

Direct Communication of Homologous and Heterologous Endocrine Islet Cells in Culture

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ABSTRACT The transfer of 6-carboxyfluorescein between islet cells in monolayer culture was observed by fluorescence microscopy, and the endocrine cells involved in this transfer were identified by immunohistochemistry and electron microscopy. The results show that carboxyfluorescein was directly exchanged between homologous B-cells and also between B- and A- or D-cells. Successive microinjections of the probe into different cells of the same cluster showed the existence of separate territories, each formed by 2–8 communicating cells. Intercellular communication was not observed after every dye microinjection, and communicating and noncommunicating islet cells were found to coexist within the same cluster. The data indicate that the exchange of exogenous cytoplasmic molecules occurs between different types of endocrine islet cells. However, within a single cluster, all islet cells are not metabolically coupled to one another, at a given time.

Ionic and metabolic cell-to-cell coupling (1) have been recently demonstrated between B-cells of the islets of Langerhans (2, 5, 8, 11). In view of the heterogeneous endocrine cell composition of the islet (14) and of the possible functional interactions between various islet cells (for review, see reference 22), it was important to assess whether coupling occurred between different islet cell types.

In the present study we have identified by immunohistochemistry and electron microscopy the islet cells involved in the direct exchange of 6-carboxyfluorescein, an exogenous fluorescent probe (376 mol wt) to which the cell membrane is impermeable (20). Our observations show that, in monolayer culture of neonatal endocrine pancreas, B-cells directly communicate not only with other B-cells but also with neighboring A- and D-cells, indicating that both homologous and heterologous coupling occur between differentiated islet cells. The metabolically coupled cells were found to form separate territories and to coexist with uncoupled cells within the same cluster.

MATERIALS AND METHODS

Islet Cell Cultures

Monolayer cultures of islet cells were prepared by a modification of a collagenase-trypsin method (6) as described (18). Briefly, the pancreases of newborn Wistar rats were enzymatically dissociated by ten successive 10-min incubations at 37°C in a Ca⁺⁺- and Mg⁺⁺-free phosphate-buffered saline (PBS) containing 5.6 mM glucose, 5 mg/ml trypsin (1:250, Difco Laboratories, Detroit, Mich.) and 0.2 mg/ml of collagenase (CLS IV, Worthington Biochemical Corp., Freehold, N. J.). The dissociated cells, collected by decantation at the end of

each of the last eight incubations, were pooled, rinsed twice by centrifugation in medium 199 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum and 16.7 mM glucose, resuspended in this medium, and plated at a concentration of 7.5×10^5 cells/ml on 100-mm plastic dishes (Falcon Plastics, Oxnard, Calif.). This primary cell suspension was cultured at 37°C in a water-saturated air/CO₂ (95/5%) atmosphere and decanted twice in new dishes, after 16 and 22 h of culture. At the time of the last decantation, the enriched suspension of islet cells was transferred to round cover glasses fitted in 35-mm Petri dishes and grown on medium 199. One-week-old cultures were used in the experiments. At that time, islet cells are differentiated as judged by their ultrastructure (15), their hormone content (6, 14), and their normal response to secretagogues (6, 18).

Microinjection Experiments

Individual islet cells were microelectrophoretically injected with 6-carboxyfluorescein (Eastman Kodak, Rochester, N. Y.) and observed by fluorescence microscopy, as previously described (4). After removal of the microinjectors, the selected cluster was photographed under both phase-contrast and fluorescence microscopy and identified on the cover glass with a diamond marker objective. In some experiments, two or three injections were carried out successively in different islet cells of the same cluster.

Immunohistochemistry

For light microscopy, the microinjected cultures were fixed in Zamboni's fluid (21), rinsed several times in PBS, dehydrated, and subsequently rehydrated through a graded ethanol series. They were then incubated for 2 h at room temperature with an anti-insulin serum (a gift from Dr. P. H. Wright) at a 1:200 dilution in PBS. After two 5-min washes in PBS, an anti-guinea pig IgG antiserum conjugated with horseradish peroxidase (RAGp/IgG [H + L] PO) (Nordic Immunological Laboratories, Tilburg, The Netherlands) was applied at a 1:50 dilution for 1 h at room temperature. After washing in PBS, the peroxidase was revealed, according to (3), by incubating the cultures in 0.05 M Tris buffer, pH 7.6, containing 100 mg% of 3,3'-diaminobenzidine and 30 μ l of 30% H₂O₂. When

adequate staining was observed, the cover glass was immersed in distilled water, mounted on a microscope slide, and examined on a Zeiss ICM 405 inverted microscope.

