PANCREATIC PEPTIDE HORMONE EXPRESSION IN THE RINm5F CELL LINE

by

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Signed by candidate

Date: 8th September 1994

To my precious son, Deon

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Table 1: 57

LIST OF ABBREVIATIONS

μg	microgram
μl	microlitre
ABC-HRP	avidin-D and biotinylated horse-radish peroxidase H reagent (Vectastain)
b-GAR-IgG	biotinylated goat anti-rabbit immunoglobulin
BSA	bovine serum albumin
DAB	3,3' diaminobenzidine
DMSO	dimethyl sulphoxide
H_2O_2	hydrogen peroxide
HEPES	N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid]
MC	mitomycin C
ml	milliliter
NaB	sodium butyrate
NGS	normal goat serum
PAF	paraformaldehyde
PBS	phosphate buffered saline
PMSF	phenylmethylsulphonyl fluoride
SA-FITC	strepavidin fluorescein
SV	simian virus
TBS	Tris buffered saline
TEMED	N,N,N',N-tetramethylethylenediamine
TH	tyrosine hydroxylase
TX	Triton X 100

ABSTRACT

Phenotypic heterogeneity is a striking feature of the pancreatic islet cell lines (RIN and MSL) derived from a rat islet cell tumour (Chick et al., 1977) which may therefore be suitable for the study of islet cell differentiation in vitro. In the present study, the RINm5F cell line (Oie et al., 1983) was investigated to determine whether or not it would be suitable for this purpose. This study presents the results from work aimed at determining which hormone-producing phenotypes were expressed under routine and modified culture conditions.

Insulin, detected by immunoperoxidase labelling at earlier passages, was not detectable in later passages of RINm5F-MRC cultures. Immunoblots and radio-immunoassay performed on cell extracts and culture medium respectively confirmed that RINm5F cells contained proinsulin and that low levels of insulin and/or proinsulin were secreted into the medium. These findings suggested that the insulin content of individual cells was at the lower limit of detection for the immunoperoxidase technique. To overcome the problem of detecting and quantifying the number of cells containing low levels of insulin by microscopy, a protocol was established for immunolabelling cell suspensions for analysis by flow cytometry. Flow cytometry confirmed that RINm5F cultures contained a small percentage (less than 2%) of cells stained for insulin and that a greater subpopulation of cells (18-38%) were positively stained for insulin after cultures had been exposed to 2mM sodium butyrate for three days. Although positively stained cells were observed by immunoperoxidase or immunofluorescence microscopy in sodium butyrate-treated cultures, staining was barely above that of background staining in peroxidase-stained preparations and positively labelled cells could only be identified at high magnification in fluorescent-stained preparations. Thus flow cytometry successfully provided an alternative approach to accurately quantify insulin expression in RINm5F-MRC cells.

Flow cytometry, immunoblotting and immunocytochemistry established that all glucagon and somatostatin-like immunoreactivity in RINm5F cultures was non-specific and was most likely caused by the binding of non-specific components present in rabbit serum. Ultrastructural studies confirmed that morphologically differentiated islet cell phenotypes were not present in RINm5F-MRC cultures. The finding that RINm5F-MRC cultures consisted mainly of agranular cells and that only a small subpopulation of cells stained for insulin, indicates that this cell line appears to be poorly differentiated. However, the increase in the number of cells containing insulin and the increased insulin secretion in response to sodium butyrate shows that at least some of these cells have the potential to differentiate. These results indicate that RINm5F-MRC cells are suitable for the study of β-cell differentiation. It remains to be determined whether or not they are suitable for studying the differentiation of other pancreatic islet cell types.

1. INTRODUCTION

1.1 General introduction:

The endocrine pancreas of mammals consists of several thousand islets of Langerhans that are scattered throughout the pancreas. The four major hormone-producing cell types have a characteristic proportion and distribution within the islets and consist of α, β, δ and PP cells that synthesise glucagon, insulin, somatostatin and pancreatic polypeptide respectively. In addition to peptide hormones, islet cells also synthesise a number of enzymes (some which are transiently expressed) which are commonly expressed by neuronal cells (Le Douarin, 1988).

Although the embryonic pancreas has been extensively investigated, little information exists on the processes that induce endocrine determination or on the mechanisms controlling islet cell differentiation. In contrast to the classic concept of development where progenitors and their progeny are committed to specific differentiation pathways, studies of embryonic pancreas in vitro have indicated that environmental factors influence endocrine cell differentiation (Rutter et al., 1964; Teitelman et al., 1987b). Furthermore, terminally differentiated β cells express embryonic traits in vivo (Teitelman et al., 1988; Teitelman, 1993) and express a neuronal phenotype in vitro (Teitelman, 1990). These findings raise the possibility that islet cells are phenotypically plastic (i.e. they have the potential to transdifferentiate). Such phenotypic plasticity may thus be the cause of the heterogeneity reported in a number of clonal islet cell lines derived from a rat insulinoma (Gazdar et al., 1980; Madsen et al., 1986; Phillipe et al., 1987a).

Cell culture models have been used as a complementary approach to in vivo studies for studying cell differentiation (Watt, 1991) and most studies on islet cell differentiation have been performed in vitro on tissue explants containing different cell types (Rutter et al., 1964; Pictet et al., 1975; Teitelman et al., 1987b). Since unequivocal evidence of transdifferentiation in a number of cell types has been obtained from in vitro experiments (Beresford, 1991), the question of phenotypic plasticity of islet cells may thus be resolved by performing experiments on cultures consisting of an homogenous phenotype. A number of clonal islet cell lines has been established from a transplantable rat islet cell tumour (Chick et al., 1977; Gazdar et al., 1980; Madsen et al., 1986; Phillipe et al., 1987) in order to study β-cell function (Chick et al., 1977).

In the present study, a continuous clonal islet cell line (RINm5F), derived from the islet cell tumour mentioned above (Gazdar et al., 1980; Oie et al., 1983), was examined in order to determine whether this cell line would be suitable for studying islet cell differentiation. To achieve this objective, it was necessary to determine which phenotypes were expressed under routine and experimental culture

conditions. This together with the pursuit of suitable immunolabelling protocols and a procedure to quantify the number of hormone-producing cells in RINm5F cultures is the subject of this thesis.

1.2 Review:

1.2.1 Morphogenesis and differentiation of the endocrine pancreas:

During embryogenesis the mammalian pancreas develops by fusion of the dorsal and ventral primordia that appear as evaginations of the duodenum (Pictet et al., 1972; Rutter, 1980). Morphogenesis of the pancreas begins by evagination of the duodenum at day 9.5 and 11 of gestation of the mouse and rat respectively (Pictet et al., 1972). Thereafter, rapid cellular proliferation and growth occur in each primordium to form cellular cords that form ductules that proliferate, branch and ultimately differentiate (Pictet et al., 1972; Rutter, 1980). The two primordia fuse at day 10.5 and 11.5 of gestation in the mouse and rat respectively. Onset of islet morphogenesis is first observed as isolated granulated cells that appear on the serosal aspect of the embryonic pancreatic ductular epithelium. Thereafter, small clusters of granulated cells are formed adjacent to the ductular epithelium. As these clusters become more populated, the cell mass extends into the mesenchyme where they eventually pinch off from the ductular epithelium to form islets (Pictet et al., 1972; Appel and Like, 1982).

It is generally accepted that the endocrine cells of the pancreatic islets originate from progenitors located in the epithelium lining the pancreatic ducts and ductules and that they originate from the endoderm (Pictet et al., 1976; Le Douarin, 1988; Dubois, 1989). At the onset of pancreatic morphogenesis in rat and mouse, cells containing endocrine-like secretory granules are present in the epithelium lining the pancreatic diverticulum and are subsequently found in all ducts and ductules during organogenesis (Pictet et al., 1972: Pearse et al., 1973). The α cell is the earliest distinguishable cell type in rat, human and mouse embryonic pancreas. Prior to the appearance of α cells, cells containing pleomorphic endocrine-like granules are present in the epithelium of the presumptive pancreatic diverticulum (Pearse et al., 1973). Alpha cells and glucagon are detectable by electron microscopy and immunocytochemistry respectively in the epithelium of the pancreatic diverticulum in 11 day rat and 12 day mouse embryos (Pearse et al., 1973; Rall et al., 1973; Taki and Baba, 1989). In the human embryo, α cells are first seen at 9 weeks in and adjacent to the pancreatic duct epithelium (Like and Orci, 1972). Beta cells are first detectable by electron microscopy in the pancreatic ductules and in small cell clusters adjacent to the ductules at 10.5 weeks in human embryos (Like and Orci, 1972) and at 15 to 16 days in rat embryos (Pictet et al., 1972; Rall et al., 1973). In the rat, insulin is detectable by radioimmunoassay (RIA) at day 11, several days before granulated β cells appear (Rall et al., 1973) and cells synthesising insulin are detected by immunohistochemistry in 14 day rat embryos (Taki and Baba 1989). In mouse embryos, β cells first appear in the epithelium

of the pancreatic ducts (Pearse et al., 1973) at day 12 at which time insulin synthesis is detected by immunocytochemistry (Alpert et al., 1988). In mouse embryos, somatostatin-producing cells appear at day 17 after islets containing α and β cells have formed (Alpert et al., 1988). Pancreatic polypeptide-synthesising cells are first detected by immunocytochemistry in one day newborn mice (Alpert et al., 1988) and in 19 day-old rat embryos (Gomez-Dumm, 1987). In a more recent study pancreatic polypeptide was identified in cells from the dorsal and ventral pancreatic buds of mouse embryos as early as 10.5 and 11.5 days respectively (Herrera et al., 1991). Other workers who used a highly purified pancreatic polypeptide antiserum and improved immunohistochemical techniques claim that pancreatic polypeptide identified in the above study was either the closely related neuropeptide Y (NPY) (Teitelman et al., 1993) or peptide YY (PYY) (Upchurch et al., 1994).

1.2.2 Islet cell differentiation:

A major question concerning differentiated islet cells is whether they are lineage-restricted (i.e. restricted to producing one hormone), or whether they retain the potential to express all islet cell phenotypes (i.e. transdifferentiate). In lineage-restricted cells, the differentiation pathway is determined by genetic programming and interaction between cells and environmental factors have little or no influence on phenotypic expression. In cells that are inherently phenotypically plastic, phenotypic expression is largely determined by complex interactions between cells and the extracellular milieu. A strategy that has been used to examine the mechanisms that regulate islet cell differentiation has been to identify the cell-lineage relationships of the different hormone-producing cell types (Teitelman, 1991). Central to this is the identification of islet cell precursors and whether individual precursors give rise to one, some or all islet cell lineages (Hellerström, 1984). Before cells containing endocrine secretory granules are detected in the developing pancreas, presumptive endocrine progenitors have no identifiable morphological characteristics. They are generally thought to reside in the pool of undifferentiated cells in the epithelium of the embryonic gut that gives rise to the pancreas, and later in the pancreatic ducts and ductules (Pictet et al., 1972). The presence of glucagon, insulin and somatostatin mRNA (detected by reverse transcriptase polymerase chain reaction) in the epithelium of the embryonic gut, suggests that progenitors committed to originating the endocrine lineages are established at least 12 hours before pancreatic morphogenesis begins (Gittes and Rutter, 1992). Co-localisation of C-peptide (a by-product of the conversion of pro-insulin to insulin (Montegue, 1983)), glucagon and the neuronal peptide, NPY, as well as the neuronal enzyme, TH, that were located in the embryonic gut epithelium in 9.5 day mouse embryos (Teitelman et al., 1993), provides strong evidence that islet cell progenitors are multipotential. The existence of multipotential islet cell progenitors is further substantiated from studies using transgenic mouse embryos, harbouring the SV 40 large T antigen (Tag), under the control of the insulin regulatory sequences (Alpert et al., 1988). These authors reported that Tag (indicating that the insulin gene had been expressed) and TH (thought

to be a marker of islet cell precursors (Teitelman et al., 1981, 1987a; Teitelman and Lee, 1987)) were co-localised in cells containing glucagon, somatostatin or pancreatic polypeptide at the onset of their appearance in the embryonic pancreas.

1.2.3 Lineage-based differentiation:

To determine whether the different islet cell types develop along separate lineages or whether they belong to the same lineage, several studies have attempted to reveal the differentiation pathway of islet cells. From numerous observations that have established the sequence of appearance of the different hormone-producing cell types during pancreas development, a few hypothetical models of differentiation pathways that could apply to islet cell differentiation have been proposed (Diagram 1). Multipotential progenitors may give rise to unipotential precursors that give rise to the α, β, δ, or PP-cell lineages (Diagram 1a). From studies of haemopoiesis in vitro, it is generally thought that haemopoietic cells differentiate according to this model (Brown et al., 1988). Because of the similarity of islet and haemopoietic cells concerning different lineages that originate from a single stem cell, it is conceivable that the different islet cell lineages arise via this route. If the different islet cell lineages arose from unipotential precursors, it is likely that differentiation would be determined by a cascade of genetic signals. Brown et al., 1988 proposed an alternative model of differentiation for haemopoietic cells by which different lineages could arise via a sequential-based differentiation pathway (Diagram 1b). According to this model, separate lineages arise from the same multipotential precursors that expresses specific differentiation potentials in an ordered linear sequence. Evidence that suggests that islet cells may differentiate according to this model is supported by the consistent sequential appearance of different islet cell phenotypes during pancreas development of the same species (Pictet et al., 1972; Pearse et al., 1973; Alpert et al., 1988; Taki and Baba, 1989). Accordingly, multipotential precursor cells would first acquire the potential to give rise to glucagon-producing cells; later as this capacity is lost, the potential for insulin differentiation is expressed. The potential to give rise to other hormone-producing cells would thus occur in a similar way. Since it is generally accepted that islet cell precursors contain hormonal peptides, the co-expressed hormones reported in individual cells during pancreas development in mice (Alpert et al., 1988; Teitelman, 1993), rats (Taki and Baba, 1987), humans and pigs (Lukinius et al., 1992) may thus represent multipotential progenitors that are changing their differentiating potentials.

Another hypothetical model of differentiation proposes that islet cells have a common relationship with a single progenitor and their developmental pathway is linked with one another via a lineage relationship (Alpert et al., 1988)(Diagram 1c). This model was proposed from studies in transgenic mice expressing Tag under the control of the insulin regulatory region (Alpert et al., 1988). In this study, glucagon, insulin, somatostatin and pancreatic polypeptide were detected in mouse embryos on

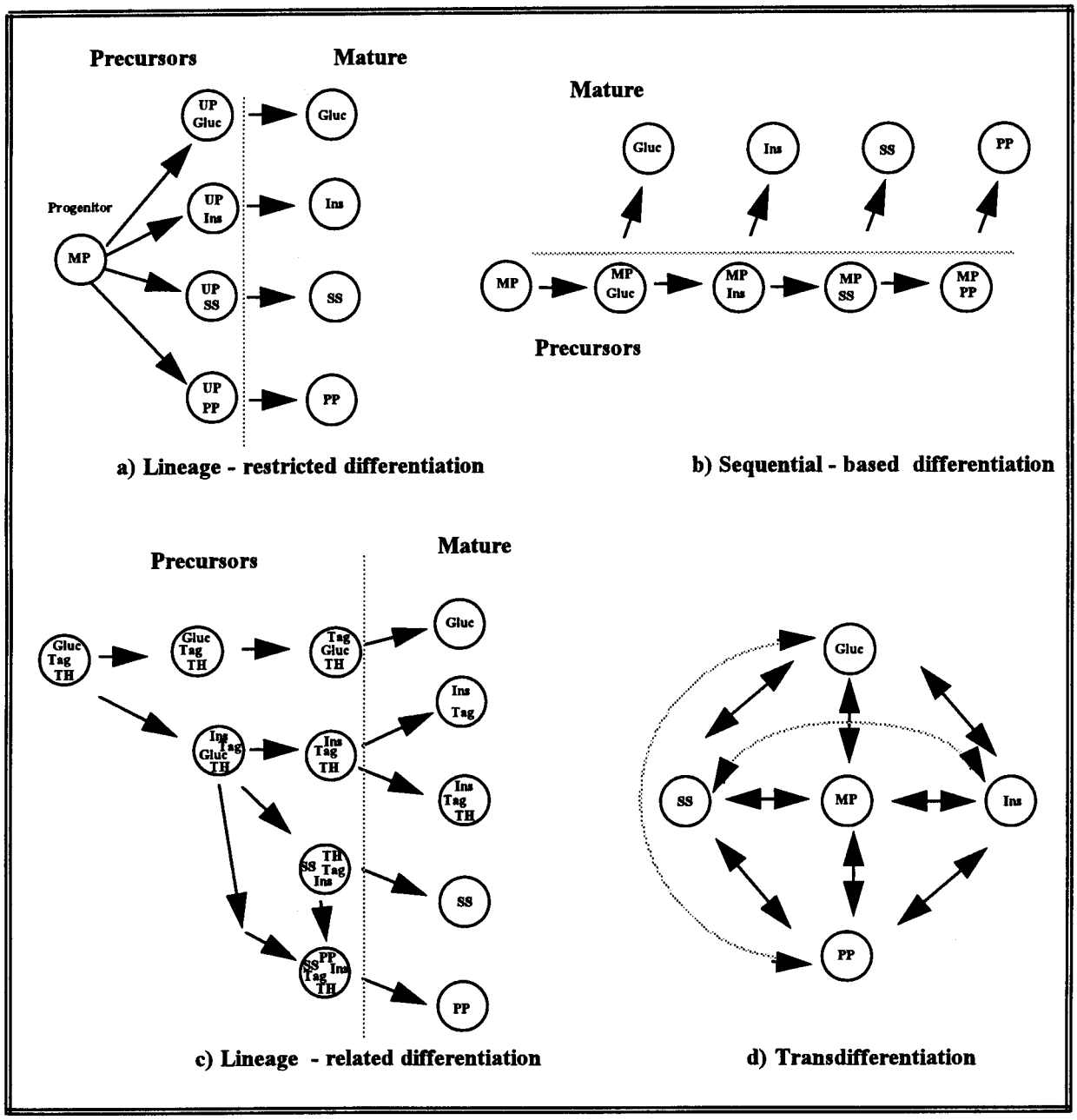


Diagram 1: Schematic diagram of hypothetical models of differentiation pathways that may apply to islet cell differentiation. a) Multipotential progenitors (MP) give rise to unipotential precursors (UP) that in turn give rise to separate islet cell lineages. b) Sequential-based differentiation (Brown et al., 1988) assumes that the different islet cell types arise from the same multipotential precursor (MP) that expresses specific differentiating potentials in an ordered sequence. c) According to this model (Alpert et al., 1988), multipotential precursors express phenotypic traits of the glucagon-producing cell type that in turn gives rise to the glucagon lineage and precursors of the other lineages. Similarly, precursor cells producing insulin give rise to the insulin lineage and precursors of the somatostatin and pancreatic polypeptide lineages. Thus the differentiation pathways of each cell type are intimately related with one another. d) Differentiation according to the transdifferentiation model (Beresford et al., 1991), assumes that cells are inherently plastic and that they can express other phenotypes through a process of dedifferentiation and redifferentiation, or by direct transdifferentiation.

Key: MP = multipotential; UP = unipotential; Ins = insulin; Gluc = glucagon; SS = somatostatin; PP = pancreatic polypeptide; TH = tyrosine hydroxylase; tag = SV 40 large T antigen that is co-expressed with insulin.

day 9.5, 11.5, 15.5 and one day after birth respectively. In addition, tyrosine hydroxylase (TH) was detected in all islet cell types when they first appeared in the embryonic pancreas. When insulin-producing β cells first appeared, they all transiently co-expressed glucagon. When somatostatin and pancreatic polypeptide were first observed in islet cells, these cells also transiently expressed the insulin genes. Based on these results these authors concluded that all islet cell precursors synthesise glucagon and that they give rise to the α and β cell lineages. The β cell lineage in turn forms the δ and PP cell lineages. Because individual mature islet cells contained only one hormone, these authors concluded that cells containing more than one hormone represented precursors that would give rise to cells expressing a single hormone at maturity. The above models depicting lineage-based differentiation, illustrate that precursors follow a progressive sequence of steps towards terminal differentiation. It is generally assumed that this process is irreversible and that once islet cells express differentiated characteristics that these traits remain stable (Teitelman, 1991; Teitelman, 1993).

1.2.4 Transdifferentiation:

Another issue that has bearing on this work is the concept of transdifferentiation. (Beresford, 1991). Transdifferentiation occurs when cells with a stable working character switch their phenotype (Diagram 1d). Fundamental to this concept is that the development of cell phenotypes is not pre-determined, but occur as a consequence of alterations within the cell's environment. Since all islet cell lineages would arise from a common precursor in the latter two models, it is likely that differentiation is controlled by interactions between the cells and extracellular factors and can be direct or indirect (Beresford, 1991). Direct transdifferentiation occurs when cells switch their phenotype without undergoing cell division. Direct transdifferentiation has been shown in vivo by the expression of phenotypic traits of juxtaglomerular cells in non-dividing arterial smooth muscle cells of rat kidneys in response to reduced arterial blood flow (Cantin et al., 1977 - reviewed by Beresford, 1991). Absence of cell division in this case was verified by the exclusion of[^{3}H]-thymidine (that had been injected into rats before the experiment) in these cells. Others have shown that adrenal chromaffin cells (endocrine cells) of adult humans and neonatal rats are converted to neurons in response to nerve growth factor in vivo and in vitro (Ogawa et al., 1986). Conversion of avian embryonic ganglionic neurons to the chromaffin cell phenotype occurred when neurons were cultured in vivo in the region occupied by the adrenal medulla (Le Duoarin et al., 1982 - reviewed in Ogawa et al., 1986).

Because direct transdifferentiation is difficult to establish in vivo without the use of proliferation markers, unequivocal evidence of direct transdifferentiation has been mainly acquired from in vitro studies, particularly in cells derived from the central nervous system (Reviewed by Beresford, 1991). When cultures of glial progenitor cells from rat optic nerve were grown in medium (DMEM) enriched with foetal calf serum, these cells expressed glial fibrillary acidic protein, a differentiated characteristic

of astrocytes. When these cells were subsequently cultured in a different culture medium containing serum, proliferation arrested cells adopted characteristics of oligodendrocytes by expressing galactocerebroside (Temple and Raff, 1985 reviewed in Beresford, 1991). In another study, non-dividing adult oligodendrocytes spontaneously expressed the astrocytic phenotype after two weeks in culture (Kim et al., 1985 - reviewed in Beresford, 1991). Others have shown that retinal pigmented epithelial cells are converted to neurons cultured on a laminin substratum, (Reh et al., 1987).

Indirect transdifferentiation arises when mature cells dedifferentiate (that is regress along the differentiation pathway) and redifferentiate into a new phenotype. Dedifferentiated cells often regain their proliferative potential as they approach the phenotype of precursors. This model of transdifferentiation is generally associated with metaplasia (Beresford, 1991) and is generally thought not to occur in normal adult cells. Indirect transdifferentiation has recently been demonstrated in cultured 3T3 mesenchymal stem cells (Sparks et al., 1994). When exposed to human plasma fractions, 3T3 mesenchymal cells entered into growth arrest and acquired characteristics of partially differentiated adipocytes. When these adipocyte-like cells were exposed to foetal calf serum, they dedifferentiated and regained their capacity to proliferate. These authors could then induce the cells to redifferentiate into macrophages by growing them in the presence of clotted plasma. These results show that once cells have expressed a phenotype characteristic of a specific differentiation pathway, they are not restricted into progressing along that pathway towards terminal differentiation. Moreover, this experiment demonstrates that the differentiation process is reversible, and that transdifferentiation to another phenotype is possible through a process of dedifferentiation and redifferentiation. Furthermore, cells regain their proliferative potential during the process of dedifferentiation. Transdifferentiation when applied to islet cell differentiation proposes that differentiated mono-hormone-producing islet cells are inherently multipotential and can express other islet cell phenotypes in response to environmental cues (i.e. they are phenotypically plastic). It is thus also possible that differentiated islet cells may have the potential to re-express embryonic phenotypes (stem cell properties) in response to changed environmental conditions. Evidence to support the feasibility of the transdifferentiation in islet cells is reviewed below.

1.2.5 Evidence for phenotypic plasticity in islet cells:

A condition essential to the transdifferentiation hypothesis is that phenotypic expression is controlled by environmental factors. Evidence that islet cell differentiation is not pre-determined but is controlled by environmental factors is supported by in vitro experiments on pancreas tissue (Rutter et al., 1964; Pictet et al., 1975; Teitelman et al., 1987b). In an experiment in which pancreatic primordia were cultured in vitro, the normal pattern of endocrine differentiation only took place if the primordia were exposed to conditioned medium from mesenchymal cells (Rutter et al., 1964; Pictet et al., 1975). This

showed that a factor crucial to islet cell differentiation is produced by connective tissue cells. In contrast to their late appearance during pancreas development in mice (Alpert et al., 1988), PP cells differentiated before somatostatin-producing cells were detected in embryonic mouse pancreas when cultured in vitro (Teitelman et al., 1987a). Thus the temporal appearance of hormone-producing cells during development in vivo is most likely to be determined by the environment. Other effects of environmental factors on the phenotypic expression of islet cells have been revealed in adult mice in which the normal physiology was disturbed by the presence of an insulinoma or by the diabetic environment (Teitelman, 1988; Teitelman, 1993). In both cases there was a significant increase in the number of β cells expressing tyrosine hydroxylase (TH) and an apparent increase in the number of glucagon-producing cells (Teitelman et al., 1988; Teitelman, 1993).

An assumption relevant to lineage-based development, is that maturation along the differentiation pathway is progressive and irreversible. The expression of embryonic traits in mature β cells of the adult pancreas (Teitelman et al., 1987; Alpert et al., 1988) are incompatible with this concept but are in keeping with the concept of transdifferentiation. Because TH is expressed in all endocrine cells at the onset of their appearance during pancreatic development (Alpert et al., 1988), it was assumed that cells synthesising TH were islet cell precursors (Teitelman and Lee, 1987). To further corroborate this, cells containing TH were detected in the duct epithelium or in cell clusters closely apposed to the ducts and were not observed in islets in the embryonic pancreas (Alpert et al., 1988). Furthermore, when insulin-producing cells first appeared in the embryonic pancreas, these cells all contained glucagon (Alpert et al., 1988). If the lineage-based concept of differentiation is applied to the above scenario, as these precursors progress along the differentiation pathway, they would cease producing TH and would eventually express a mono-hormonal phenotype. However, a subpopulation of β cells located in the islets of adult mouse pancreas expressed TH (Teitelman and Lee, 1987; Alpert et al., 1988). In contrast to embryonic cells that proliferate, β cells expressing TH in islets of adult mice were proliferation arrested and were thus thought to be terminally differentiated (Teitelman, 1993). Furthermore, these authors found that a subpopulation of mature β cells in adult mouse islets also contained glucagon. This phenomenon occurs when β cells first appear in the embryonic pancreas (Alpert et al., 1988). Thus cells containing more than one hormone reported in adult rats (Kaung, 1985), frogs (Kaung and Elde, 1980), mice (Rombout et al., 1987) and baboons (Wolfe-Coote et al., 1988), may indicate transdifferentiating mature islet cells responding to physiological cues rather than activated islet cell precursors.

A striking feature of islet cells that strongly supports their ability to transdifferentiate, is their biochemical and functional similarities with cells derived from the neuroectoderm. Similarities between β cells and neurons is that they are excitable (they release secretory granules through depolarisation

(Teitelman, 1991 - review)) and contain several neuronal enzymes such as tyrosine hydroxylase (Teitelman and Lee, 1987), neuron-specific enolase (Polak et al., 1984), and synaptophysin (Weidenmann et al., 1986). In addition, the ganglioside A2B5 (Eisenbarth et al., 1982) and the high and low affinity receptors for nerve growth factor (NGF) (Scharfmann et al., 1993) that are typically found in neurons are also present in β cells (Scharfmann et al., 1993). A unique feature of neurons is their ability to extend neurites and to synthesise neurofilament proteins. When dissociated islets from the pancreas of adult mice were grown in vitro for two weeks, a subpopulation of insulin-producing cells formed neurites that contained neurofilament proteins (Teitelman 1990). Because these cells originated from adult pancreas, it is likely that this phenotypic conversion occurred without cell division. This strongly suggests that β cells have undergone direct transdifferentiation. Since neurites were not formed if islets remained intact, the maintenance of the β cell phenotype appears to be dependant on direct contact of the β cell with the extracellular matrix. The many common characteristics shared by islet cells and cells derived from the neurectoderm as well as the striking examples of transdifferentiation shown by cells of the central nervous system reviewed above, strongly supports the notion that islet cells are inherently phenotypically plastic. Furthermore, based on common traits with neurons, Teitelman, 1991 has proposed a differentiation pathway that follows the concept of transdifferentiation described above; that is islet cell progenitors express a set of neuroendocrine genes that are selectively repressed during development but which can be re-expressed under appropriate stimulatory conditions. However, unequivocal evidence of direct transdifferentiation requires the following criteria; i.e. cells must acquire a stable working character, then alter their phenotype without undergoing cell division during the phenotypic conversion (Beresford, 1991). Revealing transdifferentiation potentials in islet cells would thus require cultures containing cells of a single phenotype such as a clonal cell line.

1.2.6 Continuous clonal islet cell lines:

The quest for obtaining continuous cultures of pancreatic cells arose primarily out of the need to study the function and biochemistry of β cells and to overcome the limitations in maintaining isolated islet cells for extended periods in culture. Clonal islet cell lines have been established from an X-irradiation-induced insulinoma generated in rats (Chick et al., 1977; Gazdar et al., 1980; Madsen et al., 1986; Phillipe et al., 1987a), oncogene-induced β-cell tumours generated in transgenic mice (Hanahan, 1985; Alpert et al., 1988; Efrat et al., 1988), and cultured hamster pancreatic cells immortalised by the syrian virus (SV40) (Santerre et al., 1981).

History of clonal islet cell lines:

The islet cell tumour (insulinoma) induced by X-irradiation of a New England Deaconess Hospital (NEDH) rat (Chick et al., 1977) is the origin of the RIN and MSL clonal cell lines. The RINm and RINr parent cell lines were established from subcutaneous tumours arising

from inoculations of cell suspensions of the abovementioned insulinoma in NEDH rats and athymic nude BALBc mice, respectively (Gazdar et al., 1980). Insulin-secreting clonal sublines such as RINm5F were established from these parent cultures. Unlike the RIN cell lines that were established from primary islet cell tumours, the parent MSL cell line was established from secondary islet cell tumours that had metastasised to the liver of NEDH rats inoculated with the insulinoma cells (Madsen et al., 1986). The daughter clone MSL-G expresses a β-cell phenotype. The βTC cell line, established from β-cell tumours that were genetically inherited in transgenic mice harbouring the chimeric insulin/Tag gene (Hanahan, 1985; Efrat et al., 1988), synthesises and secretes only insulin. The clonal HIT cell line was established by syrian virus transformation in vitro of isolated Syrian hamster pancreatic islets (Santerre et al., 1981). The HIT-T15 clone is a pure insulin-secreting cell line. The βTC and HIT-T15 clones continuously express the β cell phenotype in culture and respond to numerous compounds known to modulate insulin release in vivo (Santerre et al., 1980; Lambert and Atkins, 1989).

Despite their clonal origin, phenotypic heterogeneity is a striking feature of all cell lines derived from the rat insulinoma induced by X-irradiation. In a number of clones that are descendants of the RINr lineage, immunolabelling studies revealed a subpopulation of cells containing glucagon, somatostatin or angiotensinogen (Phillipe et al., 1987a,b). Similarly, in the MSL cell line, cultures consisted of cells containing insulin, glucagon, somatostatin or cholecystokinin (Madsen et al., 1986). Common to both cell lines, the majority of cells did not contain any secretory peptides. The spontaneous generation of a different clone (MSL-G2) expressing glucagon and CCK that arose in a culture of an insulin-producing cell line (MSL-G) (a subclone of the MSL parent culture) (Madsen et al., 1986) suggests that insulin-producing cells have transdifferentiated to a glucagon-producing phenotype. The glucagon-producing subclone had a notably faster proliferation rate, a feature in keeping with the concept of indirect transdifferentiation. When the glucagon-producing MSL-G2 cells were inoculated into rats, they formed insulin-secreting tumours (Madsen et al., 1986). The re-expression of the β cell phenotype induced by factors present in vivo illustrates the transdifferentiation potential at least of β cells. Similarly, a glucagon-producing clone was spontaneously generated in a culture of insulin-producing HIT-T15 cells, (Shennan et al., 1989). Since the HIT-T15 cell is a pure insulin-producing line (Santerre et al., 1981), this occurrence of phenotypic switching provides substantial evidence that differentiated β-cells have the potential to transdifferentiate. It is interesting to note that in the HIT-T15 and MSL-G cell lines, the switch in phenotype was towards the glucagon-producing phenotype, the phenotype of the proposed islet cell precursor (Teitelman et al., 1981; Teitelman and Lee., 1987; Alpert et al., 1988).

All insulinoma-derived cell lines demonstrate phenotypic heterogeneity (hence the possibility that they are pluripotent) and are predominantly populated with cells not expressing any secretory peptides (Madsen et al., 1986; Philippe et al., 1987a; Powers et al., 1988). This could be due to in vitro conditions that may lack appropriate physiological signals to maintain the differentiated functional state of β cells. If this was true, then a similar decline in the expression of the β-cell phenotype (insulin production and secretion) would occur in other insulin-producing cell lines. In contrast, both the βTC and HIT-T15 cell lines have maintained a functional β-cell phenotype after extensive periods in culture (Santerre et al., 1981; Efrat et al., 1991). Therefore it seems likely that non-hormone-producing cells in RIN and MSL cultures are a result of dedifferentiation (regressive differentiation) or transdifferentiation to express the phenotype of undifferentiated islet cell progenitors. Since islet cell precursors are generally accepted to be multipotential, some authors have proposed that the X-irradiation-induced rat insulinoma may have been generated from a transformed multipotent stem cell or that transformation of differentiated islet cells may have caused them to partially dedifferentiate (Madsen et al., 1986; Phillipe et al., 1987a,b). Evidence that suggests that RIN cultures consist of islet cell precursors is provided by Halban et al., 1988. These authors reported that the R2D6 monoclonal antibody, that specifically binds to gangliosides on the surface of β cells, only bound to a small proportion of RINm5F cells. Since these cells had a lower DNA content, this suggested that R2D6 was only expressed in non-dividing cells (Halban et al., 1988). Furthermore, RINm5F cells that lacked the ganglioside (recognised by R2D6) were restricted to the extreme periphery of cell aggregates which had formed after dissociated islet cells and RINm5F cells were mixed together. In contrast, pancreatic β cells were found in the centre of the aggregates (Halban et al., 1988). If these and other RIN lineages express the phenotype of islet cell precursors, the above phenomenon suggests that islet cell precursors may reside near the periphery of the islets in vivo. It is interesting to note that glucagon-producing cells are also located in this region in vivo.

