proper release of insulin [19,27]. Controversy exists in the literature regarding the detection of non-B cells in the periphery of the islets following the enzymatic isolation procedure. Tahir et al. [32] did not report secretory granules characteristic of A, D or PP-cells in their isolated islet preparations, when examined ultrastructurally. El-Naggar et al. [5], however, demonstrated the presence of A, D and PP-cells at the periphery of the isolated islets, when stained immunohistochemically for light microscope. We could not detect in the literature any investigation reporting the presence of A, D and PP-cells in cultured islets. The findings of the present work confirm the presence of the four cell types in the cultured pancreatic islets of the rat, when stained with immunogold and

examined ultrastructurally. B-cells were present in the central part of the islet, whereas A, D, and PP-cells were peripherally situated, indicating intact cellular architecture of the cultured pancreatic islets.

The results obtained in the present study revealed that the secretory granules of B, A, D and PP-cells in cultured islets bear several similarities to as well as differences from those reported for the intact islets. Whereas mature secretory granules were reported in the literature to be the most predominant form in the cells of the intact islets, our results showed that the immature forms predominate in the cells of the cultured islets.

Mature and immature forms of B-secretory granules could be detected in our specimens of cultured islets. The mature form was found to have a central or eccentric electron dense core, surrounded with a wide electron-lucent halo of a variable size between the core and the limiting membrane. This was similar to the classically described secretory granules for B-cells of the intact [2,4,9,18,23,26,28,32] and iso-