Electron Microscopy

For ultrastructural studies, the microinjected cultures were fixed at room temperature in 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.4, postfixed for 1 h in 1% osmium tetroxide, dehydrated in graded ethanols, and embedded in Epon. After removal of the bottom of the dish by immersion in liquid nitrogen, the identification mark surrounding the injected cluster was localized on the embedded cover glass under a phase-contrast microscope. A small block of Epon supporting the microinjected cluster was then cut from the sample, immersed for 5 min in warm (85°C) water and, subsequently, rapidly

transferred to liquid nitrogen. This procedure usually resulted in a clean separation of the cover glass from the polymerized plastic without damage to the cells. The injected cluster, sometimes stained in bloc (13), was recognized by comparison with the initial photograph taken in the Petri dish and serially sectioned, parallel to the support plane, with a LKB Ultratome (LKB-Products AB, Bromma, Sweden). Sections were collected on single-hole carbon-coated grids, stained with uranyl acetate and lead citrate, and observed in a Philips EM 301 electron microscope.

Identification of Communicating Islet Cells

At the light microscopic level, communicating B-cells were readily identified by comparing the phase-contrast and fluorescence photographs of each injected cluster with photographs of the same field after immunoperoxidase staining.

TABLE I
6-Carboxyfluorescein Microinjections in Monolayer Cultures of Islet Cells

Number of microinjected clusters	Number of intracellular microinjections	Absence of intercellular communication	Presence of intercellular communication	Number of communicating islet cells	Coexistence of communicating and noncommunicating islet cells in the same cluster*	Presence of separate territories of communicating islet cells*
96	172	57 (33.1)	115 (66.9)	3.5 ± 0.2‡	18 (45)	37 (92.5)

* In these columns, values in parentheses are expressed as percent of the number (n = 40) of clusters in which two or more islet cells were successively microinjected.

‡ Expressed as mean ± SEM. Values in parentheses are expressed as percent of the total number of microinjections.