Since differentiation of phenotypically plastic cells is determined by environmental signals, cells with "precursor-like" phenotypes should redifferentiate when introduced into an appropriate environment. Redifferentiation of RINm5F cells was shown by Flatt et al., 1988 who inoculated rats with "dedifferentiated" RINm5F cells and found that they formed subcutaneous tumours. After several passages in vivo, cells isolated from these tumours were functionally differentiated (i.e. responded to secretogogues that evoke insulin release) and produced large quantities of insulin. However, after 20 days in culture, these cells lost the β-cell attributes gained by their exposure to the in vivo environment. In RIN 1056 cultures (which are descendants of the RINr clones), there was a substantial increase in the number of cells containing insulin or glucagon in response to sodium butyrate that is known to induce differentiation in many cell types (Powers et al., 1988). Since sodium butyrate inhibited cell proliferation, these authors concluded that the responding cells were recruited from the

pool of undifferentiated cells (progenitors) in the culture. In another report, RINm5F cells exposed to nerve growth factor (NGF) or laminin expressed the neuronal phenotype with characteristic neurite-like processes that contained neurofilament protein (Polak et al., 1993). Interestingly, the majority of the cell population expressed the neuronal phenotype. Recently, receptors to NGF have been identified in RINm5F cells and on β-cells of the pancreas. In contrast, the insulin secreting βTC-cell line does not express the neuronal phenotype when exposed to NGF. Because βTC and HIT-T15 cells are thought to be terminally differentiated, the response of RINm5F to NGF supports the opinion that RIN cell lines express the phenotype of islet cell progenitors.

From the above review, it is apparent that cell lines derived from the X-irradiated insulinoma may be potentially useful for the study of islet cell differentiation. Because the βTC and HIT cell lines arose from β cells immortalised by the SV40 virus oncogene (Large T antigen), it is thought that the oncogene may interfere with normal gene expression (Saulnier-Michel et al., 1992). These authors proposed that cell lines derived from the insulinoma induced by X-irradiation would be more suitable for this purpose. The inherent heterogeneity and change in phenotypic expression in response to the in vivo environment or by factors such as NGF or sodium butyrate in vitro confirms that RIN cells retain the potential to differentiate. For the purpose of the present study, the RINm5F cell line was obtained to examine whether it was suitable for studying islet cell differentiation.

The RINm5F cell line is an clonal descendant of the RINm parent culture the origin of which has been described above. RINm5F cultures secreted high quantities of insulin and small amounts of somatostatin (Oie et al., 1983). Immunocytochemical analysis revealed that 80 to 85% of the cells in RINm5F cultures synthesised insulin (Oie et al., 1983). Furthermore, analyses of cytosolic extracts of RINm5F cultures by radio-immunoassay showed that they also contained negligible amounts of glucagon and somatostatin (Praz et al., 1983). As a result, the RINm5F cell line has been used as a β-cell model for studying mechanisms of insulin secretion. This cell line has not been used to study islet cell differentiation. Although the RINm5F cell line produces insulin, the cellular insulin content is less than 1% that of native β cells (Praz et al., 1983). In addition, insulin content and basal insulin secretion of continuously cultured RINm5F cells in vitro are substantially lower than those of the insulinomas that were generated from RINm5F cells that had been inoculated into rats (Flatt et al., 1988). Moreover, cellular insulin content and insulin secretion in cultures established from these insulinomas could only be sustained for 20 days in vitro (Flatt et al., 1988), after which they declined to values similar to those of continuously cultured RINm5F cells. A similar decline of insulin secretion over time occurred in all cloned cell lines that were derived from the RINm parent culture (Gazdar et al., 1980). These characteristics indicate that in vitro conditions do not sustain β-cell function or the β-cell phenotype. Exposure to high concentrations of glucose (greater than 16mM) elicits a

primary response of insulin secretion and a secondary response of stimulation of pro-insulin synthesis and transcription of the insulin gene in native β cells in vitro (Itoh and Okamoto, 1980; Brunstedt and Chan, 1982; Nielsen et al., 1985; German et al., 1990). In contrast, RINm5F cells demonstrate a poor insulin secretory response to high concentrations of glucose (Praz et al., 1983; Swanston-Flatt and Flatt, 1987; Valverdi et al., 1987;Dereli et al., 1988; Dobs et al., 1989). The GLUT2 glucose transporter is specifically expressed by β cells and mediates glucose uptake in native β cells (Chen et al., 1990; Thorens et al., 1993; Tiedge et al., 1993). Since glucose stimulates expression of the β-cell phenotype in native β cells, the GLUT2 glucose transporter is thus an essential component of functional differentiated β cells (Thorens et al., 1992). RINm5F and other RIN cell lines only express the GLUT1 glucose transporter that mediates the passage of low concentrations of glucose into the cells. However, when RINm5F cells were stabile transfected with the GLUT2 cDNA and exposed to glucose, prepro-insulin mRNA, insulin synthesis and insulin secretion were markedly raised above that of untransfected RINm5F cells (Tiedge et al., 1993). These findings indicate that the RINm5F and other RIN cell lines that do not respond to high millimolar concentrations of glucose consist of non-β or undifferentiated phenotypes.

Although immunocytochemical analysis has revealed that RINm5F cultures contain a high percentage of insulin-producing cells, immunolabelling studies were done shortly after the cell line had been established (when they would have been more differentiated). Since then, immunocytochemical studies have not been performed on the RINm5F cell line to determine whether they contain other hormone-producing cell types and undifferentiated phenotypes. Cells containing insulin, glucagon, somatostatin or pancreatic polypeptide have been revealed by immunolabelling techniques in the RIN2A cell line that is a clonal descendant of RINm5F (Powers et al., 1988). As noted in other RIN cell lines, the majority of cells in RIN2A cultures did not contain peptide hormones. Since RIN2A and RINm5F are related, it is likely that RINm5F cultures are similarly heterogeneous and contain undifferentiated cells. Further support that RINm5F cultures are populated mainly by non-β cells is provided by Halban et al., 1988 who found that only 3 to 15% of cells from RINm5F cultures express a cell surface protein (recognised by the R2D6 antibody) which is found on the surface of β cells but not on non-β cells.

The loss of β-cell function or phenotype in RINm5F cultures has initiated studies that have investigated means of restoring β-cell specific expression. To identify β-cell-specific expression, parameters such as insulin secretion, pre-pro-insulin mRNA, expression of cell surface molecules and cellular hormone content have been used to assess whether β-cell differentiation has been induced in RINm5F cultures under experimental conditions. RINm5F cultures, grown on a basement membrane matrix in the presence of hormone-defined medium, showed improved insulin secretion in response

to high concentrations of glucose (Muschel et al., 1986). Other authors have reported an increase in the number of RINm5F cells that expressed the A2B5 reactive gangliosides (expressed in differentiated pancreatic islet cells) on their cell surface in cultures treated with sodium butyrate (Bartholomeuz et al., 1989). This increase in A2B5 expression correlated with a markedly raised insulin secretion. In another study, sublines were produced from untreated RINm5F cultures that differentially expressed A2B5 (Bartholomeuz et al., 1990a). Compared to the parent RINm5F culture and other sublines expressing low levels of A2B5, sublines that had the greatest surface expression of A2B5, contained more insulin mRNA, cellular insulin and secreted higher levels of insulin . When all sublines were exposed to sodium butyrate, those expressing the highest levels of A2B5 contained the greatest quantities of intracellular insulin and secreted higher amounts of insulin (Bartholomeuz et al., 1990b). However, these studies have not revealed whether sublines expressing high levels of A2B5 consisted purely of β cells or whether sodium butyrate had induced the differentiation of glucagon, somatostatin or pancreatic polypeptide-producing cells. Others have reported that sodium butyrate did not stimulate somatostatin production in RINm5F cultures (Green and Shields, 1984). In cell lines derived from the RINr lines that contained cells producing insulin, glucagon, somatostatin and angiotensinogen (Phillipe et al., 1987a), sodium butyrate stimulated insulin and glucagon gene expression and suppressed transcription of the angiotensinogen gene (Phillipe et al., 1987b). These authors did not establish whether the raised insulin or glucagon mRNA levels in response to sodium butyrate were due to up-regulation of gene transcription in differentiated cells or whether insulin and glucagon gene expression had been induced in undifferentiated cells. However, others have reported that sodium butyrate induced non-hormone producing cells in similar RINr-derived cell lines to produce insulin or glucagon (Powers et al., 1988).

1.3 Motivation and objectives:

Despite the high percentage of β cells in cultures at the time the RINm5F cell line was established, the reports reviewed above provide evidence to show that the RINm5F cell line dedifferentiates in culture and that it consists mainly of undifferentiated cells that may represent islet cell progenitors. Several experiments have shown that β-cell differentiation can be induced by sodium butyrate and by culture on extracellular matrix and in defined medium. Furthermore, responsiveness to induction has been demonstrated in RINm5F cells by the expression of neuronal characteristics in response to nerve growth factor (Polak et al., 1993). Since the "dedifferentiated" RINm5F cell line appears to be similar in nature to other RIN cell lines, it is feasible that RINm5F cultures contain, or, have the potential to produce cells which synthesis other islet hormones. Since it is generally accepted that the same cell line may display different characteristics when cultured in different laboratories, it is preferable to establish their characteristics before subjecting the cultures to experimental conditions. This problem was emphasised by McEvoy et al., 1986, who reported that RINm5F cultures obtained from different

laboratories expressed different growth and insulin secretory rates when cultured under identical conditions in their laboratory. The RINm5F cell line was acquired by our laboratory to determine if it could be used as a model to study islet cell differentiation and to examine the possibility that differentiated islet cells have the potential to transdifferentiate either directly or indirectly.

Since it was evident that RINm5F cultures displayed variable characteristics in different laboratories, the aim of this study was to characterise the RINm5F cell line in our laboratory (RINm5F-MRC). The objective was to establish growth rate, cell morphology in culture, ultrastructure and intracellular peptide content by immunocytochemistry in cultures grown under routine culture conditions. The above investigation also served to rule out the possibility that rogue cells may have accidentally been introduced into the cell line or that phenotypic selection, due to the transport of cells from their source to this laboratory, had taken place. From the above review, a change in the status of differentiation can be identified by parameters other than their hormone content. Determination of growth rate of RINm5F-MRC cells served to establish appropriate seeding densities and culture periods for proposed experiments. In addition, growth rates were examined under experimental conditions to determine if they could reflect a change in the status of differentiation in the cultures. This rationale was used because markedly reduced or arrested growth rate has been reported in RINm5F cultures in which β-cell phenotype was restored (Muschel et al., 1986; Phillipe et al., 1987b; Bartholomeuz et al., 1990a). Madsen et al., 1986, also reported that clones that did not produce hormones had higher proliferation rates than those that synthesised peptide hormones. Furthermore, these authors reported that a glucagon-producing clone that spontaneously arose in an insulin-secreting cell line, had a markedly faster growth rate compared to its insulin-secreting counter part (Madsen et al., 1986). Therefore, cell morphology of RINm5F-MRC cells in culture was examined to see whether this correlated with the expression of a specific phenotype that may have been induced by modified culture conditions.

Since insulin content of RINm5F cells was less than one percent than insulin content of native β cells (Praz et al., 1983), it was possible that intracellular peptide content in individual cells may not be detected by immunocytochemical techniques. To ensure that the RINm5F cell line in our laboratory had not been accidentally contaminated with non-peptide-producing rogue cells, the cultures were examined by electron microscopy to observe whether or not the cells had structural characteristics that were common to insulinoma or to native islet cells. Since immunocytochemistry of whole cells in culture had successfully revealed hormone-producing cells in other RIN cell lines (Madsen et al., 1986; Phillipe et al., 1987a; Powers et al., 1988; Saulnier-Michel et al., 1992), a similar approach was used to determine which peptides were produced by our RINm5F cultures. This required optimisation of immunolabelling protocols that would suitably detect and quantify the number of hormone-producing cells in the RINm5F cultures. In this study, immunolabelling protocols were

applied to RINm5F cultures and systematically assessed by light microscopy, fluorescence microscopy, or flow cytometry to achieve these objectives.

2. MATERIALS AND METHODS

2.1 Materials:

Reagents were obtained from the sources listed below.

2.1.1 Immunoreagents:

Rabbit anti-porcine glucagon (Lot nos. 087 and 052), rabbit anti-human somatostatin (Lot nos. 077D and 072), guinea-pig anti-porcine insulin (Lot nos. 038 and 032) antisera, normal goat serum (Lot no 071), normal guinea-pig serum (Lot no. 105) and normal rabbit serum (Lot no. 043) (DAKO Corporation, Carpinteria, CA 93013 USA); human insulin (Lot no. 020661) and human glucagon (Lot no. 010439) (Peninsula Laboratories, Inc. Belmont, California, 94002, USA); somatostatin (VB150 SRIF) (Lot no. 311/01) (UCB Bioproducts, B-1420 Braine-L'Allued, Belgium); biotinylated goat anti-rabbit IgG(H+L) (Lot no B1202) and avidin-D and biotinylated horse-radish peroxidase H reagent (Vectastain) (Vector Laboratories, Burlingame, CA 940101, USA); strepavidin fluorescein (Boehringer Mannheim, D-68298, Mannheim, Germany).

2.1.2 Cell culture reagents:

Penicillin/Streptomycin; RPMI 1640 culture medium, and sodium butyrate (Sigma Chemical Company, St Louis, MO 63178, USA); foetal calf serum (Lot 965) (Highveld Biological, Sandton, Johannesburg, RSA); mitomycin C (Boerhinger Mannheim, D-68298, Mannheim Germany); trypsin (DIFCO Laboratories, Detroit 1, Michigan, USA); N-[2-hydroxyethyl]piperazine-N'[2-ethanesulphonic acid] (HEPES) and dimethyl sulphoxide (DMSO) (Sigma Chemical Company, St Louis, MO 61378, USA).

2.1.3 Electron microscopy reagents:

Epon/Araldite (BIORAD, Watford, UK); glutaraldehyde (AGAR Scientific, Essex, UK); osmium tetroxide (PGM Chemicals, Germiston, RSA).

2.1.4 General reagents:

2-Methylbutane (isopentane), paraformaldehyde and sodium dodecyl sulphate (E-Merck, Darmstadt, Germany); aproteinin, acrylamide, ammonium persulphate, bacitracin, 3,3' diaminobenzidine (DAB), lupeptin, mercaptoethanol, N,N'-methylene-bis-acrylamide, pepstatin, phenylmethylsulphonyl fluoride (PMSF) poly-L-lysine (Sigma Chemical Company, St Louis, MO 61378, USA); N,N,N',N-tetramethylethylenediamine (TEMED) (Stratagene, La Jolla, USA).

2.2 Cell line and culture conditions:

2.2.1 Cell line and routine propagation:

The RINm5F cell line used in this study (RINm5F-MRC) was derived from a nude mouse heterotransplant of a transplantable islet cell tumour (Chick et al., 1977) and was received as a generous gift from Prof C Hellerström of the University of Upsala, Sweden. Cells were transported by air parcel post in a culture flask filled with medium as described below. RINm5F-MRC cells were routinely propagated in RPMI 1640 medium containing 11.1mM glucose, supplemented with 10% heat inactivated foetal calf serum, 100 IU/ml penicillin and 100μg/ml streptomycin and 20mM HEPES at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2.1.1 Trypsinisation and storage:

Semiconfluent cultures (approximately 1 x 10^6 cells/cm^2) were routinely expanded twice weekly by trypsinisation as follows: Medium was aspirated and cells were rinsed briefly with trypsin/EDTA buffer (pH 7.4) containing 0.05% trypsin and 0.02% ethylenediaminetetraaceteic acid (EDTA), 0.8% NaCl, 0.04% KCl, 0.01% dextrose, 0.058% NaHCO3, and 0.002% phenol red and bathed in a volume of 0.01ml /cm^2 of the trypsin/EDTA buffer for 5-10 minutes at 37°C. The action of trypsin/EDTA was inhibited by the addition of 10 volumes of medium containing serum. Cell suspensions were pelleted at 300g for 3 minutes, and reseeded at 1 x 10^5 cells/cm^2. Cell stocks were stored under liquid nitrogen in antibiotic-free RPMI 1640 supplemented with 40% foetal calf serum and 10% DMSO at a density of approximately 1 x 10^7 cells/ml.

2.2.1.2 Microscopy and photography of cell cultures:

Cultures were viewed and photographed on an Olympus CK2 inverted microscope fitted with an Olympus OM101 camera. Micrographs were recorded on Kodak Technical Pan 100 ASA film.

2.2.2 Determination of cell growth:

2.2.2.1 Cell growth under routine culture conditions:

RINm5F-MRC cells from different passages (p) were seeded in triplicate, at 10^4, 3 x 10^3 and 10^3 cells/well (for RINm5F-MRC p22) or at 10^5, 3 x 10^4 and 10^4 cells/well (for RINm5F-MRC p-32), into multiwell plates, each well having a diameter of 1.5cm. Medium (see **2.2.1**) was changed every 48 hours or every 24 hours if the culture medium showed signs of exhaustion (i.e. changed colour).

Medium was aspirated and the cells were rinsed in phosphate buffered saline (PBS) and thereafter were incubated in trypsin/EDTA solution (see **2.2.1.1**) for 5-10 minutes at 37°C. Single cell

suspensions were obtained by gently pipetting cells using a micropipette. Aliquots of cell suspensions were added to equal aliquots of trypan blue and counted using a haemocytometer. The mean and standard error of the mean of triplicate cultures for each seeding density were calculated and plotted using the SigmaPlot™ software (Jandel Scientific, Corte Madera, USA). Error bars are not visible on data points where the standard error of the mean is very small.

2.2.2.2 Cell growth in the presence of mitomycin C:

RINm5F-MRC cells were seeded at 1 x 10^5 cells/well in multiwell dishes were cultured for three days (see **2.2.1**) after which they were treated with mitomycin C dissolved in methanol. Triplicate cultures were incubated overnight in fresh medium containing mitomycin C, at concentrations of 1μg, 0.5μg, 0.2μg, 0.1μg, or 0.05μg/ml. Control cultures were incubated overnight in medium containing the equivalent volume of methanol. The following day, fresh medium without mitomycin C was added to all cultures and cells were grown for 6 days.

To determine cell viability and inhibition of growth rate, cell counts were performed in the presence of trypan blue on Day 0 (just prior to the addition of mitomycin C) and thereafter on the 2nd, 4th and 6th day after exposure to mitomycin C. Medium (excluding mitomycin C) was replaced every 48 hours. Cell counts were calculated for each culture well and the means and standard error of the means were calculated and plotted using the SigmaPlot™ software (Jandel Scientific, Corte Madera, USA). Error bars are not visible on data points where the standard error of the mean is very small.

2.2.3 Culture conditions for immunolabelling studies:

2.2.3.1 RINm5F-MRC cells cultured under routine conditions for whole-mount immunolabelling:

Cells were cultured in medium (see **2.2.1**) on glass coverslips placed in 35mm diameter culture Petri dishes. Glass coverslips were sterilised by immersing in 70% ethanol for 30 minutes and flamed before placing into sterile Petri dishes. Cells were seeded at a density of 1 x 10^5 cells/cm^2 and were cultured for 3 days before fixation (see section **2.4.1**).

2.2.3.2 RINm5F-MRC cells cultured in the presence of sodium butyrate for whole-mount immunolabelling:

Cells were seeded onto sterilised coverslips in 35mm dishes (see **2.2.3.1**) and were cultured for three days in RPMI 1640 containing 0.8mM glucose and antibiotics. Cells were either cultured for a further three days in RPMI 1640 containing 0.8mM glucose (untreated cultures) or in RPMI 1640 containing 11.1mM glucose and 2mM sodium butyrate (sodium butyrate-treated cultures). Aliquots of concentrated stocks of 200mM sodium butyrate in PBS were stored at -20°C.

2.2.3.3 RINm5F-MRC cells cultured in the presence of mitomycin C or sodium butyrate.

Cells, seeded at 5 x 10^6 cells/cm^2 and 2.5 x 10^6 cells/cm^2, were cultured overnight in RPMI 1640 containing 11.1mM glucose. [The lower seeding density was used for control dishes in order to avoid overgrowth of the culture at the end of the experimental period.] Cultures were subsequently grown overnight in medium containing 0.1μg/ml mitomycin C, after which they were cultured for a further 3 days in medium excluding mitomycin C. Other cultures were grown in RPMI 1640 medium containing 11.1mM glucose and 2mM sodium butyrate for three days and control cultures were grown in medium excluding sodium butyrate for the same period. The number of viable cells was determined on duplicate cultures after the second and third day by trypan blue exclusion (see **2.2.2.1**). Cultures were examined and photographed as in **2.2.1.2**.

2.2.3.4 RINm5F-MRC cells cultured with or without sodium butyrate for analysis by flow cytometry:

Cells were seeded at 1 x 10^5 cells/cm^2 and 5 x 10^4 cells/cm^2 and cultured for three days in medium containing 0.8mM glucose (non stimulatory conditions). Cultures seeded at 1 x 10^5 cells/cm^2 were grown for another three days in medium containing 11.1mM glucose and 2mM sodium butyrate (stimulatory conditions). Untreated cultures, seeded at 5 x 10^4 cells/cm^2 to avoid overgrowth of the culture at the end of the experiment, were grown in medium containing 0.8mM glucose until the end of the experimental period.

2.3 Electron microscopy:

2.3.1 Preparation of RINm5F-MRC cells for electron microscopy:

RINm5F-MRC-MRC cells were cultured in medium (see **2.2.1**) in 100mm dishes until cultures were nearly confluent. Cells were harvested by scraping them off the culture dishes using a rubber policeman, collected in 0.1M phosphate buffer (pH 7.2), pelleted by gentle centrifugation, rinsed twice in 0.1M phosphate buffer and fixed for 30 minutes in 0.1M phosphate buffered 2% glutaraldehyde and 3% paraformaldehyde. Cells were pelleted by centrifugation between each step and all steps of the processing procedure were performed at room temperature unless otherwise specified.

Cells were rinsed in buffer three times in 15 minutes, after which cells were post-fixed for 45 minutes in 0.1M phosphate buffered 1% osmium tetroxide, rinsed for 10 minutes in distilled water and stained en bloc, in 2% aqueous uranyl acetate, for 15 minutes. Cells were dehydrated in two changes of 8 minutes each, in each of 70%, 85% and 95% ethanol and two changes of 15 minutes in absolute ethanol. Prior to infiltration with Epon/Araldite, ethanol was exchanged three times with propylene oxide in 30 minutes and infiltrated with increasing concentrations of Epon/Araldite for three hours in

each of 1:3 and 1:1 mixtures of resin:propylene oxide, and for a further 18 hours in a mixture of Epon/Araldite:propylene oxide at a ratio of 2:3. Cells were infiltrated with three changes of Epon/Araldite at 40°C of three hours each and embedded cells were polymerised at 60°C for 18 hours.

2.3.2 Preparation of rat pancreas for electron microscopy:

The pancreas was rapidly excised from a NEDH rat killed by decapitation, cut into $1mm^3$ blocks and placed into 0.1M phosphate buffered 2.5% glutaraldehyde (pH 7.4) for two hours at room temperature. After rinsing three times in 0.1M phosphate buffer in 15 minutes, tissue was further processed using the same processing reagents and times (see **2.3.1**) in a Lynx microscopy tissue processor. Embedded samples of pancreas were sectioned, stained, viewed and photographed as in section **2.3.3** below.

2.3.3 Sectioning and viewing:

Thin sections (90nm) were cut on a Reichert OM 3 ultramicrotome, mounted onto 300 mesh copper grids and stained for 10 minutes in 2% aqueous uranyl acetate and three minutes in Reynold's lead citrate (Reynolds, 1963). Sections were viewed and photographed using a Philips 420 transmission electron microscope operating at 80kV with 50μm condenser and objective apertures. Electron micrographs were recorded on Agfa Scientica film.

2.4 Whole-mount immunocytochemistry:

2.4.1 Fixation:

Glass coverslips or microscope slides to which RINm5F-MRC cells, cultured as in section **2.2.3.1** or **2.2.3.2**, were attached, were rinsed in phosphate buffered saline (PBS), fixed in 2% paraformaldehyde in PBS (pH 7.4) for 30 minutes at room temperature, rinsed three times in PBS for 5 minutes each and dipped briefly into distilled water. Coverslips were drained, air-dried and were stored in airtight boxes at -80°C until required.

2.4.2 Preliminary immunolabelling protocol:

Frozen whole-mounts of fixed (see **2.4.1**) RINm5F-MRC cells cultured on sterilised microscope slides (see **2.2.3.1**), fixed and stored as above, were thawed in 20% normal goat serum (NGS) for 20 minutes to block non-specific binding of the antibodies. The immunolabelling procedure was performed at room temperature unless otherwise specified. Excess NGS was drained from the slides and cells were covered with a 1/200 dilution of anti-insulin (Lot no. 038), a 1/400 dilution of anti-glucagon (Lot no. 087) or a 1/200 dilution of anti- somatostatin (Lot no. 077D) Slides were rinsed

in 50mM Tris-buffered saline (pH 7.2)(TBS) by jet washing excess antisera off the slides and further rinsed for 10 minutes in the same buffer. All further rinsing procedures were performed as above. Whole-mounts were further labelled with a 1/220 dilution (6.25μg/ml) of biotinylated goat anti-rabbit IgG (b-GAR-IgG) for 30 minutes, rinsed in TBS and biotinylated complexes were amplified (stained) with avidin D-biotinylated horse-radish peroxidase H complex (ABC-HRP) for 60 minutes. After a final rinse in TBS, peroxidase activity was detected by exposure to Tris-buffered 0.05% DAB activated with 0.01% H_2O_2 for 5 minutes. The slides were rinsed in tap water for 5 minutes, air dried, cleared in xylene and mounted in DPX. Slides were viewed and photographed (see **2.4.3.3**).

2.4.3 Immunolabelling of RINm5F-MRC cells grown under routine culture conditions using the Vectastain ABC (ABC-HRP) detection system:

Cells cultured on coverslips (see **2.2.3.1**), fixed and stored (see **2.4.1**) were thawed in 20% NGS at room temperature for 20 minutes. All further steps were performed at room temperature and all rinses in PBS were performed three times each for 5 minutes between every step of the labelling protocol unless otherwise specified. Excess NGS was drained and whole-mounts were stored overnight at 4°C in serial dilutions (1/200 , 1/400 and 1/800) of insulin (Lot no. 032), glucagon (Lot no. 052) or somatostatin (Lot no. 072) antiserum. The following day, coverslips were rinsed in PBS and further labelled with b-GAR-IgG for 30 minutes as before (see **2.4.2**).

After rinsing in PBS, coverslips were flooded with freshly prepared 100mM aqueous periodic acid for 4 minutes to block endogenous peroxidase. [Periodic acid was used in preference to H_2O_2 after it had been established by others in the present laboratory that it effectively blocked endogenous peroxidase without reducing the levels of antigenicity (Louw, 1990)]. After rinsing in PBS, coverslips were stained with ABC-HRP and treated as before (see **2.4.2**).

2.4.3.1 Staining and specificity controls:

Staining and specificity controls, performed on the whole-mounts in section **2.4.2** to determine specificity, were labelled in parallel with immunolabelled preparations making one of the following changes to the protocol:

Staining controls:

a) Cells or sections were incubated with the antiserum diluent buffer (0.01% bovine serum albumin (BSA) in PBS containing 0.02% sodium azide) that substituted for the primary antiserum. A positive reaction in this control would indicate non-specific binding of the secondary antibody. These controls are further referred to as "method control" in the rest of this document.

b) Cells were incubated in the diluent buffers for the antisera in place of the primary and

secondary antisera. A positive reaction in this control would indicate the presence of endogenous peroxidase or non-specific binding of b-GAR-IgG in peroxidase-stained preparations, or background fluorescence or non-specific binding of strepavidin FITC in fluorescent-stained preparations. These controls are further referred to as "background control" for the rest of this document. Staining due to the presence of endogenous peroxidase would be revealed by exposure to DAB in preparations not exposed to any antisera and ABC-HRP.

Specificity controls:

c) The primary antiserum was substituted with normal serum from the species in which the antiserum was raised i.e. normal guinea-pig (for insulin) or rabbit (for glucagon and somatostatin) sera. A positive reaction in this controls would indicate non-specific binding of the primary antiserum. These controls are referred to as "non-immune control" for the rest of this document.

The total protein concentration of normal sera was matched to that of the primary antisera as follows: Total protein concentration of normal rabbit and guinea-pig sera was determined spectroscopically at 280nm to be 28.43 and 39.25mg/ml respectively. Normal guinea-pig and rabbit sera were diluted such that they matched the total protein concentration of the 1/200 diluted antibodies.

d) The primary antisera were substituted with pre-absorbed antisera. Theses controls are referred to as "absorption control" in the rest of this document. Pre-absorbed sera were prepared as follows:

Pre-absorbed primary antisera were prepared by overnight incubation at 4°C with their corresponding peptides: A 1/200 dilution of anti-insulin, anti-glucagon or anti-somatostatin was absorbed with insulin, glucagon or somatostatin respectively at a final concentration of 100μg/ml.

e) positive tissue controls:

Since cell lines expressing high levels of insulin, glucagon or somatostatin were not available for this study, frozen sections of rat pancreas served as positive tissue controls. The pancreas was rapidly excised from NEDH rats, sacrificed by decapitation, placed into freshly prepared 2% paraformaldehyde in PBS for 6 hours, followed by rinsing three times in PBS for 15 minutes and infiltration with 20% sucrose in PBS overnight at 4°C for cryoprotection of the tissue. The next day, tissue was embedded in OCT compound (Tissue-Tek, Miles Incorporated, Elkhart, Indiana USA) frozen and 8μm thick sections prepared on a Reichart cryostat at -18°C. Sections were mounted onto poly-L-lysine-coated glass microscope slides (prepared by spreading a 1mg/ml dilution of poly-L-lysine (MW 288) across the slide) and

stored in airtight boxes at -80°C until required. For immunolabelling, sections were thawed in 20% NGS for 20 minutes, excess NGS was drained after which sections were labelled with 1/200 dilution of insulin (Lot no. 032), glucagon (Lot no. 052) or somatostatin (Lot no. 072) antisera. The rest of the labelling procedure was performed as in section **2.4.2**. Sections were lightly counterstained in 0,05% aqueous methylene blue in 0.05% borax.

2.4.3.2 Light microscopy and photomicrography:

Slides were viewed under bright field and photographed on a Zeiss optical microscope using Fuji 100 ASA colour film.

2.4.4 Immunofluorescence labelling protocol:

Cells, cultured as in **2.2.3.2**, fixed and stored as in **2.4.1**, were thawed in 3% BSA in PBS containing 0.05% Triton X (TX) for 30 minutes at room temperature. All further steps were performed at room temperature and all rinses were performed in PBS containing 0.05% TX three times, each for 5 minutes between every step of the labelling protocol unless otherwise specified.

Excess buffer was drained off the coverslips and cells were incubated in 1/25, 1/50 and 1/100 dilutions of insulin antiserum overnight at 4°C, rinsed in buffer and exposed to b-GAR-IgG for 60 minutes as before (see **2.4.2**). Whole-mounts were stained with 10μg/ml Strepavidin fluorescein (SA-FITC) for 60 minutes in the dark, followed by four rinses in buffer. Coverslips were mounted in antifade (2.5% w/v 1,4,Diazobicyclo(222)octane (Sigma) in 0.02M Tris buffered glycerol (pH 8)) on glass microscope slides, viewed and photographed (see **2.4.4.1**).

2.4.4.1 Fluorescence microscopy:

Immunolabelled whole-mounts or frozen sections stained with strepavidin fluorescein (SA-FITC) were viewed and photographed under epifluorescence on a Leitz Laborlux 12 optical microscope using a fluorescent iso-thiocyanate (FITC) bandpass filter block. Due to the low intensity of the fluorescent signal, micrographs were recorded on Fuji 1600 ASA colour film.

2.4.5 Comparative immunolabelling of RINm5F-MRC cells cultured with or without sodium butyrate stained with Vectastain ABC (ABC-HRP) or strepavidin fluorescein (SA-FITC).

2.4.5.1 Detection using ABC-HRP:

Whole-mounts were thawed in 20% NGS diluted in 0.01% BSA and 0.05% Triton X 100 in PBS (PBS/BSA/TX buffer) for 30 minutes at room temperature. Excess NGS/buffer was drained off the

coverslips and cells were stored overnight at 4°C in a 1/200 dilution of insulin (Lot no. 032), glucagon (Lot no. 052) or somatostatin (Lot no. 072) antisera in PBS buffer containing 0.1% BSA and 0.05% TX. Triton X 100 was included in all buffer rinses to further enhance penetration of the antibodies and staining molecules and whole-mounts were rinsed three times each for 5 minutes between every step of the labelling protocol. Further labelling steps were performed at room temperature. After incubation in b-GAR-IgG for 60 minutes (see **2.4.2**), endogenous peroxidase activity was inhibited by exposure to 100mM aqueous periodic acid for 4 minutes. Whole-mounts were stained with ABC-HRP for 60 minutes and peroxidase activity was detected with DAB, air-dried, and mounted in DPX (see **2.4.2**). Slides were viewed and photographed as before (see **2.4.3.2**).

2.4.5.2 Detection using SA-FITC:

Whole-mounts were thawed in 3% BSA and 0.05% Triton X 100 in PBS for 30 minutes at room temperature. Excess buffer was aspirated and cells were stored overnight at 4°C in a 1/50 dilution of insulin, glucagon or somatostatin antisera in PBS/BSA/TX buffer (see **2.4.5.1**) and were further immunolabelled as in section **2.4.5.1**.

2.5 Immunolabelling of frozen sections of RINm5F-MRC cultures grown in the presence of 0.1μg/ml mitomycin C or 2mM sodium butyrate:

2.5.1 Fixation and sectioning:

At the end of the experimental culture period (see **2.2.3.3**), cells from each experimental condition were harvested by trypsinisation, rinsed twice in PBS and pelleted at 300g for three minutes. Pelleted cells were gently mixed with an equal volume of PBS and 20μl aliquots were embedded in OCT compound mounted on small cork stubs and rapidly frozen in melting isopentane (Merck). Frozen cell pellets, attached to cork stubs, were stored in Nunc vials under liquid nitrogen until sectioned. Prior to sectioning, mounted cell pellets were warmed to -25°C. Sections (10μm thick) were mounted on poly-L-lysine-coated slides, air dried and stored in air tight boxes at -20°C until required.

2.5.2 Immunolabelling protocol:

Sections were immunolabelled using the same protocol and dilutions of antisera used for routine immunostaining on frozen sections of pancreas in our laboratory. Sections were rinsed three times each for 5 minutes, in TBS between each step of the labelling protocol unless otherwise specified. Sections were thawed and fixed in 0.1M phosphate-buffered 2% paraformaldehyde for 20 minutes at 4°C. After rinsing in 0.1M phosphate buffer, endogenous peroxidase activity was inactivated by exposure to 3% H_2O_2 in methanol for 30 minutes. Thereafter sections were rinsed and stored in 20% NGS for 20 minutes. Excess NGS was drained and sections were incubated in 1/400 dilution of anti-

insulin or anti-glucagon serum for 30 minutes at room temperature. After rinsing in TBS, sections were further labelled with b-GAR-IgG for 30 minutes and stained with ABC-HRP for 60 minutes as before (see **2.4.2**). Peroxidase activity was detected with DAB, and mounted sections were viewed and photographed (see **2.4.2** and **2.4.3.2**).

2.6 Flow cytometry:

2.6.1 Flow cytometry and data analysis:

Cells were analysed on a FACScan (Becton Dickenson) flow cytometer equipped with a 15mW argon ion laser operating at an excitation wavelength of 488nm. Green fluorescence was collected through a 530/30nm bandpass filter set and was measured using a logarithmic amplifier. Fluorescence was collected from 10 000 cells. Primary gating was only used if there was significant scatter, determined by the forward scatter dot plot, outside the main cell population.

