

**ENDOGENOUS GLUCAGON-CELLS ARE NECESSARY FOR THE GLUCOSE-
RESPONSIVENESS OF THE HIT-T15 HAMSTER β -CELL LINE**

by

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ABSTRACT

The role of glucagon in glucose-stimulated insulin release was investigated using the hamster insulinoma cell line, HIT-T15, as a model of the pancreatic β -cell.

Glucose-stimulated insulin release from HIT-T15 cells was concentration-dependent over a range of glucose concentrations between 0 and 15 mM, with immunoreactive-insulin release (IRI) rising significantly above the basal level (zero glucose) when cells were exposed to glucose concentrations of 5, 10 and 15 mM ($p < 0.02$). Minimum (zero glucose) and maximum (15 mM glucose) insulin release was 1.6 ± 0.5 % and 12.0 ± 2.9 % of total cell content (TCC), respectively. In HIT-T15 cells, glucagon secretion was 5-9 % of total cell content during a 1 h release experiment. There was no significant difference between glucagon released in the presence of zero- or high (15 mM)-glucose.

The addition of a glucagon antibody completely abolished glucose-stimulated insulin release, while antibodies raised against somatostatin and glucose-dependent insulinotropic polypeptide (GIP) had no effect. When HIT-T15 cells were incubated in conditions of zero-glucose and glucose plus glucagon-Ab there was no significant difference in insulin release ($p > 0.05$). Furthermore, glucagon-Ab inhibited glucose-stimulated insulin secretion over the full range of glucose responsiveness (5 to 15 mM; $p < 0.03$). The abolition of glucose-stimulated insulin secretion by an antibody to glucagon indicates that glucose-stimulated insulin secretion is dependent on glucagon, probably acting via a receptor-dependent pathway. HIT-T15 cells that were co-cultured with glucagon producing InR1-G9 cells, displayed basal insulin release that was elevated, with an absence of glucose potentiation.

HIT-T15 cells were immunoperoxidase stained for the following peptide hormones: insulin, somatostatin, glucagon, glucagon-like polypeptide-I, GIP, secretin, pancreatic polypeptide (PP) and peptide-tyrosine-tyrosine (PYY). Staining was positive for all peptide hormones tested except somatostatin, secretin and GIP. Insulin, glucagon and PYY were the

dominant peptides. Double staining of HIT-T15 cells for insulin and glucagon demonstrated colocalization of both peptides within single cells. Additionally, HIT-T15 cells were immunoreactive for the cell surface adhesion molecule uvomorulin.

Immunocytochemical studies of InR1-G9 cells demonstrated the presence of glucagon, GLP-I and PP, with PP immunoreactivity present in every cell. However, no immunoreactivity for insulin, somatostatin, GIP, secretin, PYY or uvomorulin was seen. Equal amounts of HIT-T15 and InR1-G9 cells were co-cultured, and stained with fluorescent and immunoperoxidase techniques. HIT-T15 cell clusters appeared to be surrounded by InR1-G9 cells. These cell lines appeared healthy when cultured together, in spite of the fact that these two cell lines are derived from different strains of hamsters, by different vectors.

Electron microscopic examination revealed differences in HIT-T15 cell insulin secretory granule number with different culture conditions, including: low-glucose media (0.8 mM), high-glucose media (11.1 mM) and high-glucose media (9.1 mM) plus InR1-G9 cells. Most importantly, HIT-T15 cells cultured in high-glucose (11.1 mM) were extensively granulated, while those cultured in high-glucose (9.1 mM) in the presence of InR1-G9 cells were agranular.

In conclusion, glucose-stimulated insulin secretion from the HIT-T15 cell line is glucagon-dependent, and endogenous glucagon containing cells (HIT-T15-G) can provide the glucagon. The addition of glucagon secreting cells (in large numbers) led to chronic stimulation of HIT-T15 cell secretion, and secretion could not be potentiated by glucose. Furthermore, co-culturing HIT-T15 cells with equal numbers of InR1-G9 cells led to a complete degranulation of HIT-T15 cells, most likely by continuous stimulation. The HIT-T15 cells are a heterogeneous cell population, expressing multiple peptides, some of which are colocalized, as in the case of insulin and glucagon. InR1-G9 cells appeared to be relatively homologous with respect to peptide production. These cells extended neuron-like processes, which contained the majority of the secretory granules.

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HYPOTHESIS

Glucose-stimulated insulin secretion, from the HIT-T15 hamster pancreatic β -cell clone, is influenced by endogenously secreted glucagon.

SPECIFIC OBJECTIVES

1. To demonstrate that insulin secretion from HIT-T15 cells can be regulated by glucagon. To enhance this insulin release by introducing glucagon secreting cells. Also, to provide evidence for paracrine or autocrine modulation of glucose-stimulated insulin release by glucagon in HIT-T15 cells, using the immunoneutralization technique.
2. To characterize the HIT-T15 and InR1-G9 cell lines with respect to peptide production.
3. To examine the ultrastructural appearance of HIT-T15 cells cultured in the presence or absence of the glucagon secreting cell line InR1-G9.

RATIONALE

HIT-T15 cells have been used extensively as a pancreatic β -cell model since their creation in 1981, however, they have not been thoroughly characterized during this time. More recently there has been the discovery of a 'contaminant' cell in the HIT-T15 cell line. This 'contaminant' consists of glucagon producing cells which have been designated as the 'HIT-T15-G' cell (85). The purpose of these studies was three fold. Firstly, glucagon immuno-neutralization was used to determine the effect of HIT-T15-G cells on insulin secretion in the HIT-T15 cell line. Secondly, characterization of HIT-T15 cell line peptide production was performed in order to determine whether or not HIT-T15 cells express regulatory peptides, other than insulin and glucagon, which may also affect insulin secretion from this cell line. Thirdly, this peptide characterization in conjunction with studies of HIT-T15 cell ultrastructure were used to determine the validity of the HIT-T15 cell line as a β -cell model.

CHAPTER ONE - BACKGROUND

I. Islet of Langerhans Background.

A. Islet Structure and Cell Distribution.

Nutrient homeostasis is one of the most important of all physiological functions. The task of maintaining an adequate fuel supply for the body's cells falls mainly on the islets of Langerhans. The islets of Langerhans (islets) are cell clusters of insulin (β), glucagon (α), somatostatin (δ) and pancreatic polypeptide (PP) containing cells. These four islet cell types are probably differentiated from a peptide-tyrosine-tyrosine (PYY) progenitor cell, as the four major islet peptides are often co-localized with PYY during development (22, 73, 151). Islets are found in the pancreas of mammals (40, 46, 64, 69) as well as the duodenal wall of rats (12). The average adult human has approximately 500,000 islets, accounting for 1 to 2% of total pancreatic volume (39). Islets range in size from 40 to 700 μm , with most being 100 to 200 μm in diameter (158). Within the islet, β -, α -, δ - and PP-cells account for about 70%, 10-15%, 5-10% and 5% of cells, respectively (39). In adult rats the core of the pancreatic islet contains mainly β -cells, surrounded by a peripheral mantle of α -, δ - and PP-cells (108). However, in humans, α - and δ -cells penetrate the β -cell core as intermixed cords (24); while, in the common tree shrew (Tupaia glis), the α - and δ -cells compose the islet core, and are surrounded by β -cells (7). The proportions of specific islet cells varies between islets from different regions of the pancreas, as demonstrated in the rat (147). Islets isolated from the rat dorsal pancreas are rich in α - and δ -cells when compared to islets from the ventral pancreas which contain a greater proportion of PP-cells.

The complex arrangement of α -, β - and δ -cells is thought to be important in the normal functioning of the islet, possibly by facilitating gap-junction and/or paracrine communication between cells (108). The highly ordered structure of the islet is partly due to differential cell

adhesion molecules among the islet cells. Different levels of Ca^{2+} -dependent (uvomorulin) and Ca^{2+} -independent cell adhesion molecules are found on heterogeneous islet cell types (10, 51, 125). These cell adhesion molecules allow dispersed islet cells to re-aggregate into organized islet-like structures similar to non-disrupted islets (51, 125). Both glucose and cyclic-adenosine-monophosphate (cAMP) promote the adhesion of β -cells (87, 103). As suggested above, it is believed that islet adhesion and/or junctional communication play a role in the regulation of hormone secretion from individual β -cells (133). Cell adhesion molecules are involved in the regulation of gap-junctional communication in other systems, such as mouse epidermal cells where communication is directly regulated by a Ca^{2+} -dependent cell adhesion molecule (E-cadherin). E-cadherin is involved in post-translational assembly and/or function of the gap-junction protein connexin-43 (65).

In addition to the complex arrangement of islet cells, there exists a complex islet microvasculature. Studies by Stagner and associates suggest that this microcirculation flows from β - to α - to δ -cells in the rat (19, 137) and dog islet (136). Additionally, human islet β -cells may also be perfused before α - and δ -cells (138). On the contrary, a study of the rat islet by Lui and associates describes blood flow from mantle to core to mantle, as follows (84):

"The arterioles first reach the surrounding mantle of the islet where they divided into capillaries that go to other portions of the mantle or the core of the islet, the flow then traverses the core and returns to different portions of the mantle. The flow is nonhomogeneous in that flow in one portion of the islet can stop, then move on, while other portions flow freely."

Another study in rats suggested that different modes of microcirculation exist in different sized islets, for example (158): (a) small islets (40-150 μm in diameter) had serial vasculature only, (b) midsize islets (160-250 μm) were found to have serial vessels, in addition to postcapillary collecting venules that were directly continuous with larger interlobular veins,

indicating parallel microcirculation, and (c) all large islets (260-700 μm) were also found to have both serial as well as parallel microcirculatory patterns within the same islet. In the tree shrew, the insular arteriole, a branch of the interlobular artery, penetrates deeply into the core of the islet before branching off into the glomerular capillary network supplying the islet (7). These capillaries reunited at the periphery of the islets to become vasa efferentia and then gave off capillaries to anastomose with those in the exocrine part of the pancreas.

This ordered perfusion of cells within the islet suggests that islet hormones may act within the islet in an endocrine manner. This view is further supported by an electron microscopic study of rat islet δ -cells, where 75 % of granules were polarized to the capillary end of the cell, suggesting that pancreatic somatostatin is released into islet capillaries (5). The precise nature of these endocrine effects appears to differ with species and islet size.

Glucagon, insulin, somatostatin and pancreatic polypeptide are also secreted from α -, β -, δ - and PP-cells found within the pancreatic duct epithelial lining of the normal adult rat, and human (109, 162). Alpha- and δ -cells found in the mammalian gastrointestinal tract are another source of glucagon and somatostatin, respectively (8, 60, 74). It is possible that hormones secreted from these duct and gastrointestinal endocrine cells play a role in the regulation of pancreatic-islet secretion and/or nutrient metabolism throughout the body.

B. Islet-Cell Ultrastructure.

1. Beta-Cells.

Beta-cells have numerous electron dense secretory vesicles that are about 300 nm in diameter (80, 98). The electron dense core is composed of rhomboidal or polygonal crystalloids, believed to be insoluble insulin (80, 98). A second insulin secretory vesicle, that is spherical and shows no subunit structure, exists (80, 98). These two secretory granule types probably represent different stages of granule maturity (80, 98).

Beta-cells have nearly spherical mitochondria that are more numerous than those of the α -cell (80, 98, 126). The Golgi apparatus is also more extensive in the β - than the α -cell (80, 98). Additionally, the rough endoplasmic reticulum (RER) is sparse in β -cells when compared with acinar cells (80, 98), but is greater than that of α -cells (126). Microtubules and microfilaments within the cytoplasm are concentrated near the capillary pole of the cell, with microtubules arrayed in association with insulin secretory vesicles probably playing a role in secretory granule exocytosis (80, 98).

2. Alpha Cells.

Identifying features of the α -cell are filamentous mitochondria and small Golgi apparatus (80, 98). Alpha-cell nuclei are indented or lobulated (80, 98). Secretory vesicles are uniform in diameter, at approximately 250 nm, with a highly electron dense core embedded in a matrix of less dense material. The secretory vesicles are closely surrounded by a limiting membrane (80).

C. Insulin, Glucagon and Somatostatin Receptors in the Islet.

Receptor binding and internalization of radiolabelled insulin, glucagon and somatostatin are time- and temperature dependent in islet cells (2). Endocytosis and lysosomal degradation of radio-labeled peptides occurs freely in cells heterologous, but poorly in cells homologous for the peptide. For example, receptor bound glucagon is rapidly endocytosed and degraded in δ - and β -cells, but only slowly in α -cells (4, 155). There are, however, specific low-affinity receptors for insulin, glucagon and somatostatin on cells homologous for these peptides (9). The presence of insulin, glucagon and somatostatin receptors on α -, β - and δ -cells supports the possibility of paracrine and/or autocrine regulation within the pancreatic-islet.

D. Paracrine Interactions Between α -, β - and δ -cells.

The existence of 'parakrinen' (paracrine) hormones was suggested by Feyrter in 1953 (42). Paracrine hormones are substances which act locally via the interstitial fluid rather than in a humoral (endocrine) manner. Somatostatin is thought to act, via cytoplasmic processes, as a paracrine inhibitor of gastrin and acid secretion within the mammalian stomach (60, 74, 76). Furthermore, somatostatin has also been suggested to be a paracrine inhibitor of α - and β -cells within the islet (2). The cytoplasmic processes found on some δ -cells have been reported on the α -cells of amphibians (104, 166) and mammals (8, 109). Cells possessing these cytoplasmic processes are sometimes called pseudo-neurons, because of their neuron-like appearance.

Paracrine interactions in the islet may be facilitated by tight-junctions, which might function to prevent diffusion of released hormone away from its 'target' (106, 107). The number of tight-junctions has been shown to increase with increasing glucose concentration and exposure time, suggesting an involvement of tight-junctions in the regulatory process of glucose-induced insulin secretion (130). However, the existence of tight-junctions in normal islets has been brought into question, as they were not seen in one study, that used freeze-fracture replicas of *in situ* rat or human islets kept in long term culture (58). It was suggested that the disruption of islets with collagenase and trypsin during isolation led to tight-junction formation, as a protective measure, intended to seal and protect islet micro-domains against sudden perturbations in local interstitial fluid (58).

II. The Structure and Function of Insulin and Glucagon.

The hormones insulin and glucagon appear to play the dominant role in nutrient homeostasis, within the body. Insulin and glucagon oppose one another in almost all of their respective nutrient homeostatic actions.

A. Insulin.

Insulin is a 51 amino acid polypeptide (m.w. 5800) which is secreted from the β -cell (108). The insulin gene, which is located on human chromosome 11, is transcribed to yield an insulin precursor (preproinsulin) with a molecular weight of 11,500 (16). Preproinsulin contains four regions (signal, A, connecting and B). The signal region is cleaved, and the remaining regions fold upon one another, with the A and B regions aligning and forming disulfide bridges. The connecting region is then cleaved to yield insulin and C-peptide.

Insulin release is stimulated under conditions of fuel excess and is inhibited under conditions of fuel deficiency. Fasting, basal and postprandial plasma insulin concentrations are approximately 5, 10 and 30-100 μ U/ml, respectively (16). Circulating insulin has a half-life of 6 to 8 min (16). The main functions of insulin are to promote the uptake, utilization and storage of nutrients, in liver, muscle and adipose tissues (150). Insulin acts through receptor-regulated glucose transporters, to stimulate glucose uptake into muscle and adipose tissues (16). In muscle, fuel utilization is increased by an increase in muscle glycolysis and glucose oxidation (143). Increased fuel storage is accomplished by increasing glycogenesis and protein anabolism (29, 62, 143). Additionally, increased storage is promoted by a decrease in glycogenolysis and gluconeogenesis (37). Insulin further reduces circulating nutrients by acting as a CNS satiety factor (16). Finally, hyperinsulinemia is often associated with obesity (63), and in the absence of insulin (diabetes) the body's cells literally starve as high levels of glucose in the blood stream can not be utilized.

B. Glucagon.

Alpha-cells secrete glucagon (m.w. 3485), a 29 amino acid polypeptide, (107, 150), that is a member of a highly conserved family of peptides. The glucagon family of peptides includes glucagon, glucagon-like peptide-I and II (GLP-I and GLP-II), glucose-dependent insulintropic polypeptide (GIP) and secretin (16, 35, 78, 153). These peptides are derived from the same

(glucagon, GLP-I and GLP-II), or closely related gene(s), and undergo post-translational processing to their respective forms (35). The glucagon gene is expressed in α -cells of the pancreas, and in endocrine L-cells of the gastrointestinal tract (111), and in the brainstem and hypothalamus (33). The mRNA transcripts that arise from these genes are identical (33). The apparent molecular weight of the preproglucagon molecules is approximately 16,000 Daltons (132). Preproglucagon is processed to proglucagon a polypeptide precursor containing glucagon, GLP-I and GLP-II, and an intervening peptide (111). The preproglucagon sequence encodes a 20-amino acid signal sequence followed by a 69 amino-acid sequence for the peptide glicentin. Glicentin contains the amino-acid sequence for glucagon. The glicentin sequence is followed by two additional glucagon-like peptides (83).

Basal plasma glucagon levels are approximately 100 pg/ml, with glucagon having a half-life of 6 min (16). Glucagon is important in normal promotion of fuel mobilization from liver, muscle and adipose tissues. It is also important in preventing hypoglycemia during serious injury, shock and starvation (150). In times of stress, it could be said that glucagon is the protector of the central nervous system as neurons have an absolute need for glucose, or ketones after prolonged starvation, to survive (43). During starvation when glycogen stores are exhausted, glucagon-dependent mechanisms convert free fatty acids to ketones which can be used in place of glucose by the CNS (43). In the maintenance of normal nutrient homeostasis glucagon maintains cellular fuel levels via receptor mediated mechanisms that: 1) increase glycogenolysis, gluconeogenesis and β -oxidation of free fatty acids (FFA), and 2) reduce triacylglyceride synthesis and glycogenesis (16). Interestingly, glucagon, like insulin, has been reported to regulate appetite by acting on the CNS (16). Antisera against glucagon lead to a decrease in food intake and body weights in rats (86), thus glucagon probably stimulates appetite.

III. Insulin and Glucagon Secretion.

A. Insulin Secretion from the β -Cell.

1. Secretory Stimuli.

The regulation of islet β -cell secretion is multi-faceted, and involves numerous neural, hormonal and nutrient inputs.

During the cephalic phase of digestion, the thought, sight, smell and taste of food can cause the autonomic nervous system (ANS), to stimulate insulin secretion (16). This predominantly vagal ANS stimulation involves sympathetic (β -adrenergic) and parasympathetic (acetylcholine, muscarinic) pathways (27, 44, 57, 77, 121). Adrenal medullary epinephrine also stimulates β -adrenergic pathways (16).

Following the cephalic phase, and during the gastric and intestinal phases of digestion, direct enteric innervation of the pancreas and/or islets may also play a role in the regulation of insulin secretion (70). The gastric and intestinal phases of digestion are partly regulated by peptide hormones which are secreted from the gastrointestinal tract and the pancreas. These insulinotropic enteric peptides, known as incretins, include the following: enteroglucagon (16), truncated forms of glucagon-like polypeptide-I (GLP-I; 7-36 amide and 7-37) (41, 121), GIP (25, 66, 105, 129), secretin (45) and cholecystokinin (121). Other hormones that stimulate insulin secretion are pancreatic glucagon (127) and epinephrine secreted from the adrenal medulla (16, 27, 44, 77).

Circulating nutrients that stimulate insulin secretion include: glucose, amino acids, free fatty acids and keto acids. The amino acids that stimulate insulin secretion are leucine, lysine, alanine or arginine (114, 115, 127). Glucose is the primary nutrient stimulus for insulin secretion, with half-maximal and maximal stimulation at glucose concentrations of 150 and 300-

500 mg/dl, respectively. In order for glucose to act as a nutrient for the β -cell it must first enter the cell. Rat islets express the GLUT-2 glucose transporter isoform, which is a glucose carrier with a low affinity for glucose but a high capacity for glucose transport (165). To a lesser degree, a lower capacity glucose transporter isoform (GLUT-1) is expressed (165). In isolated islets the levels of GLUT-1 and GLUT-2 mRNA are increased by high-glucose when compared with low-glucose culture conditions (165). Additionally, a decreased expression of glucose transporters in diabetic rat β -cells is associated with impaired glucose sensing characteristics that may contribute to the diabetic state (144).

Briefly, when glucose is metabolized, a rise in ATP leads to the closure of an ATP-sensitive potassium channel, which in-turn leads to depolarization of the β -cell (18, 125). This glucose stimulated depolarization opens voltage-dependent Ca^{2+} -channels, which in-turn leads to an increase in intracellular Ca^{2+} and subsequently insulin secretion (64).

Receptor coupled G-protein (G_s) pathways can lead to a rise in cAMP and protein kinase-A (PKA) activity, which potentiate glucose-stimulated insulin release in the β -cell (49, 122). Glucagon acts potently and rapidly through this cAMP pathway (127). However, glucose on its own does not increase cAMP levels in purified β -cells, but can enhance glucagon-induced cAMP formation (127). Therefore, glucose-dependent rises in cAMP levels are not considered to be a nutrient-induced mediator for hormone release but rather as a minor amplification of the glucagon-dependent signal. (127). Neither insulin nor PP affect cAMP formation in pancreatic B cells (127).

In addition to cAMP-PKA mechanisms, inositol triphosphate (IP_3), diacylglycerol (DAG) and protein kinase-C (PKC) are also reported to be involved in the secretion of insulin from the β -cell (14, 120). Similar to PKA, PKC is also thought to act on voltage-gated Ca^{2+} channels to increase intracellular Ca^{2+} levels, and activate calcium-dependent secretory mechanisms (163). Intracellular Ca^{2+} stores found primarily in the endoplasmic reticulum, are released via an IP_3 -dependent pathway, to further increase free intracellular Ca^{2+} levels (15, 119, 124).

2. Inhibitors of Secretion.

The above second messenger systems act in concert to stimulate insulin secretion from the islet β -cell, but are antagonized by the inhibitory systems described below. There are numerous inhibitors of insulin secretion from the islet, including the following: somatostatin (27), PYY(17), insulin (16), norepinephrine- and epinephrine-activation of α -adrenergic receptors (27, 67, 77), galanin (27, 77) and prostaglandin- E_2 (77, 122).