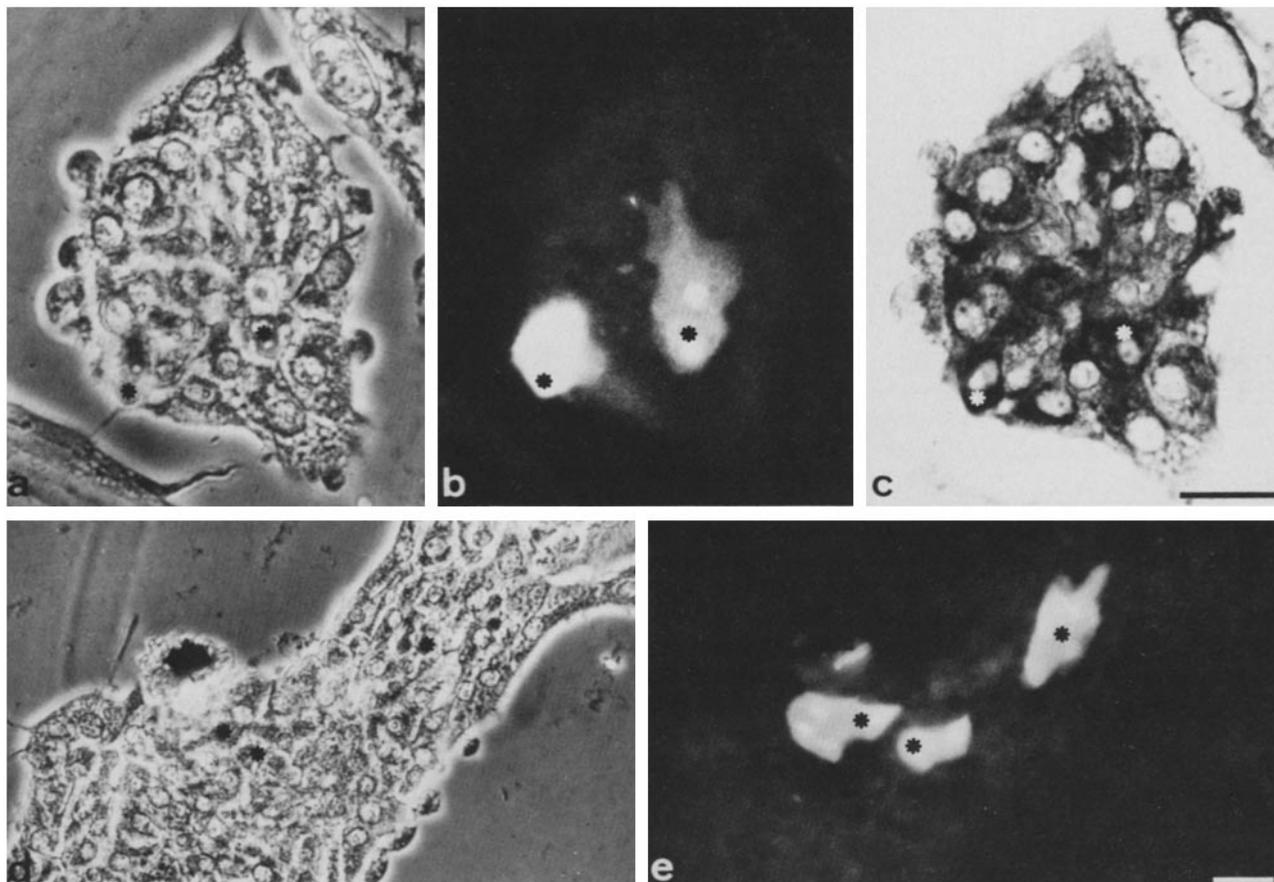


FIGURE 1 (a) In this cluster, two different islet cells (asterisks) were successively microinjected with 6-carboxyfluorescein. (b) After both injections, the dye was rapidly transferred from the injected into 2-3 neighbouring islet cells, delineating two separate territories of communicating cells. In this photograph, taken at the end of the experiment, the fluorescence of the first injected territory (right) has slightly faded whereas that of the second territory (left) is bright. (c) After immunoperoxidase staining using an anti-insulin serum, dark peroxidase reaction product stains most of the cells forming the injected cluster, including those of the two communicating territories. Thus, in this case, carboxyfluorescein was directly exchanged between insulin-containing B-cells. (a, b, and c: Bar, 25 μ m. \times 540). (d) Three islet cells (asterisks) of this large cluster were successively microinjected with 6-carboxyfluorescein. (e) In the three cases, the dye spread from the injected into some of the adjacent islet cells and delineated, within the cluster, three separate territories of coupled cells. (d and e: Bar, 25 μ m. \times 330).

At the ultrastructural level, communicating islet cells were identified by comparing the electron microscopic reconstruction of the whole injected cluster with its phase-contrast and fluorescence photographs. In all communicating islet cells that could be positively identified, a swelling of the rough endoplasmic reticulum and mitochondria and a decreased electron density of all cellular compartments was observed. As these alterations showed a decreasing gradient from the injected to the adjacent communicating cells and contrasted with the good preservation of the neighboring noncommunicating cells, we used them as an additional marker to localize the coupled cells within each cluster. A-, B- and D-cell types were identified on the basis of the characteristic ultrastructural appearance of their respective secretory granules (14, 15), a procedure that is consistent with the immunocytochemical localization of the corresponding islet hormones (19; unpublished data for the monolayer cultures).

RESULTS

As shown in Table I, 67% of the microinjections of carboxyfluorescein were followed by the transfer of the dye from the injected into some of the adjacent endocrine islet cells (Fig. 1 *a-e*). However, in 33% of the cases, no intercellular transfer of the dye was observed (Fig. 2 *a-f*). When intercellular transfer occurred, carboxyfluorescein did not usually spread throughout the entire cluster but rather delineated small territories formed by 2-8 endocrine cells (Table I). Successive microinjections of different islet cells showed that several communicating territories, usually not contiguous, could exist within the same cluster (Fig. 1 *a-e*). In addition, the coexistence of communicating and noncommunicating islet cells was observed (Fig. 2 *d-f*).