Data were analysed using the LYSIS II software on a Hewlett Packard computer linked to the FACScan flow cytometer. A histogram depicting fluorescence intensities emitted from each of 10 000 cells was generated for each sample. Thus the data as shown in Figs 26-28 represent histograms of fluorescence of individual samples from one particular experiment. Typical histograms obtained for samples not exposed to primary antiserum (method control) and from samples incubated with anti-insulin serum are shown in Diagram 2). Because there was no clear distinction (i.e. two distinct fluorescence peaks on the histogram) between immunonegative and immunopositive populations from the sample exposed to anti-insulin serum (Diagram 2b), data from several experiments were analysed in two ways:

a) A cursor (Diagram 2 - marker) was positioned at the edge of the histogram from unstained (background control) or method control samples. Thus fluorescence from preparations exposed to primary antiserum, pre-absorbed antiserum, or non-immune serum that fell to the left of the histogram marker was regarded as non-specific background fluorescence. The number of positively stained cells in these preparations was calculated from the portion of the histogram that fell within the marker region (Diagram 2b). Because it was not possible to place the marker at the same position on background or method control histograms from separate experiments, the mean of the number of "positively" stained cells from these experiments could not be calculated. However, because non-specific background fluorescence was constant in individual labelling runs, the number of "positively" stained cells could be compared amongst different samples from the same experiment. Data shown in this format (Figs 25 and 30) are from one representative experiment (n=1).

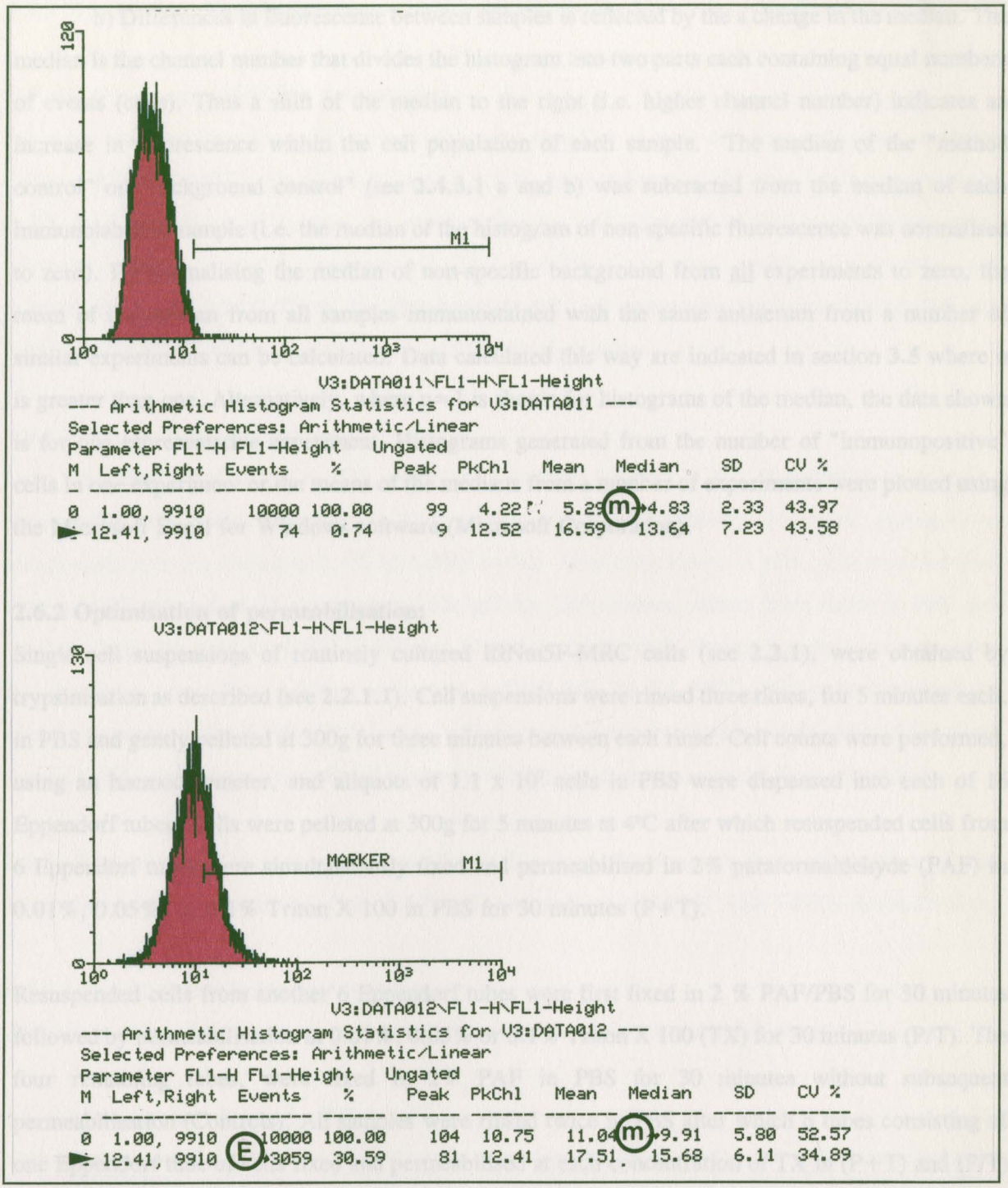


Diagram 2: Representative histograms of cell number (Y-axis: Events/channel) versus fluorescence (X-axis) from sodium butyrate-treated cells incubated in antiserum diluent buffer (method control) (a), or immunolabelled with anti-insulin serum (b).
(a) A cursor (MARKER M1) is set up on the histogram from the method control to demarcate the channel number (arrow head) that separates non-specific fluorescence (background) from that arising from positively stained cells. (b) The number of positively stained cells (E) in all immunostained samples from the same experiment are calculated from the portion of their histograms that fall within this marker region. The value of the median (M) was used to assess the overall fluorescence of the sample and fluorescence of immunostained samples (labelled with the same antiserum) from different experiments were compared by subtracting the median of the method control from that of the immunostained sample (i.e. the median of background was normalised to zero).

b) Differences in fluorescence between samples is reflected by the a change in the median. The median is the channel number that divides the histogram into two parts each containing equal numbers of events (cells). Thus a shift of the median to the right (i.e. higher channel number) indicates an increase in fluorescence within the cell population of each sample. The median of the "method control" or "background control" (see **2.4.3.1** a and b) was subtracted from the median of each immunolabelled sample (i.e. the median of the histogram of non-specific fluorescence was normalised to zero). By normalising the median of non-specific background from all experiments to zero, the mean of the median from all samples immunostained with the same antiserum from a number of similar experiments can be calculated. Data calculated this way are indicated in section **3.5** where n is greater than one. Alternatively, where n=1 is shown for histograms of the median, the data shown is for one representative experiment. Histograms generated from the number of "immunopositive" cells in one experiment or the means of the medians from a number of experiments were plotted using the Microsoft Excel for Windows software (Microsoft Corporation).

2.6.2 Optimisation of permeabilisation:

Single cell suspensions of routinely cultured RINm5F-MRC cells (see **2.2.1**), were obtained by trypsinisation as described (see **2.2.1.1**). Cell suspensions were rinsed three times, for 5 minutes each, in PBS and gently pelleted at 300g for three minutes between each rinse. Cell counts were performed, using an haemocytometer, and aliquots of 1.1 x 10^7 cells in PBS were dispensed into each of 16 Eppendorf tubes. Cells were pelleted at 300g for 5 minutes at 4°C after which resuspended cells from 6 Eppendorf tubes were simultaneously fixed and permeabilised in 2% paraformaldehyde (PAF) in 0.01%, 0.05%, or 0.1% Triton X 100 in PBS for 30 minutes (P+T).

Resuspended cells from another 6 Eppendorf tubes were first fixed in 2 % PAF/PBS for 30 minutes followed by permeabilisation in 0.01%, 0.05% or 0.1% Triton X 100 (TX) for 30 minutes (P/T). The four remaining tubes, were fixed in 2% PAF in PBS for 30 minutes without subsequent permeabilisation (Controls). All samples were rinsed twice in PBS after which 6 tubes consisting of one Eppendorf tube of cells fixed and permeabilised at each concentration of TX in (P+T) and (P/T) protocols, and as well as two of the unpermeabilised controls were stored in 100μl 0.1% BSA/PBS at 4°C overnight. From the remaining 8 duplicate Eppendorf tubes, cells were resuspended in 100μl 0.1% BSA/PBS for 75 minutes at room temperature.

All samples were subjected to 10 repeats of rinses in PBS followed by pelleting at 300g for 5 minutes after each rinse to simulate the total number of rinses and centrifugations in the immunolabelling protocol. Cell counts were performed to determine the cell yield at the end of a typical immunolabelled run. Histograms were generated from these data using the MicroSoft Excel software.

2.6.3 Preliminary immunolabelling of RINm5F-MRC cells for analysis by flow cytometry:

2.6.3.1 Pre-immunolabelling preparation:

All steps were performed at 4°C unless otherwise specified. Single cell suspensions of routinely cultured RINm5F-MRC cells (see **2.2.1** and **2.2.1.1**), were pelleted at 300g for 5 minutes and fixed in 2% PAF in PBS for 30 minutes, by resuspending the cell pellet in 5ml PBS to which was added an equal volume of freshly prepared 4% PAF in PBS. Cells were rinsed twice in PBS, and permeabilised with 0.05% TX in for 30 minutes at 4°C. All rinses were performed using PBS and each rinse was followed by pelleting cells at 250g for 5 minutes.

2.6.3.2 Immunolabelling protocol:

Cells were rinsed three times in PBS, dispensed into equal aliquots of 2 x 10^6 cells/Eppendorf tube, resuspended and stored overnight at 4°C in 200μl of a 1/50 dilution of insulin, glucagon or somatostatin antisera diluted in 0.1% BSA/PBS buffer. After three rinses in PBS, cells were labelled with a 1/220 dilution (6.25μg/ml) of b-GAR-IgG for 120 minutes, rinsed three times in PBS and incubated with 20μg/ml SA-FITC in PBS for 30 minutes in the dark. Staining controls were samples that were not exposed to primary antiserum, b-GAR-IgG, or SA-FITC (background control) or samples exposed to 0.1% BSA/PBS instead of primary antisera (method control). After three more rinses in PBS, cells were resuspended in one ml PBS prior to analysis on the flow cytometer.

2.6.4 Reduction of excessive non-specific background fluorescence by optimisation of the biotinylated goat anti-rabbit IgG (b-GAR-IgG) and strepavidin FITC (SA-FITC) concentration:

2.6.4.1 Pre-immunolabelling preparation:

Cells were cultured, harvested, fixed and permeabilised as in section **2.6.3.1** above.

2.6.4.2 Immunolabelling protocol:

Cells were rinsed three times in PBS, dispensed into equal aliquots of 2 x 10^6 cells/Eppendorf tube and resuspended in 0.1% BSA/PBS overnight. The next day, cells were rinsed 3 times in PBS, and resuspended in 1/220, 1:440, or 1:880 dilutions (6.25μg, 3.37μg, and 1.68μg/ml respectively) of b-GAR-IgG for 120 minutes, rinsed three times in PBS and incubated with 20μg, 10μg, or 5μg/ml SA-FITC in PBS for 30 minutes in the dark. Background control preparations were incubated in neither IgG nor SA-FITC. After three more rinses in PBS, cells were resuspended in one ml PBS for analysis.

2.6.5 Titration of SA-FITC:

Cell suspensions were prepared, fixed and permeabilised as in section **2.6.2.1** and were dispensed into

12 aliquots of 2 x 10^6 cells/Eppendorf tube. Cells were resuspended in 1/50 dilution of either insulin or glucagon antiserum or 0.1% BSA/PBS (4 tubes for each solution) overnight. All preparations were rinsed three times in PBS and resuspended in 1/220 dilution (6.25μg/ml) of b-GAR-IgG for 120 minutes. After three rinses in PBS, cells were resuspended in 20μg, 10μg, 5μg, or 2μg/ml SA-FITC for 30 minutes rinsed as before and analysed.

2.6.6 Immunolabelling of RINm5F-MRC cells grown with or without sodium butyrate:

At the end of the culture period, single cell suspensions of untreated and sodium butyrate-treated cultures (see **2.2.3.4**) were harvested, fixed and permeabilised (see **2.6.3.1**). All subsequent rinses and incubations were performed at 4°C unless otherwise specified and cells were pelleted at 250g for 5 minutes at room temperature between each rinse and step of the labelling procedure.

Cells were rinsed twice in PBS and resuspended in 3% BSA in PBS and incubated for 1-2 hours after which equal volumes of suspensions containing 4 x 10^6 cells were dispensed into separate Eppendorf tubes. Cells were pelleted, and resuspended and stored overnight at 4°C in 1/50, 1/250, and 1/500 dilutions for each antiserum (insulin, glucagon or somatostatin antiserum). The next day, cells were rinsed three times in PBS, and further labelled with a 1/220 dilution (6.25μg/ml) of b-GAR-IgG for 60 minutes, rinsed three times in PBS and resuspended in 5μg/ml SA-FITC in PBS for 30 minutes in the dark. Samples were rinsed and analysed as before.

2.6.7 Specificity controls:

To determine specificity of immunoreactivity of immunolabelled RINm5F-MRC cells for analysis by flow cytometry, cells were cultured (see **2.2.3.4**), harvested, fixed and permeabilised as before (see **2.6.3.1**). Cells were immunolabelled with a 1/50 dilution of either the insulin antiserum, a 1/250 dilution of the glucagon antiserum or a 1/250 dilution of the somatostatin antiserum. Cells serving as non-immune serum controls (see **2.4.3.1 c**), were incubated in either normal guinea-pig or normal rabbit serum instead of the primary antiserum. Absorption controls (see **2.4.3.1 d**) were incubated with pre-absorbed antisera and unlabelled controls were incubated in 0.1%BSA/PBS (see **2.4.3.1 a**). Immunolabelled cells and controls were stored overnight at 4°C in the above solutions and further subjected to the protocol (see **2.6.6**) and analysis by flow cytometry.

2.7 SDS-polyacrylamide gel electrophoresis and immunoblotting:

2.7.1 Preparation of cell extracts:

Medium was aspirated from the dishes containing cells cultured as in section **2.2.3.4**, cells were rinsed three times in PBS, after which they were scraped off the dish in PBS, using a rubber policeman, and

pelleted at 500g for 5 minutes. The resulting pellet volume was approximately 150μl (equivalent to 2 x 10^7 cells). To this was added 200μl of lysis buffer containing 50mM HEPES, pH 7.4, 1% Triton X 100, 5mM EDTA, 0.15mM NaCl, 2mM PMSF, 40μg/ml aproteinin, 2μg/ml lupeptin, 5μg/ml pepstatin and 200μg/ml bacitracin. Bacitracin was included in the lysis buffer as others had shown that it was more effective in preventing proteolytic degradation of insulin (Bathena et al., 1985). Cells were mixed with the lysis buffer by pipetting and further incubated at 4°C for 60 minutes after which they were pelleted at 10 000g for 15 minutes at 4°C. The supernatant was stored in 50μl aliquots at -20°C until required.

2.7.2 Electrophoresis:

SDS-PAGE electrophoresis was performed according to the method of Laemmli (1970). Aliquots of cell extracts (50μl), 10μl of insulin or glucagon (equivalent of 5μg protein) and a protein molecular weight marker (containing proteins with molecular weights of 96 000, 67 000, 43 000, 30 000, 20 000 and 14 000 daltons) were denatured in reducing sample buffer (pH 8.3) containing 50mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0,1% bromophenol blue and 0.5M mercaptoethanol, by heating in boiling water for 10 minutes. Cell extracts for immunoblots to be labelled with anti-somatostatin and 10μl somatostatin (equivalent of 5μg protein) were electrophoresed under non-reducing conditions (without mercaptoethanol in the sample buffer) as below. Samples loaded onto a 15% SDS-polyacrylamide mini-gel containing 14.6% acrylamide, 0.4% N,N'-methylene-bis-acrylamide, 0.38mM Tris (pH 8.8), 0.1% SDS, 0.1% ammonium persulphate (APS) and 0.01% TEMED with a stacking gel of 3% acrylamide/bis-acrylamide, 125mM Tris (pH 6.8), 0.1% SDS, 0.1% APS and 0.1% TEMED.

Gels were electrophoresed at 25mA in buffer containing 50mM Tris and 100mM glycine until the dye front had migrated three quarters of the length of the gel. Gels were stained in 0.5% Coomassie blue in 50% methanol and 10% acetic acid at 50°C for several hours and destained in 50% methanol/10% acetic acid at room temperature overnight. Proteins were transferred from unstained gels to nitrocellulose (Hybond C; Amersham, UK) with a pore size of 0.45μm, by electroblotting at 400mA for 150 minutes.

2.7.3 Immunoblotting:

The portions of the blot containing the molecular weight markers were removed and stained in amido black for 5 minutes. The remaining blots were blocked in buffer containing 3% BSA, PBS and 0.05% Tween 80 for 3 hours, after which they were stored in 1/500 dilutions of either insulin, glucagon or somatostatin antisera at 4°C for 15 hours with continuous agitation. Blots were rinsed in buffer containing 0.3% BSA, PBS, 0.05% Tween 80 and 0.2% NP40 (Nonidet), and incubated in a 1/220

dilution (6.25μg/ml) of b-GAR-IgG diluted in the same buffer for 30 minutes at room temperature. Each buffer rinse was done for a period of 5 minutes and rinses were performed between each step of the labelling procedure.

Blots were rinsed twice in the above rinsing buffer, followed by two more rinses in PBS containing 0.05% Tween 80 (PBS/Tween) before incubating in ABC-HRP solution diluted in the same buffer for 40 minutes. Thereafter, blots were rinsed twice for 5 minutes each in PBS/Tween buffer followed by two more rinses of 5 minutes each in 50mM TBS (pH 7.4). Blots were stained with 0.5mg/ml α chloro-l-naphthol in 50mM TBS containing 0.005% H_2O_2 for 30 minutes. Further development of staining was inhibited by rinsing blots in PBS for 10 minutes after which they were rinsed in water and air-dried. Blots were photographed using Kodak Technical Pan 100 ASA film.

A standard curve was generated from measurements of the distance migrated by the individual proteins of the molecular weight markers against their relative molecular mass. The molecular weights of the proteins in the stained bands were obtained by plotting their distance migrated in the gel against the standard curve.

3. RESULTS

3.1 Growth and morphology of RINm5F-MRC cells in culture:

3.1.1 Growth and morphology under routine culture conditions:

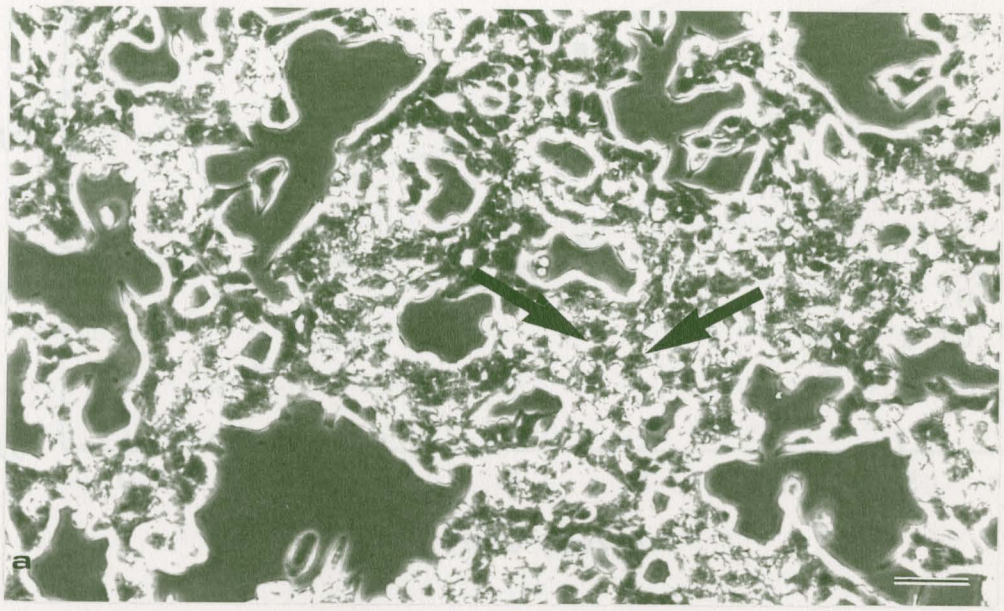
3.1.1.1 Morphology:

Since morphology may indicate the presence of rogue cells or a change in phenotype, RINm5F-MRC cultures were examined to determine whether their morphology, when grown in our laboratory under routine culture conditions (see **2.2.1 - 2.2.1.2**), was similar to that found in other laboratories. RINm5F-MRC cells formed semi-compact colonies of epithelial-like cells. The central regions of the colonies were covered by clusters of compact cells (Fig 1a) that were less adherent to each other than those attached to the dish. Monolayers of flattened cell profiles were found at the periphery of colonies (Fig 1a,b). A number of cells demonstrated cytoplasmic processes extending from free cell surfaces (Fig 1b) that were more clearly visible in less dense cultures. A moderate number of small compact non-viable cells (verified by the uptake of trypan blue) were continuously shed into the medium. The maximum culture density attained by RINm5F-MRC cultures was never more than 85-90% of the surface area of the culture dishes, and sheets of cells detached readily from the culture dish if cultures were grown further without re-seeding at lower densities.

3.1.1.2 Growth:

A growth curve for RINm5F-MRC cells at an early phase of this study (MRC passage 22) was established (see **2.2.2.1**) to determine the population doubling time (proliferation rate) so that appropriate seeding densities and period in culture could be calculated for experiments. The population doubling time also served to determine whether sub-optimal culture conditions during transportation may have promoted the selection of a phenotype with a doubling time beyond the range previously reported for RINm5F cells. Because insulin immunoreactivity had changed at later passages (see section **3.3**), the growth rate of RINm5F-MRC cultures at this higher passage number was examined to see whether it too had altered during the course of this study.

The doubling time for RINm5F-MRC cells (RINm5F-MRC p-22), calculated from a growth curve established at an early phase of the present project, was 31, 30 or 27 hours for cultures seeded at a density of 10^3, 3 x 10^3 or 10^4 cells/dish respectively (Fig 2a). The growth curve established at a later phase of this project (RINm5F-MRC p-32), indicated that the proliferation rate of RINm5F-MRC cells had increased with doubling times of 20.8, 19.1 or 19.1 hours for cultures seeded at 10^4, 5 x 10^4, or 10^5 cells/dish respectively (Fig 2b). Although the range of seeding densities for the early and late



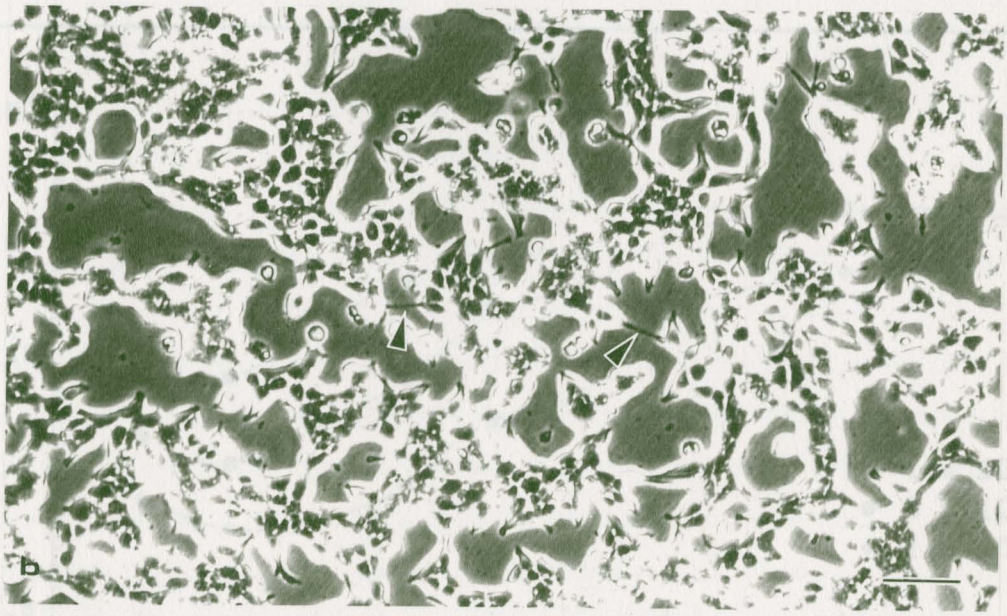
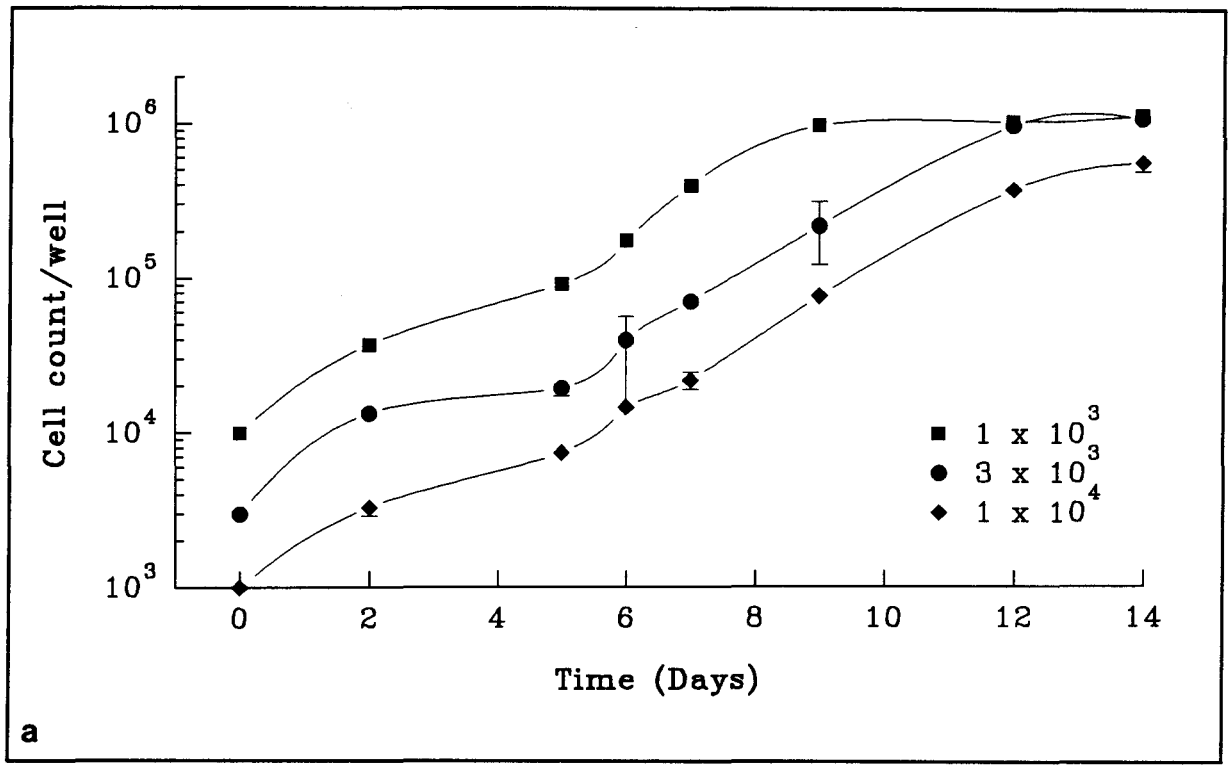


Fig 1a. Culture morphology of a subconfluent RINm5F-MRC culture demonstrating clusters of compact cells over the central regions of the colonies (arrows). Images were recorded under phase contrast. Bar = 50μm

Fig 1b. RINm5F-MRC cells at a lower culture density. The dense cell clusters, seen in Fig. 1a, have been removed by mild agitation and replacement of medium to reveal the deeper layers of the colonies. Cytoplasmic processes extend from free cell surfaces in a number of places (arrow heads). Bar = 50μm



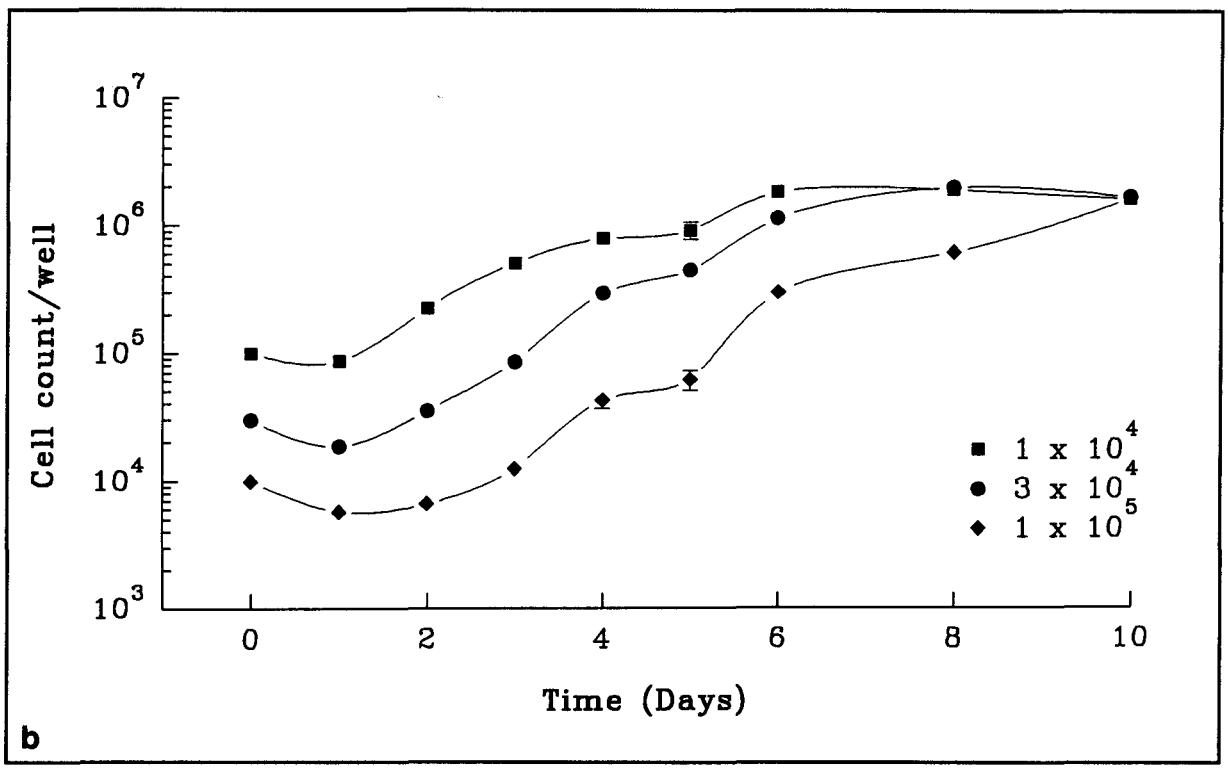


Fig 2. Growth curves established during an early phase (a) and at a later phase (b) of the present study: a) The doubling time of RINm5F-MRC cells (p-22) calculated from the log proliferation phase was 27 hours for cultures seeded at 10^4 cells/well.
b) The doubling time of RINm5F-MRC cells (p-32) from cultures seeded at 10^4 cells/well has been reduced to 19.1 hours.

growth curves differed, a seeding density of 1 x 10^4 was common to both and doubling time had decreased from 27 to 19.1 hours. Hence the difference in doubling time between the growth curves appeared to be independent of the seeding density of the culture and may reflect a change in the status of differentiation of the RINm5F-MRC cells.

3.1.2 Growth and morphology in the presence of mitomycin C:

The purpose of exposing RINm5F-MRC cells to mitomycin C was to determine whether growth arrest would increase the expression of the peptide hormones as gene expression may have been suppressed by continuous proliferation. This is because the RINm5F cell line had been transformed by irradiation (Chick et al., 1977). Consequently, cells continuously proliferate in culture until the medium becomes exhausted. Therefore, mitomycin C, that inhibits cell division by interrupting the synthesis of DNA without affecting gene transcription, was used to obtain a non-dividing population of RINm5F-MRC cells. Cultures were exposed to a range of concentrations of mitomycin C to establish the least toxic concentration that would effectively inhibit cell proliferation (see **2.2.2.2**).

3.1.2.1 Morphology:

There was a marked reduction in the number of cells layered over the central regions of the colonies and less coverage of the surface of the culture dish in all cultures exposed to mitomycin C in comparison to untreated cultures (Fig 3). Furthermore, there was a proportional decline in the number of attached cells with increasing concentrations of mitomycin C. In contrast, colonies continued to expand in untreated cultures and were covered with large clusters of small cells (Fig 3a). The size of cells in mitomycin C-treated cultures was markedly greater (Fig 3b) than that in untreated cultures (compare Figs 3b and 1b). Fig 1b is an untreated culture of low density in which individual, isolated cells (arrows) are more clearly visible than in densely populated untreated cultures (Fig 3a). The increase in the size of cells from mitomycin C-treated cultures was also clearly seen when cells were in suspension (data not shown). A number of cells in mitomycin C-treated cultures contained more than one nucleus (Fig 3b). A large proportion of cells had cytoplasmic processes some of which were approximately twice the length of the diameter of the cell bodies (Figs 3b and c). Cultures treated with concentrations of mitomycin C greater than 0.1μg/ml, demonstrated a marked reduction in the size of colonies compared to controls. In contrast, although the surface area covered by cultures treated with 0.05μg/ml mitomycin C was less than that of controls, small clusters of cells were still present over most colonies (Fig 3d). When these cultures were grown for longer than 6 days, active formation of new colonies was seen in those exposed to 0.05μg/ml mitomycin C (not shown). This suggested that cell proliferation still occurred although to a lesser extent than in untreated cultures. Thus, these morphological observations indicated that mitomycin C used at a concentration of 0.05μg/ml was too

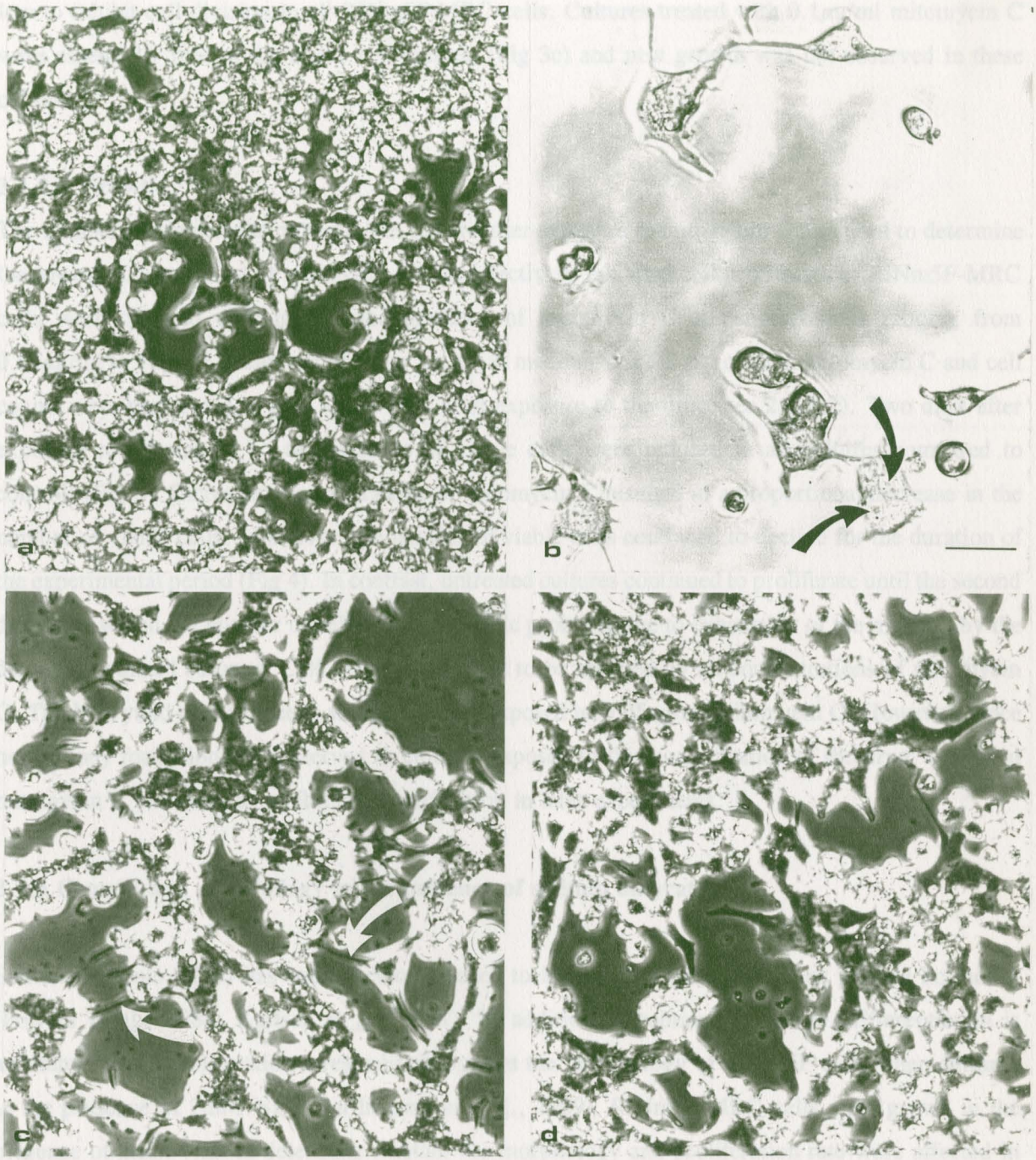


Fig 3. RINm5F-MRC cells 6 days after exposure to medium a) without mitomycin C (untreated culture) ; b) 0.5μg/ml mitomycin C; c) 0.1μg/ml mitomycin C; and d) 0.05μg/ml mitomycin C. Images were recorded under phase contrast. Bar = 50μm.

a) Cultures are almost confluent and colonies are covered with clusters of densely packed cells.
b) Cultures are sparsely populated and cells are markedly enlarged compared to untreated cultures. Most of the cells have prominent neurite-like cytoplasmic processes and one cell contains three nuclei (arrows).
c) Colonies are substantially smaller than those in controls and are not covered with clusters of small cells. Numerous cells have neurite-like cytoplasmic processes (arrows).
d) Cultures are more sparsely populated than in controls and clusters of small cells are present over most colonies.

low to inhibit cell division in all RINm5F-MRC cells. Cultures treated with 0.1μg/ml mitomycin C were essentially free of the above cell clusters (Fig 3c) and new growth was not observed in these cultures.