Somatostatin is an important intra-islet inhibitor of glucagon, insulin and somatostatin secretion (2, 71, 139). Somatostatin inhibits insulin release at multiple sites of the β -cell secretory mechanisms, including: 1) reduced intracellular Ca^{2+} , 2) reduced cAMP, and 3) cell hyperpolarization. The reduction in intracellular Ca^{2+} is due to inhibition of dihydropyridine-sensitive (L-type)- and ω -conotoxin-sensitive (N-type) voltage-dependent Ca^{2+} -channels on the β -cell. This inhibition is mediated via a pertussis toxin-sensitive inhibitory G-protein called G_i (1, 54, 97, 149). Pertussis toxin irreversibly ADP-ribosylates G_i , rendering it nonfunctional (79, 117, 122), for at least five days following toxin removal (28). Somatostatin counteracts glucagon-induced cAMP production in purified β -cells (128), by the same pertussis toxin-sensitive G_i (27, 53, 54, 122, 149). Pertussis toxin acts at a common site to block somatostatin-induced Ca^{2+} inhibition, hyperpolarization and a reduction in cAMP levels (36, 72, 161). Hyperpolarization of β -cells inhibits insulin release by closing voltage-sensitive Ca^{2+} channels and possibly by closing gap junctions. In some systems somatostatin-dependent inhibition of Na^+ - H^+ exchange can cause an increase in extracellular and a decrease in intracellular pH (46). Interestingly, both of these pH alterations are known to reduce gap junction coupling in some systems. Finally, Nilsson and associates report that there is a decreased sensitivity of the secretory machinery of the β -cell to Ca^{2+} , and a direct inhibition of the exocytotic process, mediated by a pertussis toxin-sensitive G_i (100).

It is plausible that receptor mechanisms for prostaglandin- E_2 , α_2 -adrenoceptors, and galanin involve a common G protein(s)/potassium channel complex which transduces the

inhibitory signals. A common property of receptors which activate potassium channels via G_i and inhibit Ca^{2+} channels through G_o and/or G_i is that they also inhibit adenylate cyclase (77). G_i mediates decreases in intracellular cAMP caused by inhibitors of insulin secretion, e.g. epinephrine, somatostatin, prostaglandin- E_2 , and galanin (36). G-proteins also regulate ion channels, phospholipases and distal sites in exocytosis (122).

3. Modes of Secretion.

Insulin secretion occurs in two phases, as follows: phase-I is an initial peak which is followed by phase-II a sustained plateau (16). Possible explanations for this biphasic release are as follows: 1) two storage compartments that contain granules with different sensitivities to glucose, and 2) the second phase of insulin secretion is *de novo* synthesized insulin stimulated by glucose. It is likely that both of these mechanisms are active.

Additionally, protein secretion from endocrine cells can occur via several different means. Constitutive release refers to the secretion of proteins as fast as they are synthesized, without intracellular storage in typical granules (68). Typically most post-translational processing takes place prior to exit from the medial Golgi stack, however, hormones released via constitutive means do not undergo full post-translational processing, as occurs in the secretory granule prior to secretion.

Regulated secretion refers to secretion of previously produced proteins, that have been stored at high concentrations in secretory vesicles (68). Hutton and associates state that the insulin secretory granule of the pancreatic β -cell is a complex intracellular organelle comprised of many proteins with different catalytic activities and messenger functions (55). Secretory granules form at the trans-Golgi network (TGN) by envelopment of the dense-core aggregate of regulated secretory proteins by a specific membrane. This dense-core may be created via selective aggregation of the secretory protein, or sorting of the secretory protein by a sortase

(69). At this stage they are referred to as immature secretory granules (145). The immature secretory granule then undergoes a maturation process (post-translational modification) which gives rise to the mature secretory granule (146). Proinsulin is cleaved to biologically active insulin by two distinct Ca^{2+} -dependent endopeptidases that are found in the insulin secretory granules (6, 30). Type I cleaves between the B-chain and the C-peptide junction; Type II on the C-peptide/A-chain junction. Davidson and associates, report that there are specific Ca^{2+} and pH requirements for each of these proteases. These specific requirements indicate that Type I could only be active in the intragranular environment and type II in the Golgi apparatus or secretory granule. In a cell where both constitutive and regulated protein secretion occur, mechanisms exist for sorting the correct secretory protein into the correct secretory vesicle (69).

Following this, the secretory vesicle must then be delivered to the appropriate region of plasma membrane (68). Targeting to the cell surface involves the actin-based cytoskeleton and small GTP-binding proteins. Calcium-dependent contractile events involving cytochalasin B-sensitive microfilamentous structures provide the motive force for both the intracellular translocation and exocytotic release of beta granules (154). Secretion occurs when mature secretory granules fuse with the plasma membrane.

4. Heterogeneities of Secretion.

In addition to heterogeneities in cell types within the islet it has recently become apparent that the population of β -cells is not homogeneous. Beta-cell properties such as 1) hormone responsiveness, 2) insulin secretion, 3) insulin biosynthesis, 4) electrophysiology, and 5) ion (K^+ , Na^+ and Ca^{2+}) current all show variability (20, 53, 99). Although insulin secretory responses may vary between β -cells, they are usually homogeneous within a single β -cell upon successive stimulations (20, 47). Furthermore, β -cells that are undergoing *de novo* protein synthesis release insulin preferentially during stimulation (20). Finally, differences in acetylcholine-stimulated rises in intracellular Ca^{2+} have been reported in normal rat pancreatic β -

cells (156).

The heterogeneity in biosynthesis and secretion of insulin is reduced with β -cell contact (20). This reduction in heterogeneity could in part be due to a phenomenon termed '*recruitment*', which refers to the coordination of '*units*' of cells via the sharing of various intracellular signals through gap-junctional communication. The less responsive cells are *recruited* into the secreting *unit*, where they act in a homologous manner, possibly by decreasing their average threshold for stimulation. Longo and associates report patterns of Ca^{2+} change within islets that are consistent with the recruitment of cells (82). They state that there is a coexistence of oscillations with similar periods in insulin secretion, oxygen consumption, and cytosolic free Ca^{2+} , within communicating β -cell units. Additionally, stimulation of β -cells appears to lead to an increase in the size of coupled *units*. An example of altered coupling upon stimulation is seen with prolactin, a glucose-dependent insulin secretory hormone, which increases coupling (135). Prolactin appears to decrease the apparent glucose threshold for insulin release, while it increases the extent of dye coupling among β -cells (96, 134).

When compared with intact islets, single β -cells and re-aggregated β -cells respond promptly to glucose, but exhibit an elevated basal insulin secretion, and profoundly lowered peak- and total-insulin secretion (81, 87). Although weaker than whole islets, re-aggregated β -cells have a slightly stronger glucose-stimulated insulin secretion than isolated β -cells. An improved response is restored by simple cell-to-cell contact, but a lowered basal insulin secretion requires gap-junctional contacts between re-aggregated cells (113). Conversely, heterologous β -cell to non- β -cell contact was not effective in enhancing the recruitment of β -cells or increasing their individual secretion (21).

B. Glucagon Secretion from the α -Cell.

Islet α -cells are electrically active, in the absence of glucose (159), and require Ca^{2+} for glucagon release (114, 115). Glucagon secretion is stimulated by amino acids, enteric peptides and neural pathways. Stimulatory amino acids include arginine, alanine, and glutamine (114,

115, 128). Oral nutrients stimulate glucagon release via enteric hormones (GIP, gastrin and CCK). Additionally, cAMP analogues and epinephrine can further amplify nutrient-stimulated glucagon release from α -cells (114, 115). Stressors, including infection, toxemia, burns, tissue infarction and major surgery, all promptly increase glucagon secretion. This phenomenon is probably mediated by the adrenergic nervous system via sympathetic outflow from the ventromedial hypothalamus to α -adrenergic receptors in the β -cells. Rat islets are innervated by enteric neurons, which probably play a role in normal islet regulation (70).

Amino acid-stimulated glucagon release is inhibited by glucose through direct mechanisms that potentiate insulin's inhibitory actions (16, 114, 115). Free fatty acids act to further inhibit glucagon release. Similarly, pancreatic somatostatin is an important mediator of the suppression of glucagon secretion (71). In addition to glucose, somatostatin and insulin, PYY also exerts inhibitory effects on α -cell glucagon secretion in mammals (22).

Most newly synthesized proglucagon is membrane-associated (86-88%), while glucagon exhibits much less membrane-association (24-31%). Suggesting that association of newly synthesized proglucagon with intracellular membranes could be related to the facilitation of proteolytic processing of proglucagon and/or transport from the site of synthesis to the secretory granules (102).

C. Insulin/Glucagon Interactions.

Pipeleers and associates showed that isolated β -cells were less glucose-responsive than β -cells coupled with other β - or α -cells, and that glucose-responsiveness was restored by adding glucagon or α -cells (113, 127). Alpha-cells may influence β -cell secretion via endocrine (19) or paracrine (150) pathways. Glucagon cell cytoplasmic processes originally demonstrated in the gut, have more recently been demonstrated on islet α -cells (8, 104, 109, 166). These cytoplasmic processes may be reaching out to 'pass a message on', as is suspected for gut

somatostatin cells, which also extend cytoplasmic processes (76). Thus, it is possible that glucagon stimulates insulin release via a paracrine mechanism. Furthermore, α -cells may modulate β -cells via electrical- or second messenger-coupling through gap-junctions (89-93). In the rat islet cAMP has been shown to increase gap-junction surface expression (58), another stimulus of insulin secretion. Glucagon is known to increase cAMP levels in β -cells (127). Gap-junction coupling has also been shown between β - and α -cells within the islets of various species (96, 108). Thus it is probable that β - and α -cells regulate one another through electrical and second messenger coupling through gap junctions.

Local intracellular rises in Ca^{2+} concentration have been observed with fluorescence imaging. These local rises may induce a rise in Ca^{2+} from a second Ca^{2+} store, creating a *wave* of Ca^{2+} . Calcium waves may traverse gap junctions to co-ordinate extended networks of cells, as is seen in pancreatic acinar *units* (94, 110). Gylfe and associates (50), reported the propagation of cytoplasmic Ca^{2+} waves in clusters of pancreatic β -cells in response to glucose. Similarly, cAMP waves are seen in the amoeba, *Dictyostelium discoideum*, which forms communicating colonies (110). There is the intriguing possibility that IP_3 , nucleotides and/or other intracellular molecules may act in this manner, as well. Studies using the movement of fluorescent tracers (lucifer yellow) have estimated that small molecules with molecular weights up to 1000 daltons (approx. 1.5-2.0 nm) can pass through gap junctions (148). In the rat islet, the tracer dye, 6-carboxyfluorescein has been used to identify separate territories consisting of 2-8 coupled α -, β - and/or δ -cells (95). Beta-cells located in the periphery of the islet appear to have twice as many gap junctions per unit membrane area as the β -cells situated in the islet center (89). This apparently non-random clustering of gap junctions on the β -cell membrane may play a significant role in regulation of insulin release.

IV. Tumor Cell Background.

A. HIT-T15 Cells.

The HIT-T15 cell line was established by Simian virus 40 (SV40) transformation of Syrian hamster pancreatic islet cells (125). HIT-T15 cells secrete insulin in response to glucose with reported half-maximal and maximal stimulation, at glucose concentrations of 1 mM (120) and 7.5 to 10 mM (125), respectively. Glucose-stimulated insulin secretion in the HIT-T15 cells is passage dependent with a 30-fold reduction between passages 41 and 88 (125), thus it is important to either standardize experiments or perform experiments on a single passage. The loss of HIT-T15 cell function is caused by a loss of insulin mRNA, insulin content, and insulin secretion and is preventable by culturing HIT-T15 cells in low-glucose conditions (123). Insulin secreted from HIT-T15 cells is reported to have a half-life of 36 h (32), significantly greater than the 6 to 8 min half-life of circulating insulin (16).

In the HIT-T15 cell line there appears to be abnormal glucose handling, as there is an elevated level of GLUT-1 and GLUT-3, but a depressed level of the major glucose transporter (GLUT-2) found in islet β -cells (23). Additionally, HIT-T15 cells chronically exposed to high glucose containing media, exhibit lowered glucose transporter mRNA levels, glucose transport and glucose-induced insulin secretion, when compared with cells cultured in low-glucose media (118). In HIT-T15 cells, GLUT-2 mRNA, glucose uptake activity, and the glucose-responsiveness of insulin secretion correlates with glucose-induced changes in glucose uptake activity (56). Although glucose handling does not appear to be normal in the HIT-T15 cell line, it remains a glucose-responsive cell line.

Glucose stimulated depolarization of HIT-T15 cells increases intracellular Ca^{2+} (116, 120), most likely by ATP-sensitive K^{+} -channel associated depolarization, as described above in the normal β -cell (99). Similarly, glucagon stimulates the secretion of insulin from HIT-T15 cells in the absence and presence of glucose (75), possibly via a rise in cAMP that leads to an increase in protein kinase-A activity, and Ca^{2+} influx through voltage-dependent Ca^{2+} channels (116). GIP and GLP-I potentiate glucose-stimulated insulin secretion by increasing extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels (84). Increasing glucose leads to an increase in preproinsulin mRNA (48). Similarly, glucagon, forskolin and dibutyryl cAMP also

increase content of preproinsulin mRNA approximately two fold, and stimulate insulin release in HIT-T15 cells in the presence and absence of glucose (48, 75, 125). Unlike normal β -cells, it is unlikely that DAG and PKC play an important role in insulin secretion from HIT-T15 cells (120).

Glucose- and glucagon-stimulated insulin release from HIT-T15 cells is inhibited by somatostatin and PP (75, 149). The somatostatin-dependent inhibition of insulin release occurs via G-protein mediated mechanisms (149). Furthermore, the predominant somatostatin receptor prefers somatostatin-28 (142).

As mentioned above, cultured HIT-T15 cells have been shown to contain a subpopulation of glucagon expressing and secreting cells designated HIT-T15-G (32, 131). These HIT-T15-G cells had a 2-fold increase in glucagon mRNA following forskolin or phorbol ester treatment (131). Glucagon levels in acid extracts were found to be 0.72 ± 0.15 pmol/mg protein, with secreted glucagon having a half-life of 18 hr (32), significantly greater than the 6 min half-life of circulating glucagon (16). In addition to glucagon, HIT-T15-G cells also process proglucagon into the peptides GLP-I and GLP-II, and the major proglucagon fragment (MPF) (131). Forskolin, adrenaline, arginine and KCl stimulate glucagon release from HIT-T15-G cells (131), while arginine-stimulated glucagon secretion is inhibited by somatostatin in this subset of cells (32, 131). Shennan and associates, suggest that HIT-T15-G cells may represent a less differentiated form of the parental HIT-T15 cell line in which the α -cell phenotype is dominant but not complete (131). Finally, HIT-T15 cells are also known to transiently express and secrete secretin (160).

It has been reported that HIT-T15 cells grow in dome shaped clusters, similar to hamster islet monolayers (125). Unlike the normal β -cell, uvomorulin has not been reported on the HIT-T15 cell surface.

B. InR1-G9 Cell Background.

The InR1-G9 cell line is a BK virus transformed hamster islet cell line, that undergoes post-translational processing of proglucagon to glucagon, GLP-I and II (35). Phorbol esters and sodium butyrate, agents that increase glucagon gene transcription in RIN1056A cells, have no effect on glucagon mRNA levels in InR1-G9 cells, but secretion of glucagon and the glucagon-like peptides is stimulated by phorbol esters (35). Insulin negatively regulates glucagon secretion as well as glucagon gene expression (112). The InR1-G9 cell line does not contain secretin, another member of the glucagon family of peptides (160), and they are not responsive to glucocorticoid stimulation (157). In the rat endocrine pancreas, the glucagon gene is regulated by a PKA-dependent pathway, however, InR1-G9 cells lack PKA regulation of glucagon secretion (34). In the InR1-G9 cells a 50 base-pair region in the 3'-flanking sequence of the glucagon gene is important for the accurate processing of proglucagon mRNA transcripts (78). Glucagon gene sequences are not amplified, but appear to be hypomethylated in the InR1-G9 cells when compared to hamster liver or kidney gene sequences (35). The InR1-G9 glucagon mRNA species is 1300 base-pairs (35), similar to the 1200 base-pairs found in mammals (83).

In the following studies, the InR1-G9 cell line provides a tool for studying HIT-T15 cell secretion and ultrastructure in the presence of exogenous glucagon-secreting cells. The effects of InR1-G9 cell secreted GLP-I and GLP-II were not studied.

V. The Immunoneutralization Technique.

Due to the non-availability of specific glucagon antagonists, immunoneutralization becomes the technique of choice when examining the role of glucagon in the β -cell and the HIT-T15 cell line. The term immunoneutralization refers to the binding of an antibody (Ab) to the

active site of a molecule in order to abolish biological activity. The technique of immunoneutralization requires an Ab that will prevent antigen binding to the receptor. When using an Ab to immunoneutralize a peptide such as glucagon it is important to ensure that cross-reactivity for glucagon-like peptides does not exist. Finally, an immunoglobulin control must be utilized to ensure that the effect is due to the specific Ab in use, and is not just a non-specific effect of immunoglobulins.

Studies using antisera to immunoneutralize glucagon and somatostatin have been performed on the islet (141) and perfused pancreas (126), of rats. In the isolated islet the addition of anti-somatostatin serum to incubation media containing 5.5 mM or 20 mM glucose, significantly increased or had no effect on insulin secretion, respectively (141). In another study, anti-somatostatin gamma globulin augmented secretion of glucagon and insulin from pancreatic α - and β -cells, respectively (61).

In the isolated islet the addition of glucagon antiserum to incubation media containing 5.5 or 20 mM glucose, significantly increased or had no effect on insulin secretion, respectively (141). However, glucagon antiserum led to a marked increase in glucagon secretion from the islet (141). This marked increase in glucagon secretion may have been a compensatory mechanism, allowing the islet to overcome an artificially induced reduction in glucagon levels.

In the perfused pancreas glucagon antiserum had no significant affect on either insulin or somatostatin secretion, although it enhanced glucagon secretion. Another study in the arginine infused rat, demonstrated a reduction in circulating C-peptide levels when glucagon antisera prevented a rise in plasma immunoreactive glucagon levels (101). Anterograde infusion of glucagon antibodies did not effect insulin release but did decrease somatostatin secretion by about 50%, while retrograde infusion of glucagon-Ab decreased insulin secretion approximately 30%, but had no effect on somatostatin secretion (137). Glucagon-Ab administration led to no change in plasma glucose, immunoreactive insulin or immunoreactive somatostatin in rats (140).

The ability of glucagon antibodies to gain access to the inner core of the islet is questionable. If paracrine or autocrine regulation is occurring in the islet, and tight junction isolated inter-cellular spaces exist, it is unlikely that an Ab could easily enter these isolated spaces. Thus, the immunoneutralizing ability of antibodies used in the vasculature or in perfusion experiments is questionable. Additionally, the above studies did not take nervous and humoral factors into consideration. The finely balanced interactions between insulin, glucagon, and somatostatin within the islet may be able to compensate for disturbances created by the addition of antisera to one of these three peptides. Therefore, studies using glucagon and somatostatin antisera on isolated, dissociated islet cells are required to determine their effects in the absence of nervous and humoral factors.

CHAPTER TWO - MATERIALS AND METHODS

I. Cell Culture.

A. HIT-T15 Cells. The insulin-secreting hamster β -cell line HIT-T15 (passage 60), was purchased from American Tissue and Cell Culture (ATCC, Rockville, MD). Frozen HIT-T15 cells were rapidly thawed in a 37°C water bath and seeded at 3 million cells / 50 ml media in a 250 ml flask (Falcon, Becton Dickinson). Cells were cultured in Ham's F12K (Irvine Sci., Santa Ana, Ca) containing glucose (7.0 mM), L-glutamine (Sigma, 2 mM), penicillin (Gibco; 100 U/ml), streptomycin (Gibco; 100 μ g/ml) and supplemented with 10% dialyzed horse serum (DHS; Gibco) and 2.5% heat inactivated fetal bovine serum (FBS; Gibco). Additionally, long term cell culture experiments were performed using low- (0.8 mM) and high-glucose (11.1 mM) containing RPMI (Gibco) media that was supplemented with glutamine, penicillin, streptomycin and serum, as described above. HIT-T15 cell doubling time was one week, when cells were fed every three days and grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. When cells reached greater than 90% confluency they were detached from culture flasks with a 10 min incubation in a trypsin/EDTA solution (Gibco), washed and re-seeded in new culture flasks at 5 million cells per flask. Some trypsinized cells in approximately 100 μ l of media were placed on microscope slides and observed at the light microscopic level, for the purposes of determining re-aggregation characteristics. In order to eliminate variation between passages, all experiments used cells subcloned to a single passage (# 68) rather than using a series of passages.

B. InR1-G9 Cells. The glucagon-secreting hamster α -cell line InR1-G9 (passage 19), was kindly provided by Dr. D. Drucker (University of Toronto). InR1-G9 cells were seeded at 3 million cells/50 ml media in a 250 ml flask. Media contained high glucose (11.1 mM) RPMI supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml) and 5% heat inactivated fetal calf serum (FCS; Sigma). InR1-G9 cell doubling time was three days, when cells were fed every three days and grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. As above, all experiments used cells subcloned to a single passage (# 24).

C. HIT-T15 and InR1-G9 Co-cultures. Co-cultures of HIT-T15 (passage 68) and InR1-G9 (Passage 24) cells were grown in media composed of HIT-T15 and InR1-G9 culture media (1:1), under the conditions described above. Hence, glucose was 9.1 mM in the mixed media. Co-cultures were seeded at a density of one million cells per well (48 h prior to the experiment), including the following ratios of HIT-T15:InR1-G9 cells: 100:0; 75:25; 50:50 and 25:75.

II. Static Incubation Studies.

Static release experiments were performed on one million cells per well, cultured for 48 h on 12 well plates (Falcon, Becton Dickinson). After the culture period, medium was removed from HIT-T15 cells, and replaced with preincubation medium. Preincubation medium consisted of DMEM supplemented with glucose (3.0 mM). Preincubation was for 1 h at 37°C in an atmosphere of 95% air: 5% CO₂. Preincubation medium was removed and DMEM, which was supplemented with glucose (0, 3, 5, 10 or 15 mM) or glucose plus a purified Ab, was added and incubated for 1 hr under the same conditions as the preincubation. Antibodies that were specific to glucagon (26), somatostatin (164) or GIP (T.J. Kieffer and N. Dhatt, unpublished observations), were used to immunoneutralize their respective antigens. See Table 1 for further information regarding these antibodies. All test conditions were performed in duplicate. Following incubation, the medium was removed and insulin content was determined by radioimmunoassay (RIA) unless indicated as being determined by solid-phase RIA (SPRIA). Values for released insulin are expressed as a percentage of total cell insulin content, as determined by the extraction procedure described below.