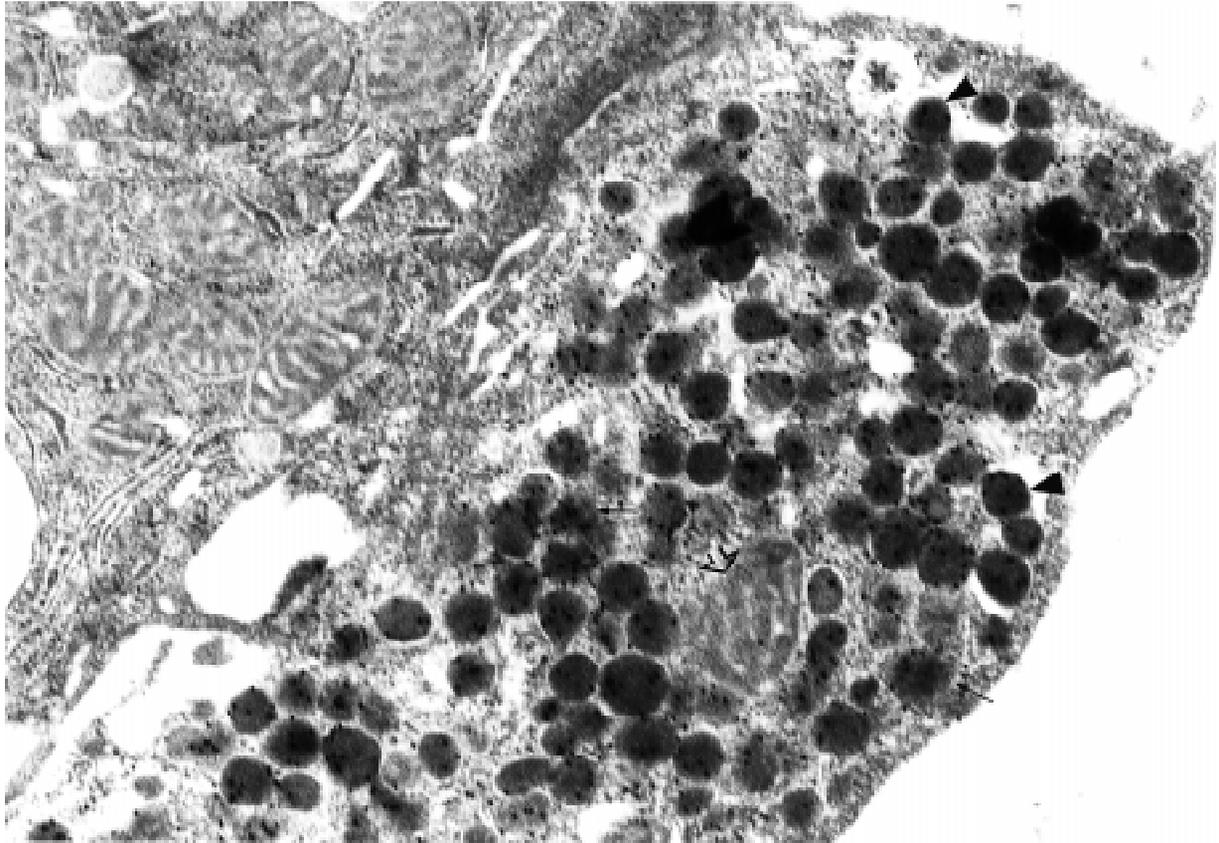


Figure 3. Electron micrograph of a D-cell of an islet cultured for 3 days. The somatostatin secretory granules are positively marked with 10 nm gold particles. The cytoplasm is well granulated, showing immature secretory granules with moderate electron density and a well-fitting limiting membrane (solid arrows). Mature secretory granules are surrounded with a narrow electron-lucent halo (arrowheads). Large swollen mitochondria with swollen cristae could be seen (open arrow). The cell at the upper left corner was not marked with gold particles. Immunogold for somatostatin. x 51,500.

lated [12,19,30,32] islets. Similar mature secretory granules were also described in monolayer cell culture [24], organ culture [6] and isolated cultured islets [10]. Although the mature secretory granules were reported to be the predominant form in the intact islets, they formed 44% of the total secretory granules in our specimens of cultured islets.

On the other hand, immature secretory granules in the B-cells of cultured islets in our specimens

formed 56% of the total secretory granules. They had a moderately dense fluffy or homogeneous core and a tightly-fitting limiting membrane. They were nearly similar to the immature secretory granules described in cultured islets by Hamaguchi et al. [10] as having a porous core and ill-defined contour. Although they formed 56% in our specimens, Hamaguchi et al. [10] mentioned that they were few. Orci et al. [24] described immature B-secretory granules as

Table 1. Diameter and axial ratio of each type of the secretory granules of the cultured islets. The number of gold particles over the immunostained secretory granules and immunogold labelling density are also shown*

Secretory granules	Profile diameter (d) [nm]	True diameter (\bar{D}) [nm]	Axial ratio	Number of gold particles/profile	Immunogold labelling density
B	272 ± 58	410	1.2 ± 0.2	5.2 ± 3.6	0.000082 ± 0.000045
A	155 ± 38	228	1.1 ± 0.1	3.5 ± 2.4	0.000160 ± 0.000111
D	104 ± 30	149	1.1 ± 0.1	4.6 ± 2.4	0.000620 ± 0.000457
PP	110 ± 25	165	1.3 ± 0.3	8.4 ± 4.0	0.000883 ± 0.000349