3.1.2.2 Growth:

The number of viable cells remaining in culture after exposure to mitomycin C was used to determine the lowest concentration of mitomycin C that effectively inhibited cell proliferation. RINm5F-MRC cells were cultured overnight in the presence of mitomycin C at concentrations ranging from 0.05μg/ml to 1μg/ml. Cells were further grown in medium that did not contain mitomycin C and cell counts were performed at 2, 4 and 6 days after exposure to the drug (see **2.2.2.2**). Two days after exposure to mitomycin C, the numbers of viable cells were reduced in all cultures compared to controls (Fig 4). Increasing concentrations of mitomycin C resulted in a proportional decrease in the number of viable cells. Moreover, the number of viable cells continued to decline for the duration of the experimental period (Fig 4). In contrast, untreated cultures continued to proliferate until the second day, after which the number of cells remained static probably due to exhaustion of the medium by the densely populated cultures. Cell division appeared to be arrested by all concentrations of mitomycin C. The best yield of viable cells was in cultures exposed to 0.05μg/ml mitomycin C. However, since new colony formation was observed in cultures exposed to this concentration of the drug, 0.1μg/ml mitomycin C was used to inhibit cell proliferation in later experiments.

3.1.3 Growth and morphology in the presence of sodium butyrate:

Sodium butyrate (NaB) has been previously used to induce RIN cells to express peptide hormones (Powers et al., 1988; Karlsen et al., 1991). In addition to peptide expression, differentiation is accompanied by proliferation arrest and changes in the morphology of RINm5F cells when cultured in the presence of 2mM NaB (Bartholomeuz et al., 1989). RINm5F-MRC cells were grown in the presence of 2mM NaB to determine whether the morphology and proliferation rate were affected in the same way as reported previously, and thus to possibly indicate the potential of RINm5F-MRC cells to differentiate.

3.1.3.1 Morphology:

Changes in the morphology of individual cells and cell colonies were seen in cultures that were grown in the presence of sodium butyrate. The colonies were substantially reduced in size compared to untreated cultures after culture for three days in 2mM NaB (Fig 5). Cell size was notably larger in a number of cells in sodium butyrate-treated cultures (Fig 5a). Long neurite-like cytoplasmic processes extending from free cell surfaces at the peripheral edges of the colonies of NaB-treated cultures

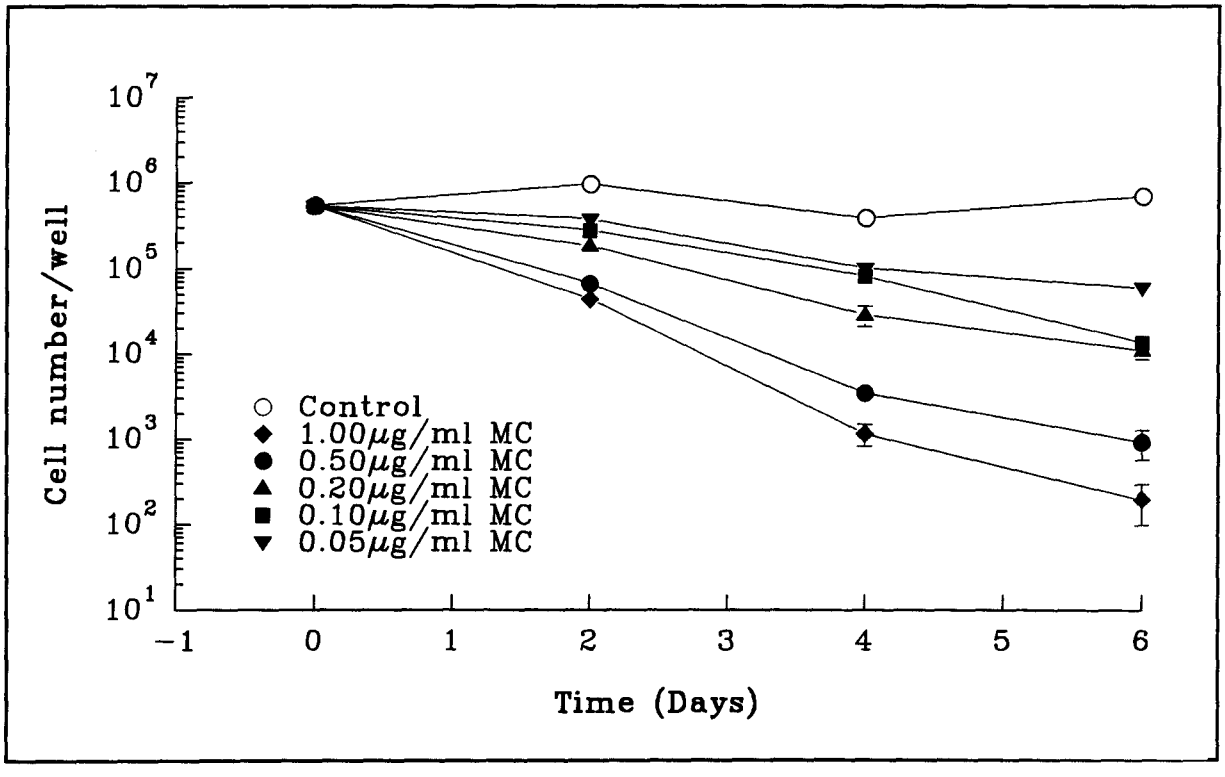


Fig 4. Graph obtained from cell counts of viable cells at 0, 2, 4 and 6 days after treatment with mitomycin C. A steady decline in the number of viable cells occurs in all mitomycin C-treated cultures. Control = untreated cultures. n=3.

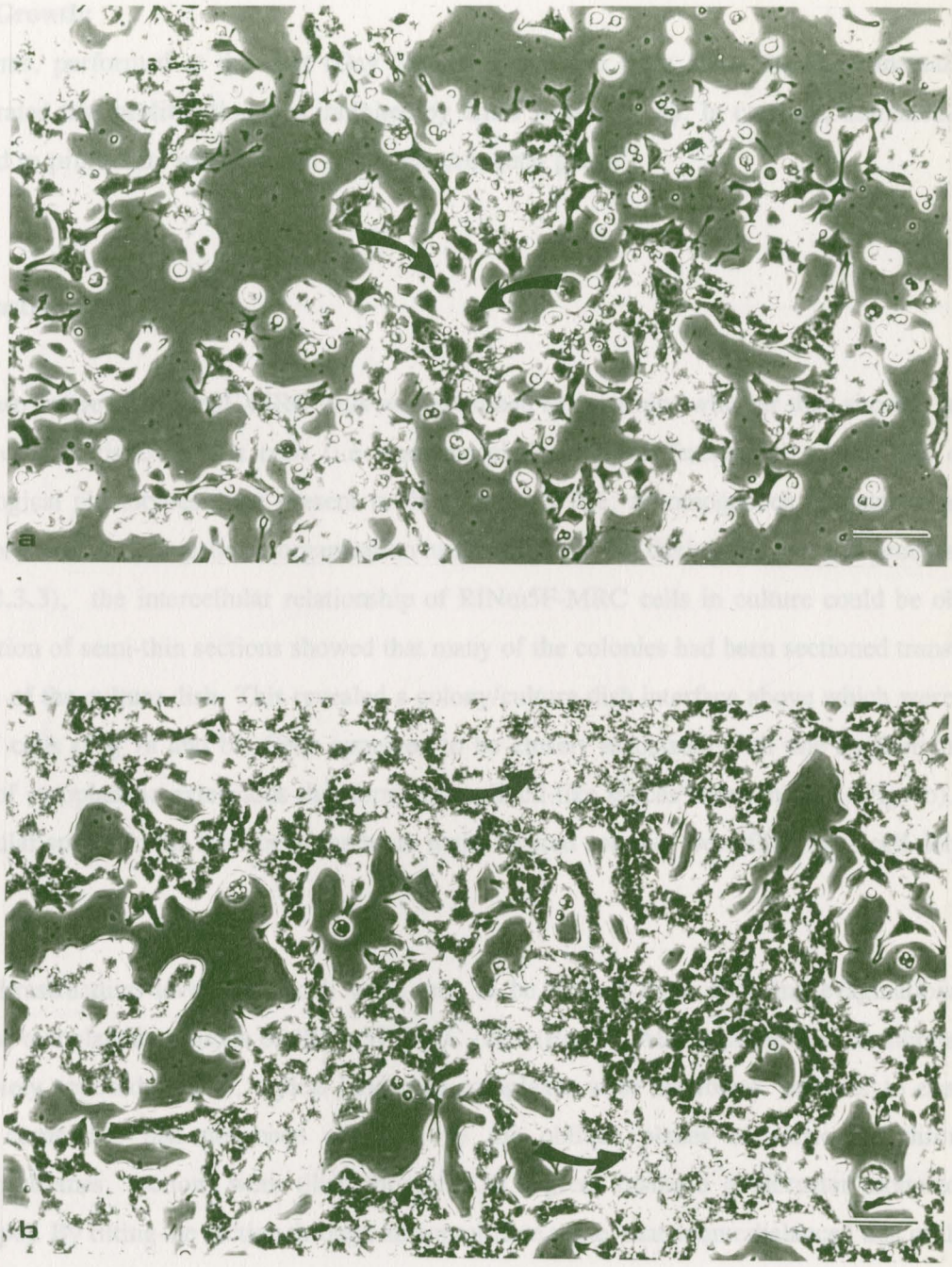


Fig 5. RINm5F-MRC cells grown for three days (a) in the presence of 2mM sodium butyrate or (b) without the drug (untreated culture) Bar = 50μm. Images were recorded under phase contrast.

(a) Cultures are more sparsely populated than in untreated cultures (below) and a notable number of cells have larger cell bodies (arrows) compared to untreated cultures.
(b) Semi-compact colonies have covered most of the surface area of the dish and colonies are covered with numerous cells (arrow).

(Fig 5a).

3.1.3.2 Growth:

Cell counts, performed at the start (Day 2), and at the end (Day 5) of the experimental period, demonstrated that proliferation was inhibited by 2mM NaB (Fig. 6). In contrast, untreated cultures continued to proliferate until the end of the experimental period.

3.2 Ultrastructure:

The ultrastructure of RINm5F-MRC cells was examined to determine whether they showed structural features characteristic of islet cells (i.e were morphologically differentiated) and if one or more morphological phenotypes were present in these cultures (i.e. a homogenous or heterogenous cell population). Because the cellular organization had been preserved during fixation and processing (see **2.3.1 - 2.3.3**), the intercellular relationship of RINm5F-MRC cells in culture could be observed. Examination of semi-thin sections showed that many of the colonies had been sectioned transverse to the plane of the culture dish. This revealed a colony/culture dish interface above which were several layers of cells (Fig 7a and b). Cells appeared to be closely associated with one another through a number of cytoplasmic extensions that formed canaliculi-like spaces between cells (Fig 7b). Larger extracellular spaces in the interior of the more multi-layered colonies were filled with cell debris (Fig 7b).

At the ultrastructural level, canaliculi were seen to be formed from apposing evaginations of the peripheral cytoplasmic regions of RINm5F-MRC cells (Fig 8). Although the cell membranes were often closely apposed (Fig 9), typical junctional complexes were not found. In order to ensure that possible complexes had not been obscured by the oblique planes of sectioning through the plasmamembranes, sections were tilted through 120 degrees (using a goniometer mounted to the microscope). By tilting the section through an appropriate angle, that counterbalanced that of the plane of sectioning, true transverse profiles of the apposing plasmamembranes could be demonstrated. This confirmed that there were no junctional complexes. A small proportion of RINm5F-MRC cells contained abundant secretory granules that were often polarized to one side of the cell (Fig 9). Granule morphology within individual RINm5F-MRC cells was extremely variable and none appeared similar to any of the secretory granules typically found in islet cells in sections of the rat pancreas (Fig 10). Granule-like structures in RINm5F-MRC cells were smaller than the secretory granules of islet cells (compare Figs 9 and 10), were highly electron dense and included spherical- and rod-shaped structures (Fig 9). In addition, secretory granules did not appear to be surrounded by a clear limiting membrane

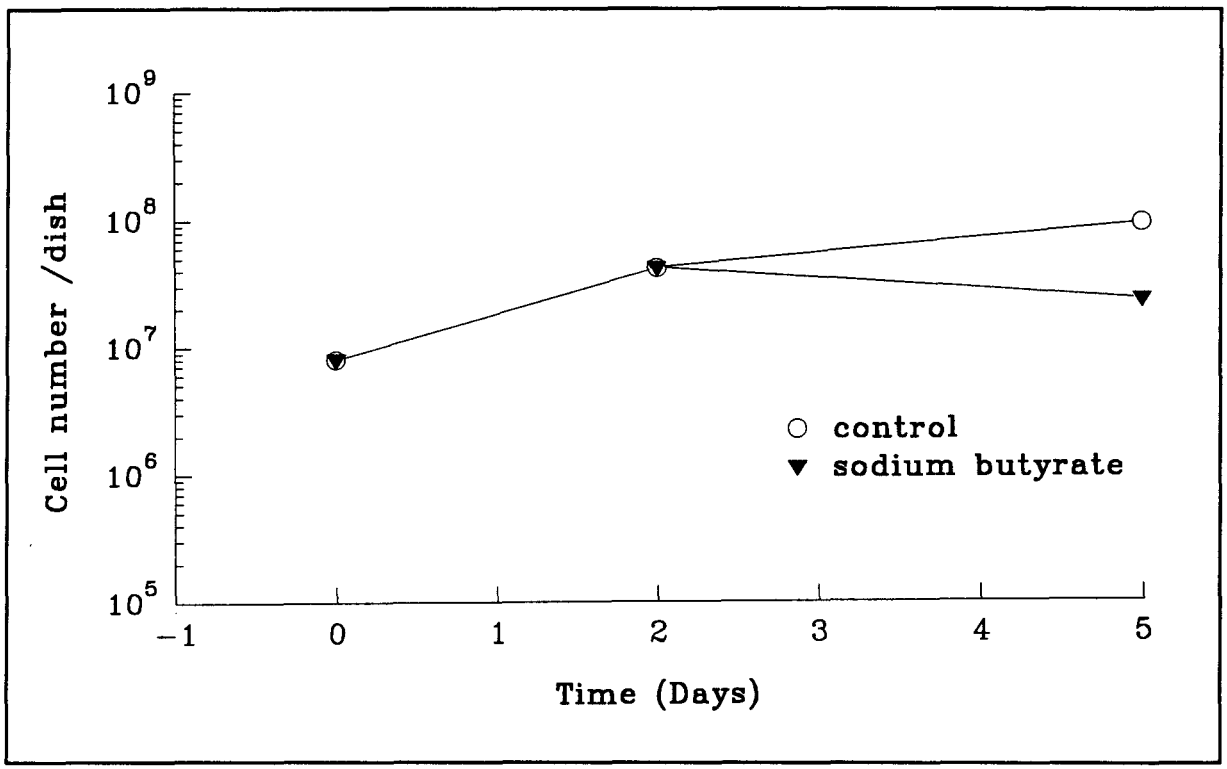


Fig 6. A graph obtained from cell counts at different stages of the experimental period demonstrating proliferation arrest by 2mM sodium butyrate. n=3

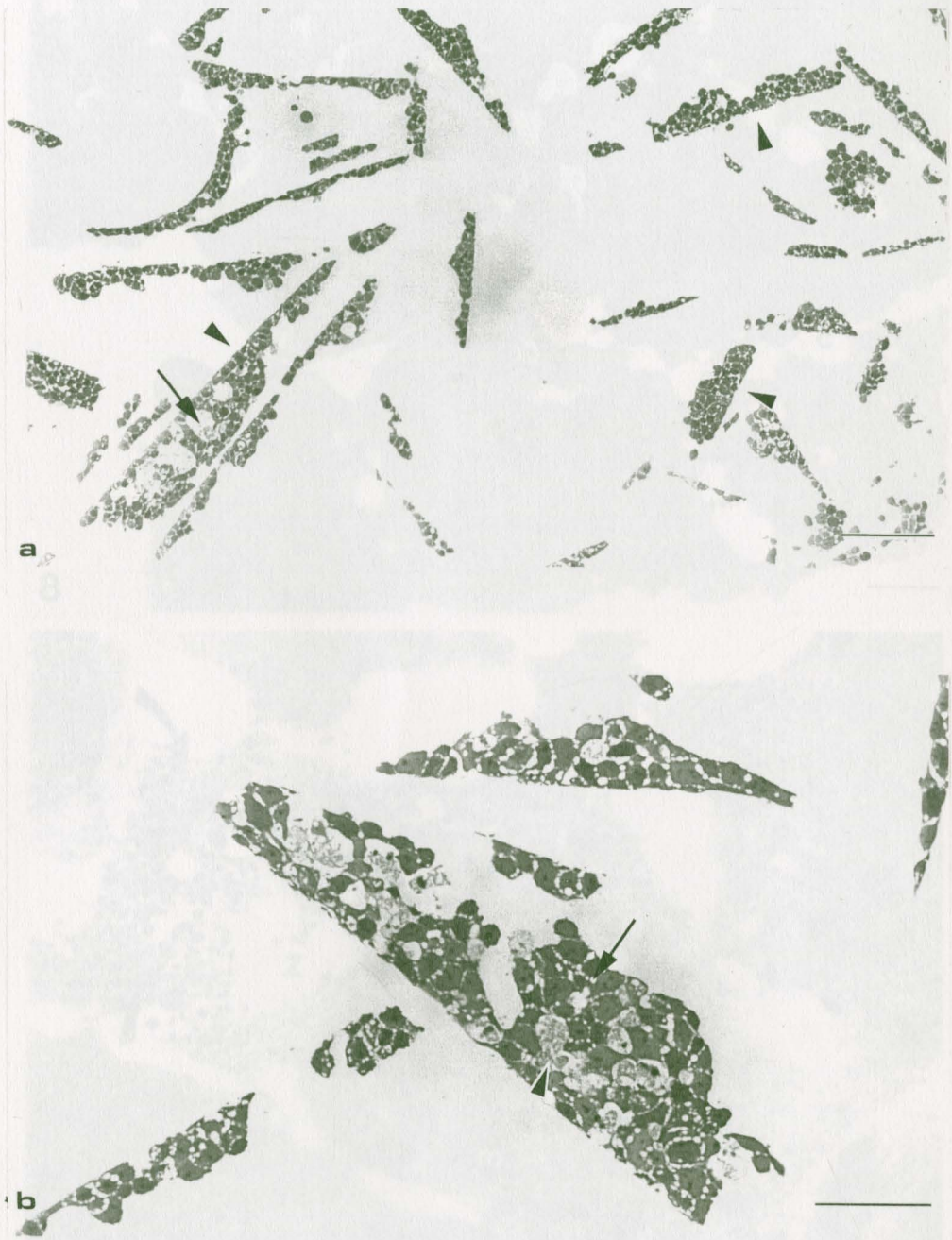


Fig 7. Light micrographs of toluidine blue-stained semithin sections of fragments of RINm5F-MRC cultures.

(a) The colony/culture dish interface (arrow heads) is present in most of the cross sections of the colonies. Colonies consist of 2 - 6 layers of cells and debris (arrow) that is seen in the deeper layers of the colony. Bar = 100μmM

(b) Canaliculi-like spaces (arrow) around all cells and debris (arrow head) can be seen in the deeper layers of the colony. Bar = 50μm.

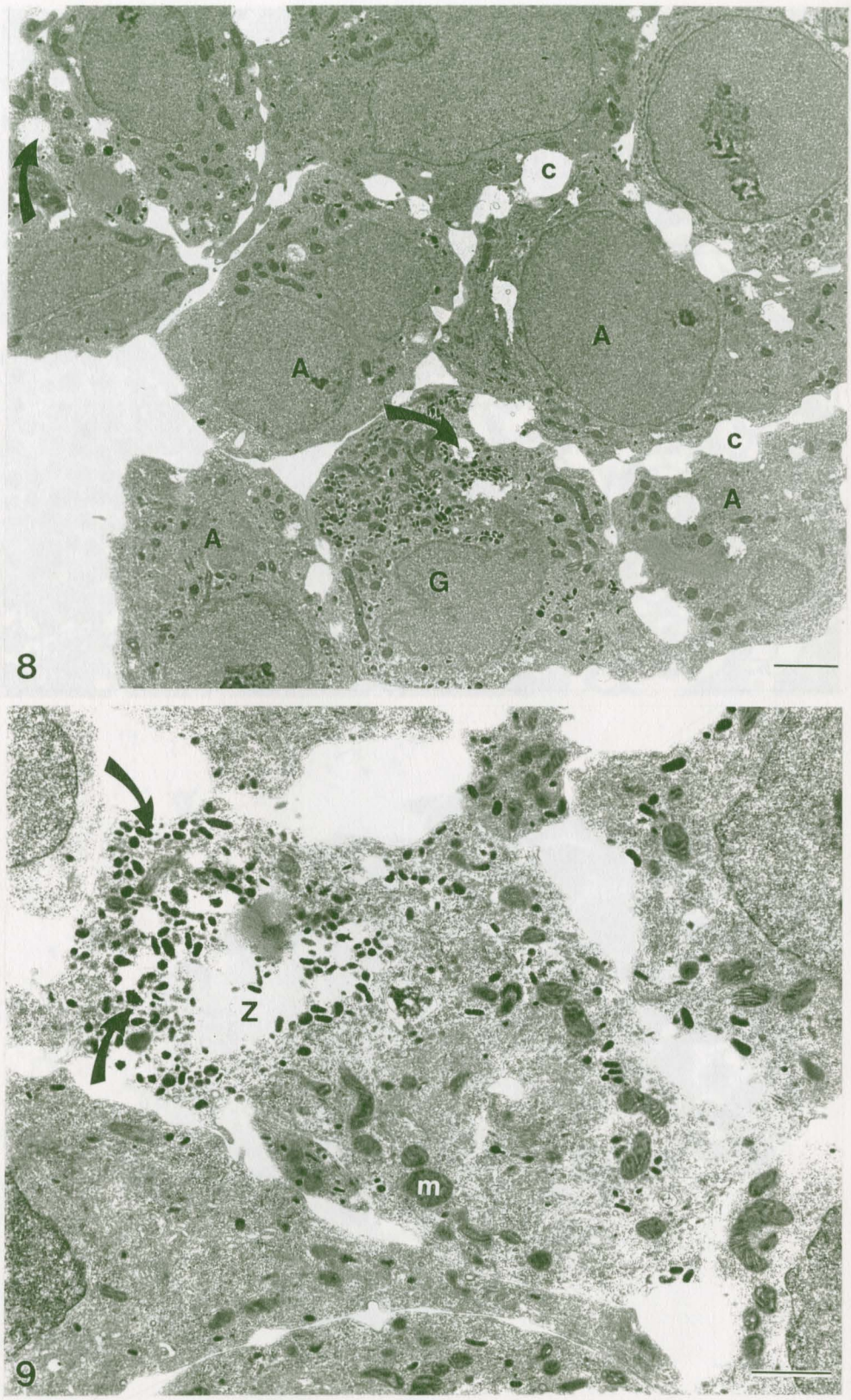
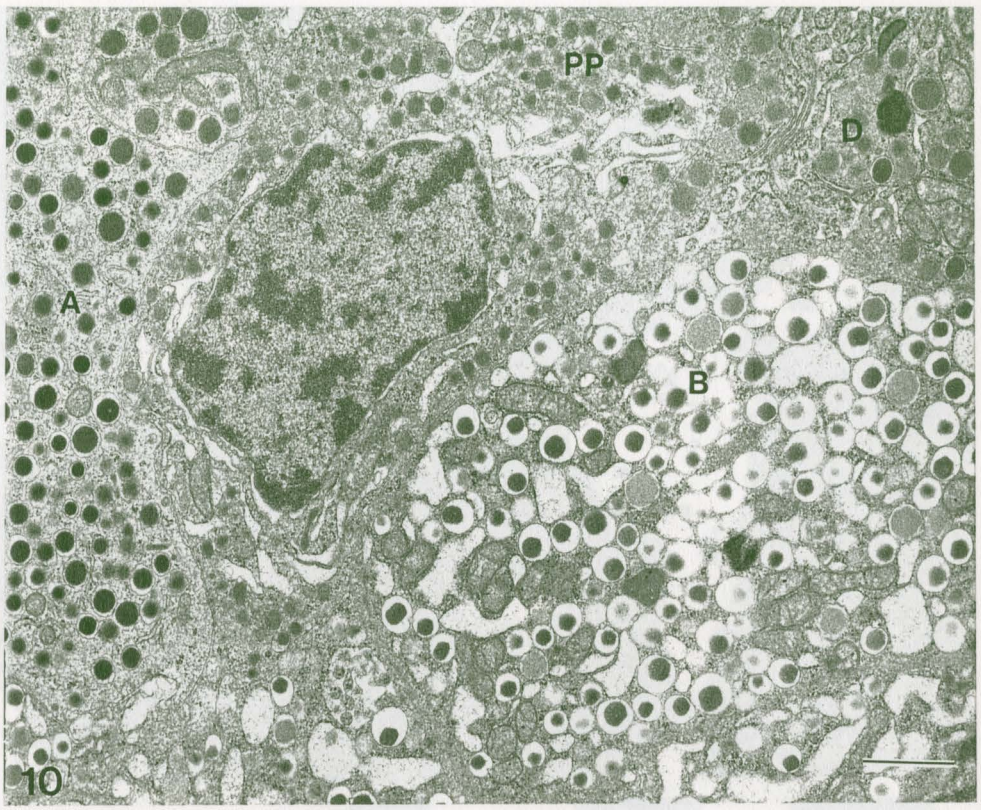


Fig 8. Electron micrograph of a cross section of cultured RINm5F-MRC cells demonstrating canaliculi (c) between all cells. A single well granulated cell (G) is surrounded by agranular cells (A). Debris can be seen in some intracytoplasmic vacuoles (arrows). Bar = 2μm.

Fig 9. Electron micrograph of a section of RINm5F-MRC cells demonstrating pale zymogen-like granules (Z) surrounded by pleomorphic, small electron dense granules (arrows). Mitochondria (m) are filled with numerous cristae. Bar = 1μm.



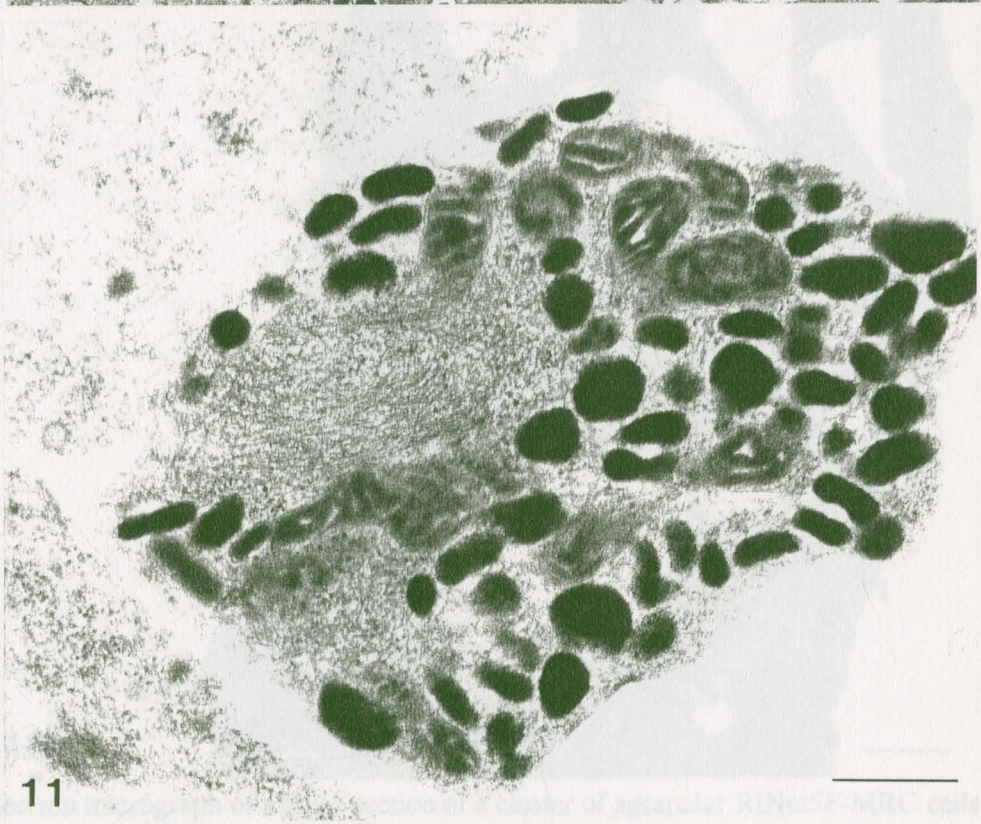
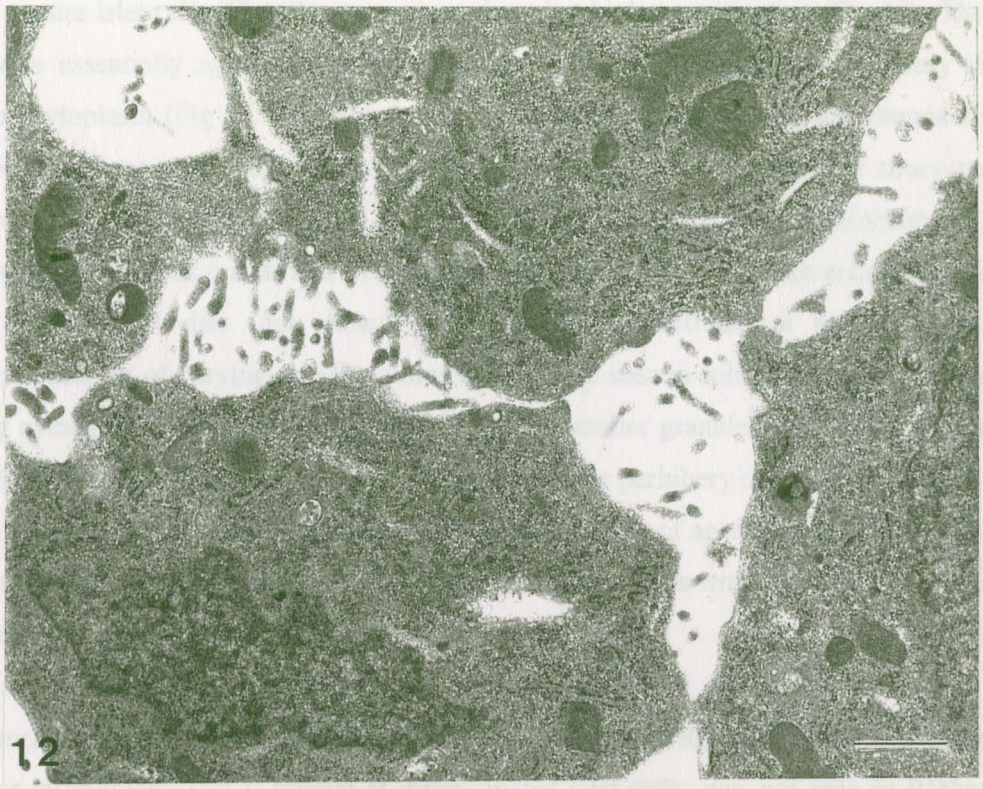


Fig 10. Electron micrograph of a section through an islet of Langerhans from the NEDH rat pancreas. The section demonstrates the ultrastructural morphologies of the secretory granules of an A-cell (A), B-cell (B), D-cell (D) and PP-cell (PP). Bar = 1μm.

Fig 11. Electron micrograph of a portion of a RINm5F-MRC cell demonstrating very electron dense, pleomorphic granule-like structures that do not appear to have a limiting membrane. Bar = 1μm



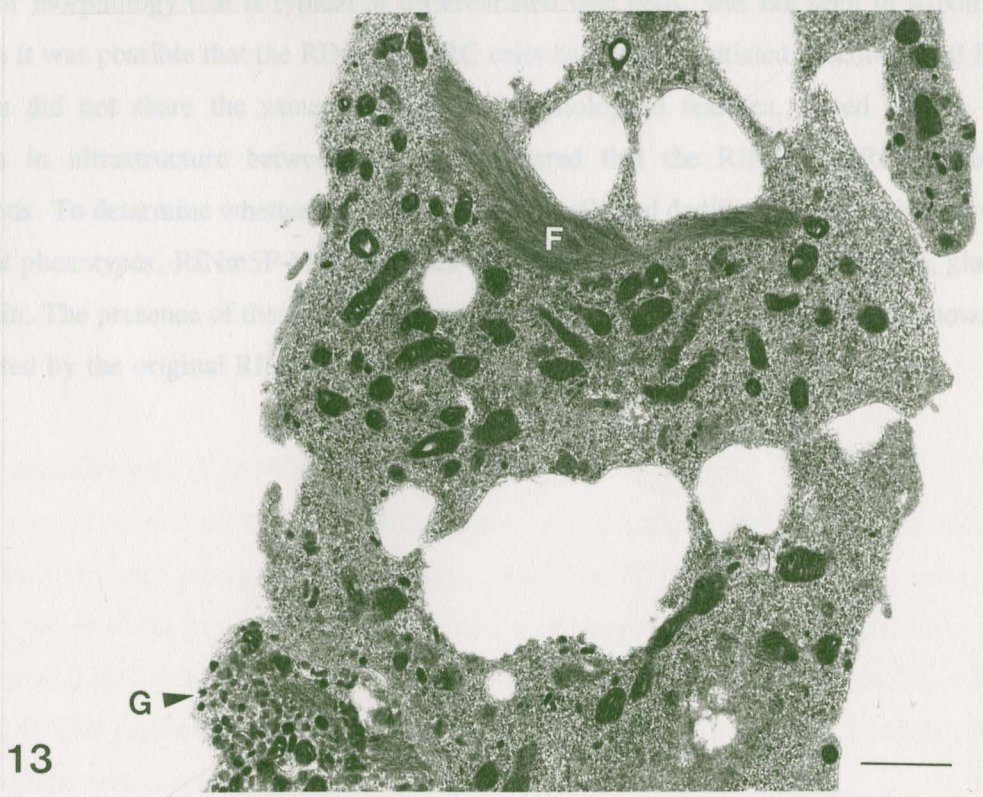


Fig 12. Electron micrograph of a cross section of a cluster of agranular RINm5F-MRC cells that have prominent microvilli extending into the canaliculi. Bar = 2μm.