III. Microscopy.

Tumor cells used for immunocytochemistry or electron microscopy were cultured for 48 h on ethanol sterilized glass coverslips (25 mm x 1 mm; Fisher, Canada) in 6 well plates (Falcon, Becton Dickinson) at a density of one million cells per well.

A. Light Microscopy (LM)

1. Fixation. Cells which adhered to coverslips were fixed in Bouin's solution for 5 min and washed in phosphate buffered saline (PBS; pH 7.3) at room temperature before immunostaining. The ability to stain cells fixed with the electron microscopic fixatives paraformaldehyde (2%) or glutaraldehyde (0.5%) with or without 1% osmium tetroxide was tested at the light microscopic level.

2. Staining Procedure. Peptide antigens were localized with specific primary antibodies (see Table 1). The bound primary antibodies were subsequently detected with secondary antibodies to either rabbit, mouse or guinea pig immunoglobulins. Secondary antibodies conjugated to a fluorescent label or peroxidase were visualized with fluorescence or light microscopy, as described below. Prior to incubation with primary antibodies, endogenous peroxidase activity was blocked by incubating slides with 0.3% hydrogen peroxide in methanol, for thirty minutes. Fixed cells on coverslips were washed three times with phosphate buffered saline (PBS), then non-specific binding of Ab was blocked with 10% solution of normal swine serum (NSS), in PBS. The PBS solution was composed of NaCl (137 mM), KH_2PO_4 (1.5 mM), Na_2HPO_4 (8.1 mM), KCl (2.7 mM), NaN_3 (3.0 mM), at pH 7.3. Excess blocking solution was washed off with PBS. Cells were incubated with primary antibodies (for 48 hr, at 4°C), diluted with NSS:PBS (1:10). Unbound primary antibodies were removed by washing three times with PBS prior incubation with secondary antisera.

3. Fluorescence Staining. Primary-Ab labeling for insulin and glucagon was localized using fluorescein isothiocyanate (FITC)-conjugated or rhodamine isothiocyanate (RITC)-conjugated secondary antibodies. Incubation with these secondary antibodies was for 1 h at room temperature in a humid atmosphere. The stained cells were then examined under a Zeiss Universal Microscope equipped for epifluorescence. The FITC and RITC conjugated secondary antibodies are listed in Table 2. Stained cells on coverslips were mounted on glass slides (VWR Sci. Inc., San Francisco, CA) with 10% glycerol in PBS, and photographed.

4. Immunoperoxidase Staining. The diaminobenzidine (DAB)/glucose oxidase immunoperoxidase staining method, as described by Buchan et al. (26), was used to localize primary antibodies bound to desired antigens. Slides were washed three times with PBS, followed by a 30 min incubation with 1:10 NSS. Excess NSS was rinsed off with three washes of PBS and a biotinylated secondary-Ab (diluted in 1:10 NSS) was applied for 1 h. Biotinylated secondary antibodies are listed in Table 3. Following the secondary-Ab, the cells were washed three times with PBS, and further incubated with complexed A and B (ABC Vectastain, Dimension Lab. Inc., Mississauga, Ont.) in 1:10 NSS, for 1 h. Three final washes with PBS were performed prior to development of the staining with a DAB/glucose oxidase solution. The DAB/glucose oxidase solution consists of dextrose (11.1 mM), ammonium chloride (0.4 mM), glucose oxidase (20 mg/l) and DAB (12.5 mg/ml) in a 0.1 M TRIS buffer. Staining developed within 10 to 60 minutes, and was then washed for 5 min in running tap water. Some coverslips were counterstained (for 30 sec) with filtered hematoxylin. Following a wash in running tap water the stain was differentiated in acid alcohol (30 sec), and again washed (5 min) in running tap water. Coverslips were dehydrated through graded alcohols (70, 90, 100 and 100%), followed by 2 x 3 min in xylene. Finally, coverslips were permanently mounted on slides with Eukitt, and photographed.

5. Photography. Stained cells were photographed with a Zeiss Axiophot microscope using a built-in 35 mm camera, or a Zeiss Axiovert 35M equipped with a 35 mm camera (Contax 167 MT). The types of film used for black/white and colour photography were

Table 1 - Details of Primary Antibodies

Antigen	Host	Source	Dilution	Dilution-IN	Type
* Glucagon	Mouse	Gregor	10 µg/ml	10 µg/ml	Ascites
Glucagon	Rabbit	Milab	1:2,500		Serum
* GIP	Mouse	RPG	1:5,000	1:1000	Ascites
GLP-I (C-terminal)	Guinea pig	RPG	1:1,000		Serum
PP (C-terminal)	Rabbit	Buchanan	1:2,000		Serum
PYY	Rabbit	McDonald	1:1,000		Serum
Secretin 53	Rabbit	Polak	1:500		Serum
Insulin	Guinea pig	RPG	1:1,000		Serum
* Somatostatin	Mouse	CURE	1:10,000	1:1000	Ascites
Uvomorulin	Rat	Sigma	1:500		Monoclonal

Dilution-IN - dilution/concentration of Ab used in immunoneutralization studies.

Buchanan - Dr. K. Buchanan, Dept. Med., University Hospital, Belfast, Ireland.

CURE - Dr. J. Walsh, Center for Ulcer Research and Education.

Gregor - Dr. M. Gregor, Dept. Gastroenterology, University of Dusseldorf, Germany.

McDonald - Dr. T. McDonald, University of Western Ontario, Ontario, Canada.

Milab - Milab, Sweden.

Polak - Dr. J. Polak, Royal Postgrad. Med. School, London, England.

RPG - Regulatory Peptide Group, Dept. Physiology, University of British Columbia, Canada.

Sigma - Sigma, St. Louis, MO, USA.

* Antibodies used for immunoneutralizing target peptides.

Table 2 - Secondary Antibodies used in Fluorescence Immunostaining

Antigen	Host	Label	Source	Dilution
Guinea pig IgG	Goat	FITC	Jackson	1:500
Mouse IgG	Donkey	FITC	Jackson	1:1,000
Guinea pig IgG	Goat	RITC	Jackson	1:2,000
Mouse IgG	Rabbit	RITC	Vector	1:300

Jackson - Jackson ImmunoResearch Laboratories Inc., Mississauga, Ontario, Canada

Vector - Dimension Labs, Mississauga, Ontario, Canada

Table 3 - Biotinylated Secondary Antibodies used in Peroxidase Immunostaining

Antigen	Host	Source	Dilution
Guinea pig IgG	Goat	Vector	1:300
Mouse IgG	Rabbit	Vector	1:300
Rabbit IgG	Goat	Vector	1:300
Rat IgG	Rabbit	Vector	1:200

Vector - Dimension Labs, Mississauga, Ontario, Canada

Ilford HP5 (ASA 400) and Fujichrome P1600 (ASA 800), respectively. Ilford HP5 was developed using Ilford developer (Ilford, Mobberly Cheshire, UK). The developer was applied for 10 min followed by a 5 min wash in running tap water. The film was fixed (Ilford fixer) for 5 min, and again washed for 5 min in running tap water, then hung to dry. Fujichrome P1600 was developed using the Rapid E6 process (Photo Systems Inc., Dexter, Michigan), as described in the supplied instructions.

B. Electron Microscopy (EM).

1. Tissue Preparation. HIT-T15 cell cultures (at 2, 4, 6, 8 and 10 days of culture in low- and high-glucose), and HIT-T15/InR1-G9 co-cultures were fixed for 15 min with 2.5 % glutaraldehyde in a 0.1 M phosphate buffer. Fixed tissue was washed three times with phosphate buffer, removed from the bottom of the culture plate with a rubber policeman, and centrifuged in a microtube (Eppendorf, Sigma) for 5 minutes. Pelleted tissue was further fixed with 1% osmium tetroxide, and washed three times (10 min each wash) with phosphate buffer. At this stage, tissue was stored at 4°C until embedding.

Prior to embedding, tissue was dehydrated through graded alcohols (70, 80, 90, 100, 100 and 100%; 15 min each). Dehydration was followed by a wash in propylene oxide (10 min), after which, the tissue was infiltrated with propylene oxide:epon (described below) mixtures of 1:1 and 1:3 (2 h each). After the 2 h periods the 1:3 propylene oxide:epon mixture was removed and replaced with pure epon overnight following which the epon was replaced. The epon infiltrated tissue was then placed in a 70°C oven and allowed to harden overnight. Epon consisted of Jemmed-812-resin (6 ml) and dodecenyl-succinic-anhydride (12 ml) mixed with Jemmed 812 resin (7 ml) and nadic-methyl-anhydride (6 ml), and tri(dimethylaminomethyl)-phenol as a hardener. All epon reagents were purchased from JBS Chem, Dorval, Quebec.

Tissue blocks were cut free from microtubes with a razor blade, and trimmed in preparation for sectioning with an ultramicrotome. Semi-thin sections were cut to a thickness of

5 μm and stained with 1% toluidine blue, for light microscopic examination. Following this examination tissue blocks that were determined to be embedded adequately were sectioned for EM. Sections cut for EM were $\leq 0.5 \mu\text{m}$ in thickness, as confirmed by the reflection of gold and silver light when sections were floated on a water bath. Sections were picked up with EM grids (200 mesh Ni) and allowed to dry for two days before staining.

2. Staining of EM Sections. All tissue sections were stained with a saturated solution of uranyl acetate (UA), by placing the EM grid on a drop UA solution for 5 minutes. The grid was rinsed thoroughly with distilled water, and further stained with a saturated solution of lead citrate (LC) for 1 min (in the presence of a pellet of NaOH). Sections were again washed with distilled water, and dried.

Prior to UA/LC staining some sections were stained for insulin and glucagon using a variation of the immuno-gold staining technique (13). Briefly, EM grids were placed on drops of 33% H_2O_2 (10 min) washed with distilled water, and left on drops of 1:10 NSS (30 min). Primary antibodies raised against insulin and glucagon (Table 1) were diluted in PBS containing 0.1% bovine serum albumin (BSA, Sigma). Grids were incubated on drops of Ab solution for 48 h, at 4°C. Following incubation with Ab, grids were washed in PBS, PBS-tween (PBS containing 0.05% tween-20), and PBS-tween containing 0.5% BSA for 15 min each. A 30 min incubation on drops of 1:10 NSS followed. Gold particle-conjugated secondary antibodies raised against guinea-pig (Sigma; 5 nm gold particle) and mouse (Sigma; 10 nm gold particle), were diluted 1:10 in 0.5% BSA/PBS-tween. Grids were incubated for 1.5 h on drops of Ab solution. Three 15 min washes with 0.5% BSA/PBS-tween, PBS-tween and distilled water, were followed by counterstaining with LC and UA, as described above.

3. Equipment and Photography. Prepared tissue sections were examined and photographed using a transmission electron microscope (Zeiss, High Resolution Electron Microscope, EM 10C/CR). Film negatives were developed for 3-4 min (Kodak, TMAX

developer), rinsed (1 min; running tap water), fixed (3 min; Kodak Fixer) and rinsed (30 min; running tap water), before being hung to dry. Negatives were printed in the Physiology Department with the assistance of Joseph Tay, and some kind suggestions from the staff at Lens and Shutter.

IV. Radioimmunoassay.

A. Insulin-Antibody. The insulin antibodies used for RIA and SPRIA were derived by subcutaneous immunization of guinea pigs (Gp) with crystalline human insulin (UBC Physiology Dept., Dr. R.A. Pederson, 1977). Blood was collected through cardiac puncture and plasma was separated by centrifugation. The antisera designated Gp-01 and Gp-07 were divided into 100 μ l aliquots, lyophilized and stored at -20°C until reconstituted in distilled H_2O prior to use.

B. Insulin-RIA. Insulin in the release medium or in acid extracts of HIT-T15 cells was assayed using RIA, unless otherwise stated. The assay used guinea pig antiserum (Gp-01). Iodinated bovine insulin (2000 cpm/tube), and rat insulin standards were employed. The range of the assay standards was from 5 to 160 $\mu\text{U}/\text{ml}$. A 0.01 M phosphate buffer (pH 7.5) containing 5 % charcoal extracted human plasma was used for diluting samples and controls. Samples were assayed in duplicate, and counted for 2 min on a Wallac 1277 Gammamaster. The RIA was sensitive to 10 $\mu\text{U}/\text{ml}$, when the sensitivity was determined by taking the insulin concentration twice the standard error of the zero-binding below the zero-binding value. Intra- and interassay variations were less than 2.5 %.

C. Insulin Solid Phase RIA. SPRIA for insulin was performed as outlined by Väänänen and co-workers (152). Prior to performing the insulin-SPRIA, Immulon-3 removawells in a removawell holder (Canlab, Vancouver, BC) were coated with 100 μ l of insulin-Ab (Gp-07) in Carbonate Coating Buffer (CCB), at least 18 h before use. The CCB (pH 9.6) is a solution composed of Na_2CO_3 (1.5 mM), NaHCO_3 (3.5 mM) and NaN_3 (0.3 mM). After the 18 h incubation the plates were washed three times with PBS-tween, and dried by lightly tapping the

plate on a bed of paper towels. Nonspecific binding was blocked by incubation with 200 μ l of PBS-tween containing 5% fetal calf serum (Gibco, Burlington, Ont.) for 1 to 4 h at room temperature. Plates were again washed 3 times with PBS-tween then dried and either used immediately or stored at 4°C until use. Human insulin (Novo Research, Copenhagen) standards, from 0.5 to 256 μ U/ml, were made up at pH 7.3 in DMEM containing 0.1 % BSA. Standards and unknowns were then added to triplicate wells (100 μ l/well). One hundred μ l of 125 I-insulin diluted in PBS-tween to 2000 CPM was then added to the wells immediately following the standards and samples. After a minimum 15 hr incubation at 4°C, plates were washed three times in PBS-tween and dried; the removable wells were separated and placed in borosilicate culture tubes (Canlab, Vancouver, BC). Tubes were counted for 2 min on a Wallac 1277 Gammamaster. After counting, culture tubes were emptied and recounted; any contaminated tubes were discarded, while non-contaminated tubes were stored and reused in subsequent assays.

D. Glucagon RIA. Glucagon RIA was kindly performed by Dr. R.A. Pederson's technician Leslie Cheqnita. A 0.06 M phosphate buffering system (pH 7.4) was used. Highly purified porcine glucagon (Novo) was used for standard controls which covered a range of 25 to 6,400 pg/ml in 1:2 serial dilutions. Iodinated glucagon was purchased from Amersham, and used in the assay at 2000 cpm/tube. Glucagon-Ab (Gregor MoAB 23-6-B4) was used at a final dilution of 1:160,000. Samples were assayed in duplicate, and counted for 2 min on a Wallac 1277 Gammamaster.

E. Acid Extraction Protocol. Total cell hormone content was determined by assaying acid extracts of cultured cells. The extraction procedure consisted of removing cultured cells (in 1 ml of 2N acetic acid) from the bottom of the culture plate with a rubber policeman, and placing the suspension into a microtube. The suspended cells were then sonicated and boiled for 10 minutes. Extracts were centrifuged (at 500 g) for 5 min, transferred to a fresh microtube and frozen (-20°C) until assayed. Acid extracts of HIT-T15 cells were assayed using either RIA (20x dilution) or SPRIA (50x dilution). Cells extracted for glucagon were not boiled.

F. Analysis of RIA Results. Using RIA or SPRIA, samples were assayed in duplicate or triplicate, with values of duplicates or triplicates averaged. Experiments were each performed in duplicate and the duplicate measurements were averaged. The values of each separate experiment were then averaged, and standard error of the mean (SEM) was calculated. Data sets with $p < 0.05$ (student's t-test) were considered to be significantly different. Hormone released was expressed as a percentage of total cell content (% TCC), as determined by the acid extraction protocol above.

CHAPTER THREE - STATIC INCUBATION EXPERIMENT RESULTS

I. HIT-T15 Glucose-Stimulated Insulin Release.

Figure 1 depicts a concentration-dependent rise in IR-insulin released from HIT-T15 cells (passage 68), in response to glucose. Immunoreactive-insulin release (IRI) increased significantly above the basal level (zero glucose) when cells were exposed to glucose concentrations of 5, 10 and 15 mM ($p < 0.02$). Release of IRI is expressed as a percentage of total cell content (% TCC), with TCC being $1300 \pm 51 \mu\text{U/ml}$ (mean \pm SEM; $n=6$). When the TCC from cells exposed to zero- and high-glucose (15 mM) was compared (Figure 2), however, there was slightly less intracellular-insulin in the high-glucose exposed cells. This reduction in intracellular-insulin was not significant ($p > 0.05$; $n=6$).

II. HIT-T15 Insulin Release in the Presence of Antibodies.

Antibodies raised against somatostatin and GIP had no effect on glucose-stimulated insulin release, while glucagon-Ab completely abolished insulin release (Figure 3). Somatostatin-Ab and GIP-Ab acted as immunoglobulin controls for the glucagon-Ab effect. Insulin released under conditions of zero-glucose and glucose with glucagon-Ab were not significantly different ($p > 0.05$). Insulin released in response to glucose was the same as that in response to glucose with somatostatin-Ab ($p > 0.05$) or glucose with GIP-Ab ($p > 0.05$). However, zero-glucose and glucose with glucagon-Ab conditions were both significantly different from glucose, glucose with somatostatin-Ab, and glucose with GIP-Ab ($p < 0.001$). Values depicted in figure 3 are means \pm SEM ($n = 7$).

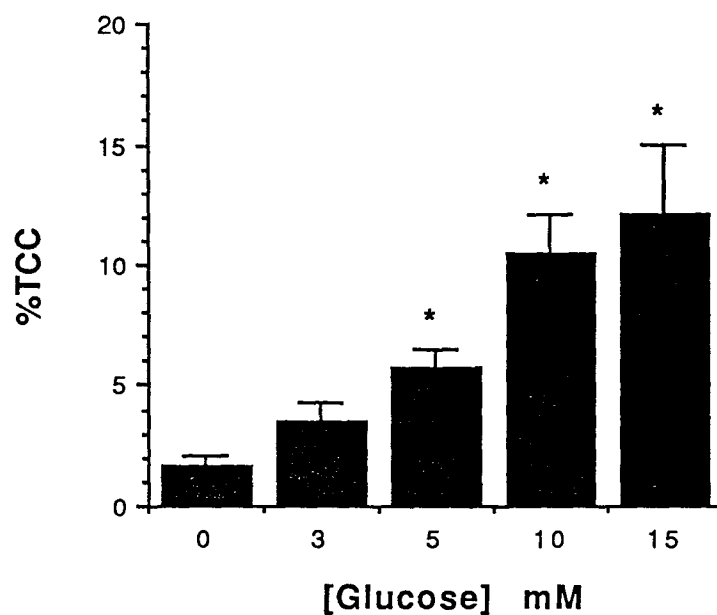


Figure 1. IR-Insulin Released from HIT-T15 Cells in Response to Glucose.

IR-Insulin release is expressed as a percentage of total cell content (% TCC). Glucose-stimulated insulin secretion is significantly different from basal (0 mM glucose) insulin release (* $p < 0.02$). Each value represents the mean \pm SEM of six experiments.

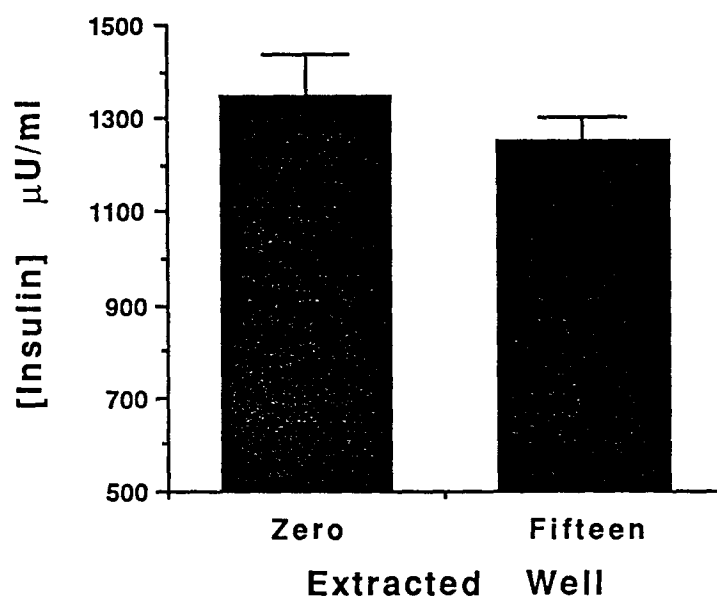


Figure 2. HIT-T15 Cell Extract Values.

Both zero- and high (15 mM)-glucose exposed HIT-T15 cells showed similar total insulin content. Each value represents the mean \pm SEM ($n = 6$). No significant difference ($p > 0.05$) between zero- and high-glucose exposed cells was detected.

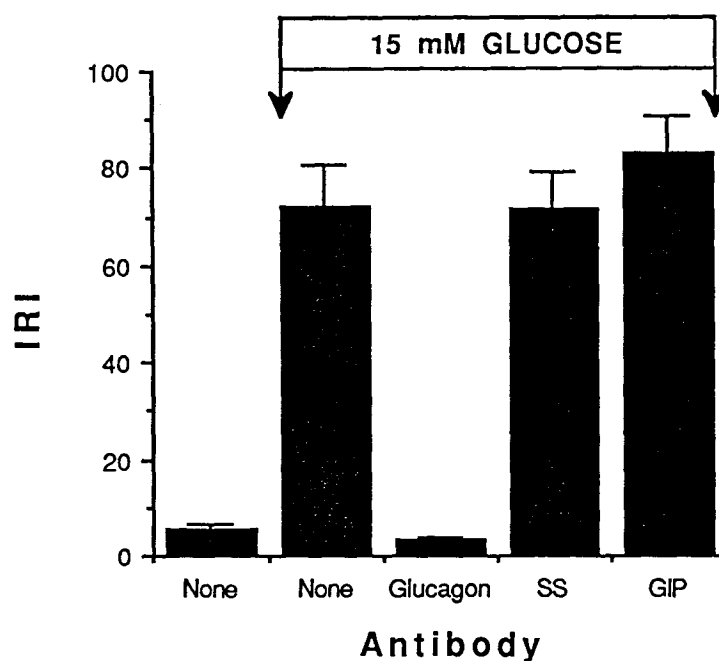


Figure 3. HTT-T15 Cell Glucose-Response in the Presence of Antibodies.

Each value represents the mean \pm SEM ($n = 7$). Insulin released from the zero glucose condition was significantly different from that of the glucose, glucose/anti-somatostatin and glucose/anti-GIP conditions ($p < 0.001$). However, no significant difference was detected between zero-glucose and glucose/anti-glucagon conditions ($p > 0.05$); or, glucose, glucose/anti-somatostatin and glucose/anti-GIP conditions ($p > 0.05$). These data were obtained using SPRIA.