Most of the communicating and noncommunicating islet

cells were identified as insulin-containing B-cells by either their immunoperoxidase staining with the anti-insulin serum (Figs. 1 *c* and 2 *c*) or the occurrence of characteristic secretory granules in their cytoplasm (Fig. 3 *b* and *c*). Fig. 3 *c* shows that junctional specializations could be found between the communicating B-cells as well as between these cells and their noncommunicating neighbors. In 3 out of the 36 microinjected clusters that were analyzed by electron microscopy, communicating islet cells were found to contain different types of specific secretory granules, indicating heterocellular communication. In two of these cases, secretory granules were typical of glucagon-containing A-cells and insulin-containing B-cells (Fig. 4 *b* and *c*). In the third case, secretory granules were characteristic of a somatostatin-containing D-cell and of several insulin-containing B-cells (data not shown).

DISCUSSION

Previous studies have shown that small exogenous and endogenous molecules to which the cell membrane is impermeable can be rapidly transferred between adjacent pancreatic islet cells in culture (5, 8). The present observations using carboxyfluorescein, another impermeant fluorescent probe of comparable molecular weight (20), show that this transfer takes place not only between homologous insulin-containing B-cells but also between B-cells and heterologous glucagon-containing A-cells or somatostatin-containing D-cells. So far, the data available indicate that the observed incidence of homologous and