Fig 13. Electron micrograph of a RINm5F-MRC cell containing prominent intracytoplasmic fibrillar structures (F) and numerous mitochondria. A portion of another cell has granule-like structures of varying electron density (G). Bar = 1μm.

as seen in mature islet cells (Fig 10), even when viewed at high magnification (Fig 11). The majority of cells were essentially agranular with a few small granule-like structures randomly distributed through the cytoplasm (Fig 9). Within a colony, individual cells that contained abundant secretory granule-like structures were usually surrounded by cells almost devoid of granular structures (Fig 8). Other cytoplasmic structures found in all cells included a moderate number of mitochondria that were characterised by numerous cristae and an electron dense matrix (Fig 9). Rough endoplasmic reticulum (Fig 12) and Golgi bodies (not shown) were clearly demonstrated at higher magnifications. Intracellular vacuoles of varying size containing debris were seen in some cells (Fig 8) and structures resembling zymogen-like granules, surrounded by other smaller granules were noted in a number of cells (Fig 9). Prominent fibrillar structures were seen near the periphery of a number of cells (Fig 13). Cells with numerous microvilli were seen in occasional clusters of agranular cells (Fig 12) in which the majority of cells were essentially devoid of microvilli. Islet cells with microvilli were also present in sections of the rat pancreas (Fig 10).

Ultrastructural examination revealed two interesting features of RINm5F-MRC cells. Firstly, the intracellular morphology that is typical of differentiated islet cells, was not seen in RINm5F-MRC cells. Thus it was possible that the RINm5F-MRC cells had dedifferentiated. Secondly, all RINm5F-MRC cells did not share the same intracellular morphological features. Based on the observed differences in ultrastructure between cells, it appeared that the RINm5F-MRC cultures were heterogenous. To determine whether the RINm5F-MRC cells had dedifferentiated or if they consisted of different phenotypes, RINm5F-MRC cultures were immunolabelled to reveal insulin, glucagon or somatostatin. The presence of these peptides was sought in this study because they are known to have been secreted by the original RINm5F clone (Oie et al., 1983).

3.3 Whole-mount immunolabelling of early and late passaged RINm5F-MRC cultures to detect the presence of insulin, glucagon and somatostatin:

3.3.1 Immunoreactivity of RINm5F-MRC cultures at an early passage:

Since insulin, glucagon and somatostatin had been detected in the culture medium of the parent RINm5F cell line (Oie et al., 1983), immunolabelling was performed on whole-mounts of RINm5F-MRC cells to determine whether or not our routine culture conditions (see **2.2.1** and **2.2.1.1**) supported the expression of these peptide hormones. Shortly after the RINm5F-MRC cultures had been established in our laboratory, a preliminary study aimed to detect the intracellular presence of insulin, glucagon or somatostatin on cultures of these cells at early passage (MRC - p22) was conducted. Because immunolabelling of whole-mount preparations had not previously been performed in our laboratory, the protocol that was used routinely in our laboratory to detect the above peptides in sections was applied to whole-mounts of RINm5F-MRC cultures. (see **2.4.2**). Staining was observed in whole-mounts labelled to detect insulin, glucagon or somatostatin (Fig 14). Staining of moderate intensity was seen in a number of cells in whole-mounts immunolabelled to reveal insulin or glucagon, whereas most cells in whole-mounts immunolabelled with anti-somatostatin serum were strongly stained (Fig 14c). Intense staining was often present in small cells from whole-mounts labelled to reveal glucagon (Fig 14b). Background staining was minimal in whole-mounts in which the primary antiserum (method control) or both the primary antiserum and biotinylated GAR-IgG (background control) were omitted from the staining protocol (Fig 14d). Due to limited availability of RINm5F-MRC cells at low passage number, further immunolabelling (that would have included specificity controls) RINm5F-MRC cultures of similar passage could not be carried out.

3.3.2 Immunoreactivity of RINm5F-MRC cultures at later passages:

Because immunoreactivity of RINm5F-MRC cultures was investigated by other means (see **3.5** and **3.6**) on cells from later passages in this study, RINm5F-MRC cultures of similar passage number (MRC-p32) grown under routine culture conditions, were immunolabelled to reveal insulin, glucagon or somatostatin (see **2.4.2** and **2.4.3.1**). Furthermore, since there were sufficient stocks of RINm5F-MRC cells at later passages, immunolabelling experiments included specificity controls (absorption and non-immune sera controls) as well as a range of dilutions for each antibody.

The intensity and staining pattern differed markedly between whole-mounts immunolabelled to reveal insulin, glucagon or somatostatin (Fig 15). Although staining intensity decreased proportionally in preparations incubated with higher dilutions of antisera, the staining pattern in preparations labelled with increasing dilutions of the same antiserum remained constant. In contrast to that observed in

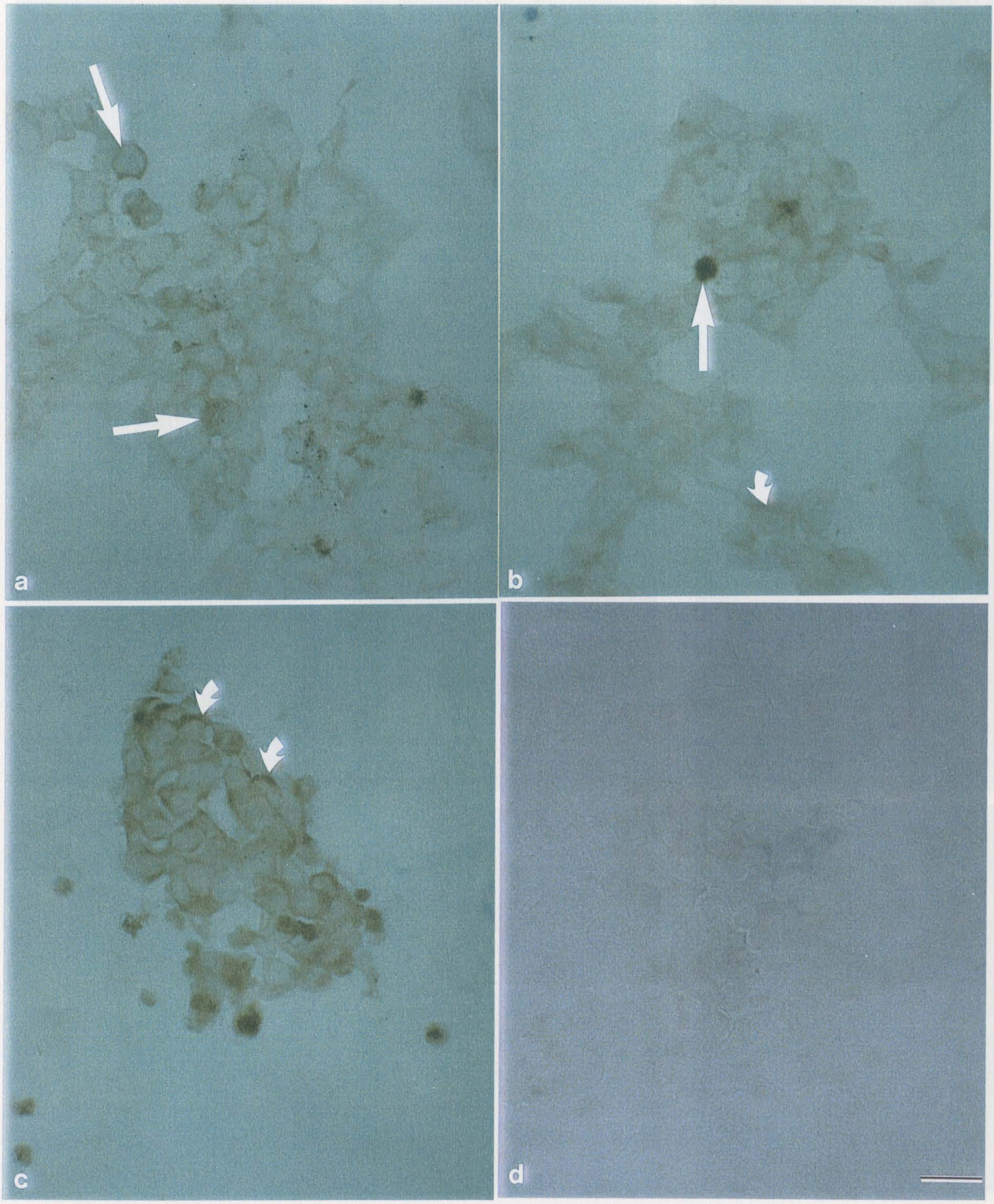


Fig 14. Light micrographs of whole-mounts of RINm5F-MRC cultures (MRC passage no. 22) immunolabelled with (a) anti-insulin, (b) anti-glucagon, (c) anti-somatostatin and (d) antiserum diluent buffer (method control). Whole-mounts were not counterstained. Bar = 20μm.
(a) Staining of moderate intensity is present in a few cells (arrows) and is evenly distributed throughout the cytoplasm. (b) Moderate to strong staining is seen in occasional cells (arrows). (c) Strong immunoreactivity can be seen in most cells particularly near the cell borders (arrows). (d) Absence of staining in the method control shows that the above immunoreactivity is not due to endogenous peroxidase or non-specific attachment of the ABC-HRP label.

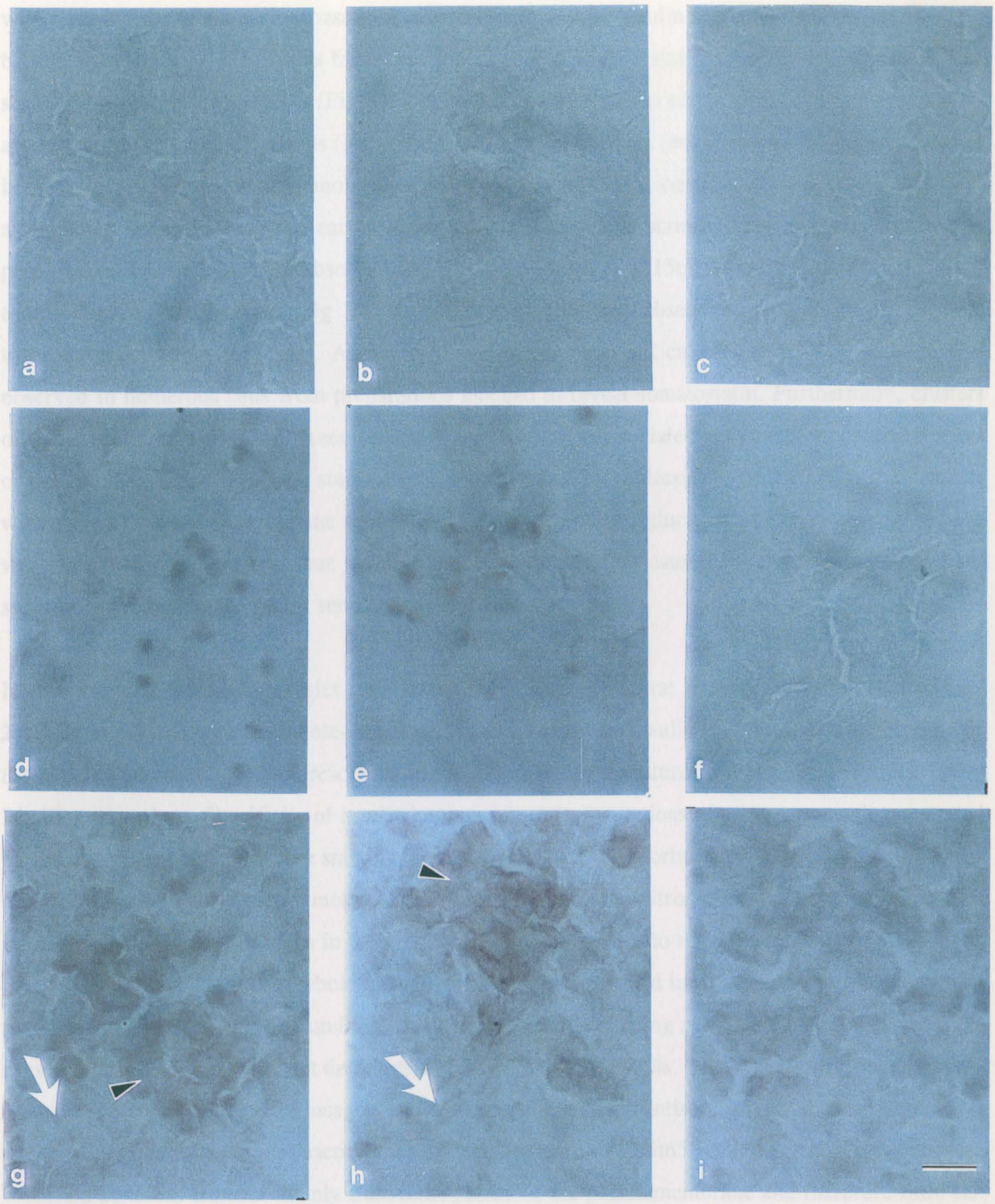


Fig 15. Light micrographs of whole-mounts of cultured RINm5F-MRC cells (MRC passage no. 32) immunolabelled with (a) anti-insulin, (b) pre-absorbed anti-insulin, (c) non-immune guinea-pig serum, (d) anti-glucagon, (e) pre-absorbed anti-glucagon (f) diluent buffer for primary antisera (method control), (g) anti-somatostatin, (h) pre-absorbed anti-somatostatin, and i) non-immune rabbit serum. The dilution of primary antiserum was 1/200 and whole-mounts were not counterstained. Bar = 20μm. (a-c) Staining is similar in all preparations and is barely above that of the method control (f). (d-e) Staining of similar intensity occurs in small cells in both preparations and is significantly above that of the method control. (g-h) Moderate to strong staining is seen over regions where cells are densely packed (arrow head). Where cells occur as monolayers, staining is absent (arrow).
(i) Although staining intensity is less than in (g) and (h), the distribution of stain is similar to that in the somatostatin absorption control and in the whole-mount stained with anti-somatostatin serum.

whole-mounts from the earlier passaged RINm5F-MRC cells, insulin immunoreactivity appeared to be absent in RINm5F-MRC cells from later passages (Fig 15a). Staining intensity was similar to that seen in the absorption controls (Fig 15b) or non-immune serum controls (Fig 15c) and was barely above that of the method controls (Fig 15f) or background controls (not shown). The highest staining intensity in whole-mounts immunolabelled to reveal glucagon was confined to small cells (Fig 15d) as noted in cultures from the earlier passage (Fig 14b). This staining was neither abolished in preparations labelled with pre-absorbed anti-glucagon serum (Fig 15e), nor in preparations exposed to non-immune rabbit serum (Fig 15i). In contrast, staining was absent in preparations not exposed to primary antiserum (Fig 15f). As noted in the earlier passaged cultures (Fig 14c), staining was observed in numerous cells from preparations labelled to reveal somatostatin. Furthermore, clusters of stained cells were frequently seen and these appeared to be associated with the more central regions of the colonies (Fig 15g). Such staining was not abolished by replacement of the primary antiserum with pre-absorbed or non-immune sera (Figs 15h and i). Because glucagon and somatostatin antisera were raised in rabbit, it was thus possible that this staining was caused by cross-reactivity to non-specific antibodies in the rabbit serum.

Intense staining was seen in islet cells from frozen sections of rat pancreas immunolabelled (see **2.4.3.1**) in parallel with the whole-mount preparations to reveal insulin (Fig 16a) This suggested that the absence of insulin immunoreactivity in the later passaged cultures did not result form a faulty labelling procedure. Specificity of insulin immunoreactivity in sections of pancreas was demonstrated by the absorption control where staining was abolished by pre-absorbtion of anti-insulin with insulin (Fig 16b) and was absent from method-control sections (Fig 17). Strong to intense staining occurred in cells at the periphery of islets in frozen sections immunostained to reveal glucagon or somatostatin (Fig 16c and e). In contrast to whole-mounts, staining was abolished in frozen sections incubated with preabsorbed antisera (Figs 16d and f). Thus the non-specific staining seen in whole-mounts (possibly caused by rabbit serum) was not demonstrated in sectioned islet cells. This finding suggested that the non-specific staining, due to glucagon and somatostatin primary antisera, detected in whole-mounts was possibly caused by cross-reactivity with the cell surface of RINm5F-MRC cells. (This is because sectioned pancreas exposes mainly transverse planes of the plasmamembrane and therefore little cell surface is exposed. Hence few non-specific binding sites are exposed to antibodies in the rabbit serum compared to those exposed in whole-mount preparations. It is thus possible that non-specific binding on the cell surface of islet cells in sections of rat pancreas is below the limit of detection.) The staining pattern of whole-mounts exposed to non-immune rabbit serum was similar to that in whole-mounts labelled with anti-somatostatin or pre-absorbed anti-somatostatin sera yet was quite different to whole-mounts labelled with anti-glucagon or pre-absorbed anti-glucagon sera. Thus it was possible that sera in which glucagon and somatostatin were raised contained different non-specific antibodies.

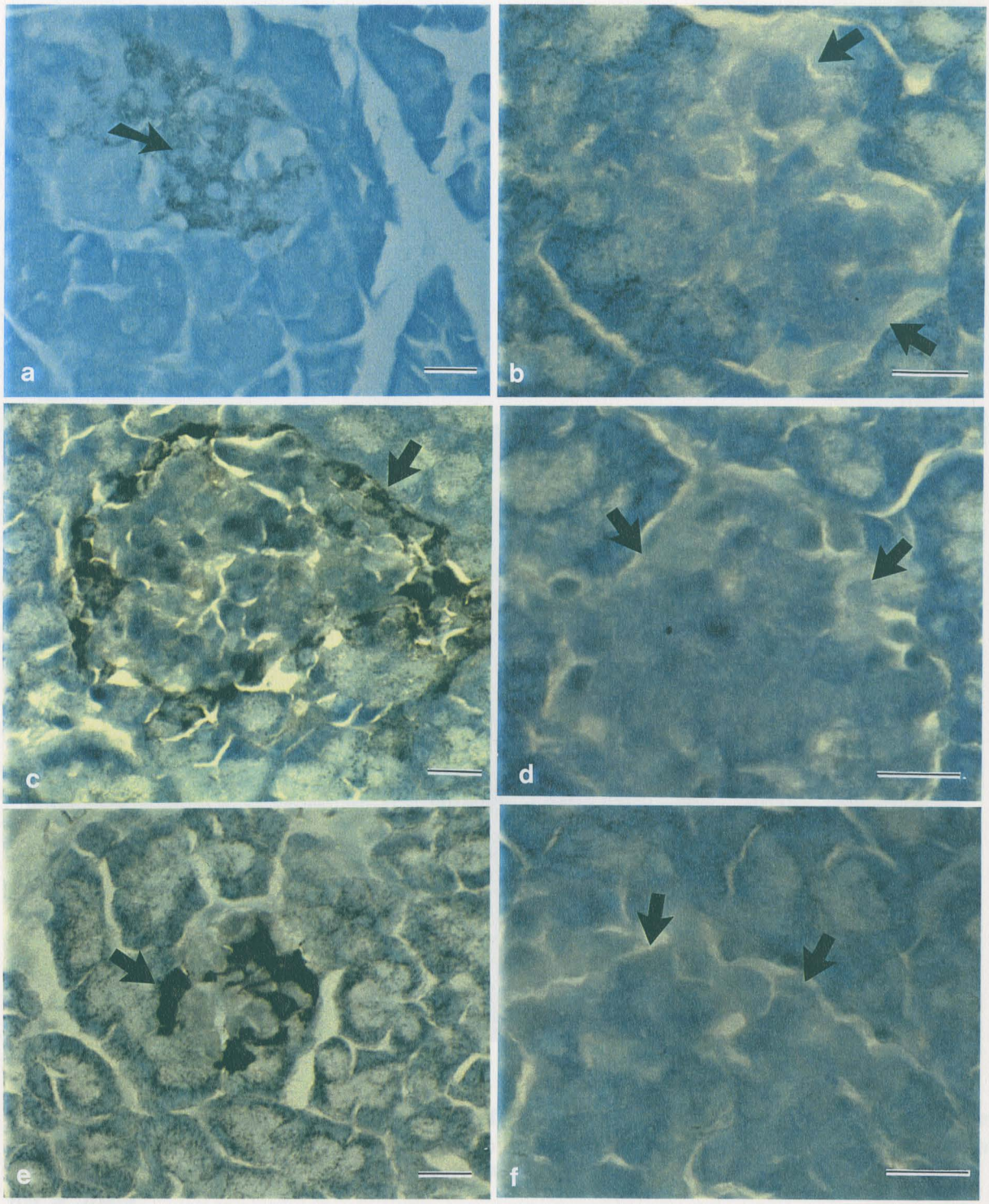


Fig 16. Light micrographs of frozen sections of NEDH rat pancreas immunolabelled with (a) anti-insulin, (b) pre-absorbed anti-insulin serum (c) anti-glucagon, (d) pre-absorbed anti-glucagon serum (e) anti-somatostatin, pre-absorbed anti-somatostatin serum. Sections were labelled with a 1/200 dilution of antiserum and were counterstained with aqueous methylene blue. Bar = 20μm .
(a) Insulin staining is present in most of the islet cells (arrow). (b,d,f) Insulin, glucagon and somatostatin immunoreactivity in islets has been abolished in sections incubated with the pre-absorbed antisera. Arrows indicate islet periphery. (c) Strongly stained cells are found at the islet periphery (arrow) in sections labelled to reveal glucagon. (e) Cells near the islet periphery are intensely stained in sections labelled to reveal somatostatin.

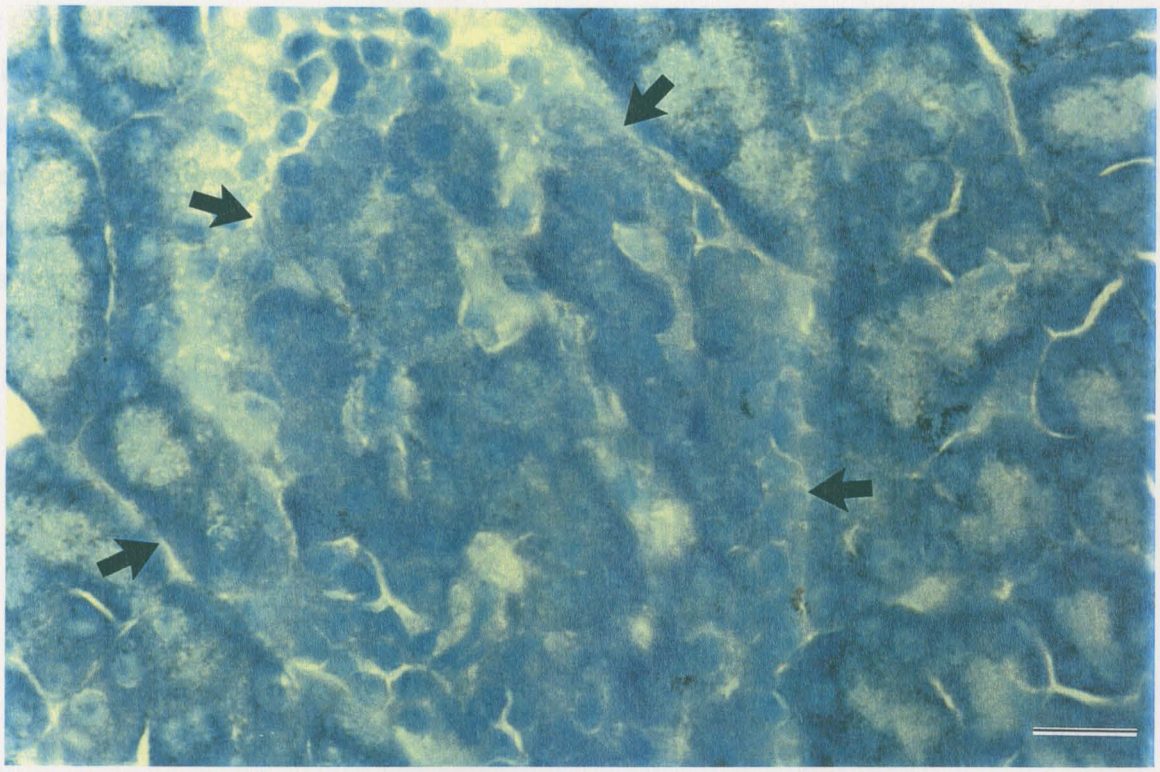


Fig 17. Light micrograph of a frozen section of rat pancreas exposed to antiserum diluent buffer (method control) demonstrates the effective inhibition of endogenous peroxidase and the absence of non-specific binding of the secondary antibody. Arrows indicate the islet periphery.
Bar = 20μm.

The substantially reduced staining intensity of insulin-labelled cells from early passaged RINm5F-MRC cultures compared to that in frozen sections, suggested that RINm5F-MRC cells contained extremely low levels of insulin. The absence of cells demonstrating insulin immunoreactivity in later passaged cultures suggests that RINm5F-MRC cells may have dedifferentiated. Alternatively, the insulin content of later passaged cultures was below the limit of detection of the Vectastain ABC staining system. Furthermore, non-specific immunoreactivity seen in whole-mounts labelled for glucagon and somatostatin appeared to be caused by non-specific staining by rabbit serum and staining appeared to be associated with the cell membrane. A problem related to the detection of immunoreactivity in individual cells within multilayered colonies was revealed in immunolabelling of whole-mounts. This was seen particularly in those cultures labelled with anti-somatostatin serum where central regions of the colonies appeared to be more intensely stained (Fig 15g). It was not possible to determine whether staining was due to the additive effect of a number of less intensely stained cells within a multilayered colony, or to individual cells that had stained more intensely. Thus, in the following experiment, an attempt was made to improve detection of the above peptides by application of alternative immunolabelling methods, and by inducing RINm5F-MRC cells to differentiate thus raising the possibility of detecting intracellular peptides.

3.4 Immunostaining of frozen sections of RINm5F-MRC cultures grown in the presence of sodium butyrate or mitomycin C:

To overcome the problems of detecting immunoreactivity in individual cells (see **3.3.2.**) and the possible masking of intracellular immunoreactivity by non-specific staining of cell membranes, immunolabelling was applied to frozen sections of RINm5F-MRC cells. This was done because in sectioned cells immunoreagents do not need to pass through two membrane systems (plasmamembrane and membranes of secretory vesicles, Golgi apparatus or endoplasmic reticulum) as in the case of whole-mounts. Cells were grown in the presence of sodium butyrate (NaB) (see **2.2.3.3**) to induce expression of insulin or glucagon as shown by Powers et al., 1988 and thus to increase the possibility of detecting intracellular insulin and glucagon. At the same time, in other cultures of the same experiment, cell proliferation was inhibited with mitomycin C (MC) (see **2.2.3.3**) to establish whether cessation of proliferation would favour expression of insulin or glucagon. Immunolabelling was performed twice on frozen sections of pelleted RINm5F-MRC cells (see **2.5.2**) using the protocol and immunoreagents that were used for routine immunostaining of sections in our laboratory.

Staining intensity was extremely low in all immunostained sections of RINm5F-MRC cells under all culture conditions (Fig 18). In contrast, immunolabelled frozen sections of pancreas prepared in parallel (not shown) showed intense staining of islet cells similar to that seen in Fig 16. Faint staining was observed in very few cells in untreated cultures labelled to reveal insulin (Fig 18a). In general, the staining intensity of positively stained cells in sections immunolabelled to reveal insulin was greater in NaB-treated (Fig 18c) and MC-treated (Fig 18e) cultures compared to untreated cultures. In addition, insulin-stained cells were more frequently seen in NaB-treated and MC-treated cultures. Furthermore, staining was generally seen in cross sections of cells in which the nucleus was visible, and appeared to be evenly distributed throughout the cytoplasm (Figs 18b and c). Apparent glucagon immunoreactivity was seen in all cultures and the intensity and the number of stained cells were marginally raised compared to the intensity and number of insulin-stained cells. Furthermore, staining was more often seen in small cells in which nuclei were not clearly visible (Fig 18c). This was similar to the staining pattern seen whole-mount preparations labelled with anti-glucagon (see **3.3.2**). Although a larger number of cells staining for insulin or glucagon were seen in cultures treated with NaB or MC, the staining intensity was not significantly increased above that in untreated preparations. Thus, it appeared that intracellular levels of insulin or glucagon were not raised by NaB or MC. Because it is virtually impossible to cut frozen sections of the same thickness, the slight differences observed in staining intensities of untreated and NaB- or MC-treated cultures may have been due to differences in section thickness alone. Even when sections were not counterstained (to avoid masking of the faint immunoreactivity), the contrast between unstained and immunopositive cells remained low.

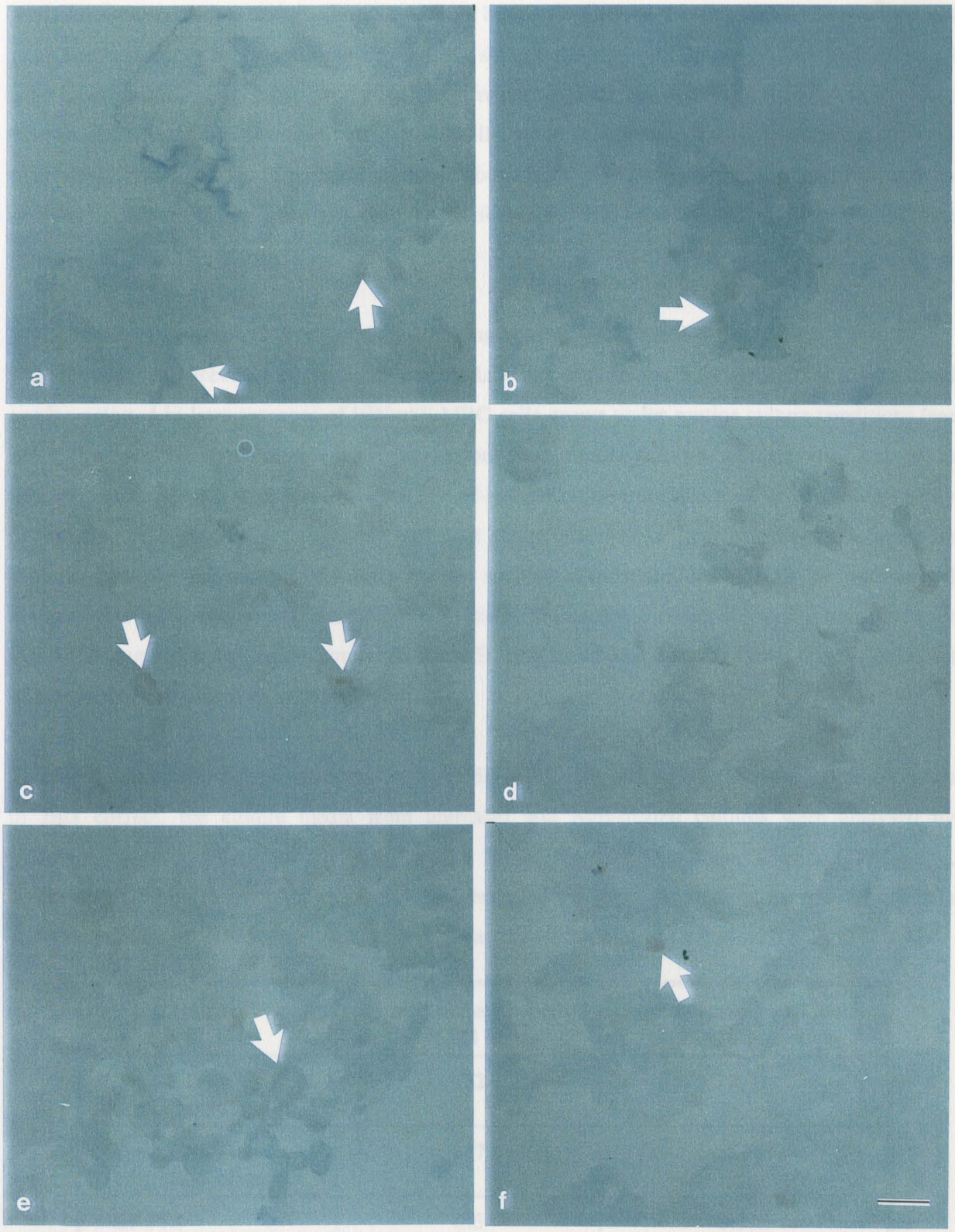


Fig 18. Light micrographs of frozen sections of RINm5F-MRC cells immunolabelled with anti-insulin (a),(c),(e) or anti-glucagon (b),(d),(f). (a) and (b) untreated cultures. (c) and (d) sodium butyrate-treated cultures. (e) and (f) mitomycin C-treated cultures.
Faint staining is seen in very few cells (arrows) and staining appears slightly stronger in sodium butyrate-treated (c) and mitomycin C-treated (e) cultures compared to untreated cultures (a). Staining is seen in occasional small cells in all cultures immunolabelled to reveal glucagon (b,d,f). Sections were not counterstained. Bar = 20μm.

Furthermore, individual unstained cells could not be resolved due to the low contrast, thus it was not possible to quantify the proportion of positively stained RINm5F-MRC cells. Although insulin and glucagon-labelled cells were poorly stained, frozen sections showed that staining was evenly distributed in the cytoplasm of RINm5F-MRC cells, whereas this could not be determined in whole-mount preparations. The difficulties mentioned above in detecting and quantifying immunoreactivity on frozen sections led to investigation of more suitable methods to assess immunoreactivity in these cells.

3.4.1 Summary of results from immunolabelling studies on RINm5F-MRC cells:

The results of the previous whole-mount immunolabelling experiments have shown that insulin could not be detected in later passages of RINm5F-MRC cells grown under routine culture conditions (see **3.3.2**) and was barely detectable in frozen sections from similar cultures. Furthermore, staining in whole-mounts labelled with anti-glucagon or anti-somatostatin serum appeared to be caused mainly by binding of non-specific components in rabbit serum (the species in which the antibodies were raised). Although the number of insulin-labelled cells detected in sections of NaB- or MC-treated cultures was marginally greater than that in untreated cultures, the intensity of staining remained very low. This suggested that insulin content in RINm5F-MRC cells was near the lower limit of detection of the ABC-HRP detection system.

To check that insulin was produced by the RINm5F-MRC cells and that the above observations were in fact correct, the culture media from untreated and NaB-treated cultures were analysed by radioimmunoassay (RIA) (kindly performed by Mr M Voges of the Dept. Chemical Pathology, Tygerberg Hospital, Parow Valley, Cape). RIA revealed insulin in the medium harvested from both cultures conditions and that the insulin content was higher in cultures treated with NaB (Table 1).