*Data are presented as means ± SD

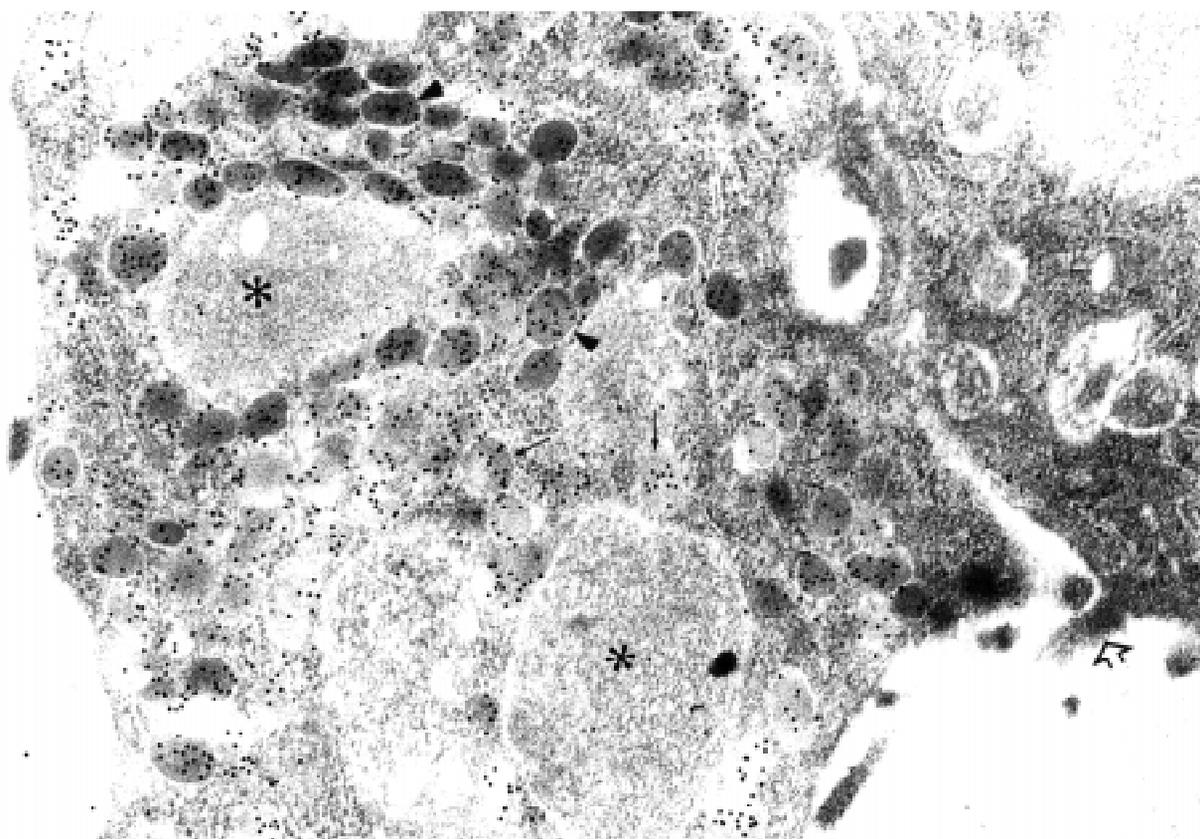


Figure 4. Electron micrograph of PP-cells of an islet cultured for 3 days. The pancreatic polypeptide secretory granules are positively marked with 10 nm gold particles. The immature secretory granules have rounded or oval homogeneous cores of low or moderate electron density (solid arrows). Mature secretory granules of high electron density and thin electron-lucent haloes could also be seen (arrowheads). Vacuolar spaces of variable sizes could be seen in the cytoplasm (stars). A cell at the right side of the micrograph appears moderately granulated with non-PP secretory granules, which are not marked by the gold particles. Microvillous processes could be seen extending from the surface of the cell (open arrow). Immunogold for pancreatic polypeptide. x 51,500.

having a less electron-dense core and tightly fitting limiting membrane. They added that they were the predominant forms in monolayer cell culture. Fukuma [6] described two forms of immature B-secretory granules. The first form was similar to the mature one, but with an amorphous less dense core and was considered the predominant one in the cultured islets. The second form was scarcely observed, having a homogeneous dense core and a narrow space between the core and the limiting membrane. Tahir et al. [32] described immature B-secretory granules in the isolated islets of the adult rat, having a less electron-dense core completely filling the limiting membrane or having a narrow electron-lucent halo. However, the incidence of the immature B-secretory granules was not mentioned in these reports. These forms of immature secretory granules were thought by Fukuma [6] to be the precursor of the mature form, as they were frequently observed around the Golgi apparatus of B-cells when stimulated by glucose. Maturation was attributed to the condensa-

tion of the secretory material in a central core accompanied by resolution of the peripheral zone.

The present study showed that the secretory granules of A-cells of the cultured islets had a large homogeneous rounded core of moderate to high electron density surrounded with a narrow lucent-halo or a well-fitting limiting membrane. Similar A-secretory granules for intact islets were reported in the literature as having a dense core with narrow halo [11,13,17,18,22] or with a well-fitting membrane [2,7,11]. Most authors, however, described A-secretory granules as having a very dense core surrounded by a wide electron-lucent halo between the core and the limiting membrane [7,26,28,29] or as dense-cored granules with a less dense surrounding halo [24]. Pleomorphism in the secretory granules of A-cells was evident in the isolated rat islet preparations of Tahir et al. [32]. The heterogeneous ultrastructural appearance of A-secretory granules given in the literature raises the possibility that these cells called A-cells by the previous authors do not all belong to the same cell type.