III. Glucose-Stimulated Insulin Release Abolished by Glucagon-Antibodies.

Glucagon-Ab inhibited glucose-stimulated insulin secretion over the full range of HIT-T15 cell glucose responsiveness (Figure 4), with significance in the 5, 10 and 15 mM glucose conditions ($p < 0.03$). The %TCC depicted in Figure 4 are means \pm SEM of seven experiments. Figure 5 depicts insulin extract values from HIT-T15 cells ($n = 6$) and glucagon-Ab exposed HIT-T15 cells ($n = 7$), with no significant difference between the two groups ($p > 0.05$). Non-specific binding of ^{125}I -insulin (and fragments) in the RIA, was unaffected by the addition of sample, sample plus glucagon-Ab or 20 times diluted acid extracts (Figure 6).

IV. Glucose-Stimulated Insulin Release from HIT-T15/InR1-G9 Co-cultures.

The glucose responses of HIT-T15 and InR1-G9 co-cultures at HIT-T15:InR1-G9 ratios of 75:25, 50:50 and 25:75 were similar, with all displaying basal insulin release that was highly elevated over HIT-T15 cells alone (Figure 7). Additionally, a marked reduction in sensitivity to glucose is also observed. Acid extracts of co-cultured cells showed parallel changes in TCC of IRI with changes in insulin cell (HIT-T15) percentage (Figure 8).

V. Glucagon Release from HIT-T15 Cells in the Presence of Glucose.

Glucagon released from HIT-T15 cells in the presence of glucose is depicted in Figure 9. The concentrations of glucagon released in response to zero-, 5- and 15 mM-glucose, were 264 ± 72 , 157 ± 66 and 165 ± 77 pg/ml, respectively. The mean glucagon concentration of wells extracted into 1 ml of 2N acetic acid was 3062 ± 150 pg/ml. No significant difference was seen between any of the glucose conditions studied ($p > 0.05$). Standard error for all conditions was high at an average of 38.6% ($n=4$).

VI. Cell viability Following Static Release.

Finally, prior to acid extraction, viability staining with trypan-blue confirmed that greater than 95% of HIT-T15 and InR1-G9 cells were viable following each of the static release experiments. Visual inspection of viability stained cells, under the light microscope, with a haemocytometer indicated that the viability of cells remained unchanged throughout the experiments.

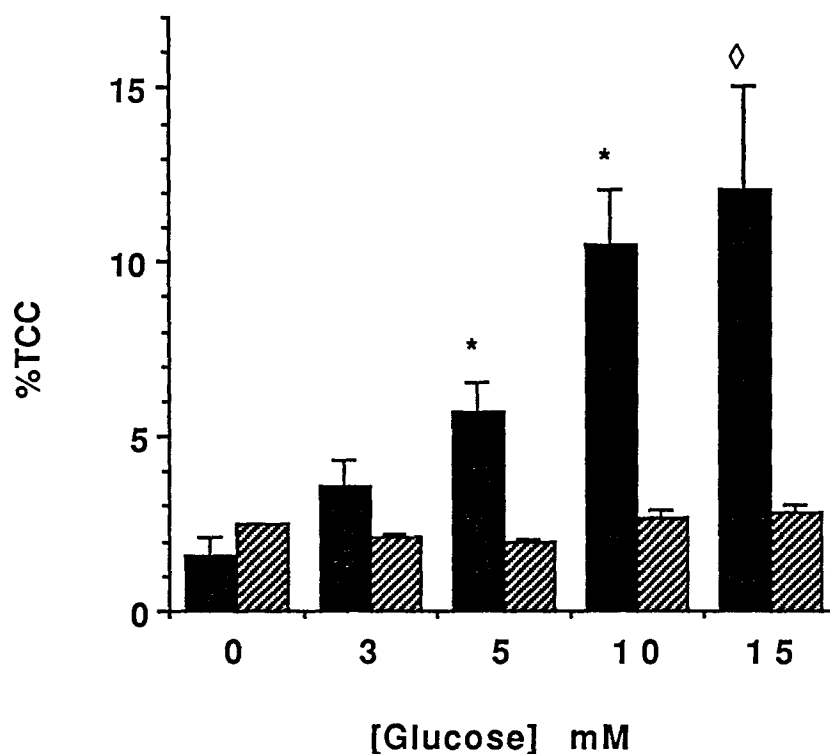


Figure 4. HIT-T15 Cell Insulin-Response to Glucose Immunoneutralized by Glucagon-Antibody

Insulin release values in response to glucose (solid black) are the mean \pm SEM of six experiments, while insulin release values in response to glucose/glucagon-Ab (hatched) are the mean \pm SEM of seven experiments. All values are expressed as a percentage of total cell content (% TCC). Glucagon-Ab significantly inhibited glucose-stimulated insulin release (* $p < 0.004$; ◇ $p < 0.03$).

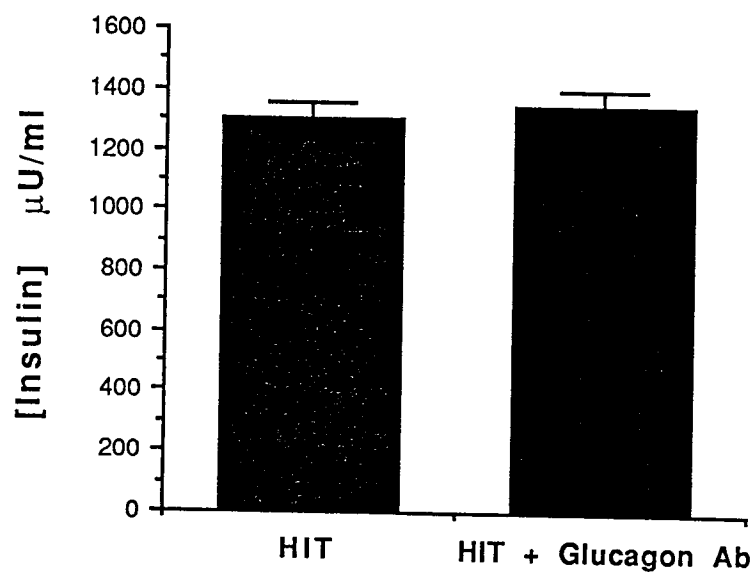


Figure 5. Comparison of HIT-T15 Cell Extract Values with/without Glucagon-Antibody.

This figure compares the extract values from HIT-T15 cells (n=6) and HIT-T15 cells exposed to glucagon-Ab (n=7). Each value represents the mean \pm SEM of experiments. These conditions are not significantly different ($p > 0.05$).

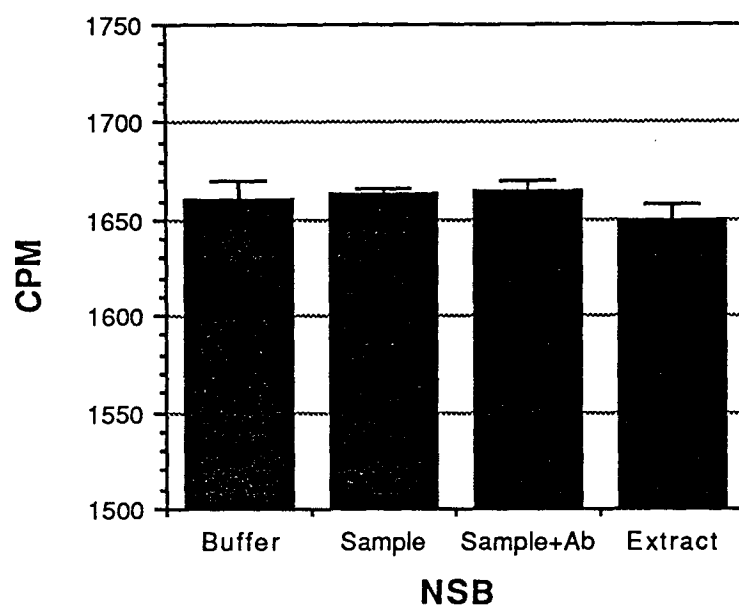
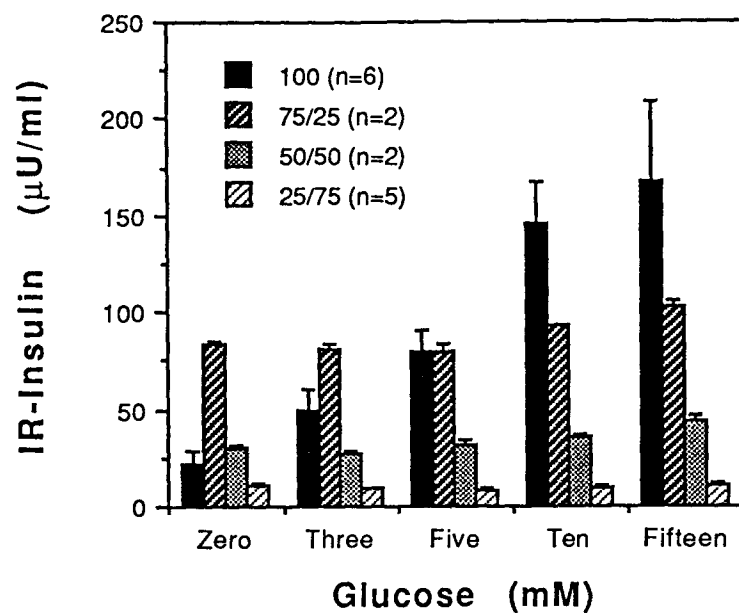


Figure 6. RIA Non-Specific Binding.

The level of nonspecific binding (NSB) was unchanged by the presence of either sample, sample containing glucagon-Ab, or 20x diluted acid extracts when compared with the assay NSB ($p > 0.05$). Each value is the mean \pm SEM of six experiments.

A



B

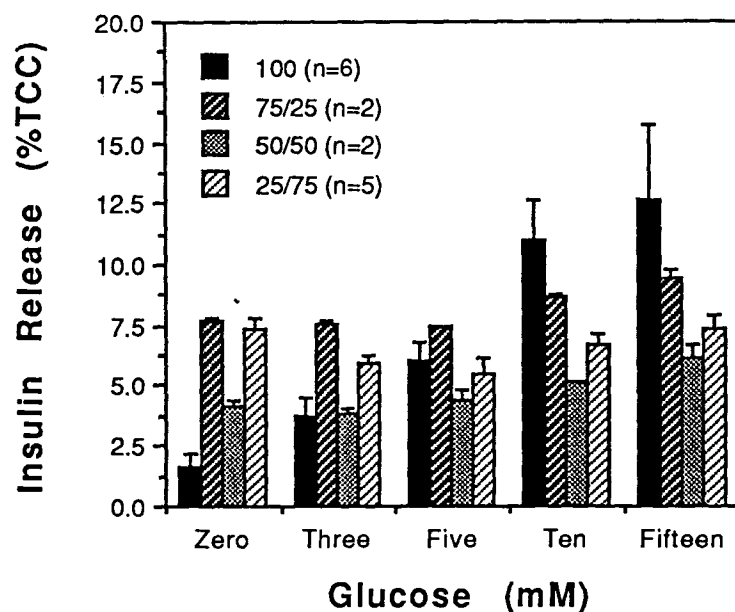


Figure 7. Glucose-Response of HIT-T15/InR1-G9 Co-cultures.

HIT-T15 and InR1-G9 cells were co-cultured at the HIT-T15:InR1-G9 ratios indicated above. Insulin release is displayed in: A) concentration (in $\mu\text{U/ml}$), or B) percentage of total cell content (% TCC). Error bars represent SEM in the 100/0 and 25/75 conditions, while they represent the range in the 75/25 and 50/50 conditions.

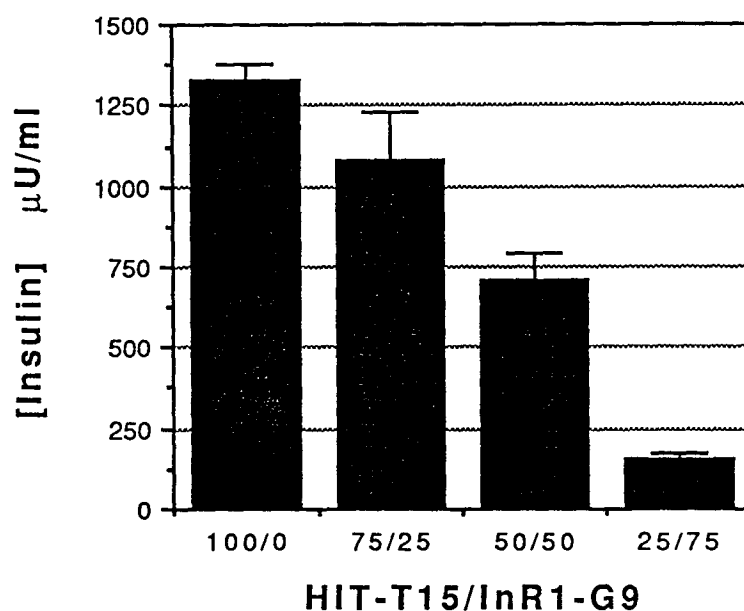


Figure 8. HIT-T15 and InR1-G9 Co-culture Extract Values

Total cell content of insulin was determined, in varying ratios of co-cultured HIT-T15 and InR1-G9 cells. The HIT-T15/InR1-G9 ratios follow, with n values in brackets: 100/0 (6), 75/25 (2), 50/50 (2) and 25/75 (5). Values represent means of experiments performed in duplicate. Error bars represent SEM in the 100/0 and 25/75 conditions, while they represent range in the 75/25 and 50/50 conditions.

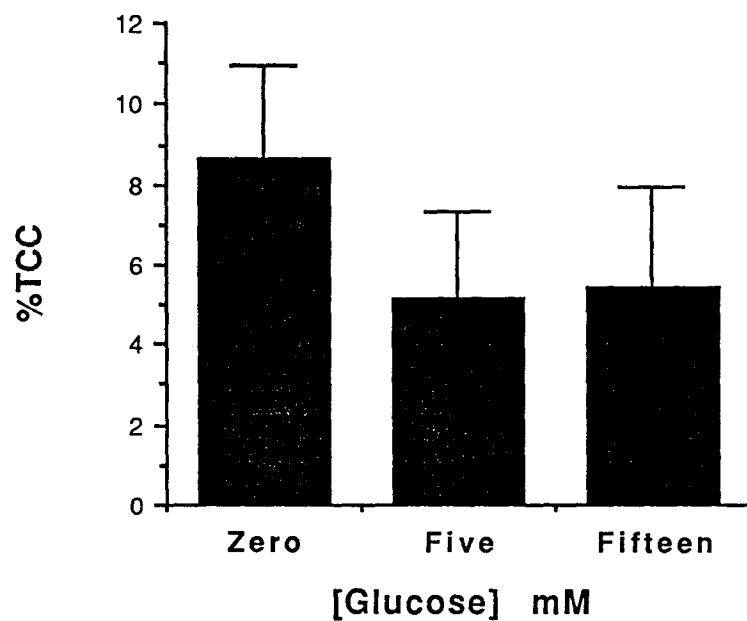


Figure 9. Glucagon Release from HIT-T15 Cells in the Presence of Glucose.

Glucagon release from HIT-T15 cells in the presence of glucose. Release is expressed as a percentage of total cell content (% TCC). There was no significant difference between values ($p > 0.05$).

CHAPTER FOUR - LIGHT MICROSCOPY RESULTS

I. HIT-T15 Cells.

A. Single Staining. Using the immunoperoxidase staining procedure described above HIT-T15 cells were stained for peptide hormones, including: insulin (Figure 10), somatostatin (Figure 11), glucagon (Figure 12), C-terminally amidated GLP-I (Figure 13), GIP (not shown), secretin (not shown), PYY (Figure 14) and PP (Figure 15). Additionally, HIT-T15 cells were stained for the cell surface adhesion molecule uvomorulin (Figure 16). Staining was positive for all peptides except somatostatin, secretin and GIP. It should be noted that cell surface staining of uvomorulin allowed the irregular surface of HIT-T15 cells to be seen. As a control, immunoperoxidase staining was performed in the absence of a primary-Ab, using either an anti-mouse or an anti-guinea pig secondary-Ab (Figure 17). The immunoperoxidase controls were both negative. The results of the above HIT-T15 cell immunostaining are summarized (Table 4).

B. Double Staining. Double staining of HIT-T15 cells for insulin and glucagon demonstrated co-localization of these peptides within single cells (Figure 18).

C. Morphological Observations. While viewed under the light microscope (at 200-400x magnification) it was observed that trypsinized HIT-T15 cells re-aggregated into islet-like masses, before attaching to the bottom of a culture plate. This re-aggregation occurred in as little as 5 to 10 min, and it was difficult to physically separate these cells once they were aggregates. With time these domed HIT-T15 cell masses combined to cover the bottom of the culture plate.

II. InR1-G9 Cells.

A. Staining. InR1-G9 cells were stained in the same manner as the HIT-T15 cells. Positive staining for glucagon, GLP-I and PP is depicted in Figures 19, 20 and 21, respectively. Staining for insulin (Figure 22), somatostatin (not shown), GIP (not shown), secretin (not

shown), PYY (not shown) and uvomorulin (not shown) was negative. As a control, immunoperoxidase staining was performed in the absence of a primary-Ab, with either an anti-mouse or an anti-guinea pig secondary-Ab (Figure 23). As with the HIT-T15 cells, the immunoperoxidase controls were unstained. Table 4 provides a summary of InR1-G9 cell staining.

B. Morphological Observations. Unlike the HIT-T15 cells, trypsinized InR1-G9 cells did not re-aggregate with one another prior to attaching to the bottom of culture flasks. However, once attached to the plate, InR1-G9 cells grew toward neighboring cells by extending neuron-like processes. In the InR1-G9 cells the intensity of fluorescent staining for glucagon was greatest near the end of these processes. These neuron-like processes were not seen in the HIT-T15 cells and served as a good marker to distinguish between HIT-T15 and InR1-G9 cells in co-culture.

III. HIT-T15 and InR1-G9 Co-cultures.

HIT-T15 and InR1-G9 cells were co-cultured at a HIT-T15:InR1-G9 ratio of 1:1 and stained with fluorescent and immunoperoxidase techniques. Figure 24 shows a co-culture stained for insulin while Figure 25 shows similar cells stained for glucagon. These two figures clearly show (based on morphology), clustering of putative HIT-T15 cells, with putative InR1-G9 cells and their processes surrounding these clusters. Fluorescence staining for insulin and glucagon further revealed the spatial arrangement of co-cultured cells (Figure 26). Within one week, InR1-G9 cells filled in all empty spaces between HIT-T15 cell clusters.

Finally, these the two cell lines generated from hamster tissue were compatible in culture, as trypan blue viability staining indicated a greater than 95 % viability. Additionally, at the electron microscopic level there was an absence of significant amounts of dead cells and phagocytic activity. These data are unlike co-cultures of β TC3 (murine derived) and InR1-G9 (hamster derived) cells which were incompatible and destroyed one another (T. Kieffer, unpublished data).

Table 4 - Summary of HIT-T15 and InR1-G9 Cell Staining

Peptide	Relative Abundance	
	HIT-T15	InR1-G9
Insulin	+++	0
Somatostatin	0	0
Glucagon	++	+++
GLP-I	++	+++
GIP	0	0
Secretin	0	0
Pancreatic Polypeptide	+	++++
Peptide-YY	++++	0
Uvomorulin	+++	0
Control	0	0
++++ - 100% of cells were immunoreactive for peptide		
+++	- > 75%	" " "
++	- > 50%	" " "
+	- < 50%	" " "
0	- < 5%	" " "

GLP-I - Glucagon-like Polypeptide-I

GIP - Glucose-dependent Insulinotropic Polypeptide, or
Gastric Inhibitory Polypeptide

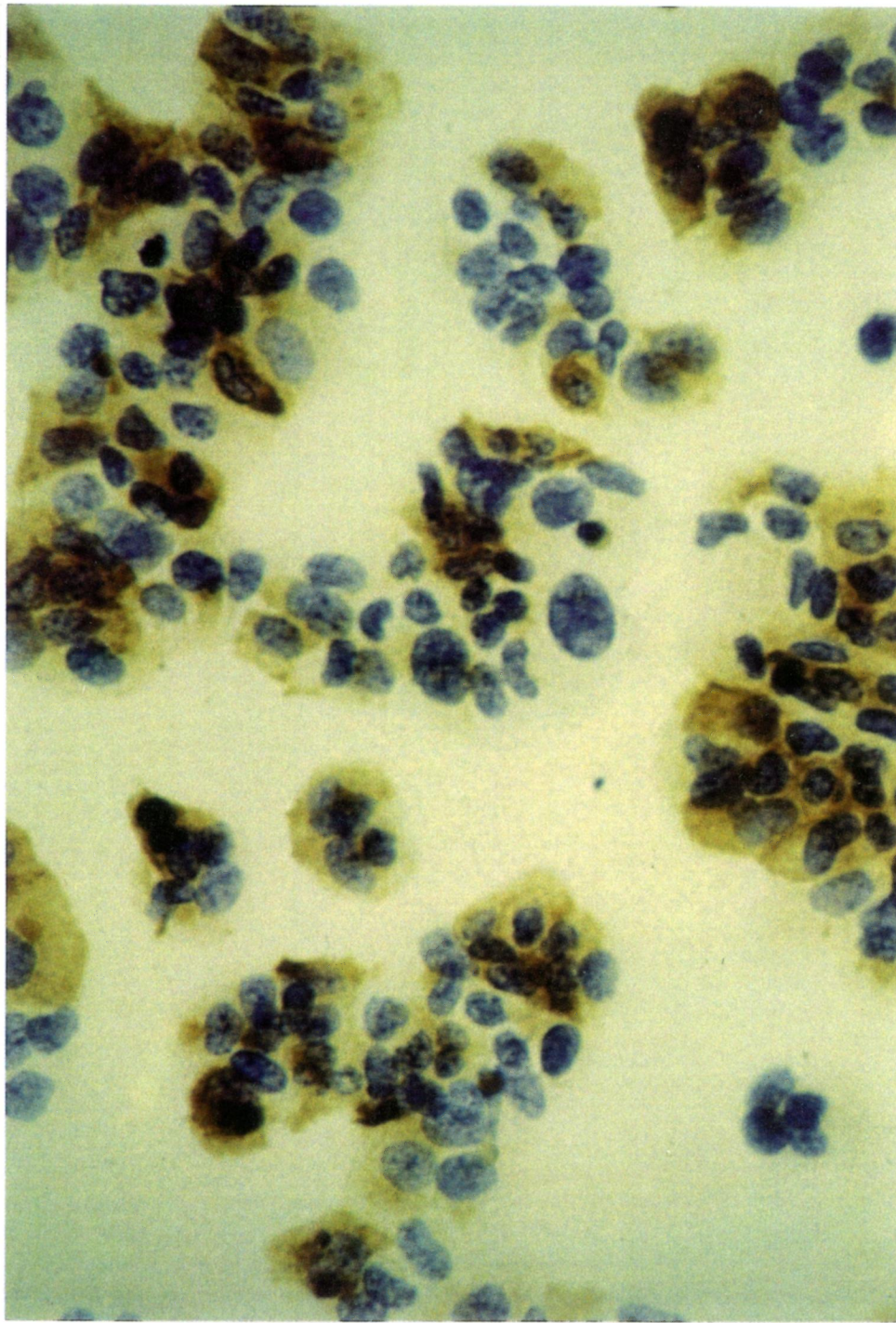


Figure 10. HIT-T15 Cells Stained for Insulin.