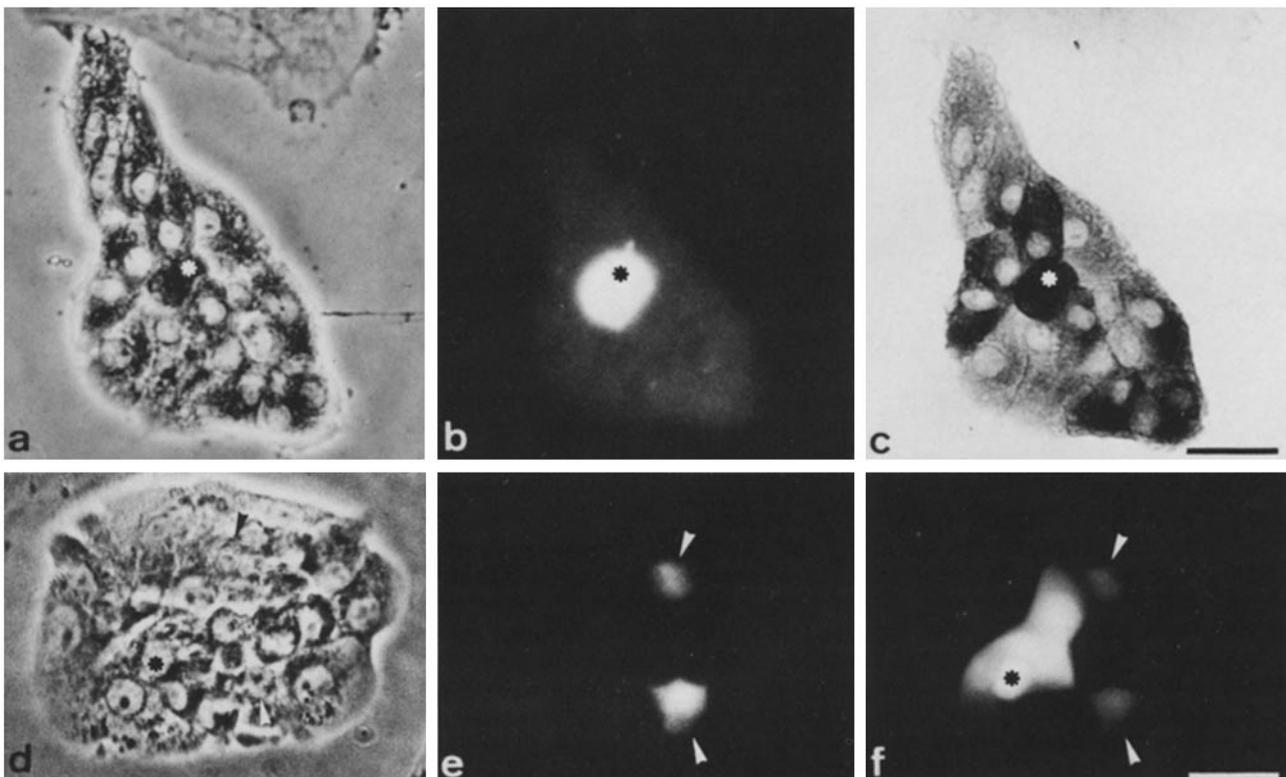


FIGURE 2 (a) An islet cell (asterisk) was microinjected in this cluster. (b) The fluorescence photograph taken several minutes after the microinjection shows that, in this case, the dye did not pass into the neighboring islet cells. (c) The dark staining after peroxidase reaction shows that both the injected noncommunicating cell and several of its neighbors were insulin-containing B-cells. (a, b, and c: Bar, 25 μm . \times 490). (d) In this cluster, 6 carboxyfluorescein was successively microinjected into three different islet cells (asterisk and arrowheads). (e) The fluorescence photograph taken after the two first injections shows that, in both cases, the dye failed to move from the injected (arrowheads) into the neighboring islet cells. (f) In contrast, the third injection (asterisk) was rapidly followed by the intercellular exchange of the fluorescent dye, showing that communicating and noncommunicating islet cells can coexist, in close proximity, within the same cluster. (d, e, and f: Bar, 25 μm . \times 500).