Ultrastructural examination of D-cells of the cultured islets in the present work showed that most of the secretory granules were having moderate electron density and a well-fitting limiting membrane. Few D-secretory granules were surrounded with a narrow electron-lucent halo. Similar D-secretory granules were described in the literature for the intact islets of the rat. Orci et al. [24] reported that D-cells contain moderately electron-dense secretory granules adhering to the limiting membrane. This was confirmed by applying the immunohistochemical techniques at the electron microscopic level [8,17]. Larsson et al. [17] reported D-cell secretory granules, each having a dense central core separated from the limiting membrane by a well-defined but narrow electron-lucent space similar to that of B-cells. The noticed controversy in the literature regarding the D-secretory granules could be attributed to the reporting of D-cells under different metabolic and secretory conditions.

PP-cells of the cultured islets in the present study were moderately granulated, showing immature secretory granules with rounded homogeneous cores of variable electron density and mature secretory granules having a core with high electron density separated from the limiting membrane by a narrow electron-lucent halo. The mature PP-secretory granules were similar to those reported in the literature. Larsson et al. [17], Pelletier [25] and Sacchi and Bani [28] reported PP-secretory granules as having a dense core separated from the limiting membrane by a narrow but well-defined electron-lucent halo. Whereas immature PP-secretory granules described in this work were the predominant form in cultured islets, the mature PP-secretory granules were reported to be the predominant form in the intact islets.

Morphometric analysis showed that the profile diameters of the B, A, D, and PP-secretory granules in the cultured islets were 272 ± 58 , 155 ± 38 , 104 ± 30 , and 110 ± 25 nm, respectively. Those of the intact islets of the rat were found by Sacchi and Bani [28] to be 443 ± 101 , 498 ± 118 , 290 ± 64 , and 394 ± 77 nm, respectively. It is evident that the secretory granules of the various cells of the cultured islets were smaller than the reported corresponding secretory granules of the intact islets. This finding is in agreement with the literature reports on the B-secretory granules of the isolated and cultured islets. They ranged from 200 to 450 nm in the isolated islets [32], 498 ± 117 nm in organ cultured pancreatic B-cells [6] and from 100 to 300 nm in cultured islets of rat [10]. They were smaller than the profile diameters of the

B-secretory granules of the intact islets, which were found to be 461 ± 93 [6] or 250–550 nm [32]. We could not detect in the literature any report for the diameters of the secretory granules of the non-B cells in isolated or cultured islets. It seems, however, that the decrease in the size of the secretory granules due to culture is uniform, except for A-secretory granules, which have suffered more decrease in size than B-secretory granules. Whereas A-secretory granules were the largest in the intact islets [28], they were shown in the present study to be smaller than the secretory granules of the B-cells in the cultured islets. It could be inferred that the mechanism of formation and maturation of the A-secretory granules is more sensitive to the culture procedure than that for the other secretory granules.

Another finding that was worthy of note in the different cells of the cultured islets concerns the widened extracellular spaces and the formation of numerous microvillous projections on the cell surface. Similar findings were reported in B-cells of the intact [14,20,36], isolated [16,32] and organ cultured islets [6]. These changes were reported, however, in the four cell types in our specimens of the cultured islets. Lacy [14], Williamson et al. [36] and Lacy et al. [16] attributed these changes to hyperactivity of the B-cells, and enhanced insulin secretion by the process of exocytosis. On the other hand, Meyer and Bencosme [20] and Fukuma [6] could not find any evidence that microvilli on the surface of pancreatic B-cells were formed as a result of granule liberation. Although in the present study some secretory granules were found in a process of exocytosis, microvillous processes were also detected without exocytotic vesicles. These projections possibly represent a device for enlargement of the cell surface to adapt to the *in vitro* environment.

From the foregoing, it is evident that the immunocytochemical method used in the present study had the advantage that it allowed distinct and easy identification of the four types of cells in the cultured islets together with satisfactory preservation of their ultrastructural architecture.

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