Immunoperoxidase staining of insulin appears brown, and hematoxylin counterstaining of nuclei is seen as blue. Magnification 400x

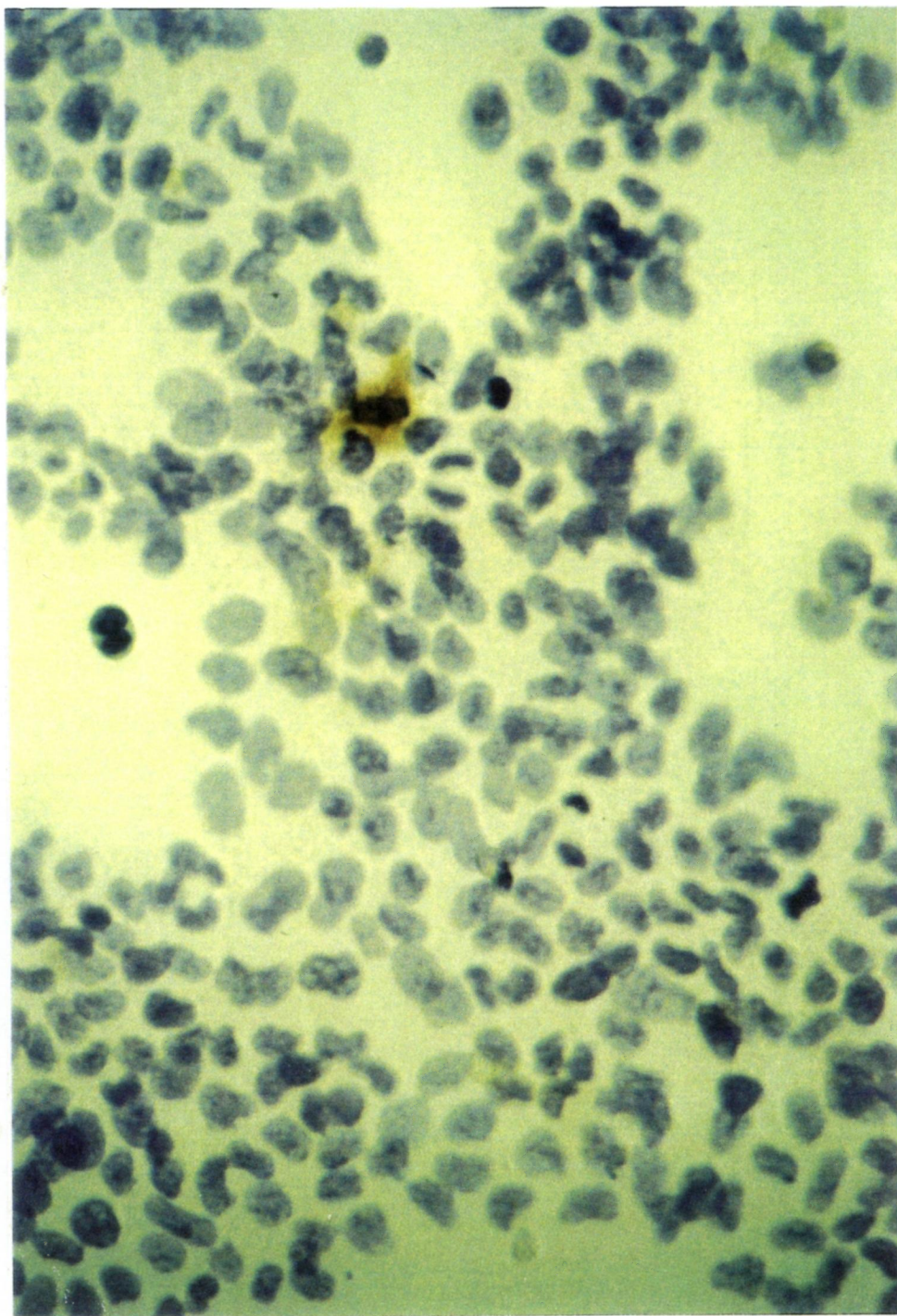


Figure 11. HIT-T15 Cells Stained for Somatostatin.

Immunoperoxidase staining of somatostatin appears brown, and hematoxylin counterstaining of nuclei is seen as blue. Magnification 400x

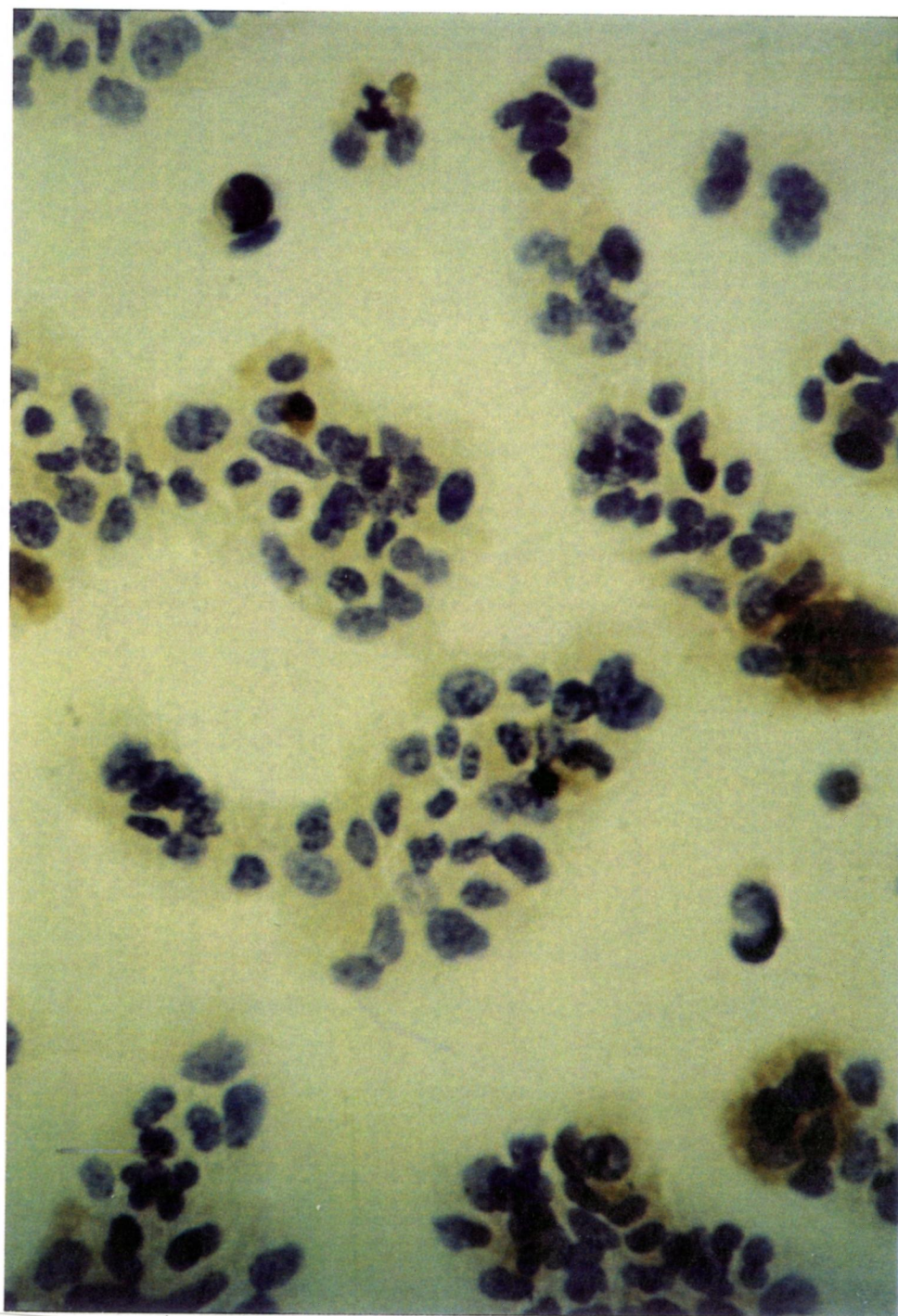


Figure 12. HIT-T15 Cells Stained for Glucagon.

Immunoperoxidase staining of glucagon appears brown, and hematoxylin counterstaining of nuclei is seen as blue. Magnification 400x

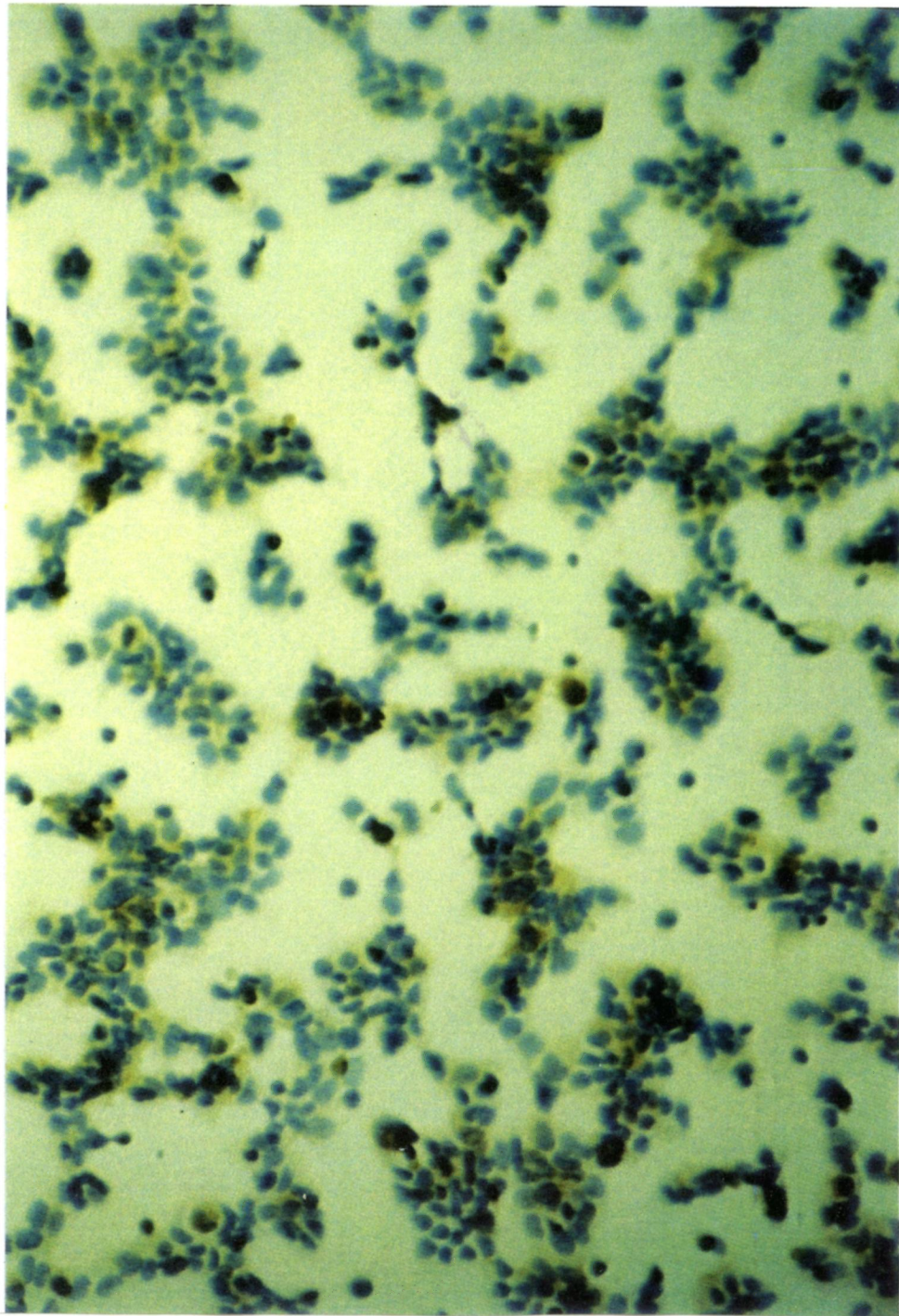


Figure 13 HIT-T15 Cells Stained for Glucagon-Like Polypeptide-I (GLP-I).

Immunoperoxidase staining of GLP-I appears brown, and hematoxylin counterstaining of nuclei is seen as blue. Magnification 200x

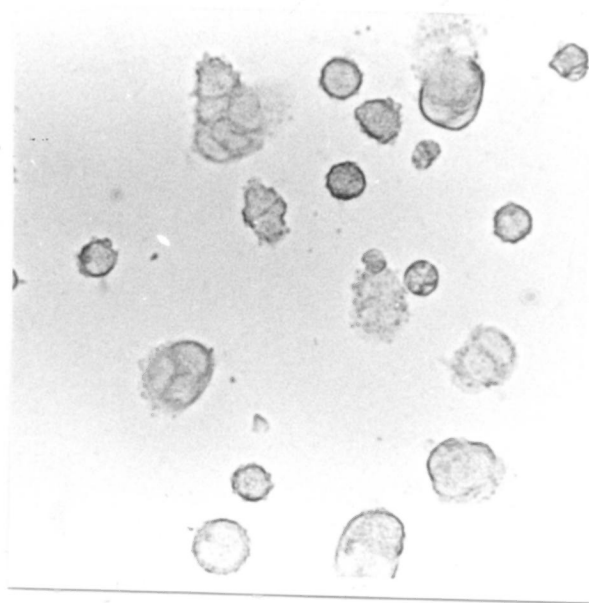


Figure 14. HIT-T15 Cells Stained for Peptide-YY (PYY).

Immunoperoxidase staining of PYY appears dark. Magnification 400x

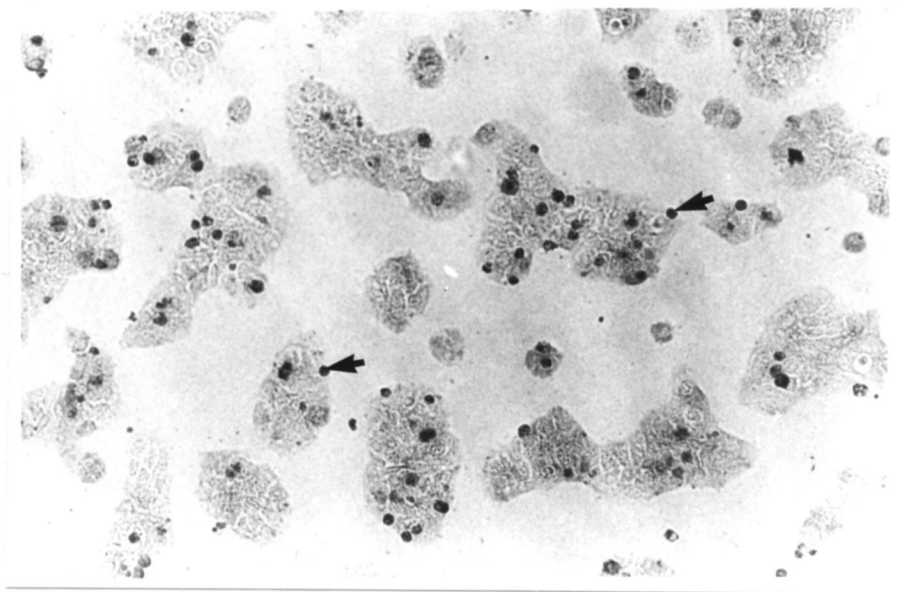


Figure 15. HIT-T15 Cells Stained for Pancreatic Polypeptide (PP).

Immunoperoxidase staining of PP appears dark (arrows). Magnification 200x

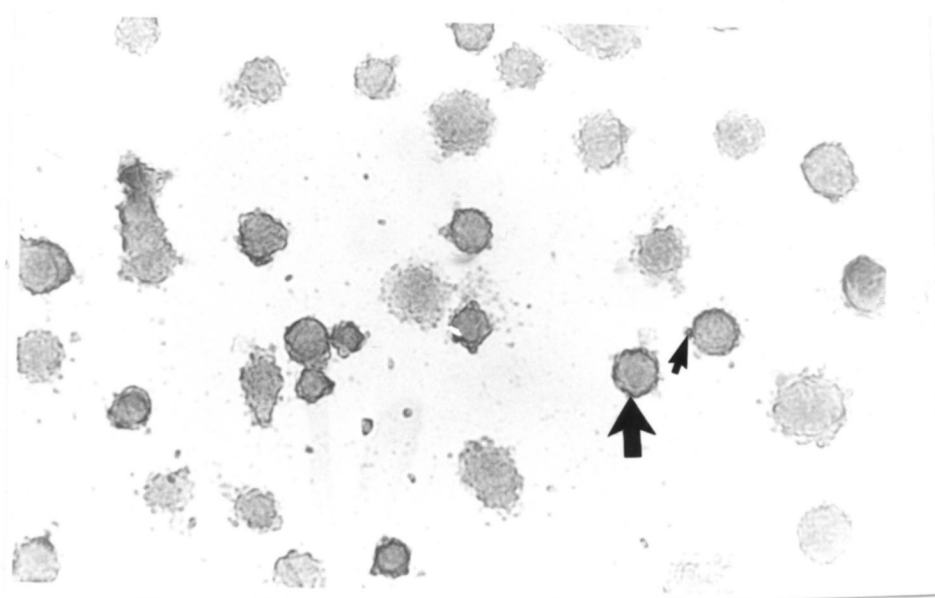


Figure 16. HIT-T15 Cells Stained for Uvomorulin.

Immunoperoxidase staining of uvomorulin (large arrow) appears dark.
Blebs protruding from the HIT-T15 cell surface (small arrow). Magnification 400x.

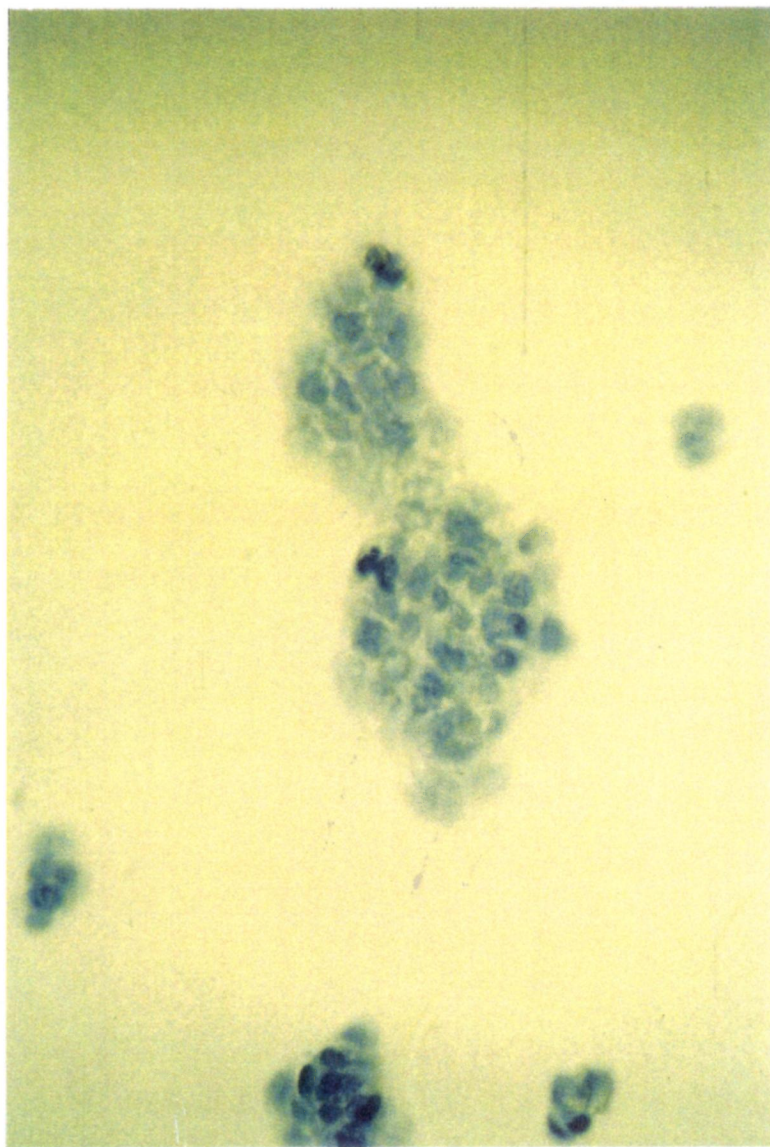


Figure 17. HIT-T15 Immunoperoxidase Control.

HIT-T15 cells stained with the immunoperoxidase method in the absence of a primary-Ab, but using a secondary-Ab to mouse immunoglobulins. Haematoxylin staining of nuclei appears blue. Note that secondary-Ab to guinea-pig showed similar results. Magnification 400x

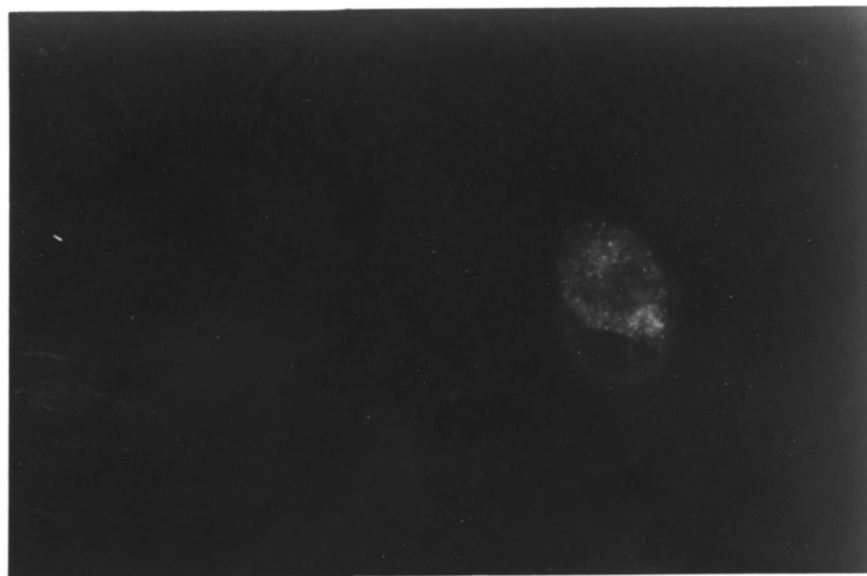
A**B**

Figure 18. HIT-T15 Cell Colocalization of Insulin and Glucagon.