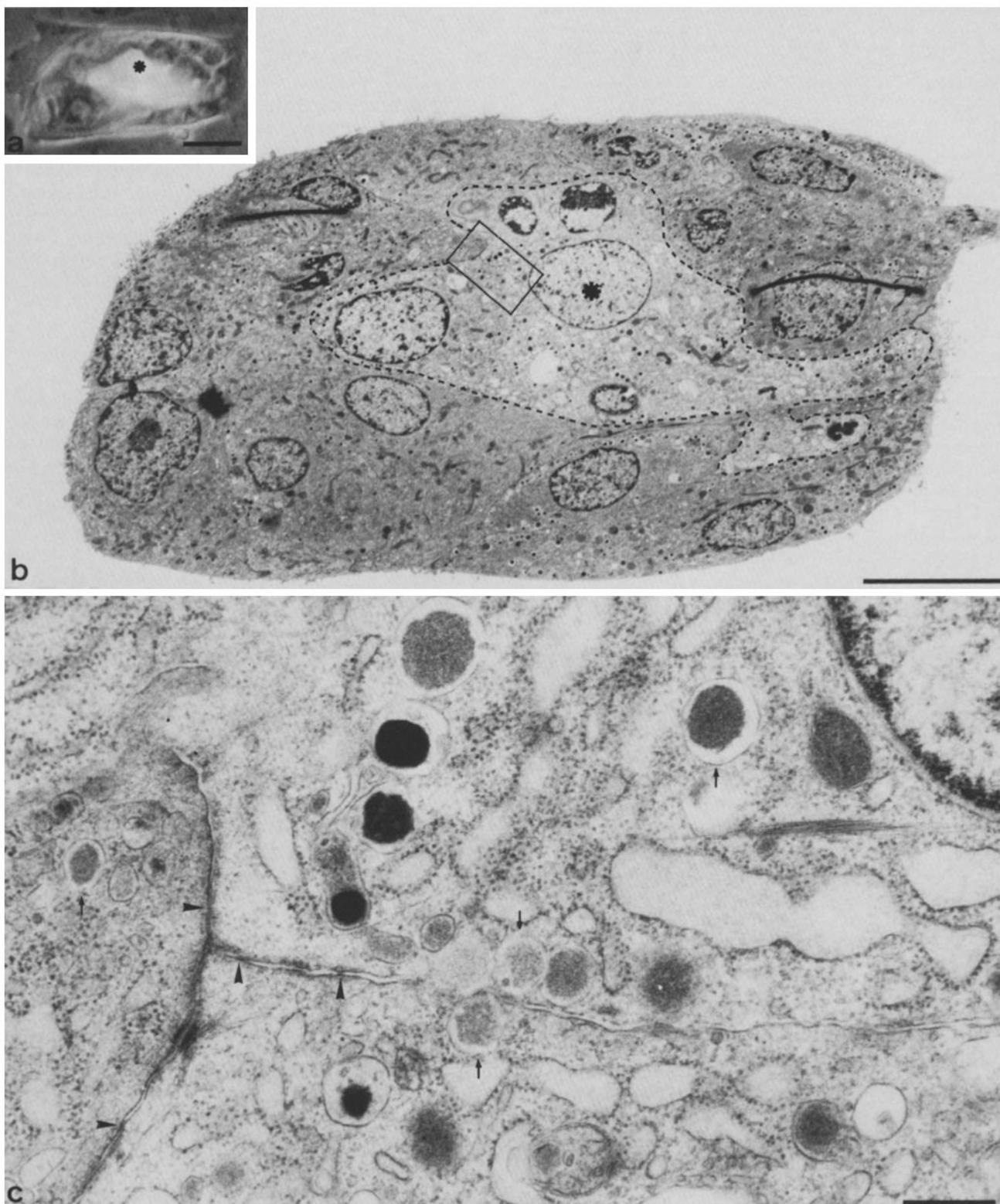


FIGURE 3 (a) 6-carboxyfluorescein was injected into an islet cell (asterisk) of this cluster. As shown in this combined fluorescence and phase-contrast photograph, the coupled islet cells which have exchanged the dye form a fluorescent territory in the center of the cluster. (Bar, 25 μm . \times 400). (b) At the ultrastructural level, the communicating territory (limited by the broken line) was identified by its central position within the cluster and by the clear appearance of the coupled cells. The area outlined by the rectangle is shown at higher magnification in C. (Bar, 10 μm . \times 2,400). (c) Higher magnification view of the area outlined by the rectangle in b shows the periphery of the microinjected cell and of two of its neighbors. Of these, only one (top) was involved in the direct exchange of carboxyfluorescein. Typical beta secretory granules (arrows) are present in the cytoplasm of the three cells that can thus be identified as differentiated insulin-containing B-cells. Note that junctional differentiations (arrowheads) connect both the two communicating B-cells and their non-communicating neighbor. (Bar, 0.5 μm . \times 32,700).