Culture condition	**Insulin secretion/dish μU/L/24 hours**	**Insulin secretion/10^7 cells μU/24 hours**
RINm5F-MRC untreated culture	**222**	**2.7**
RINm5F-MRC NaB-treated culture	**227**	**4.55**
NIH 3T3 untreated culture	**0.5**	**0.012**
NIH 3T3 NaB-treated culture	**1.5**	**0.03**

Table 1: Insulin secretion from untreated and sodium butyrate-treated RINm5MRC cells: Data were obtained from one experiment. The data are from one experiment.

Since medium contained serum in which small amounts of insulin may be present, medium from NIH-3T3 fibroblast cultures with or without NaB treatment were also analysed by RIA to serve as controls. Insulin concentration in media from these cultures was at least two orders of magnitude less than insulin levels secreted by RINm5F-MRC cultures (Table 1). The above data confirmed that RINm5F-MRC cells secreted insulin and that they had responded to NaB. Furthermore, these data supported the findings from immunolabelling studies on frozen sections of RINm5F-MRC cells (see **3.4**). Thus it appeared likely that intracellular insulin content in the RINm5F-MRC cells was low and would therefore require a more sensitive method for the detection of peptides in individual cells.

3.5 Flow cytometry:

The previous experiments had established that RINm5F-MRC cells produced insulin and that cellular insulin content was most likely at or below the lower limit of detection for the Vectastain ABC staining system. Because fluorescence is detected by photomultiplier detectors on the flow cytometer, fluorescent signal from individual cells stained by immunofluorescence, are greatly amplified, thus improving the likelihood of detecting cells containing low peptide concentrations. Furthermore, several thousands of cells are rapidly analysed and the number of positively stained cells can be accurately quantified. As protocols for immunolabelling cells to detect insulin, glucagon or somatostatin by flow cytometry have not been reported previously, a protocol for detecting these peptides in RINm5F-MRC cells was established in the present study as follows:

a) Optimising fixation and permeabilisation of RINm5F-MRC cells (see **3.5.1**)

b) Optimising the concentrations of the antisera (see **3.5.2.1- 3.5.3**) and SA-FITC

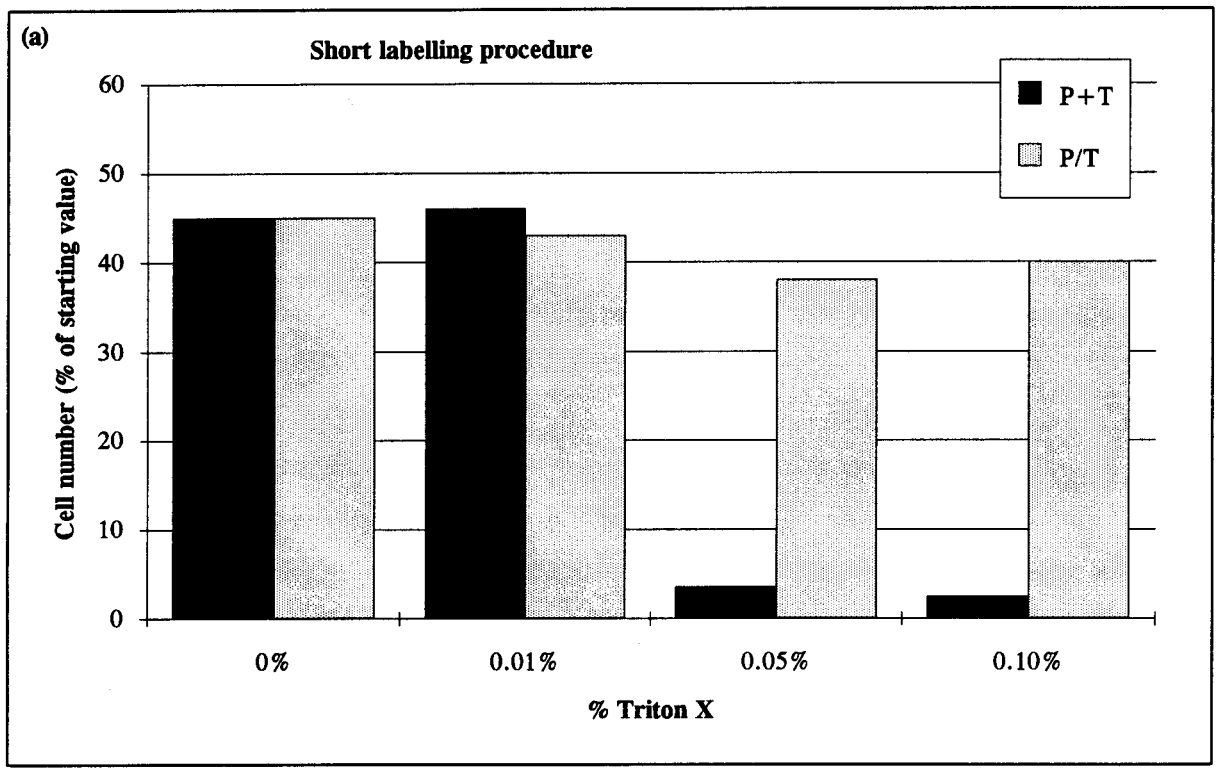
c) establishing specificity of immunoreactivity (see **3.5.2.2**)

3.5.1 Optimisation of fixation and permeabilisation:

A pilot experiment in which RINm5F-MRC cells that had been simultaneously fixed and permeabilised in 2% paraformaldehyde (PAF) and 0.1% Triton X 100 (TX) in PBS, revealed that excessive amounts of cells were lost during the labelling procedure (data not shown). The loss of cells was thought to caused by increased fragility resulting from extraction of membrane components before they could be cross-linked by the fixative. Thus it was necessary to establish a suitable procedure to fix and permeabilise cells whilst minimizing cell loss during the labelling procedure.

Cell suspensions were fixed and permeabilised (see **2.6.2**) and subjected to the same protocol used for immunolabelling (see **2.6.3.2**) except that cells were incubated in antiserum diluent buffers instead of antiserum (see **2.6.2**). Cell numbers declined with increasing concentrations of TX in all preparations (Fig 19) and were further reduced in preparations that had been simultaneously fixed and permeabilised (Fig 19 (P+T)). After overnight incubation in primary antibody diluent, loss of cells was greater in samples simultaneously fixed and permeabilised with 0.05 or 0.1% TX (Fig 19b) compared to those similarly treated and incubated for 75 minutes (Fig 19a). Because cells were centrifuged after each buffer rinse, the maximum yield obtained from samples not treated with TX (controls) ranged from 44 to 56% of the cell number at the start of the experiment.

The similar yield of cells from samples sequentially fixed and permeabilised (P/T) with 0.01% TX to that from untreated samples (0% TX), indicated that cells may not be sufficiently permeabilised to allow adequate penetration of immunoreagents. Furthermore, to maximise binding of the primary



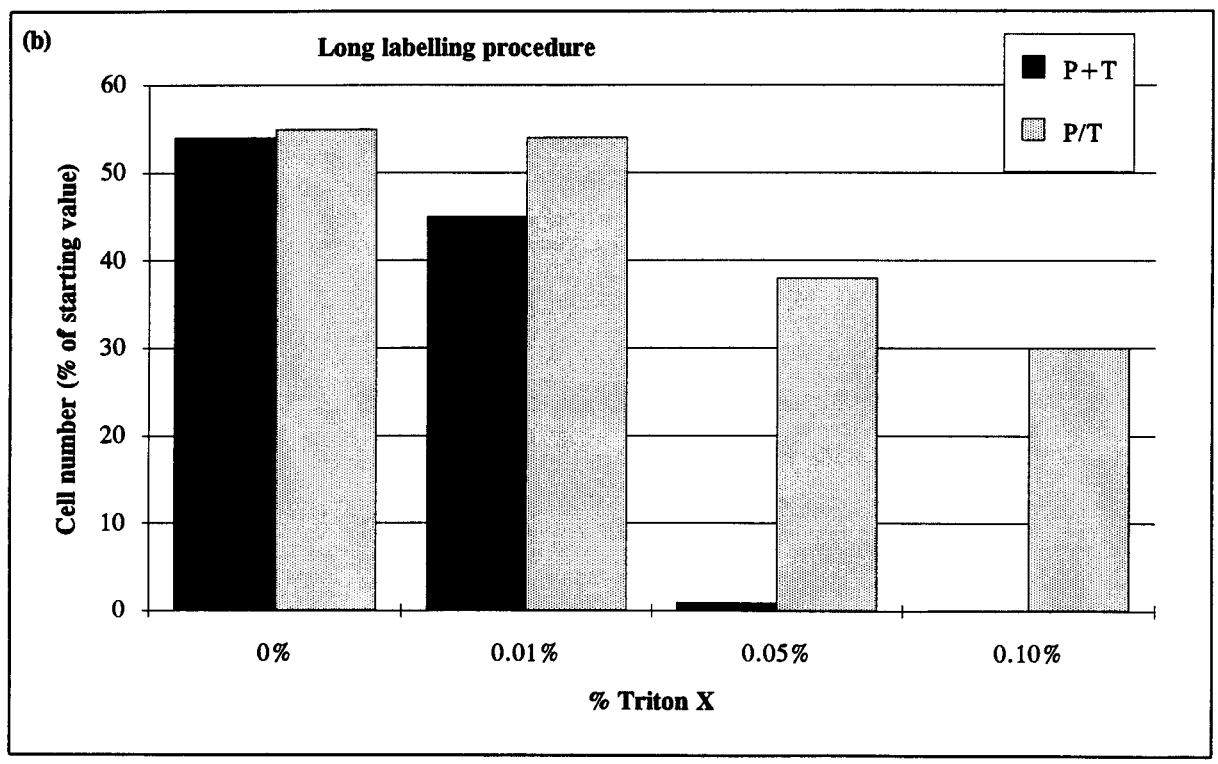


Fig 19. The effect of increasing concentrations of Triton X 100 (Triton X) on the yield of cells that were either simultaneously (P+T) or sequentially (P/T) fixed and permeabilised. Fixed control preparations were not treated with Triton X 100.

a) The percentage yield of cells subjected to a short labelling protocol (75 minutes in antibody diluent buffer). n=1 (Data are from one experiment)

b) The percentage yield of cells subjected to a long labelling protocol (overnight in antibody diluent buffer). n=1 (Data are from one experiment)

antibody during labelling, cells were to be stored overnight in primary antisera in all further immunolabelling experiments for flow cytometry. Because the yield of cells from samples sequentially fixed and permeabilised in 0.05% TX (Fig 19b (P/T)) was greater than in those permeabilised with 0.1% TX (Fig 19b (P/T)), sequential fixation and permeabilisation with 0.05% TX was used for further flow cytometry experiments in this study.

3.5.2 Optimisation of the concentrations of biotinylated goat anti-rabbit IgG (b-GAR-IgG) and strepavidin FITC (SA-FITC) on cultured RINm5F cells:

Because fluorescent signals are electronically amplified by the flow cytometer, dilutions of antisera used to detect immunolabelled cells by microscopy may not be equally suitable for labelled samples analysed by flow cytometry. Therefore analysis by flow cytometry was done on samples exposed to serial dilutions of the primary antisera, b-GAR-IgG, and SA-FITC in order to obtain the dilutions of antisera and SA-FITC that displayed the least non-specific fluorescence.

3.5.2.2 Titration of b-GAR-IgG and SA-FITC to reduce background fluorescence:

In a pilot study in which RINm5F-MRC cells were immunolabelled (see **2.6.3**) and analysed by flow cytometry (see **2.6.1**), fluorescence of samples incubated with antibody diluent buffer (method control), b-GAR-IgG and SA-FITC was markedly increased above that of unstained samples (background - i.e. samples not exposed to primary antiserum, b-GAR-IgG or SA-FITC) (Fig 20). In order to determine whether the b-GAR-IgG or SA-FITC alone could cause an increase in the intensity of fluorescence, fluorescence of cells treated with three concentrations of these reagents were compared (see **2.6.4**) to that of unstained samples. As seen in Fig 21, fluorescence of samples exposed to b-GAR-IgG and SA-FITC was markedly raised above background. Variation of the concentrations of b-GAR-IgG made little difference to the intensity of fluorescence (data not shown) whereas the intensity was markedly reduced with decreasing concentrations of SA-FITC. A concentration of 5μg/ml SA-FITC was closest to background fluorescence and appeared to be the most suitable concentration for use in further experiments.

3.5.2.3 Titration of SA-FITC:

Since primary antisera were excluded from the previous step, it was necessary to determine whether 5μg/ml SA-FITC was suitable when used to stain cells immunolabelled with anti-insulin or anti-glucagon. Cells were immunolabelled with a 1/50 dilution of anti-insulin or anti-glucagon, 6.25μg/ml b-GAR-IgG and stained with concentrations of SA-FITC ranging from 2 to 20μg/ml (see **2.6.5**).

Fluorescence declined with decreasing concentrations of SA-FITC in all preparations (Fig 22a).

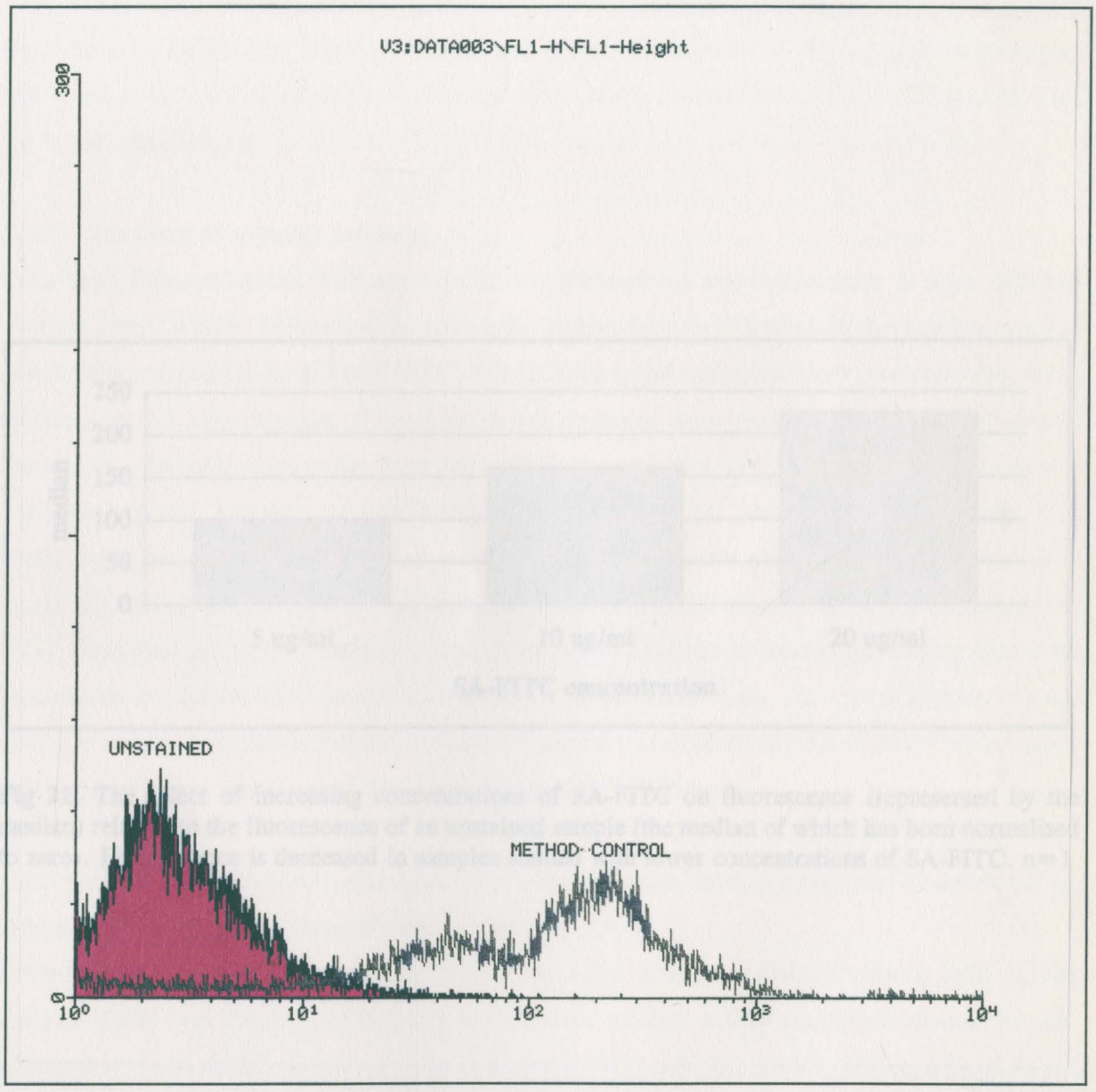


Fig 20. Histograms of fluorescence from unstained samples and preparations in which the primary antiserum was excluded from the protocol (method control). Fluorescence of the method control is markedly raised above that of the unstained sample. n = 1

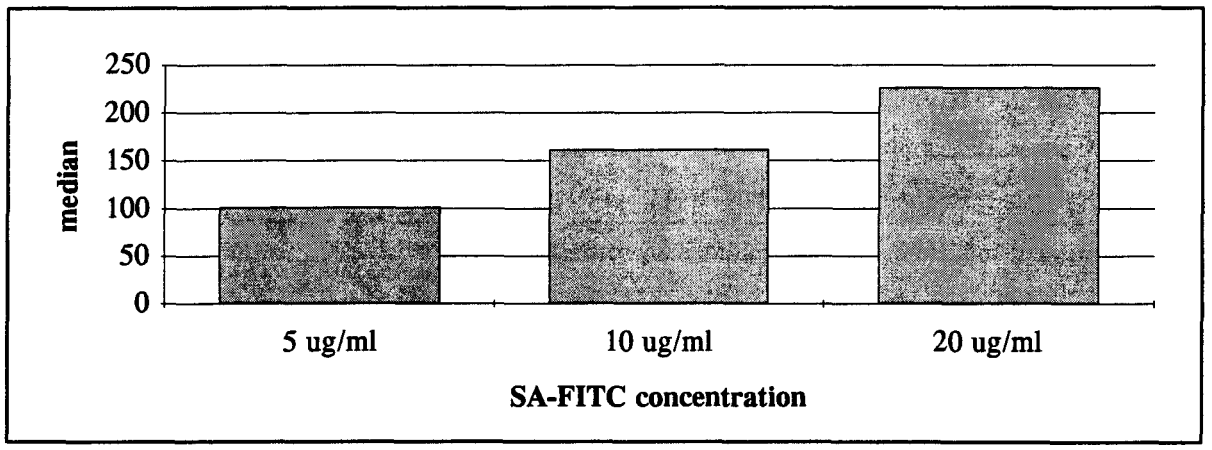


Fig 21. The effect of increasing concentrations of SA-FITC on fluorescence (represented by the median) relative to the fluorescence of an unstained sample (the median of which has been normalised to zero). Fluorescence is decreased in samples stained with lower concentrations of SA-FITC. n=1

Furthermore, there was a marked increase in fluorescence (represented by the median) of preparations immunolabelled with anti-insulin or anti-glucagon serum above that of samples exposed to antibody diluent buffer (method control) (Fig 22a) . These differences were only detectable in samples stained with and 20, 10 and 5μg/ml SA-FITC (Fig 22 b and c). In contrast, no difference in fluorescence was detected in preparations stained with 2μg/ml SA-FITC. Therefore, a concentration of 5μg/ml SA-FITC appeared to be the limiting concentration that was sufficiently sensitive to detect insulin or glucagon-like immunoreactivity whilst displaying the least non-specific fluorescence and was subsequently used for further experiments.

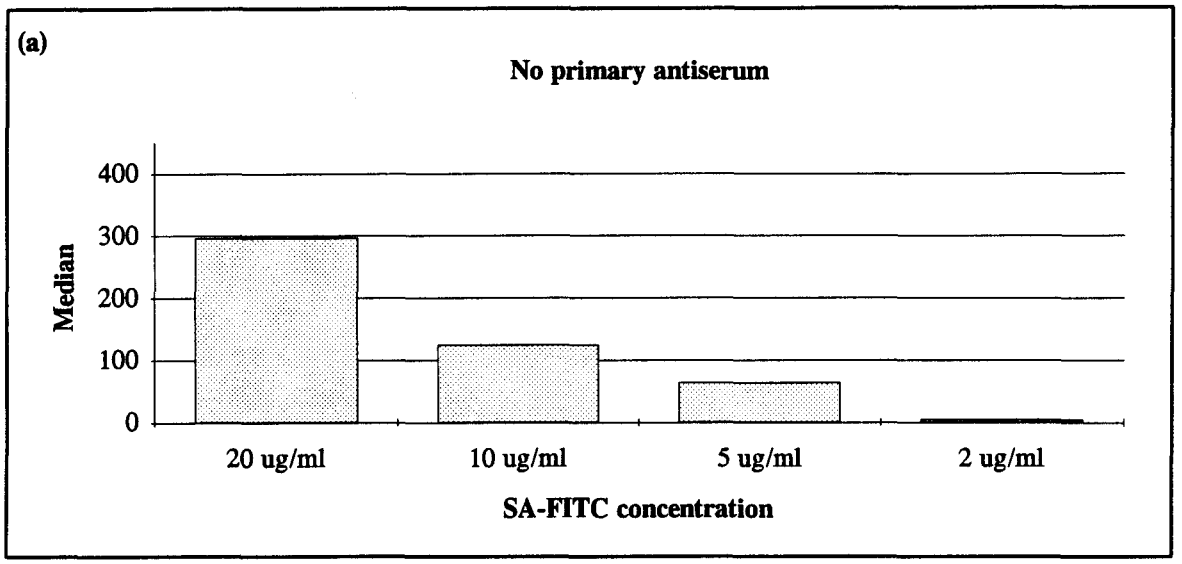
3.5.2.4 Titration of primary antisera:

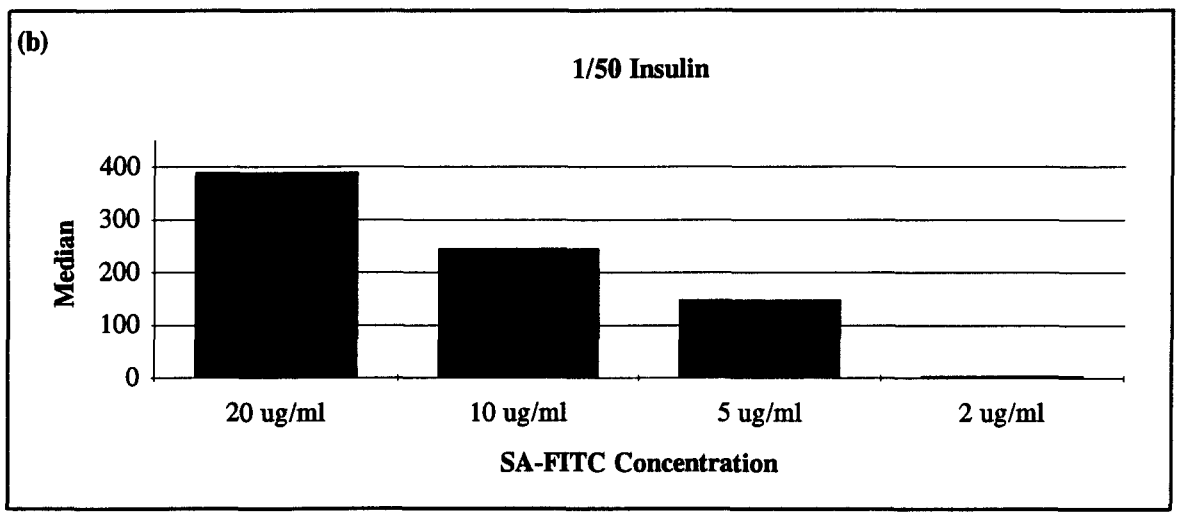
Cells were immunolabelled with anti-insulin, anti-glucagon or anti-somatostatin at three different dilutions (see **2.6.6.2**). This was to determine the highest dilution of antiserum that could be used to detect immunoreactivity in RINm5F-MRC cells cultured under non-stimulatory conditions (untreated cultures)(see **2.2.3.4**). Intensity of fluorescence was increased above background (i.e. method control samples in which the median has been normalised to zero) (Fig 23). Fluorescence of all samples labelled to reveal glucagon or somatostatin (Fig 23 b and c), was substantially greater than background. In contrast, the fluorescence intensity of samples labelled with anti-insulin was only marginally above background in samples labelled with 1/250 or 1/500 dilutions of the antiserum (Fig 23 a). Thus these dilutions of antisera were assumed to be too dilute to saturate all binding sites if the intracellular concentration of insulin had increased in stimulated cells. As a 1/50 dilution of anti-glucagon or anti-somatostatin resulted in fluorescence that was substantially above that of background, a 1/250 dilution was chosen for further immunolabelling experiments.

3.5.3 Comparison of insulin , glucagon or somatostatin-like immunoreactivity of untreated or sodium butyrate-treated RINm5F-MRC cultures:

RINm5F cells were cultured with (NaB-treated cultures) or without (untreated cultures) 2mM sodium butyrate (NaB) (see **2.6.6.1** and **2.6.6.2**) to determine whether culture conditions affected insulin, glucagon or somatostatin immunoreactivity in RINm5F-MRC cells. In addition, this experiment served to establish the optimal dilution of each antiserum for use in subsequent experiments of immunolabelled RINm5F-MRC cultures.

Intensity of fluorescence was increased above that of background (method control: the median of which has been normalised to zero) in all cultures and was notably raised in NaB-treated preparations (Fig 24). Samples labelled with a 1/50 dilution of each antiserum demonstrated the greatest difference in fluorescence between untreated and NaB-treated cultures. Fluorescence of untreated cultures labelled with a 1/50 dilution of anti-glucagon or anti-somatostatin was substantially higher than that of





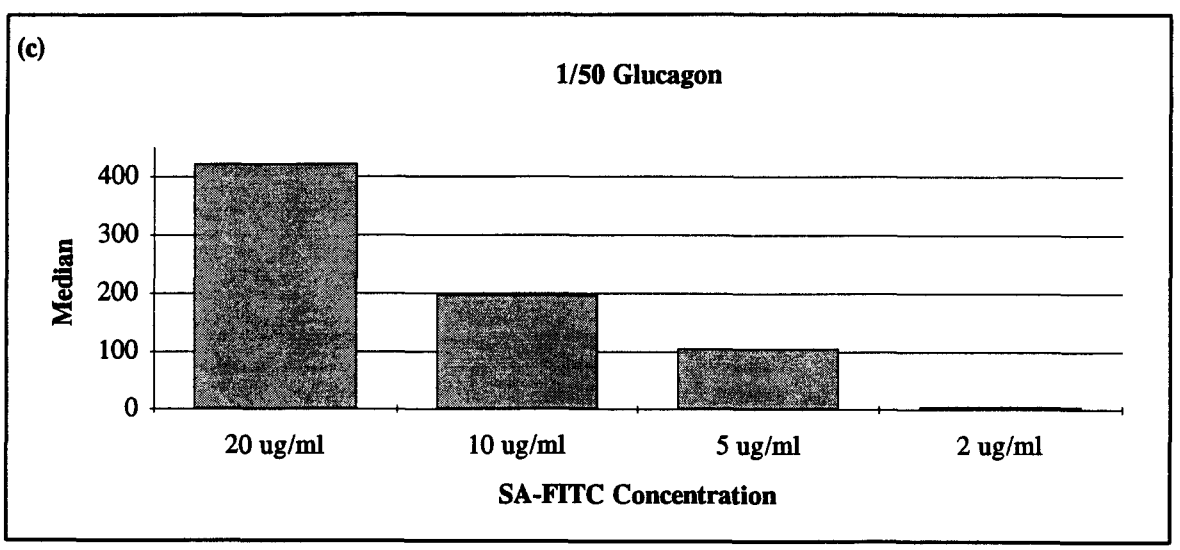
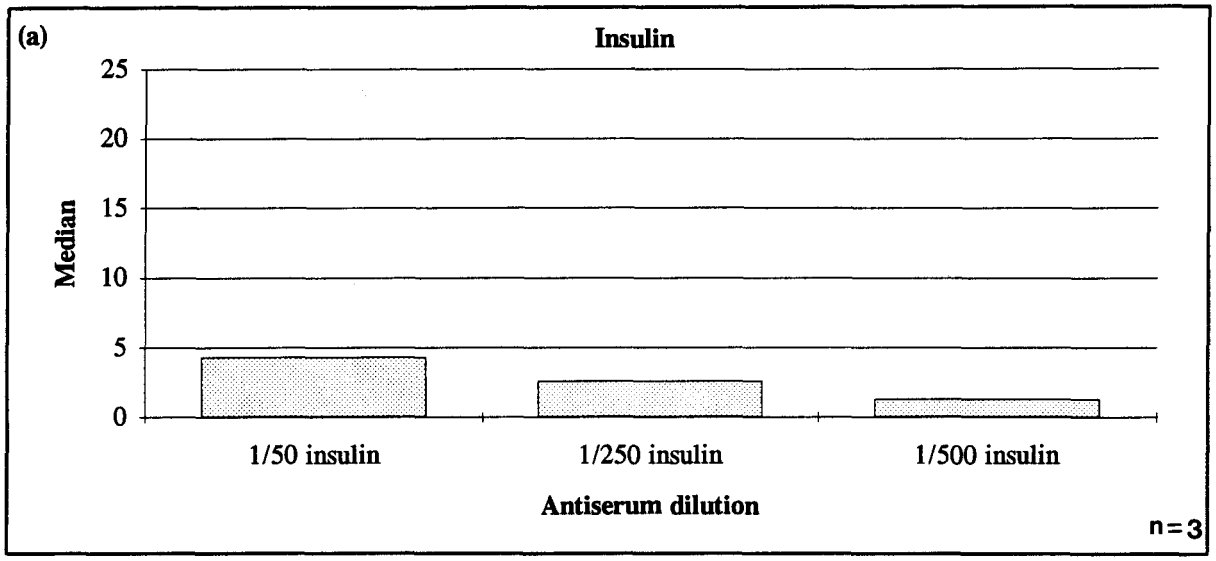
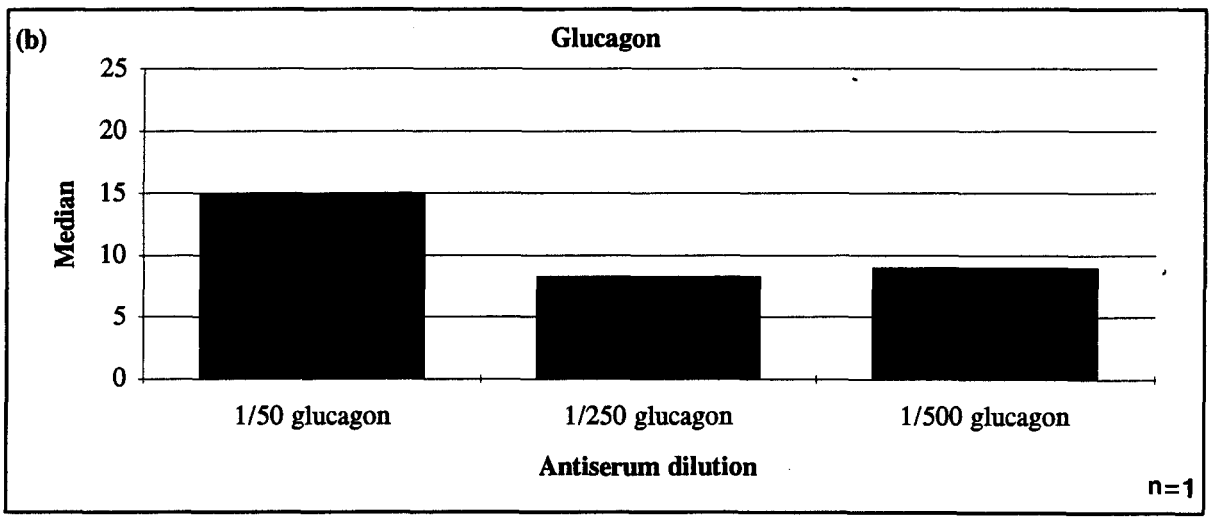


Fig 22. The effect of decreasing concentrations of SA-FITC on fluorescence in samples exposed to (a) antibody diluent buffer (method control), (b) anti-insulin or (c) anti-glucagon. Fluorescence is relative to that of unstained samples the median of which has been normalised to zero. n=1





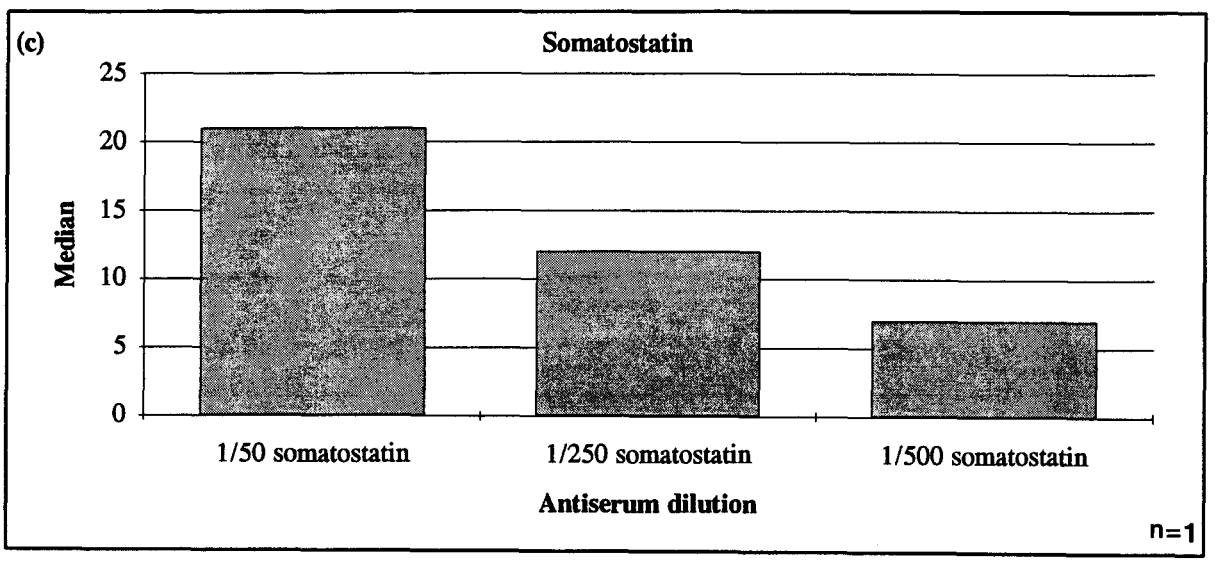
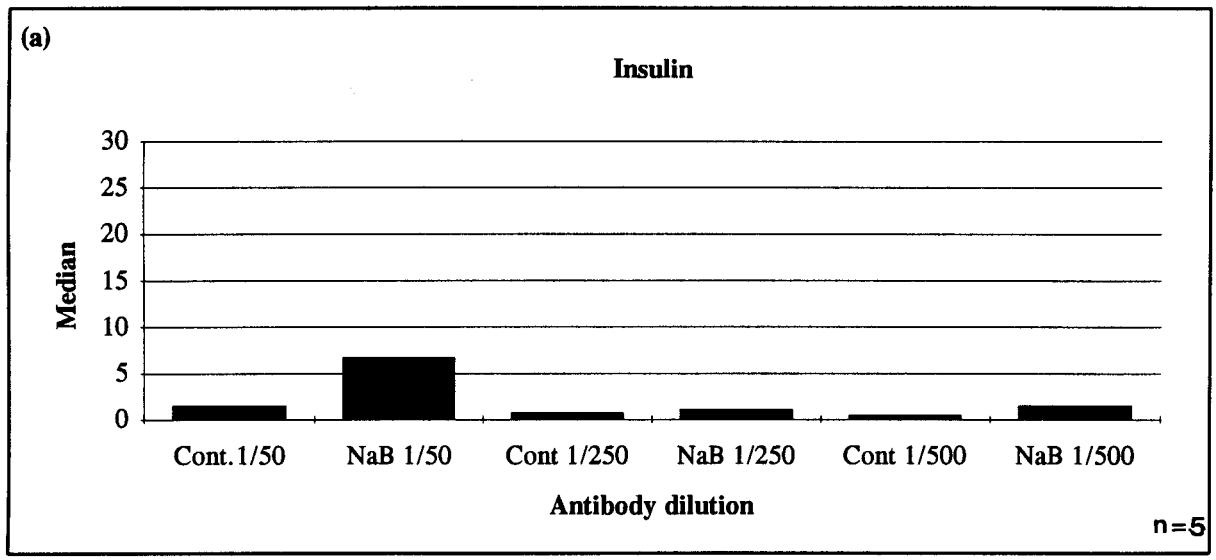
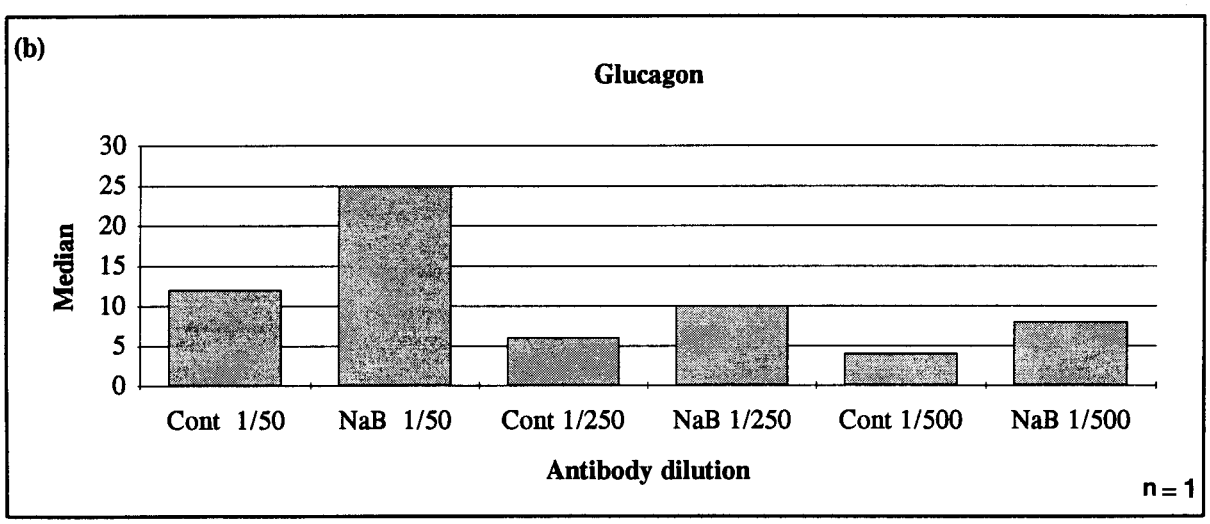


Fig 23. The effect of increasing dilutions of primary antisera on the fluorescence of samples immunolabelled to reveal (a) insulin, (b) glucagon or (c) somatostatin. The fluorescence (median) of insulin-labelled samples is markedly less than that from preparations exposed to anti-glucagon or anti-somatostatin serum. Fluorescence (median) of the method control has been normalised to zero.