This figure depicts fluorescein isothiocyanate (FITC) staining of insulin (A), and immunoperoxidase staining of glucagon (B). Magnification 1000x.

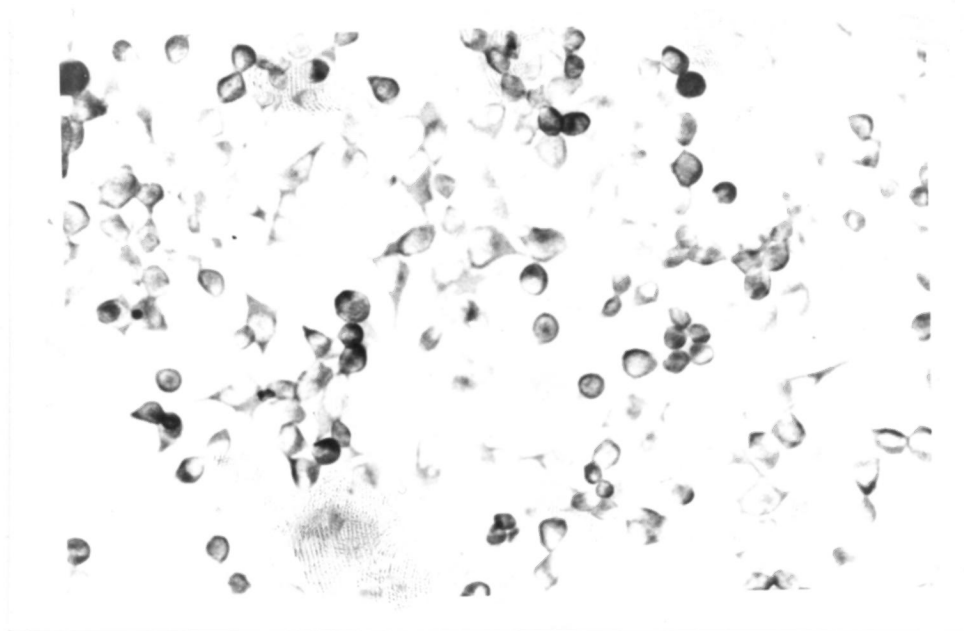


Figure 19. InR1-G9 Cells Stained for Glucagon.

Immunoperoxidase staining of glucagon appears dark. Magnification 400x

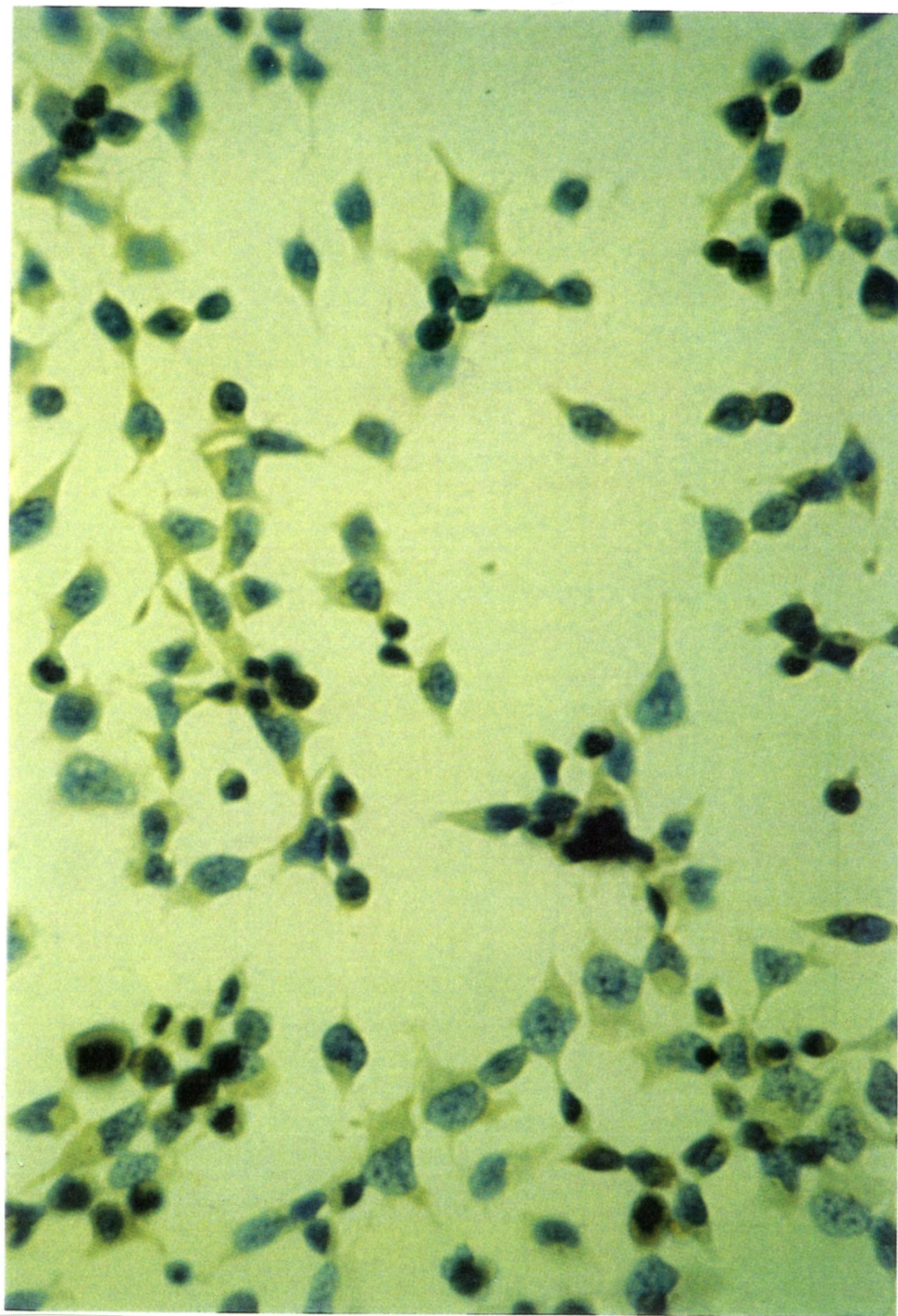


Figure 20. InR1-G9 Cells Stained for Glucagon-Like Polypeptide-I (GLP-I).

Immunoperoxidase staining of GLP-I appears dark. Magnification 400x

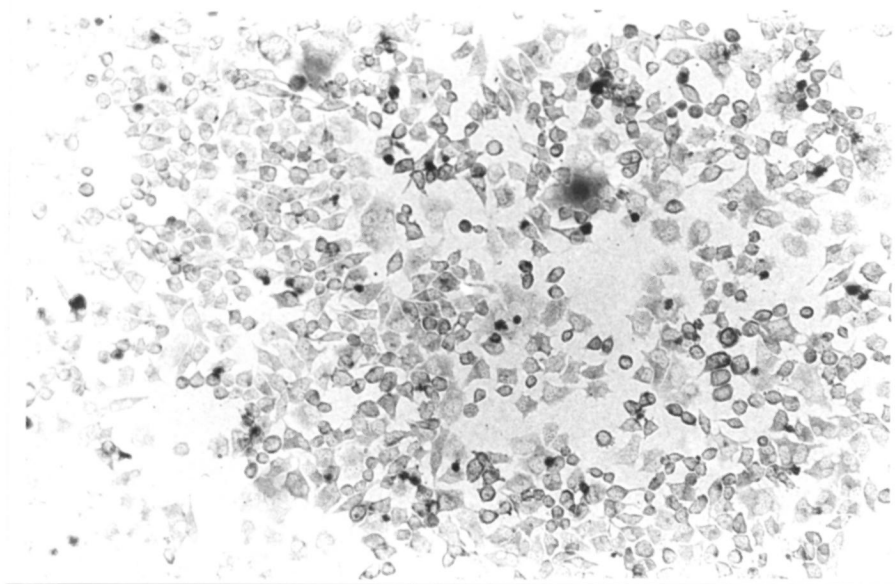


Figure 21. InR1-G9 Cells Stained for Pancreatic Polypeptide (PP).

Immunoperoxidase staining of PP appears dark. Magnification 200x

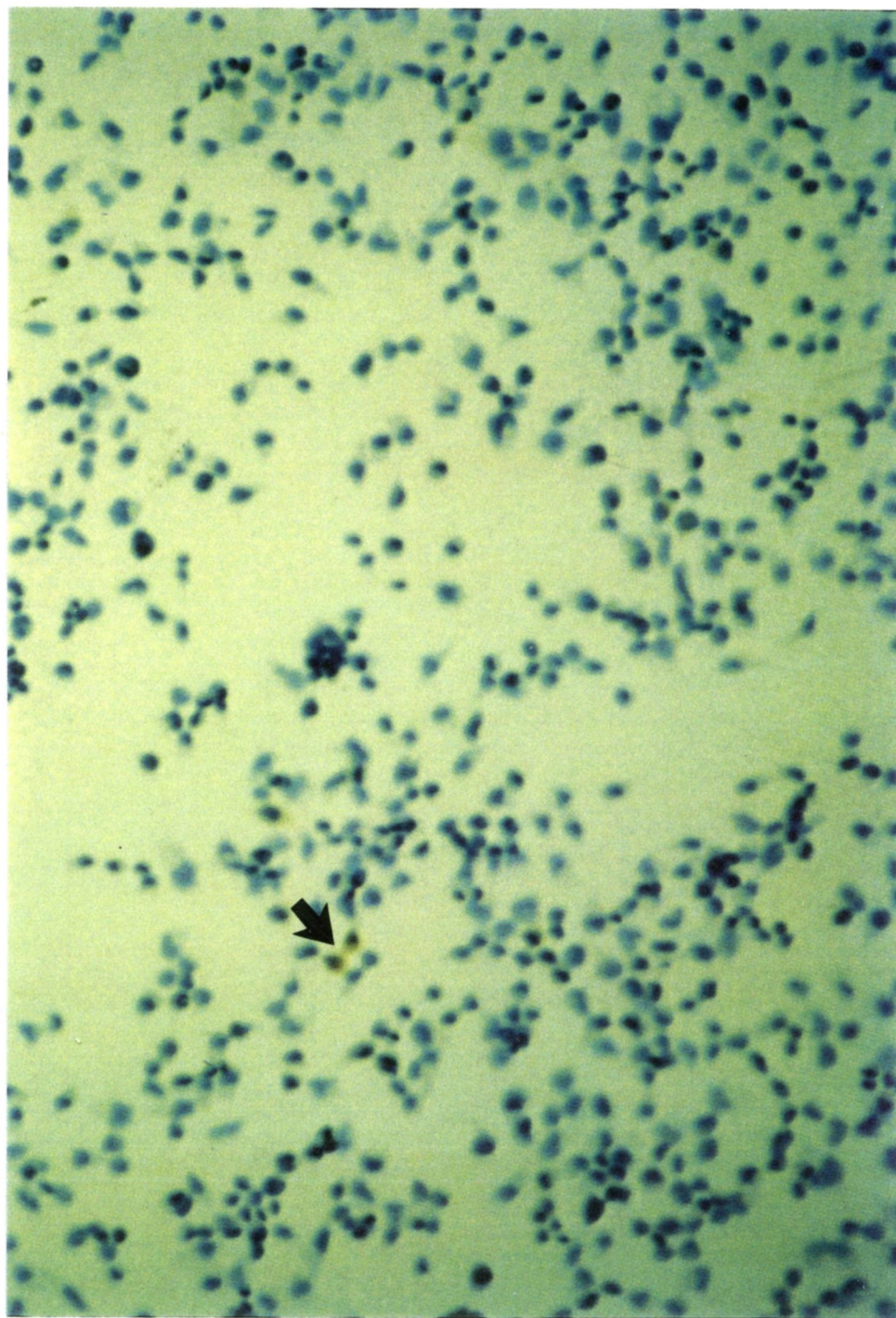


Figure 22. InR1-G9 Cells Stained for Insulin.

Immunoperoxidase staining of insulin (brown) is exceptionally rare (arrow). Haematoxylin counterstaining stained InR1-G9 cell nuclei (blue). Magnification 200x

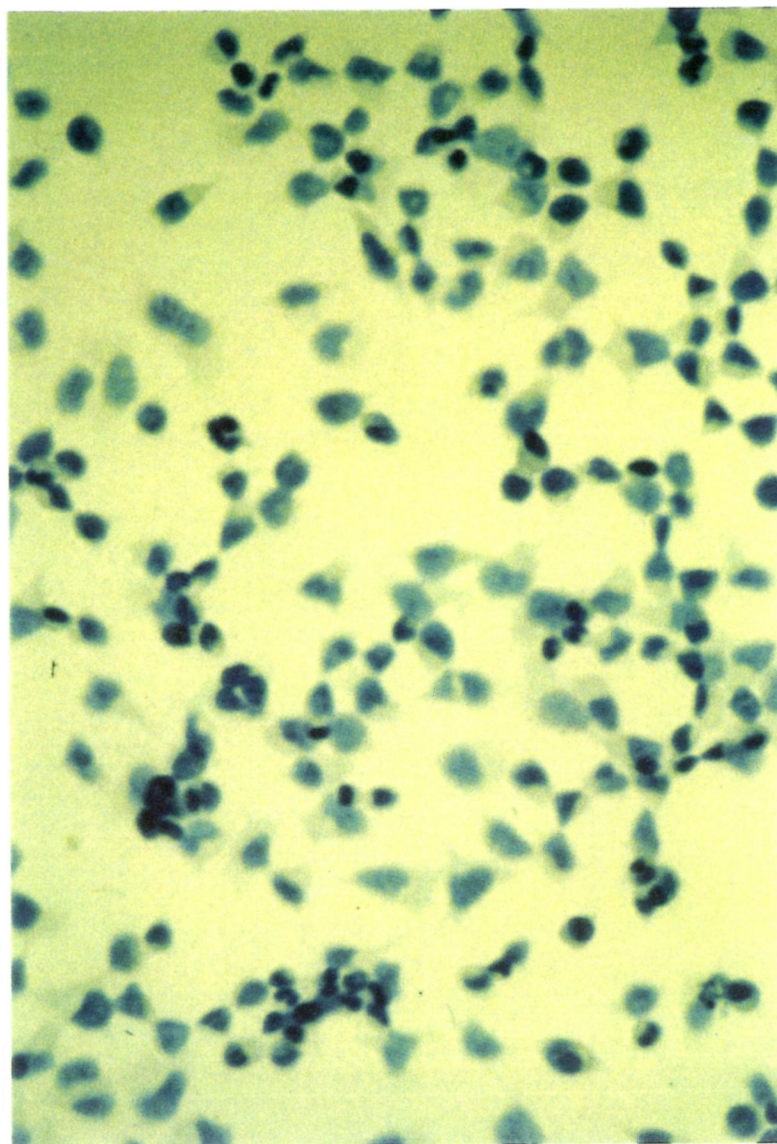


Figure 23. InR1-G9 Immunoperoxidase Control

InR1-G9 cells stained with the immunoperoxidase method in the absence of a primary-Ab, but using a secondary-Ab to guinea-pig immunoglobulins . Haematoxylin staining of nuclei appears blue. Phase contrast allows cells to be visualized. Note that anti-mouse secondary-Ab showed similar results. Magnification 400x

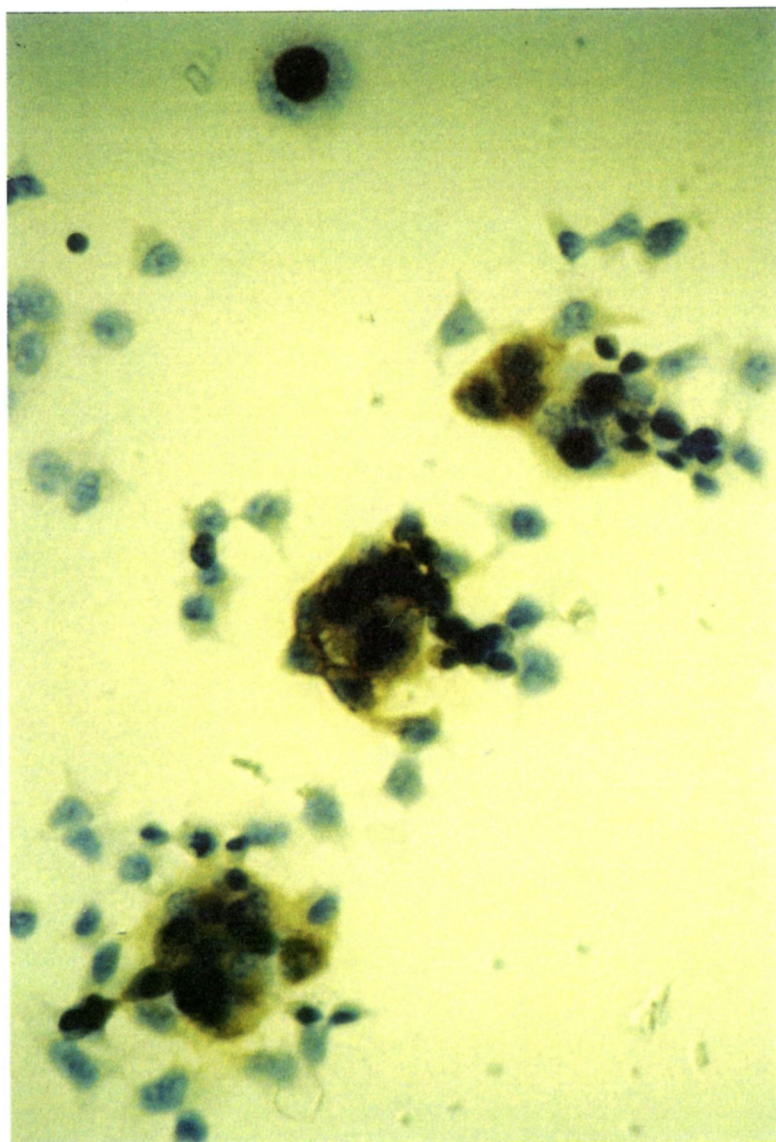


Figure 24. Co-cultured HIT-T15 and InR1-G9 Cells Stained for Insulin.

Immunoperoxidase staining of co-cultured cells for insulin appears brown, and haematoxylin counterstaining of nuclei appears blue. Magnification 400x

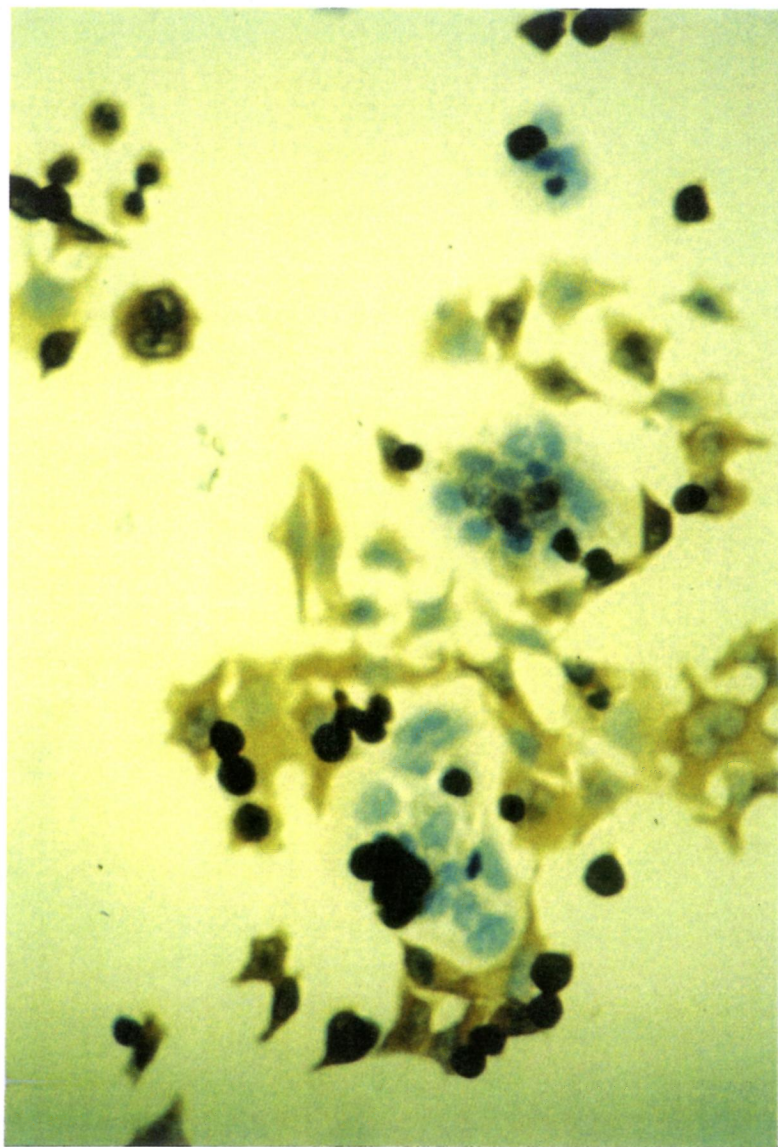
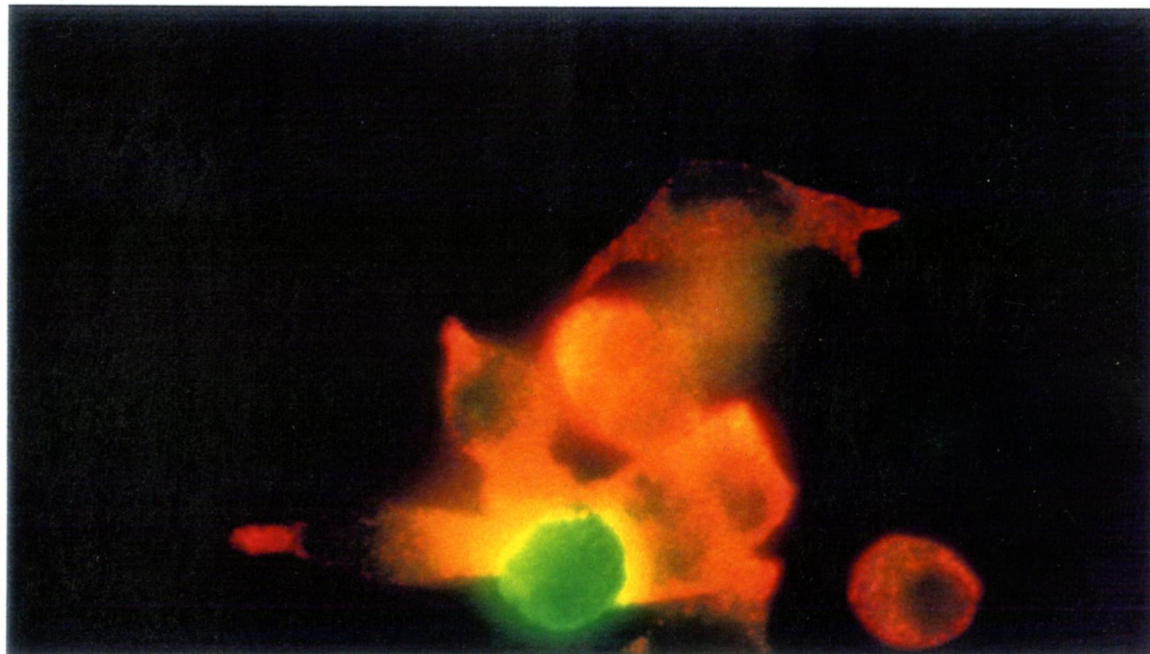


Figure 25. Co-cultured HIT-T15 and InR1-G9 Cells Stained for Glucagon.