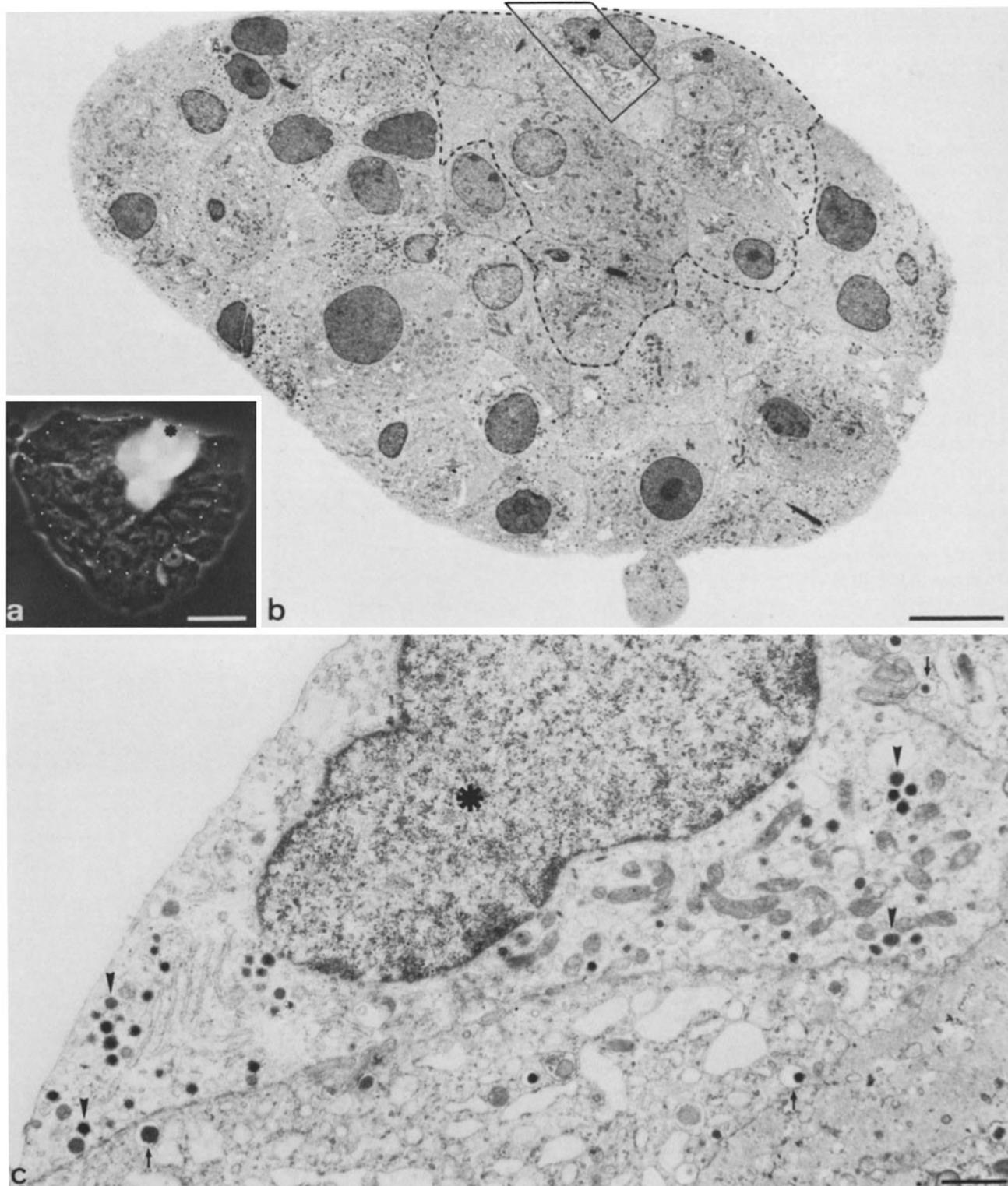


FIGURE 4 (a) After injection into a cell (asterisk) at the periphery of this cluster, 6-carboxyfluorescein spread into several islet cells. The dotted line limits the part of the cluster that is seen, after thin section, in Fig. 4 b. (Bar, 25 μm . $\times 400$). (b) At the ultrastructural level, the communicating territory (limited by the broken line) was identified by its peripheral position within the cluster and by the slight, selective alteration of the coupled cells. The area outlined by the trapezium is shown at higher magnification in Fig. 4 c. (Bar, 10 μm . $\times 1,650$). (c) Higher magnification view of the area outlined by the trapezium in Fig. 4 b and showing part of three communicating islet cells. In the peripheral microinjected cell (asterisk), secretory granules (arrowheads) are small and dense whereas in the neighboring communicating cells secretory granules (arrows) are larger and show a clear halo between their core and their limiting membrane. This different appearance, characteristic of α - and β -granules, respectively, indicates that, in this case, carboxyfluorescein was directly transferred from a glucagon-containing A-cell into several adjacent insulin-containing B-cells. (Bar, 1 μm . $\times 11,200$).

heterologous coupling (91.6 and 8.3%, respectively) is consistent with the different numerical proportions of B- and non-B-cells present in the cultures (an electron microscopic evaluation of 1,619 cells from 28 clusters of a 3-d-old culture showed that B-, A- and D-cells represented 78 ± 2 (mean \pm SEM), 9 ± 1 and $10 \pm 1\%$ of the total islet cell population, respectively).

Rat islet cells in culture were found to exchange carboxyfluorescein with only a limited number of their neighbors, and about one-third of them failed to transfer the dye after microinjection. Although, in some cases, this may reflect a damage of the microinjected cell and/or the limited sensitivity of the dye transfer method (for critical discussion, see references 7 and 20), the fact that similar observations were done with impermeant metabolites, which are spontaneously incorporated by the cells (8), suggests that, within a monolayer cluster, only some islet cells exchange cytoplasmic molecules at a given time. This apparently limited intercellular transfer might be one of the possible explanations for the existence, within individual clusters, of discrete communicating territories. An alternative explanation for these apparent compartments, i.e. that they result from the juxtaposition of undissociated cell clumps that retain their native junctions but are unable to form permeable junctions with neighboring clumps, seems unlikely. Indeed, most of the clusters studied in our cultures were derived from undissociated portions of disrupted islets much larger than the communication territories which, usually, were formed by only 2–8 cells.

The biological significance of these findings is unknown as is the relationship between our *in vitro* data and data on islet cells *in vivo*. However, the occurrence of gap junctions (16, 17) and of dye transfer (12) between cells of intact isolated islets suggests that metabolic coupling also occurs between homologous and heterologous islet cells *in situ*. A modulation of such coupling, as suggested by the observations of gap junction changes with B-cell function (9, 10), may participate in the complex regulation (22, 23) of A-, B-, and D-cell activity.

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