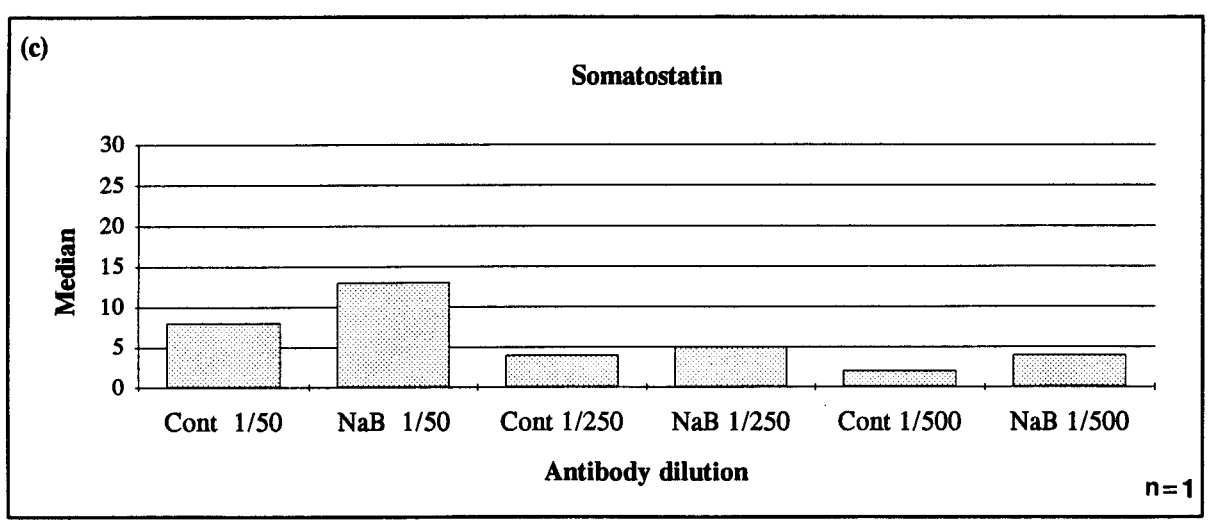


Fig 24. The effect of sodium butyrate on (a) insulin, (b) glucagon and (c) somatostatin immunoreactivity and the relative differences in fluorescence intensity between untreated (Cont) and sodium butyrate-treated (NaB) cultures with decreasing dilutions of antiserum. The median of the method control has been normalised to zero.

untreated cultures labelled with a 1/50 dilution of anti-insulin (Fig 24). Furthermore, the number of cells displaying fluorescence intensities greater than background was notably higher in samples exposed to anti-glucagon or anti-somatostatin serum (Fig 25). Because the raised fluorescence in these samples is likely to be due to non-specific immunoreactivity, a 1/250 dilution of the glucagon or the somatostatin antiserum was used for the following experiment in which the specificity of immunoreactivity was tested.

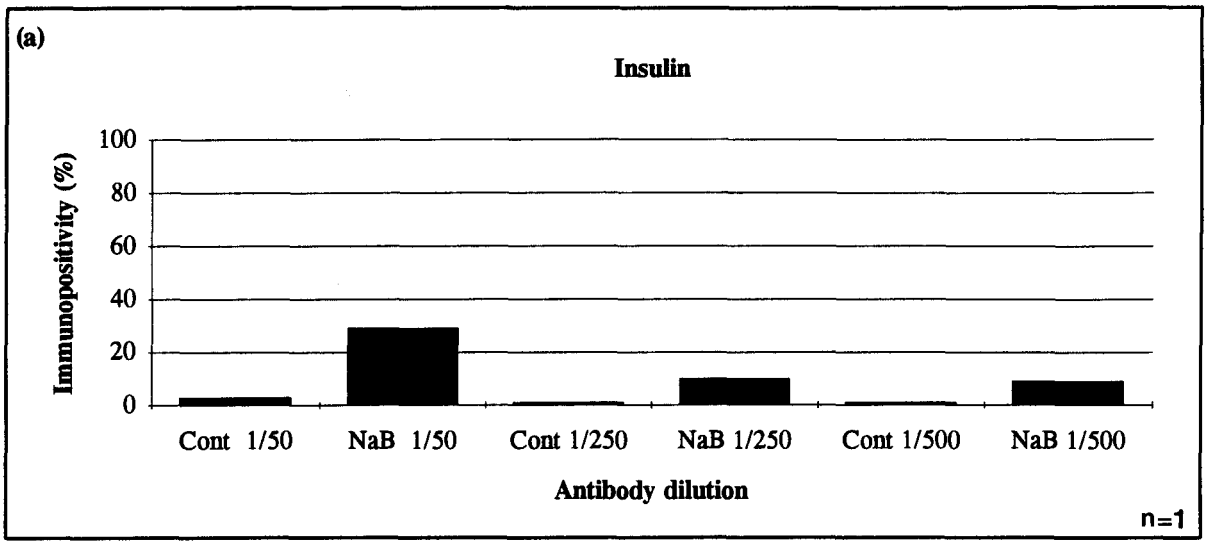
3.5.4 Determination of specificity of antiserum:

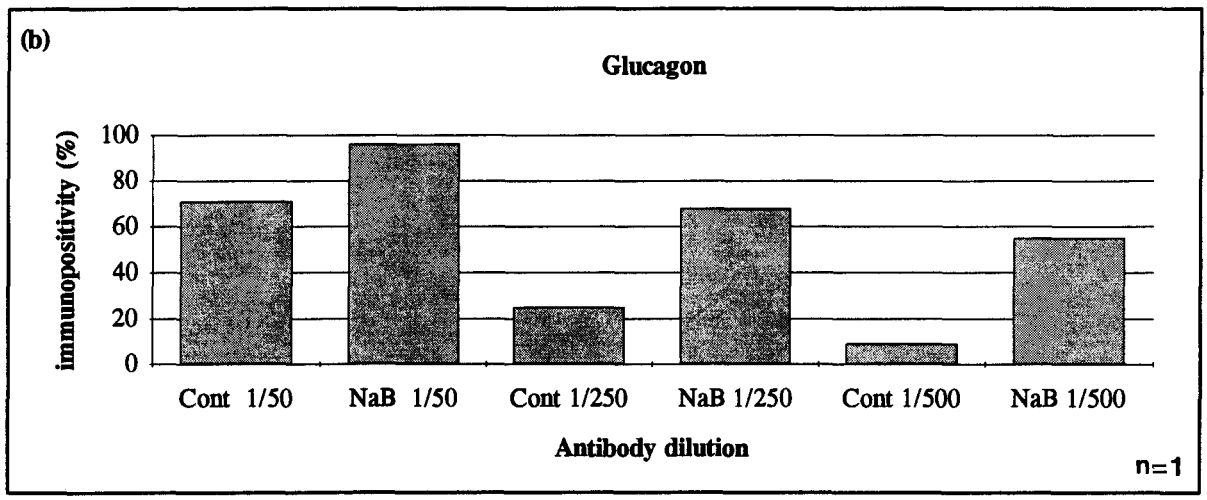
To verify the specificity of insulin, glucagon and somatostatin immunoreactivity seen in the previous experiments, RINm5F-MRC cells of untreated and NaB-treated cultures were immunolabelled with insulin, glucagon or somatostatin antisera (at dilutions established above), non-immune sera, or antisera pre-absorbed with their respective antigens (see **2.6.7**).

The intensity of fluorescence of samples from untreated cultures exposed to anti-insulin serum, to non-immune serum or to pre-absorbed anti-insulin serum was identical to that from samples exposed to antibody diluent buffer (background)(Fig 26). Thus insulin specificity could not be established in untreated RINm5F-MRC cultures. On the other hand, insulin immunoreactivity was abolished in NaB-treated samples exposed to pre-absorbed anti-insulin serum, or non-immune guinea-pig serum:hence insulin specificity was established in NaB-treated cultures. Although the median was similar in non-immune serum controls and absorption controls from both culture conditions (Fig 29), a small number of immunopositive cells were detected in the absorption controls from NaB-treated cultures (Fig 30).

In contrast, a substantial shift of the fluorescent peaks above background occurred in the non-immune serum controls, absorption controls and samples labelled to reveal glucagon or somatostatin from both culture conditions (Figs 27 and 28). An interesting observation was the increase in the number of positively-stained cells in non-immune and absorption controls above that in preparations labelled to reveal glucagon or somatostatin (Fig 30). Furthermore, the fluorescence of all preparations from NaB-treated cultures exposed to anti-glucagon or anti-somatostatin serum was markedly raised above that of untreated cultures (Fig 29).

The presence of insulin in the RINm5F-MRC cells was unequivocally established by flow cytometry analysis. Moreover, this confirmed that insulin could only be detected in cells grown in the presence of sodium butyrate. Specificity of insulin immunoreactivity was confirmed by the reduction of fluorescence to background levels in the absorption controls and no cross-reactivity of non-immune guinea-pig serum with RINm5F-MRC cells was apparent.





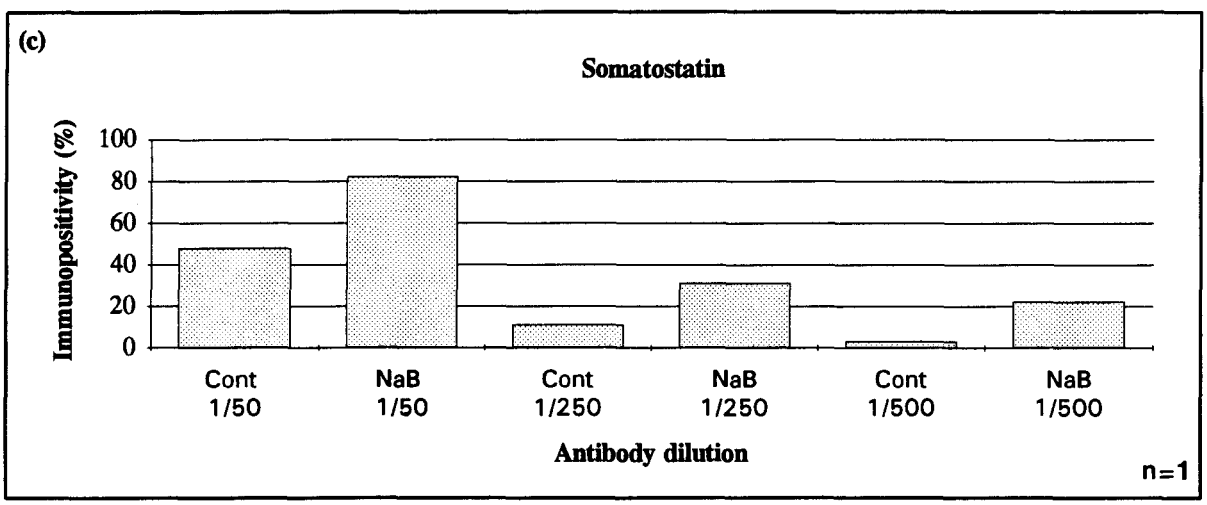


Fig 25. The effect of sodium butyrate on (a) insulin, (b) glucagon and (c) somatostatin immunoreactivity and decreasing concentrations of primary antiserum on the relative differences in the number of immunopositive cells (expressed as a percentage of the total number analysed) between untreated (Cont) and NaB-treated (NaB) cultures. The number of immunopositive cells was determined from cells displaying fluorescence intensities greater than background.

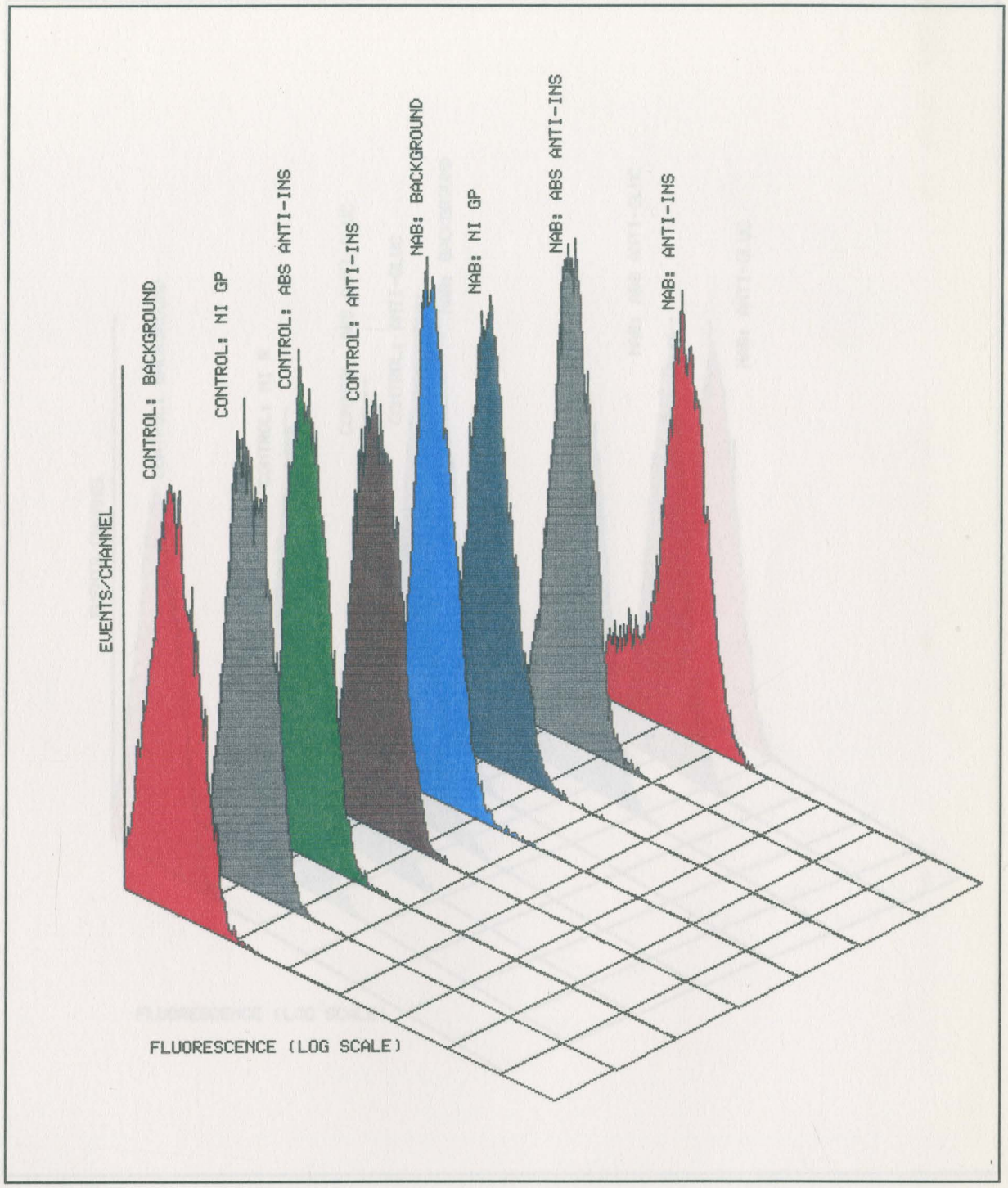


Fig 26. A histogram of fluorescence intensities of individual samples immunolabelled to determine insulin specificity in untreated (CONTR) or sodium butyrate-treated (NaB) cultures. Samples were exposed to antiserum diluent buffer (background), non-immune guinea-pig serum (NI GP), pre-absorbed anti-insulin serum (ABS ANTI-INS) or insulin antiserum (ANTI-INS). n=1

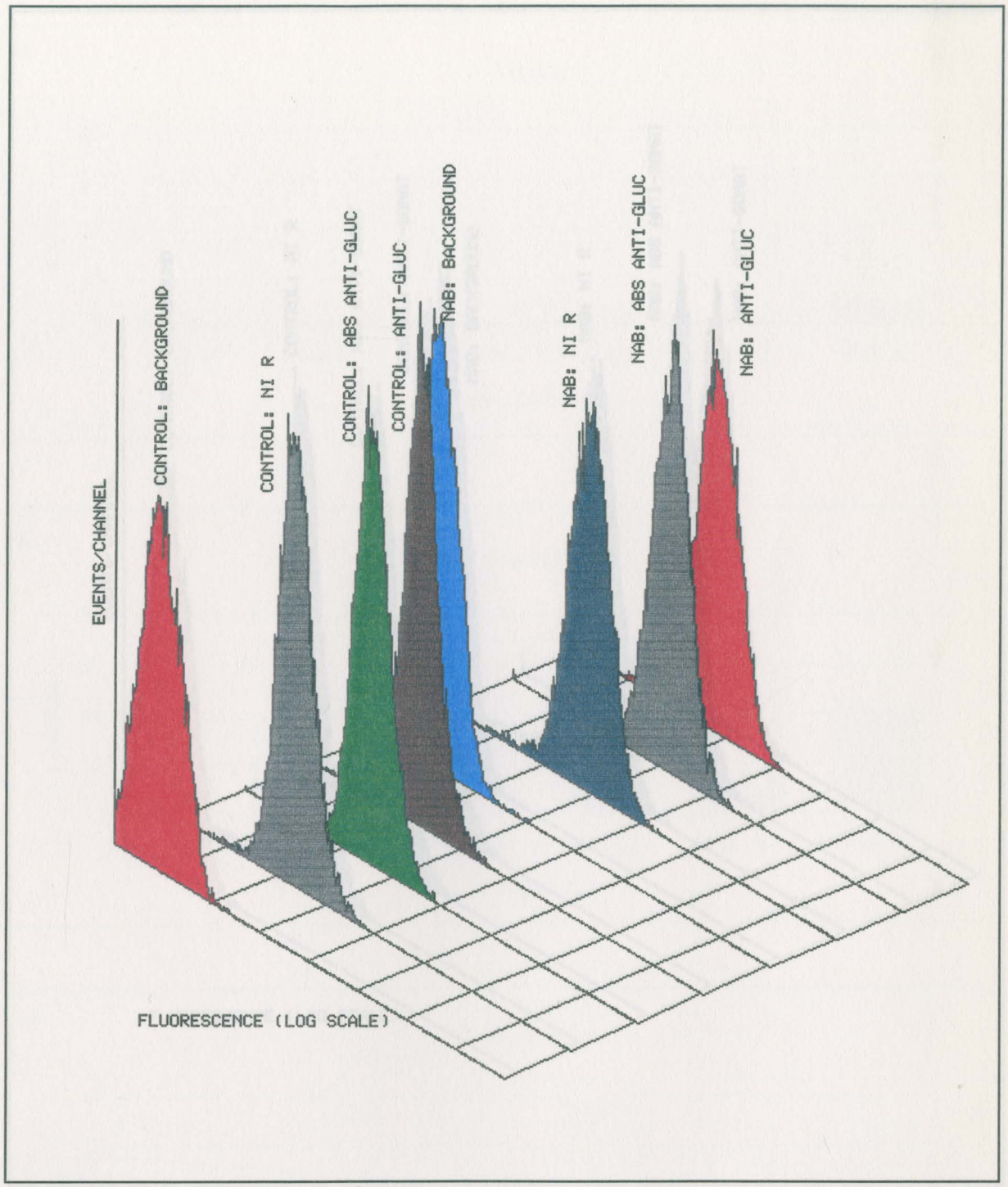


Fig 27. A histogram of fluorescence intensities of samples immunolabelled to determine glucagon specificity in untreated (CONTR) or sodium butyrate-treated (NaB) cultures. Samples were exposed to antiserum diluent buffer (background), non-immune rabbit serum (NI R), pre-absorbed anti-glucagon serum (ABS ANTI-GLUC) or glucagon antiserum (ANTI-GLUC). The marked increase in the intensity of fluorescence in non-immune or absorption controls suggests that glucagon immunoreactivity is non-specific. n=1

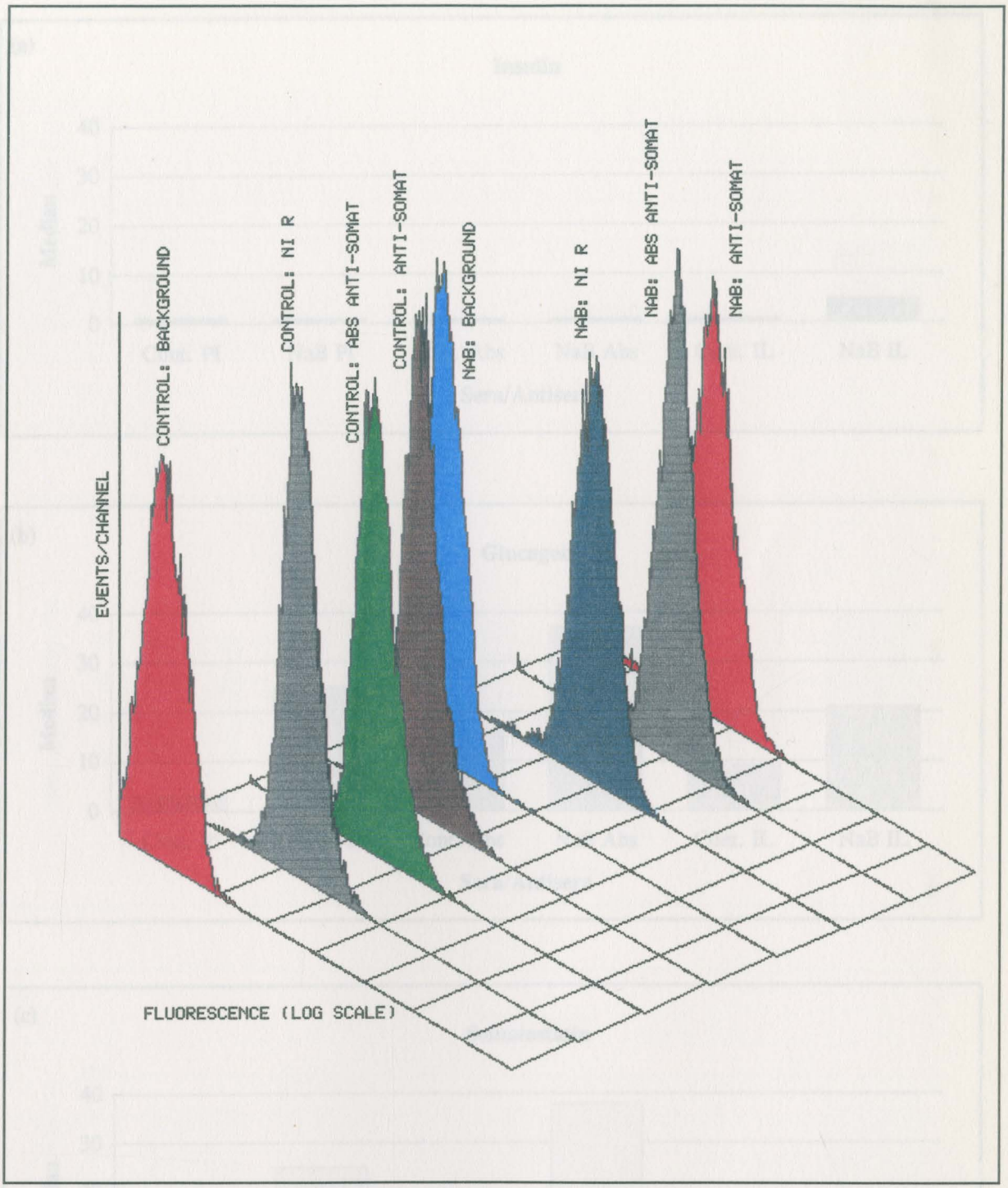
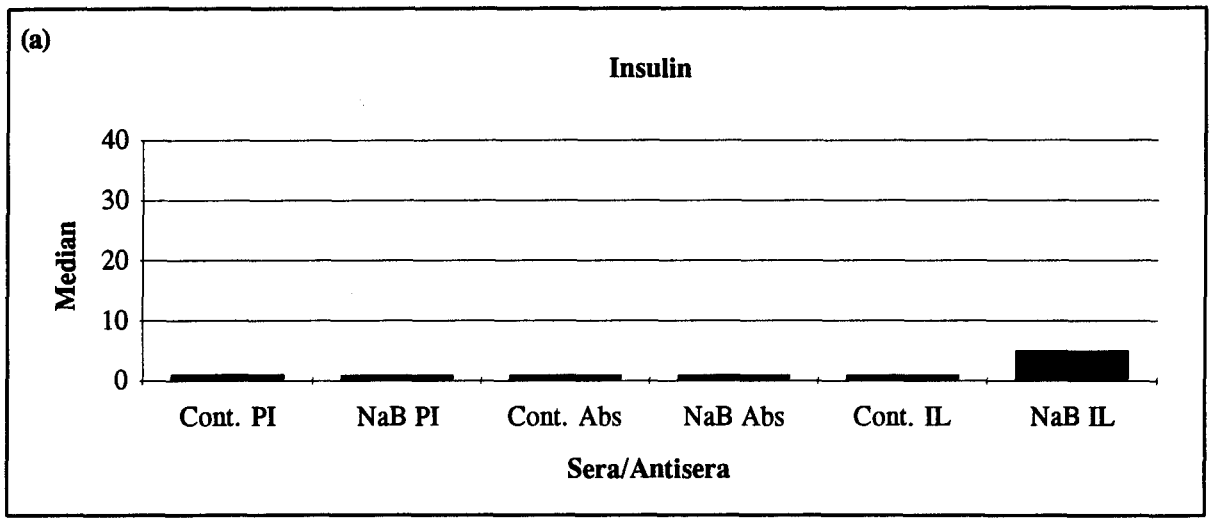
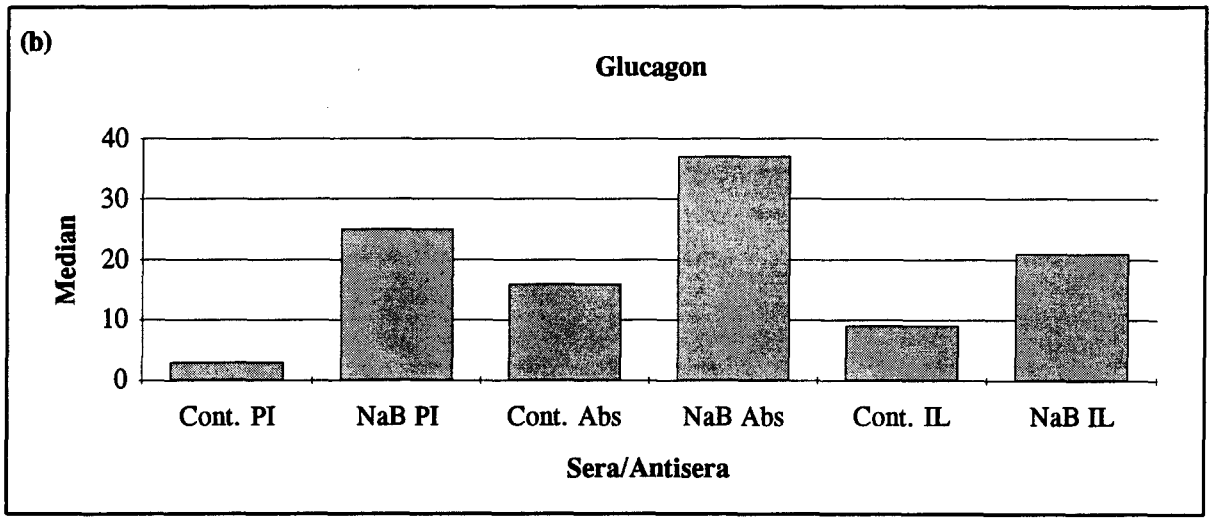


Fig 28. A histogram of fluorescence intensities of samples immunolabelled to determine somatostatin specificity in untreated (CONTR) or sodium butyrate-treated (NaB) cultures. Samples were exposed to antiserum diluent buffer (background), non-immune rabbit serum (NI R), pre-absorbed anti-somatostatin serum (ABS ANTI-SOMAT) or somatostatin antiserum (ANTI-SOMAT). The marked increase in intensity of fluorescence in non-immune and absorption controls shows that somatostatin immunoreactivity is non-specific. n=1





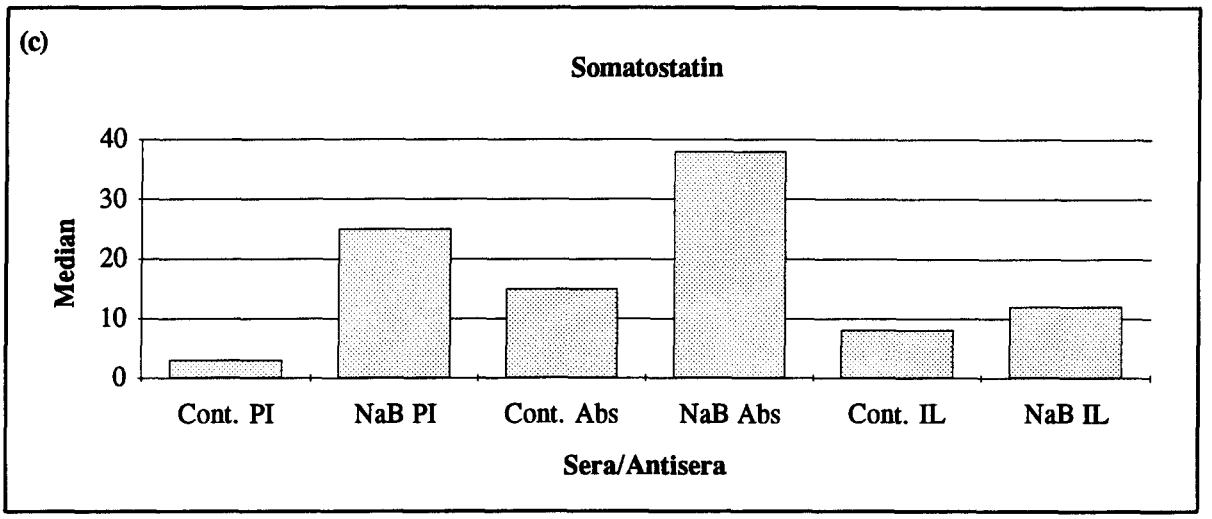


Fig 29. The effect of non-immune sera (PI), pre-absorbed antisera (Abs) or (a) insulin (IL), (b) glucagon (IL), or (c) somatostatin (IL) antiserum on the intensity of fluorescence of samples from untreated (Cont) or sodium butyrate-treated (NaB) cultures. Fluorescence of all preparations exposed to rabbit serum is markedly raised above that of cells exposed to guinea-pig serum. (n=1)

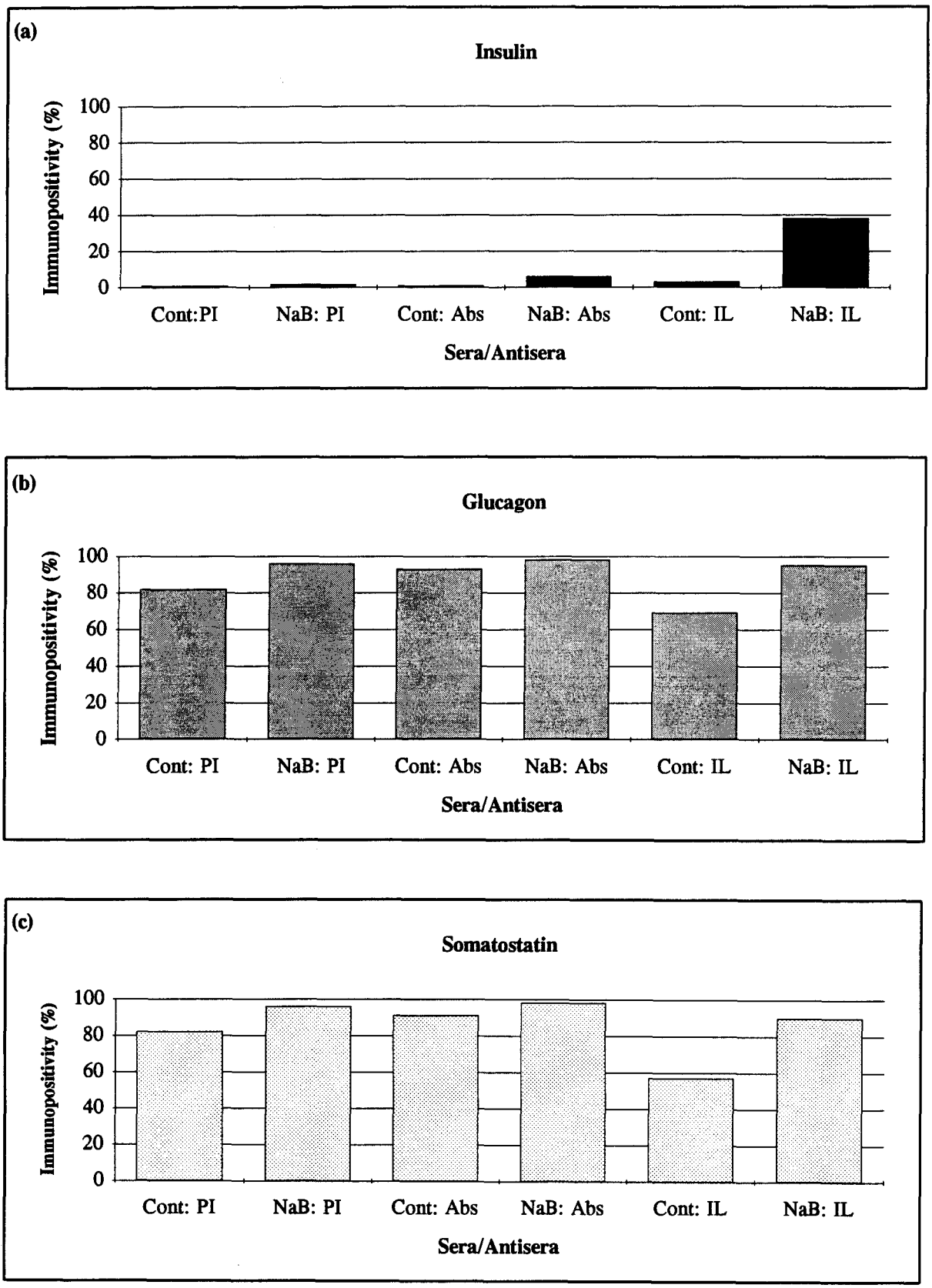


Fig 30. The number of positively stained cells (expressed as a percentage of the total number analysed) in non-immune controls (PI), absorption controls (Abs) and cells labelled to reveal (a) insulin (IL), (b) glucagon (IL) and (c) somatostatin (IL) from untreated (Cont) or sodium-butyrate-treated cultures (NaB). Most of the cells in preparations labelled with anti-glucagon or anti-somatostatin are "positively" labelled. n=1

In contrast, the presence of glucagon and somatostatin in RINm5F-MRC cells could not be established by flow cytometry. Furthermore, immunoreactivity appeared to be non-specific and was predominantly caused by cross-reactivity to rabbit serum. Probable causes of the differences in fluorescence between untreated and NaB-treated cultures are dealt with in Chapter 4 (**Discussion**).