Immunoperoxidase staining of co-cultured cells for glucagon appears brown, and haematoxylin counterstaining of nuclei appears blue. Magnification 400x

A



B

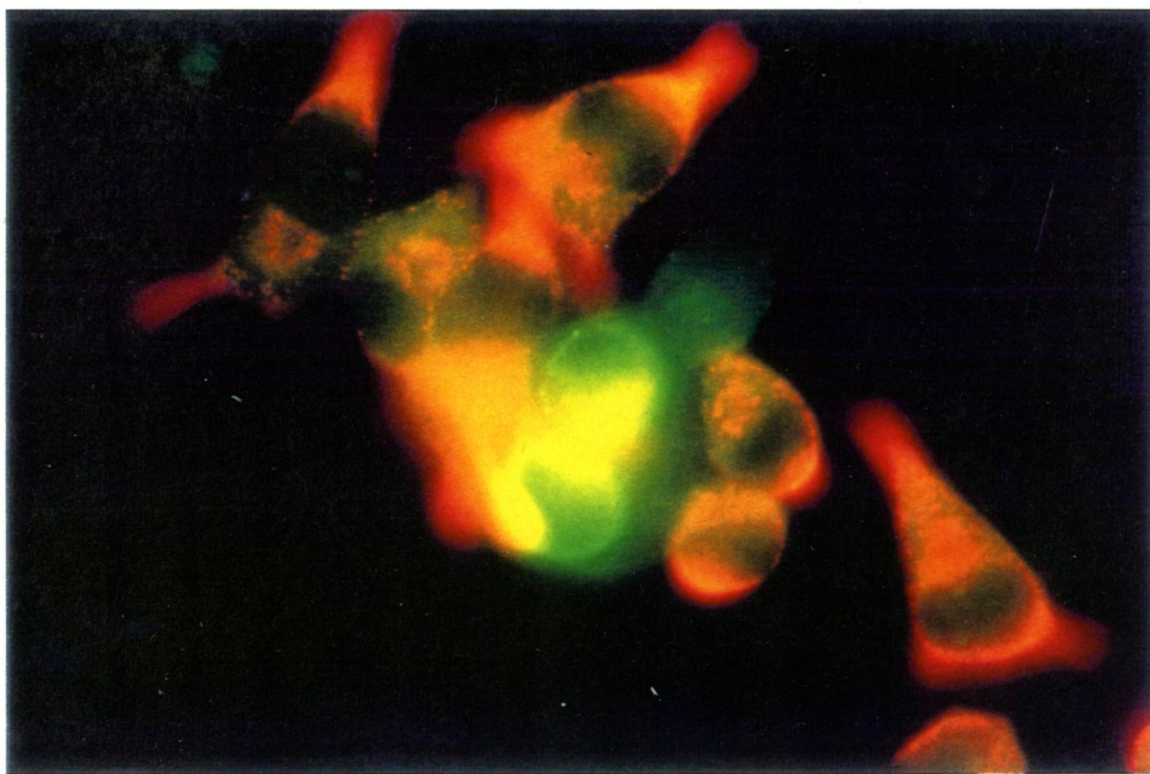


Figure 26. Co-cultured HIT-T15 and InR1-G9 Cells Stained for Insulin/Glucagon.

Staining of co-cultured cells for glucagon appears red (Rhodamine), while staining of insulin appears green (FITC). Magnification 1000x.

CHAPTER FIVE - ELECTRON MICROSCOPY RESULTS

I. Immunogold Staining.

Immunogold staining of HIT-T15 and InR1-G9 co-cultures for insulin and glucagon yielded no convincing labeling when viewed with the electron microscope. Cell cytoplasm was dotted with gold particles, however, nuclei and epon embedding plastic also showed some background. Staining of prepared HIT cell sections was complicated by fixative interference with Ab binding. Fixative interference was confirmed with immunoperoxidase staining of Bouin's, paraformaldehyde (2%), glutaraldehyde (0.5%) and glutaraldehyde (0.5%) plus 1% osmium tetroxide fixed tissue (data not shown). At the light microscopic level, Bouin's fixed tissue was stained positively using the immunoperoxidase method (Figures 10-16). On the contrary, the preferred EM-fixatives paraformaldehyde, glutaraldehyde and osmium tetroxide prevented staining, and were therefore not suitable for electron microscopic tissue preparation in these experiments (data not shown). Following this result, further attempts at immunogold staining of HIT-T15 cultures and HIT-T15/InR1-G9 co-cultures were not made. Although staining was not performed HIT-T15 and InR1-G9 cells were easily distinguished by their gross morphology, such that, HIT-T15 cells appear round while InR1-G9 cells extend neuron-like processes.

II. HIT-T15 Cell Morphology.

Electron microscopic examination revealed changes in HIT-T15 cell insulin granulation under varied culture conditions. Specifically, cells cultured in media supplemented with high-glucose (11.1 mM) had large numbers of secretory-granules (Figure 27). Furthermore, the number of granules appeared to increase with culture time (compare Figures 27 and 28). Similarly, cells cultured in low-glucose (0.8 mM) supplemented media were also extensively granulated (Figure 29). As in the high-glucose condition, culture time appeared to increase the overall number of granules (compare Figures 29 and 30). No evidence was obtained for polarity

in distribution of secretory granules, in either the low (0.8 mM)- or high (11.1 mM)-glucose culture conditions. Unlike the two previous culture conditions, HIT-T15 cells co-cultured with InR1-G9 cells (50:50) in high-glucose (9.2 mM) media were agranular. For a summary of these results see Table 5.

In all of the described culture conditions HIT-T15 cells appeared to store large amounts of glycogen (see small arrows in Figures 27-31).

III. InR1-G9 Cell Morphology.

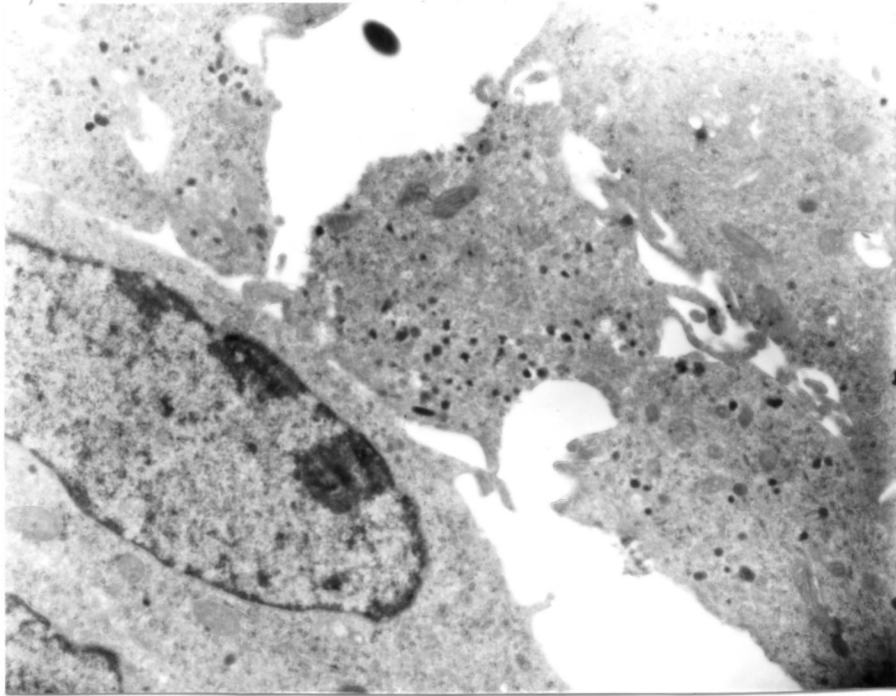
InR1-G9 cells appear to release secretory granule contents into the interstitial space between adjacent cells (Figure 31). This directed release of granule content is further enhanced by the neuron-like projections that InR1-G9 cells extend toward other cells, as these projections contain the majority of secretory granules (Figure 32). This finding supports the light microscopic observation that fluorescent staining for glucagon is most intense at the tip of InR1-G9 projections.

Table 5 - HIT-T15 Cell Secretory-Granulation Under Varied Culture Conditions

Culture Conditions		Secretory-granule		Percentage of
Glucose (mM)	Duration (days)	Number/(1 section)	Characteristics	live cells
11.1	2	25-100	Clustered	> 95
11.1	10	25-100	Clustered	> 95
0.8	2	25-100	Unclustered	75 - 90
0.8	10	25-75	Unclustered	50 - 75
* 9.2	2	0-5		> 95

* This condition consisted of HIT-T15 cells co-cultured with equal numbers of InR1-G9 cells.

A



B

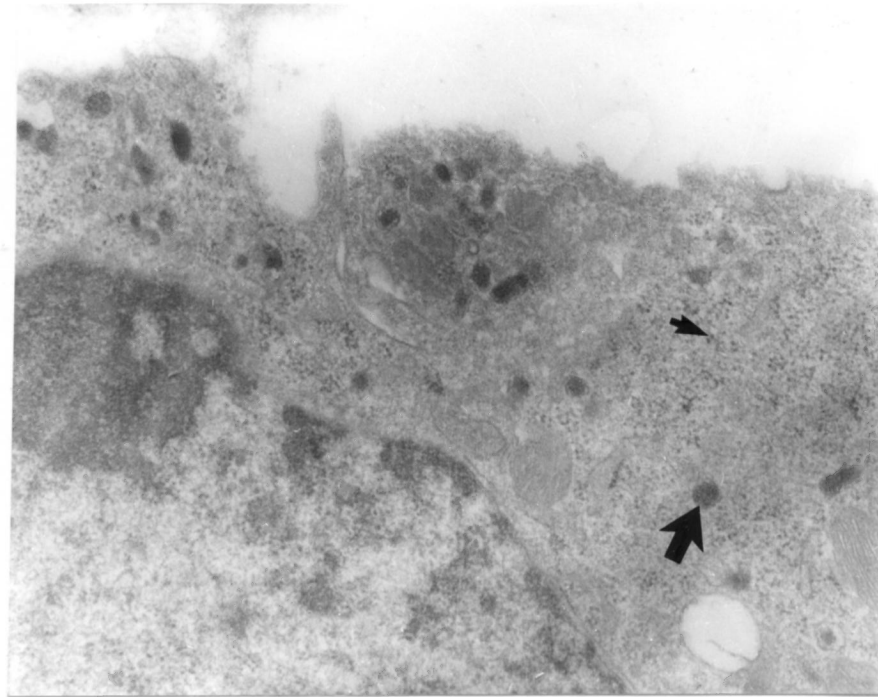


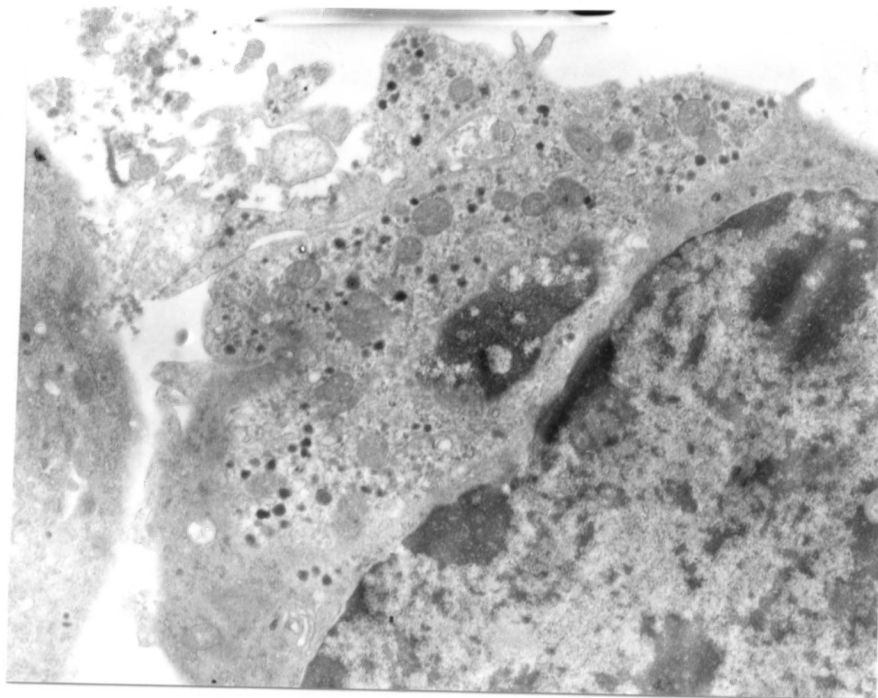
Figure 27. HIT-Cells Cultured in 11.1 mM-Glucose (2 days).

Secretory-granule (large arrow) and glycogen-granule (small arrow)

A. Magnification 6,500 x

B. Magnification 21,500 x

A



B

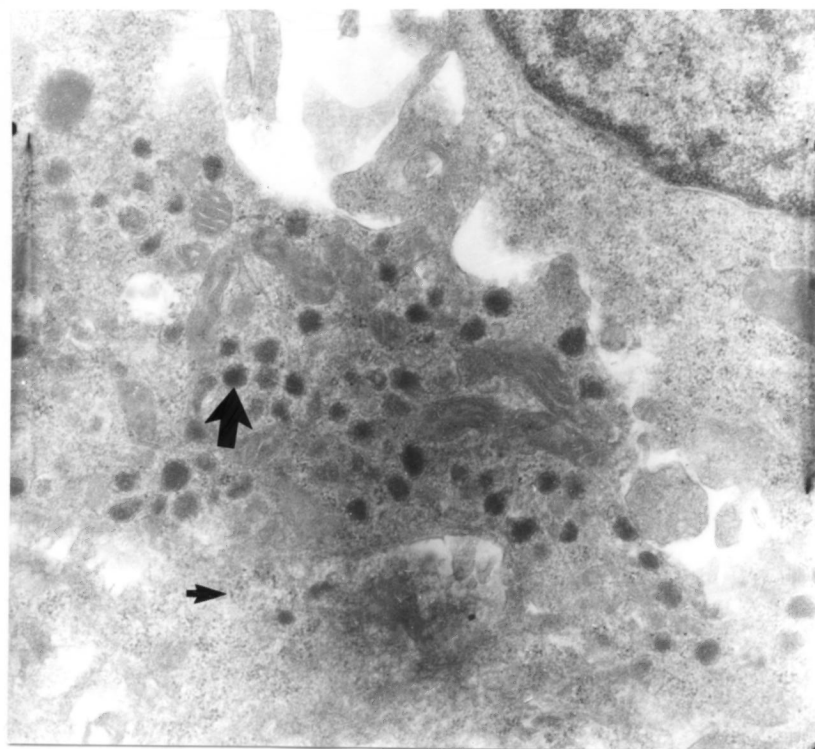


Figure 28. HIT-Cells Cultured in 11.1 mM-Glucose (10 days).

Secretory-granule (large arrow) and glycogen-granule (small arrow)

A. Magnification 10,500 x

B. Magnification 21,500 x

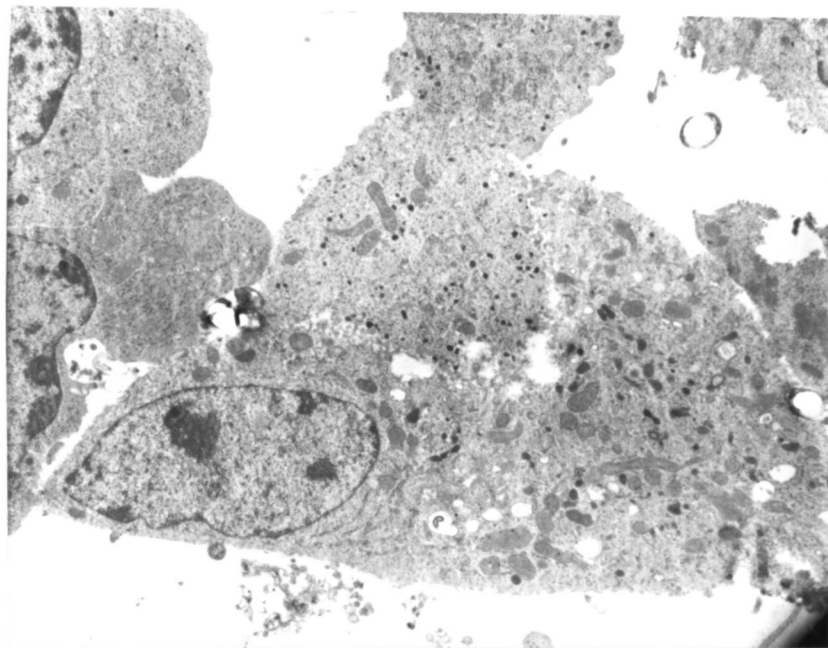
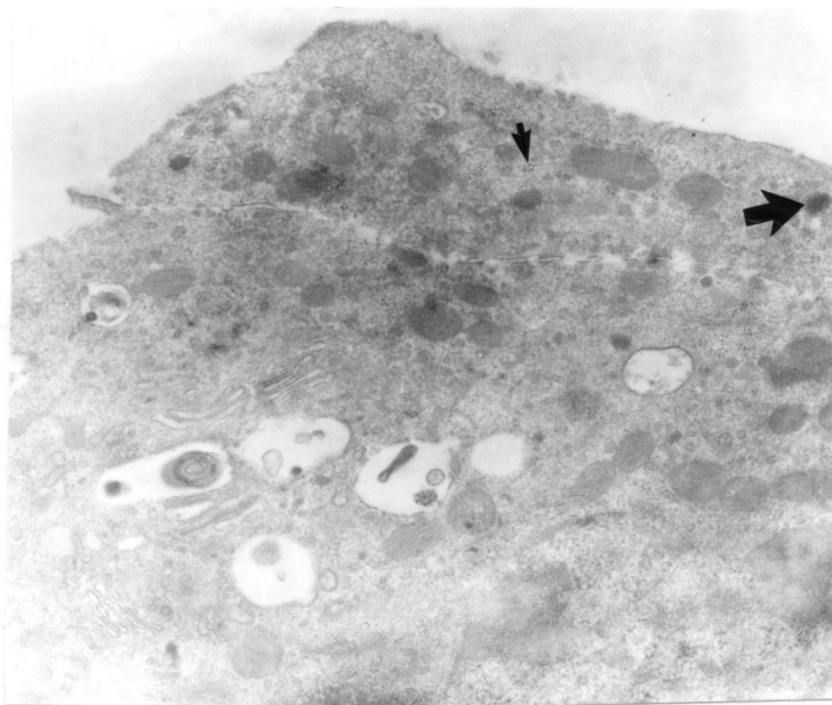
A**B**

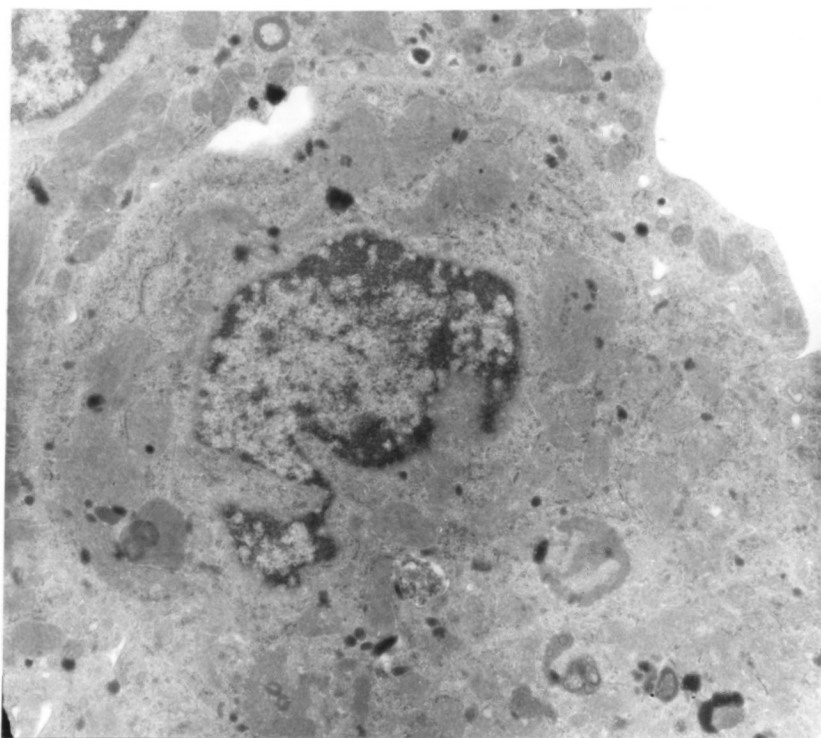
Figure 29. HIT-Cells Cultured in 0.8 mM-Glucose (2 days).

Secretory-granule (large arrow) and glycogen-granule (small arrow)

A. Magnification 5,200 x

B. Magnification 21,500 x

A



B

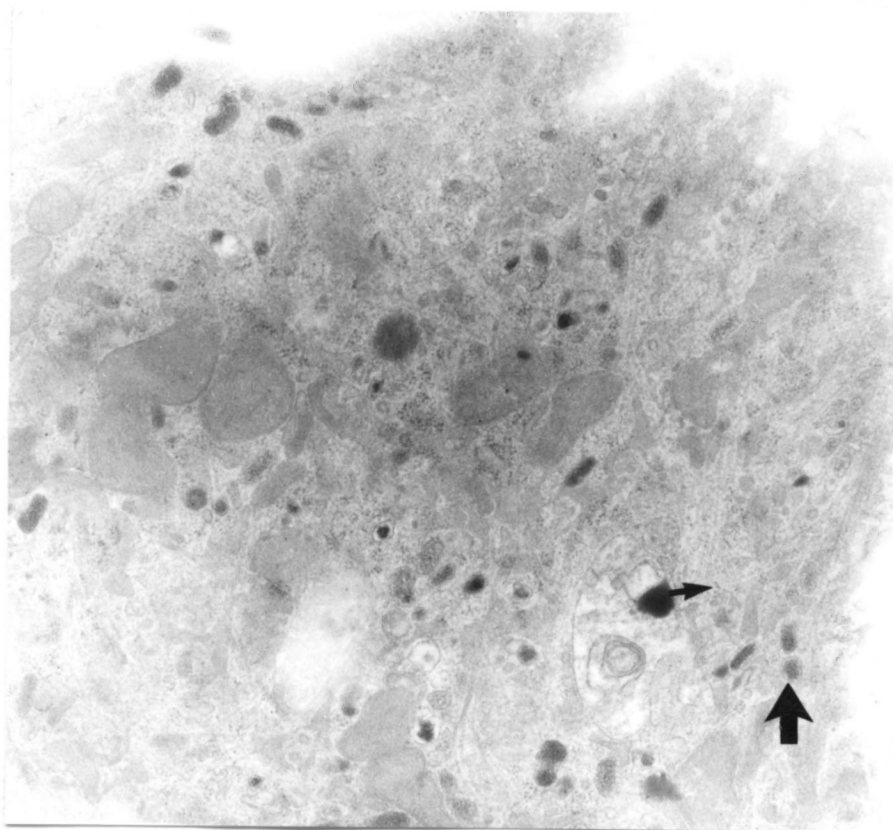


Figure 30. HIT-Cells Cultured in 0.8 mM-Glucose (10 days).

Secretory-granule (large arrow) and glycogen-granule (small arrow)

A. Magnification 10,500 x

B. Magnification 21,500 x

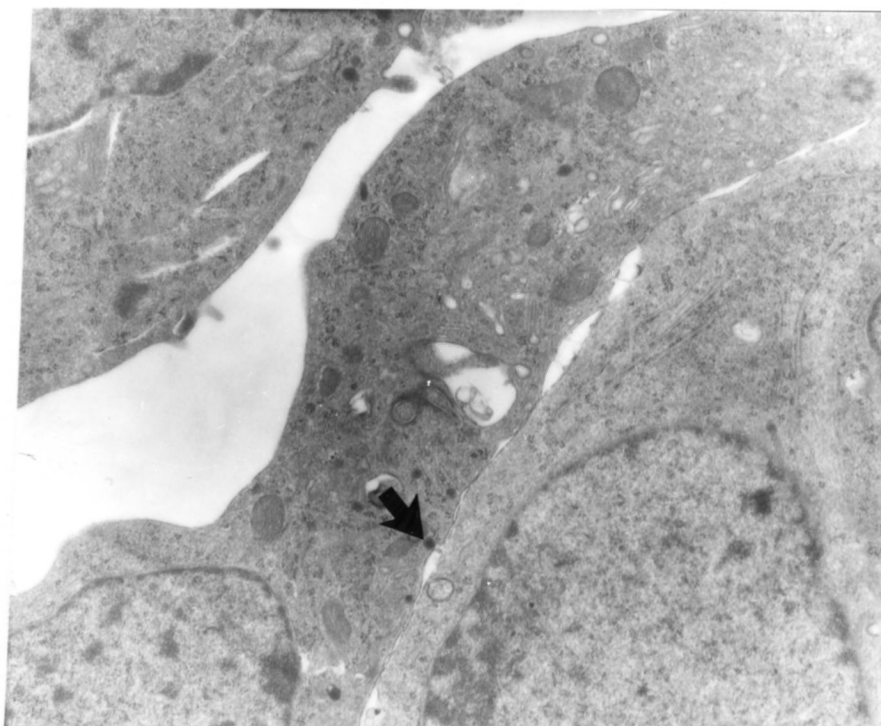
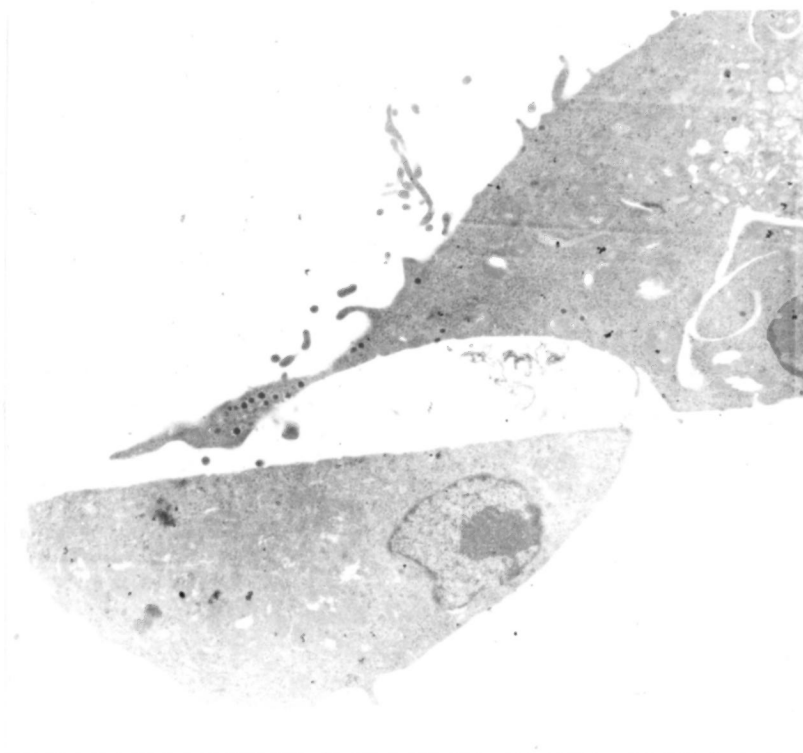


Figure 31. HIT-T15/InR1-G9 Cells Co-cultured in 9.1 mM-Glucose (2 days).

InR1-G9 projection (middle cell) in close contact with an adjacent cell. The position of some glucagon-granules (large arrow) suggests that glucagon may be released into the space between cells.

Magnification 10,500 x

A



B

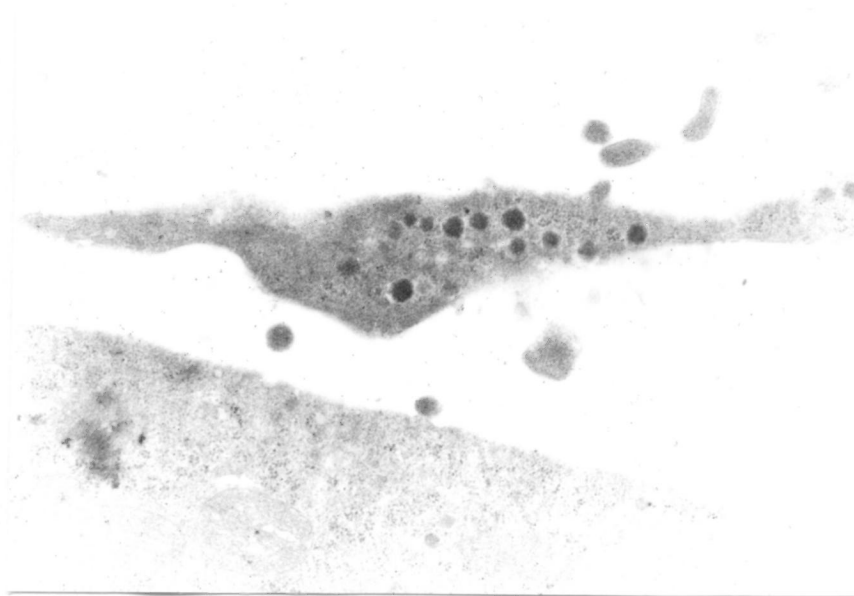


Figure 32. InR1-G9 Cell Projections and Hormone Granule Localization.

A. InR1-G9 projecting a process toward an adjacent cell. Magnification 7,000 x

B. Hormone granule localization within the process above. Magnification 20,500 x

CHAPTER SIX - DISCUSSION

I. Morphology

A. Immunocytochemistry.

HIT-T15 cells (passage 68) stained with the immunoperoxidase method were positive for the peptide hormones insulin, glucagon, GLP-I, PYY and PP. Of these peptides glucagon and GLP-I are known to have a regulatory role in insulin secretion, raising the possibility that PYY and PP may also exert effects on the HIT-T15 cells. The presence of glucagon and GLP-I immunoreactivity in the HIT-T15 cell line suggested that other glucagon family members such as GIP and secretin may also be expressed. However, no immunoreactivity for the glucagon family members GIP and secretin was found in the HIT-T15 cell line, suggesting that expression was limited to the preproglucagon gene. Another product of this gene is GLP-II, but lack of a specific antibody precluded staining for the peptide. A small percentage (< 5%) of HIT-T15 cells demonstrated immunoreactivity for somatostatin.

Interestingly, insulin and glucagon were colocalized within single cells, as demonstrated by double staining with fluorescent and immunoperoxidase techniques. Some cells showed no co-localization. However, cells with co-localization varied, such that, some stained for both peptides with one at a greater intensity, while others stained with equal intensity for both peptides.

All HIT-T15 cells stained positively for PYY, however, as with insulin and glucagon, some cells stained more intensely than others. It has been reported that the midgestational fetal pancreas (human and porcine) contains cells that co-express insulin, glucagon, somatostatin and PP/PYY (31, 85). Additionally, these hormones were localized in separate secretory granules (31) or colocalized in individual secretory granules (85), however, this co-expression was not

found in the newborn pancreas of either species (31, 85). As the HIT-T15 cells display multiple peptide expression and colocalization, in a manner similar to the fetal pancreas (31, 85) they may provide a model for the study of fetal islets and/or islet development. Additionally, these characteristics are also similar to those of human and rodent islet tumors (3, 11, 13, 38), thus HIT-T15 cells may also be a good model for studying these tumors.

The InR1-G9 cell line was immunoreactive for glucagon, GLP-I and PP. The presence of PP immunoreactivity in all cells suggests a potential regulatory role in this cell line.

B. HIT-T15 Cell Ultrastructure.

Ultrastructural studies demonstrated that HIT-T15 cell secretory-granule content varied with condition and duration of culture. Glucose levels appeared to have only minor effects on secretory-granule characteristics, however, in all culture conditions the HIT-T15 cells stored large amounts of glycogen. Hyperglycaemic mice and rat models exhibit glycogen stores that are reduced by lowering circulating glucose levels (52). It was thought that HIT-T15 cell glycogen could be due to chronic exposure to high-glucose culture conditions shunting glucose into the glycogenic pathway. However, after 10 days in low-glucose culture conditions no significant depletion of intracellular glycogen was observed.

II. Cell Culture

A. Growth Pattern of HIT-T15 Cells in Culture.

Trypsinized HIT-T15 cells re-aggregate into islet like masses. This re-aggregation occurs prior to cell attachment to the culture vessel, and can be observed under the light microscope. Within five to ten minutes clusters of three to ten cells have formed. Uvomorulin, a cellular adhesion molecule (cadherin), is probably responsible for this aggregation, as uvomorulin immunoreactivity was clearly localized to the external surface of the HIT-T15 cells. As

uvomorulin is a Ca^{2+} -dependent adhesion molecule, studies comparing the re-aggregation of cells in Ca^{2+} containing and Ca^{2+} free media, would be expected to further confirm the role of Ca^{2+} -dependent uvomorulin in HIT-T15 cell clustering.

B. Effect of Low-Glucose Culture Conditions on Cell Viability.

As mentioned above, HIT-T15 cells are reported to regain some of their responsiveness to glucose when cultured in low-glucose (118). Thus, it was important to determine the ability of these cells to grow and survive in low-glucose media. Low-glucose (0.8 mM) media culture adversely affected HIT-T15 cell viability in a time-dependent fashion. Evidence for this was, as follows: 1) following one week of culture, there were approximately four times more floating cells in culture media containing low-glucose than in culture media containing high-glucose, 2) floating cells were dead, as indicated by an inability of these cells to exclude the viability stain trypan blue, 3) electron microscopic examination revealed a greater number of dead cells in low-glucose compared to high-glucose cultures, and 4) cell doubling time was almost twice as long in the low-glucose cultured cells. These results indicate that growing HIT-T15 cells in low-glucose containing media may not be practical. However, the reported (118) restoration of glucose responsiveness may over-ride any extension in time required to perform experiments.

C. Insulin Secretion from HIT-T15 Cells.

The glucose responsiveness of HIT-T15 cells (passage 68), with maximal glucose-stimulated insulin secretion at around 10 mM glucose, corresponded well with the maximal glucose-stimulated insulin secretion for hamster β -cells (120) and HIT-T15 cells in the hands of other investigators (120, 125). However, as previously reported, when compared with hamster β -cells, HIT-T15 cells show a blunted insulin secretory response, due primarily to a low total cell content (120, 125). The total cell content of HIT-T15 cells at passage 68 (3.2 mU/ml) was one fifth of the reported value for cells at passage 41 (15.0 mU/ml), but was six times greater than that reported at passage 88 (0.5 mU/ml) (125). Thus, in view of the decreasing total cell content

previously reported, the values appeared to be within the range expected for cells at passage 68. There is a reduction in HIT-T15 cell insulin secretion with increasing passage, which parallels the reduced content. It is possible that this reduction in secretion is due to alterations in HIT-T15 cell metabolism, or changes in the ratio of insulin- to other peptide-containing cells with successive passages.

The morphological studies demonstrated co-production of glucagon within the HIT-T15 cells. Therefore, because of its known stimulatory action on insulin release, the ability of glucagon to regulate HIT-T15 cell secretion was examined.

Addition of a monoclonal glucagon-antibody, to passage 68 cells, completely abolished glucose-stimulated insulin secretion over all glucose concentrations tested. The glucagon-antibody did not affect total insulin content of HIT-T15 cells, as determined by extract values. Control experiments demonstrated that addition of either a somatostatin or GIP monoclonal antibody did not alter secretion indicating the specificity of the technique. No interference of the glucagon antibody with the insulin RIA was observed either, with no change in non-specific binding levels. Furthermore, samples from the two immunoneutralization studies were assayed using different techniques, and their results corroborated one another. These studies could be further supported by performing experiments using varying concentrations of the glucagon-Ab, and by using other glucagon-antibodies. Finally, in order to rule out this effect as a secondary one, immunoneutralization of other peptides found in the HIT-T15 cell line (GLP-I, GLP-II, PYY and PP), should be performed.

Of the remaining peptides localized to the HIT-T15 cells only the activity of somatostatin has been investigated. An antibody to somatostatin, added in the same manner as the glucagon antibody, had no effect on glucose-stimulated insulin release. These data indicate that the amount of somatostatin released into the medium was insufficient to affect insulin release.

The abolition of glucose-stimulated insulin secretion by a glucagon-antibody indicates that glucose-stimulated insulin secretion in HIT-T15 cells may be dependent on glucagon co-secreted from the cells acting through a receptor-dependent pathway. Presumably, the antibody inhibits insulin secretion by binding the functional domain of the antigen, thus rendering it incapable of binding to its receptor. It is unlikely that gap-junctional pathways could be blocked by an antibody, as it would not traverse the cell membrane and interfere with intracellular mechanisms. No attempt was made in the present studies to define the nature of potential receptor-mediated mechanisms, however, previous studies of the effect of glucagon on HIT-T15 cells indicates that they respond to glucagon via a G-protein mediated rise in cAMP which causes the release of insulin (75, 116). These data support a receptor-dependent mechanism for glucagon's effect because, it is unlikely that glucagon released from HIT-T15-G cells acts in a manner different from exogenously applied peptide. It is also unlikely that glucagon acts through cytosolic receptors, as once again, antibodies would not block this effect.

Recent studies, of β -cell clones (β -TC3), have demonstrated the presence of receptors that bind GLP-I more readily than glucagon (H. Kofod, NOVO, unpublished data). However, as mentioned in the background section, paracrine communication between cells, may not require a high-affinity receptor. The high concentrations of peptide that receptors are in contact with may be sufficient, if the peptide release is directed toward 'target' cells, rather than being released into the general circulation, thus eliminating the need for a high-affinity receptor. The effects of endogenous GLP-I on HIT-T15 cell insulin secretion were not examined in this investigation.

D. Glucagon Secretion.

Glucagon release from HIT-T15 cells was measured in response to glucose, and no significant difference was seen between zero-, low- and high-glucose conditions. This suggests that glucagon was differentially released from the HIT-T15 cells and was not regulated by glucose. It also demonstrates that glucagon and insulin are stored in separate secretory granules, because, if insulin and glucagon were stored in the same granule a parallel rise in glucagon

secretion would follow the glucose-stimulated insulin secretion. The most effective secretagogues for glucagon secretion are amino acids. However, their effect was not examined in the present study. These data do, however, provide evidence for a basal level of glucagon release from HIT-T15 cells which provides a basis for the requirement of glucagon for glucose-stimulated insulin release.

E. Co-Culture HIT-T15 and InR1-G9 Cells.

1. Insulin Secretion from Co-cultures.

Insulin release from HIT-T15 cells was stimulated by the addition of exogenous glucagon-secreting cells (InR1-G9; passage 24). The InR1-G9 cell line stimulated insulin secretion was not enhanced by glucose in any of the concentrations tested. The 25:75 condition clearly demonstrates an absence of glucose-potential of glucagon-stimulated insulin release. It also demonstrates a glucose-independent, glucagon-stimulated insulin secretion, as shown by the high level of insulin released in the zero-glucose condition. Glucagon concentrations were not measured in these experiments. The usefulness of this data would, however, be limited as all concentrations of InR1-G9 cells tested exhibited the same degree of insulin stimulation. This indicates that even at 25% of the cells, a maximal stimulation of insulin secretion was present. Co-cultures with fewer InR1-G9 cells would be useful in determining if HIT-T15 cells already possess the optimal glucagon conditions for maintaining glucose-stimulated insulin secretion.

2. Morphology of Co-cultures.

Fortunately HIT-T15 cells were easily distinguished from InR1-G9 cells by their gross morphology and growth patterns. For example, HIT-T15 cells were circular, while InR1-G9 cells extended neuron-like processes (almost without exception). In addition, HIT-T15 cells grew in dome shaped clusters, probably mediated by uromodulin (as suggested by the presence of uromodulin immunoreactivity). On the contrary, InR1-G9 cells, which were not

immunoreactive for uromorulin, grew evenly spaced apart until cell density caused them to grow together after approximately one week. These growth patterns were maintained even when co-cultured. Interestingly, InR1-G9 cells appeared to direct hormone release toward adjacent cells, by concentrating secretory granules within projections that were reaching toward neighboring cells. Further evidence for this apparent polarization of secretory granules is provided by the high intensity of fluorescent staining found at the tips of InR1-G9 cell projections. These data suggest that InR1-G9 cells, like somatostatin cells of the gut, may act in a directed paracrine fashion (74). This directed release may reduce the total amount of hormone required, for a specific effect, by concentrating the peptide to sites of action.

In co-culture, HIT-T15 cells appear to be essentially agranular, while, InR1-G9 cells had granules. The absence of secretory granules in co-cultured HIT-T15 cells is likely due to depletion caused by chronic stimulation, and an inability to replenish stores. The inability to replenish these stores could be due to the length of glucagon exposure, or an absence of phasic glucagon release. It is unknown why high glucose concentrations do not cause a similar degranulation of the HIT-T15 cells, however, this difference indicates that glucagon is probably a more potent stimulus to insulin secretion than glucose alone in these experimental conditions. In a fashion similar to the normal β -cell, glucose may play a role as a potentiator of glucagon-stimulated insulin secretion (127). It would be interesting to see if granules were depleted in low-glucose media co-cultures. Studies using high-performance liquid chromatography, could be used to determine whether or not the proinsulin/insulin ratio of degranulated co-cultures was elevated. If elevated this might provide evidence for constitutive release of insulin in this experimental condition.

III. Future Directions.

Studies to quantify relative peptide abundance between early and late HIT-T15 cell passages, would be useful. Especially if glucagon expression rose with increasing passages, as a rise in glucagon expression could be the cause of the elevated basal and reduced insulin secretory response.

Subcloning of pure insulin and pure glucagon secreting cells from the HIT-T15 cell line, and examining their peptide secretion alone and when mixed at fixed ratios, could further assist in understanding HIT-T15 cell function. Performing these experiments would require HIT-T15 cells of a very early passage, as growing subcloned cells would take them into later passages where their usefulness as a model is in question. This study would be complicated by the co-expression of peptides in the HIT-T15 cell line, however, some cells do not co-express insulin and glucagon, and these should be the cells targeted for further study.

And finally, studies of insulin release in co-cultures could also be done in a time course experiment, with observations being taken at hour intervals. These studies would help to determine how long these conditions had to exist for degranulation to occur.

CONCLUSIONS

In conclusion, glucose-stimulated insulin secretion from the HIT-T15 cell line is glucagon-dependent, and endogenous glucagon-cells (HIT-T15-G) provide a ready source of glucagon. The addition of large numbers of exogenous glucagon secreting cells (InR1-G9) led to a high-level stimulation of HIT-T15 cell insulin secretion, and this secretion was not potentiated by glucose. The absence of glucose-potentiation of glucagon-stimulated insulin secretion was probably due to degranulation of cells, due to chronic high-level stimulation by glucagon. The HIT-T15 cells appear to be different from normal β -cells in that β -cells are only partially dependent on glucagon for full glucose-responsiveness, while HIT-T15 cells are completely dependent on glucagon's presence. Further, studies are required to determine whether or not this apparent complete dependence on glucagon is a property of only the HIT-T15 cells, or a property of other cell types such as a fetal β -cell precursor. These studies suggest that the HIT-T15 cell line is not a good adult β -cell model, but may be a good developmental or cancer model.

Additionally, HIT-T15 cells are a heterogeneous cell population, expressing multiple peptides. Some of these peptides were colocalized, as in the case of insulin and glucagon. On the contrary, InR1-G9 cells appeared to be comparatively homogeneous, with immunoreactivity for PP in all cells. These cells extended neuron-like processes, which contained the majority of secretory granules.

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