3.6 Comparison of the strepavidin FITC (SA-FITC) and Vectastain ABC (ABC-HRP) immunostaining systems to detect insulin, glucagon or somatostatin immunoreactivity in untreated and sodium butyrate-treated RINm5F cultures:

Because cells containing insulin were detected by flow cytometry in cultures treated with sodium butyrate (NaB) (see **3.5.3**), immunofluorescence was performed on whole-mounts to determine whether cells containing insulin from similarly treated cultures could be detected by fluorescence microscopy. Furthermore, although non-specific glucagon and somatostatin immunoreactivity had been further substantiated by flow cytometry, whole-mounts were stained by immunofluorescence to determine whether this non-specific immunoreactivity was associated with the cell membrane as suggested by results of earlier studies (see **3.3.2**).

In a preliminary investigation (performed as in section **2.4.4.** and **2.4.4.1**)), a small proportion of RINm5F-MRC cells in whole-mounts of NaB-treated cultures were positively stained for insulin (Fig 31). Although staining was observed in whole-mounts incubated with different dilutions of anti-insulin serum, the intensity of fluorescence remained low and was not substantially affected by the dilution of anti-insulin. Therefore, further immunofluorescence was performed using the same dilution of insulin antiserum (1:50) that had successfully demonstrated insulin immunoreactivity on the flow cytometer. Staining intensity was so low that positively stained cells could only be identified when viewed at high magnification using a 63 x oil immersion objective lens of high numerical aperture. In the present study, individual positively stained cells could not be detected at all using dry objective lenses. Furthermore, due to the low overall signal intensity, acceptable images of fluorescence-stained preparations were recorded using 1600 ASA rated film. In contrast, intensely stained cells were observed using dry objective lenses (at low magnification), in islet cells of frozen sections of pancreas, immunolabelled in parallel with the cultures (Fig 32). The marked difference in staining intensity of whole-mounts and sectioned pancreas suggested that RINm5F-MRC cells contained extremely low levels of insulin.

In earlier experiments in this study, insulin was not detected in untreated cultures of RINm5F-MRC cells stained with ABC-HRP (see **2.4.3** and **3.3.2**), but was subsequently detected by flow cytometry (see **3.7.3**) and whole-mount immunofluorescence (see above) in NaB-treated cultures. Therefore whole-mounts of untreated and NaB-treated cultures were immunolabelled and stained with either ABC-HRP (see **2.4.5.1**) or with SA-FITC (see **2.4.5.2**) to determine whether insulin detection resulted from the improved sensitivity of SA-FITC above that of the ABC-HRP staining system or from a change in culture conditions. Since specificity of insulin immunoreactivity and non-specific staining of glucagon and somatostatin antisera had been established by flow cytometry (see **3.5.4**), controls to

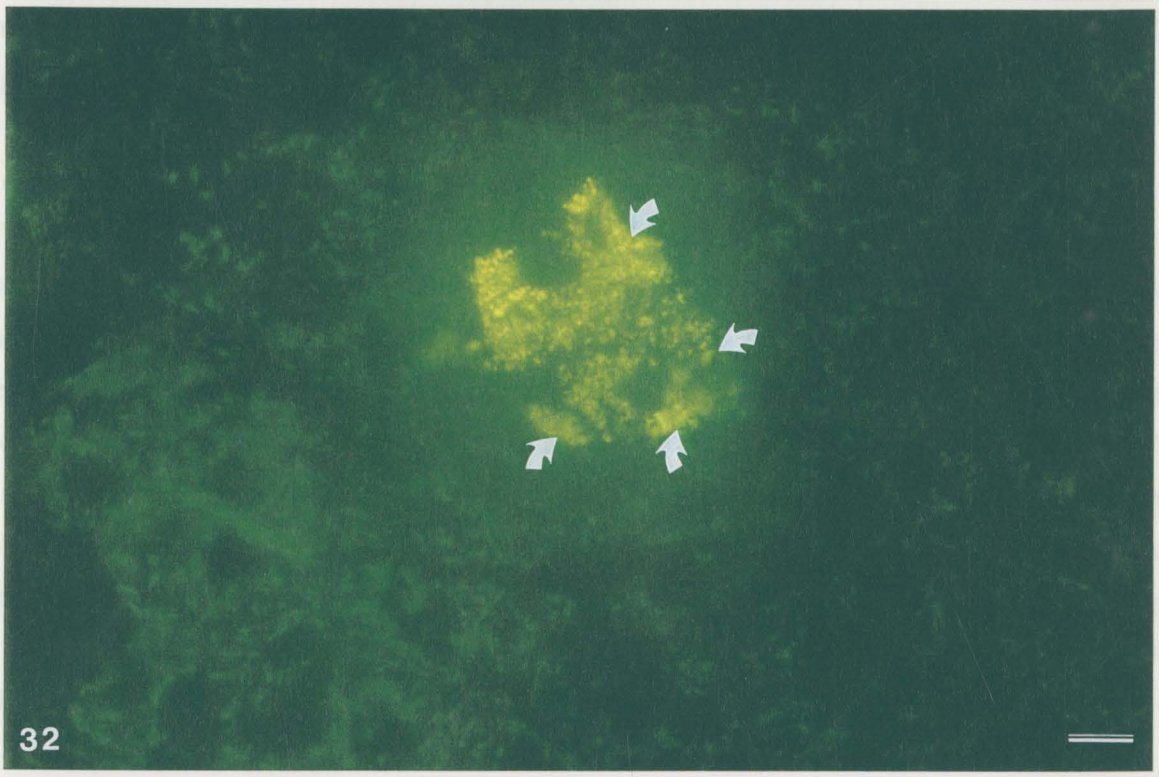


Fig 31. Fluorescent-stained culture exposed to NaB: the whole-mount was incubated in a 1/50 dilution of insulin antiserum. An optical section shows even distribution of punctate fluorescent signals of immunoreactive insulin throughout the cytoplasm of the reactive cell. Bar = 10μm.

Fig 32. Fluorescent-stained frozen section of rat pancreas demonstrating a cross section of a small islet containing four strongly stained cells containing insulin (arrows). The dilution of anti-insulin and the photographic exposure were the same as that used in Fig 31. The yellow/green signal is caused by overexposure from the high signal intensity. Note non-specific staining of granules in exocrine cells. Bar = 10μm.

check specificity of staining were included in this experiment.

3.6.1 Insulin immunoreactivity:

Neither whole-mounts from untreated cultures stained with SA-FITC nor with ABC-HRP demonstrated cells containing insulin (Figs 33b and 35a). In contrast, positively stained cells were observed in NaB-treated cultures stained with ABC-HRP (Fig 33a) and in those stained with SA-FITC (Fig 35a). Furthermore, staining was confined to a small proportion of cells from these cultures, and positively stained cells were often grouped together in small clusters. Staining intensity of insulin immunoreactivity in NaB-treated cultures stained with ABC-HRP was raised when compared to untreated cultures (compare Fig 33a and b). However, staining of similar intensity was also noted in whole-mounts of NaB-treated cultures labelled with the primary antiserum diluent (method control - see **2.4.3.1**) (Fig 33c). Nevertheless, cells marginally more intensely stained than background were visible in NaB-treated cultures incubated with anti-insulin serum (Fig 33a). On the other hand, only diffuse background fluorescence was seen in method control and unstained preparations from untreated and NaB-treated cultures stained with SA-FITC (Fig 36). Furthermore, because SA-FITC signals are characteristically punctate and emit bright green fluorescence, even cells that contained few SA-FITC signals were detectable against the diffuse background fluorescence in immunonegative cells.

3.6.2 Glucagon and somatostatin immunoreactivity:

Whole-mounts from NaB-treated cultures, labelled with anti-glucagon or anti-somatostatin serum and stained with ABC-HRP, demonstrated a marked increase in staining intensity above that of untreated cultures in whole-mounts stained with ABC-HRP (Fig 34). In NaB-treated cultures, small cells not demonstrating nuclei were intensely stained, and staining of moderate intensity was evenly distributed around nuclei of the majority of cells (Fig 34b and d). In contrast, in cultures stained with SA-FITC a marked increase in fluorescence above background was seen in both untreated and NaB-treated cultures labelled to reveal glucagon (not shown) or somatostatin (Fig 37). The differences in the general intensity of fluorescence between control and NaB-treated cultures labelled to reveal glucagon or somatostatin (not shown) were not as marked as those that seen in preparations stained with ABC-HRP.

Because the distribution of staining in cells was similar for ABC-HRP-stained whole-mounts immunolabelled to reveal insulin, glucagon or somatostatin (Fig 33), the staining reaction gave the impression that antibodies in glucagon or somatostatin antiserum bound to intracellular sites. On the other hand, optical sections of whole mounts examined by fluorescence microscopy revealed that insulin immunoreactivity was confined to the cytoplasm (Fig 31) whereas glucagon and somatostatin-like immunoreactivity was associated with the plasmamembrane (Fig 37). However, because of the

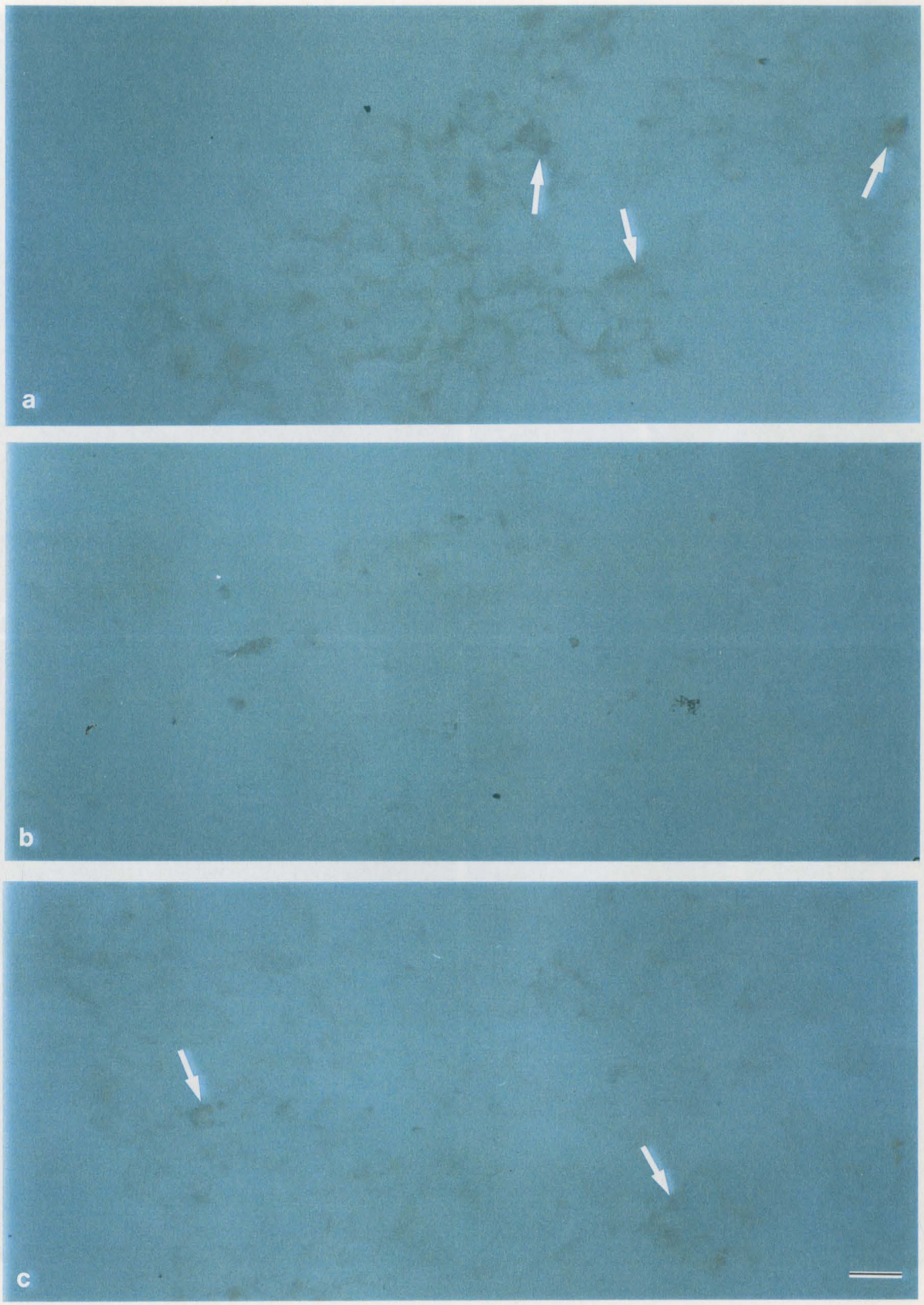


Fig 33. Light micrographs of whole-mounts immunolabelled to reveal insulin demonstrate (a) faint staining in numerous cells from NaB-treated cultures. Cells that are more intensely stained (arrows) possibly correspond to the insulin-containing cells revealed by immunofluorescence. (b) Staining is absent in cells from untreated cultures. (c) Staining is present in numerous cells from NaB-treated cultures immunolabelled without primary antiserum (method control). Preparations were not counterstained. Bar = 20μm.

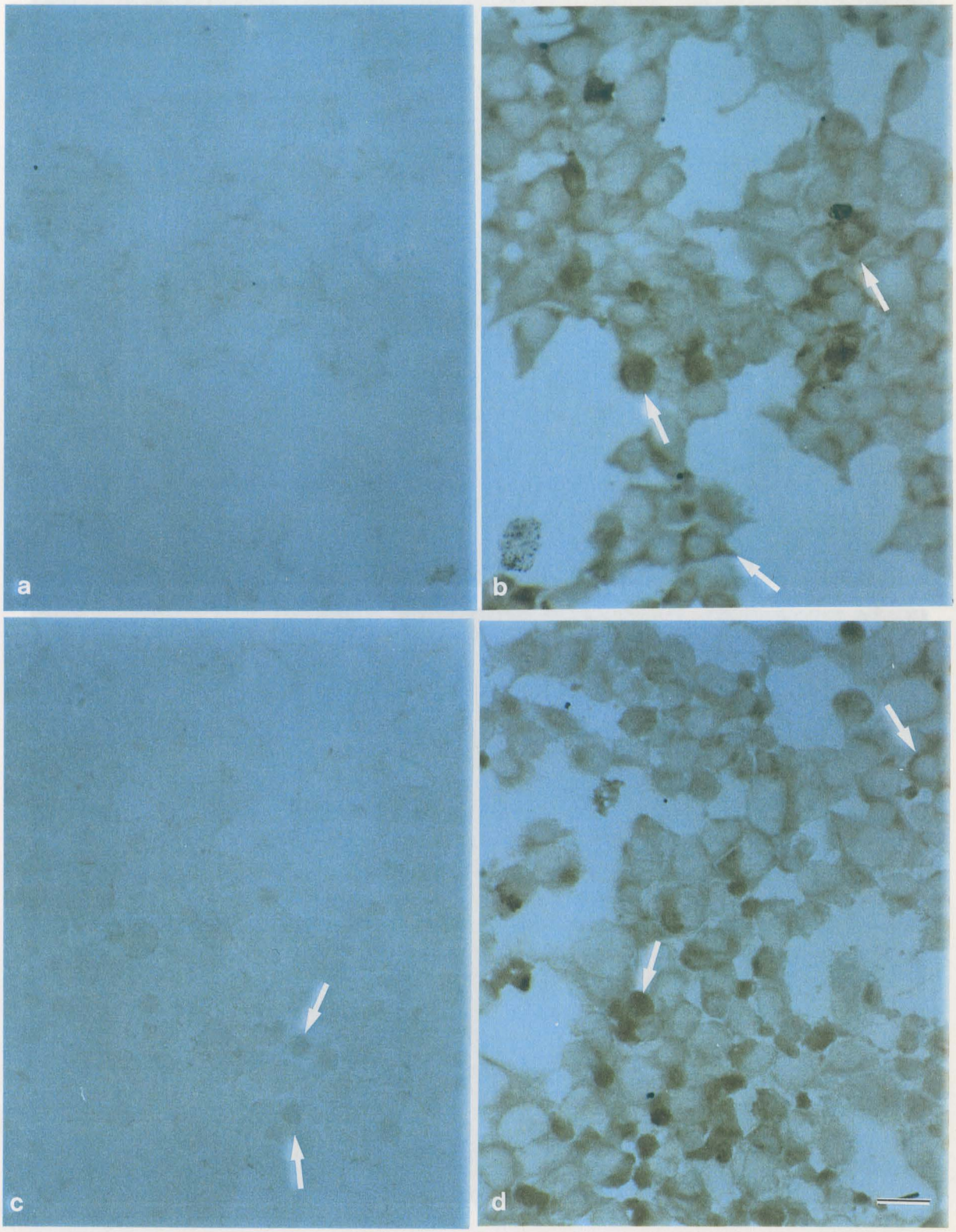


Fig 34. Light micrographs of whole-mounts immunolabelled to reveal glucagon (a and b) or somatostatin (c and d) from untreated (a and c) and NaB-treated (b and d) cultures.
(a) Glucagon immunoreactivity is absent in the untreated culture. (b) Staining is present in all cells in the NaB-treated culture and is more intense in several cells (arrows). (c) Faint staining is present in untreated cultures labelled to reveal somatostatin and is marginally heavier in small cells (arrows). (d) Staining is present in all cells in the NaB-treated culture. Intense staining is seen around nuclei of several cells and in small cells (arrows). Preparations were not counterstained. Bar = 20μm.

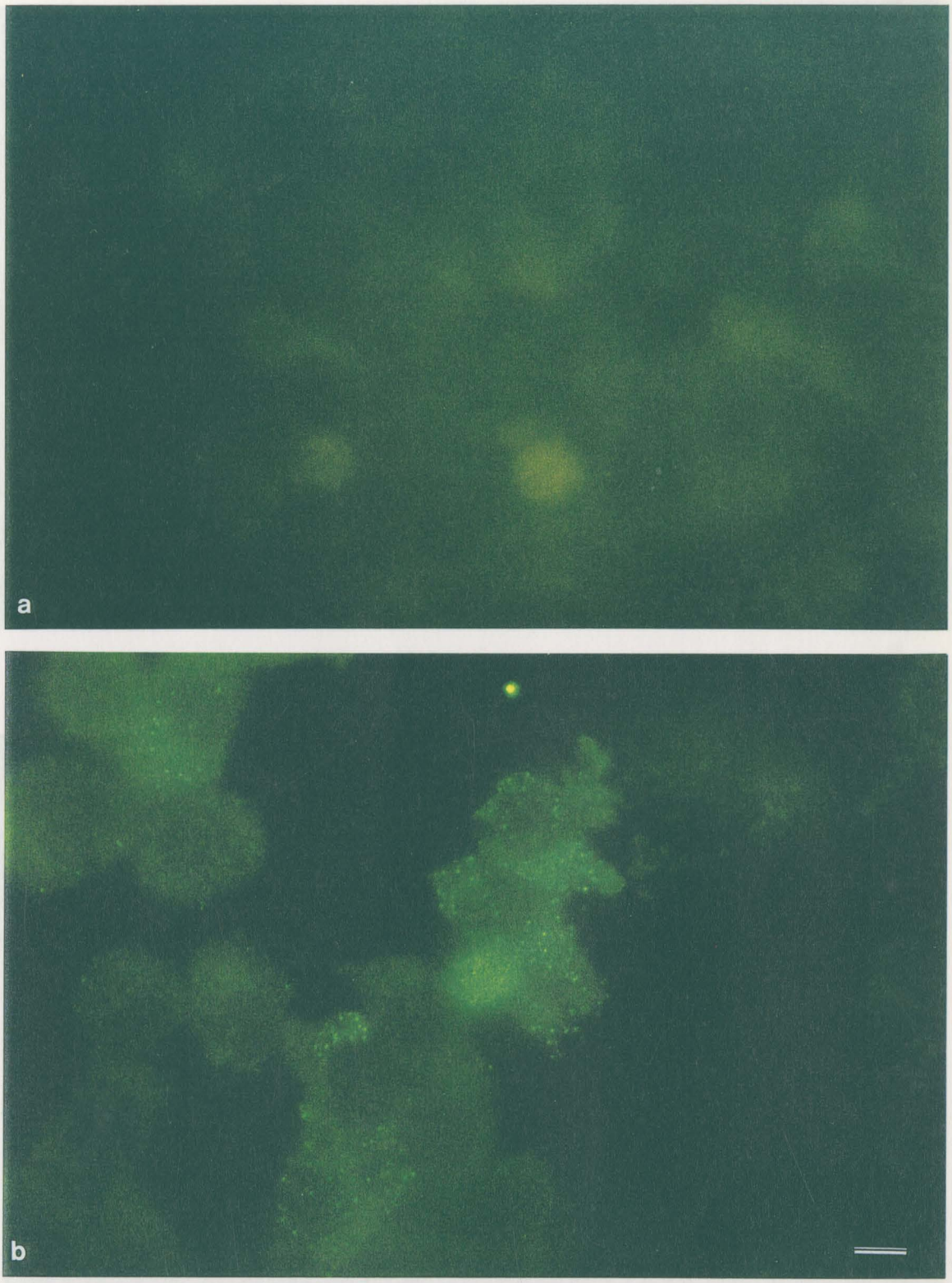


Fig 35. Fluorescent-stained whole-mounts immunolabelled to reveal insulin in untreated (a) or NaB-treated (b) cultures. (a) Staining is absent in all cells from untreated cultures. (b) Small clusters of immunopositive cells containing punctate fluorescent signals are present in NaB-treated cultures. Bar = 10μm.

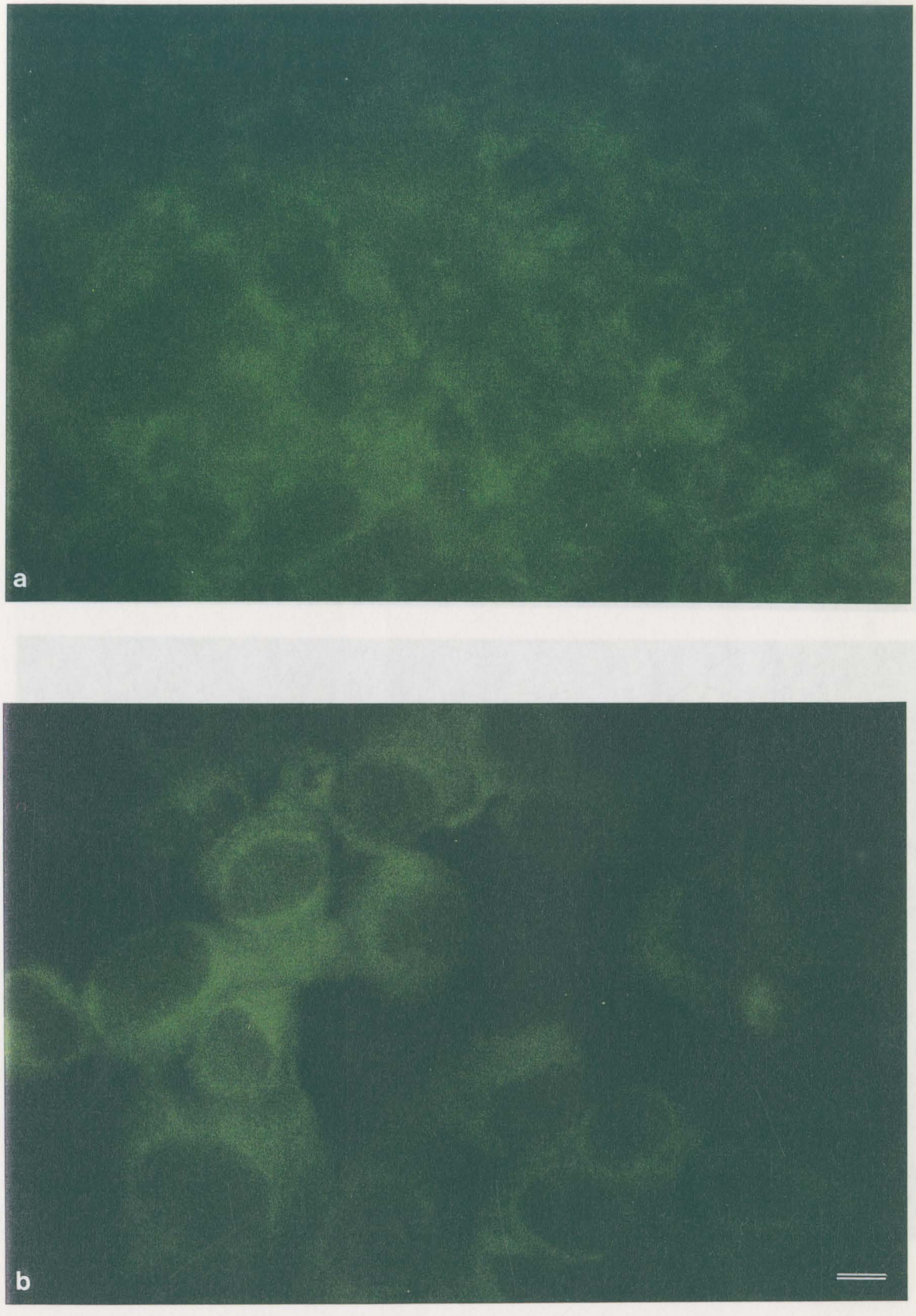


Fig 36. Fluorescent-stained untreated cultures demonstrating background staining in unstained whole-mounts (a) and in the method control (b) (incubated in primary antiserum diluent). The fluorescence in the method control is marginally raised above that of the unstained preparation and is most likely caused by residual SA-FITC. Bar = 10μm.

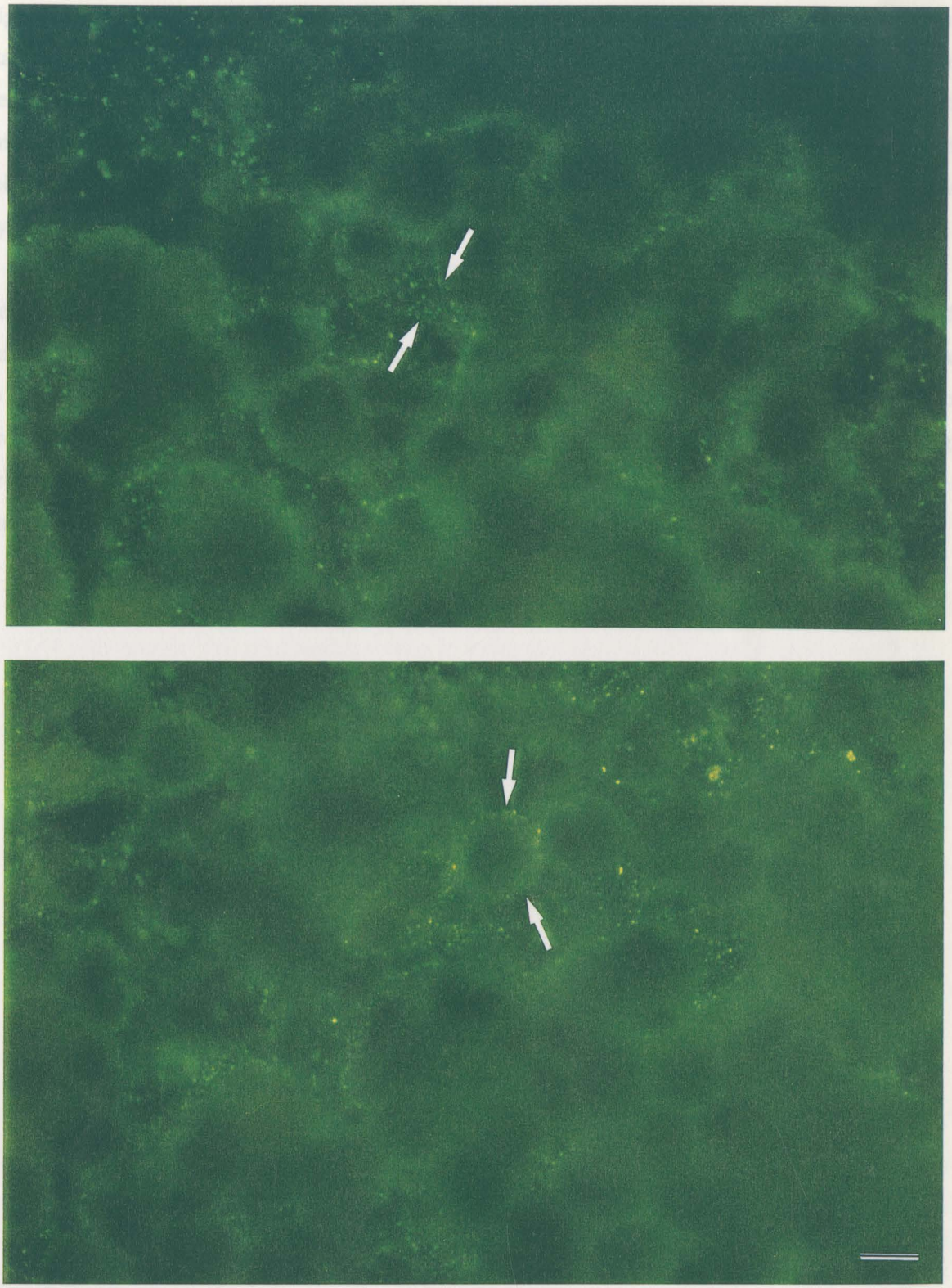


Fig 37: Two optical sections through the same field of untreated cultures immunolabelled for somatostatin demonstrating (a) a high labelling density (arrows) where the plane of focus is in the same plane as the plasmamembrane. Where the focal plane has passed through the centre of the cell (unstained centre), the signals are located only on the cell periphery.
Bar = 10μm.

high signal density and resultant out-of-focus glare in the latter preparations, possible intracellular staining was not detected. Although stained cells in preparations stained with ABC-HRP were detectable at low magnification, and thus appears to be a more sensitive technique than immunofluorescence, positively stained cells for insulin were better identified by fluorescence microscopy. In addition, the distribution of fluorescence signals at the cellular level was best resolved by the latter technique.

Because glucagon and somatostatin antisera raised in a species other than rabbit were unavailable for this study, the following experiment investigated immunoblotting techniques as an alternative means to determine the presence of glucagon or somatostatin in RINm5F-MRC cells. By separating cell proteins according to their relative molecular weights by polyacrylamide gels electrophoresis, glucagon or somatostatin would be discriminated from other immunoreactive cellular proteins on the basis of their size (see **3.7**).

3.7 SDS-Polyacrylamide gel electrophoresis and immunoblotting:

Immunoblots were performed on crude cell extracts from untreated and sodium butyrate-treated RINm5F-MRC cultures to determine whether the non-specific staining reaction in whole-mounts labelled to detect glucagon and somatostatin masked any glucagon or somatostatin immunoreactivity that was not resolved by flow cytometry or microscopy. Although insulin immunoreactivity was shown to be specific by flow cytometry and by whole-mount immunofluorescent labelling, an immunoblot labelled with insulin antiserum, was performed to verify the specificity of this antiserum and also to serve as a positive control for the immunoblotting procedure.

Electrophoresed cell extracts from untreated (Fig 38a (CON)) and sodium butyrate-treated RINm5F-MRC cultures (Fig 38a (NaB)), labelled to detect insulin, each demonstrated a single, strongly stained band of insulin-like immunoreactivity. The molecular weight of insulin-stained proteins in these bands, calculated from the standard curve, was 9000 daltons (see **2.7.3**). Since the molecular weight of proinsulin is 9,000 daltons it is likely that the insulin antibodies have recognised proinsulin. Insulin (MW 6 000 daltons) that migrates according to molecular weights of 2 300 (A chain) and 3 500 (B chain) daltons under reducing conditions, was not detected in RINm5F-MRC cells. The insulin standard (Fig 38a (INS)) shows a single band corresponding 3 500 daltons. As the molecular weights of the A and B chains are relatively close it is possible that they have nearly identical mobilities in this system. Alternative reasons for resolving only the B chain of insulin are discussed in Chapter 4. A finding that correlates with data from flow cytometry and fluorescence microscopy is that staining was more intense in extracts from sodium butyrate-treated cultures. However, although equal volumes of cell extracts, obtained from similar volumes of pelleted cells were electrophoresed, it is possible that increased levels of proinsulin from extracts of sodium butyrate-treated cultures may result from loading unequal concentrations of total proteins. These had not been quantified for this study.

In contrast, cell extracts from untreated (Fig 38b (CON)) and sodium butyrate-treated cultures (Fig 38b (NaB)) immunostained with anti-glucagon serum, demonstrated numerous strongly stained protein bands. Furthermore, regions corresponding to the glucagon control (Fig 38b (GLU)) or to proglucagon (MW 9 000 daltons) were not stained. Similarly, an immunoblot stained for somatostatin also revealed staining to only larger proteins (data not shown).

The findings of the immunoblots confirm the presence and specificity of insulin/proinsulin immunoreactivity in RINm5F-MRC and that cells appear to contain only proinsulin which is detected by the insulin antiserum. Furthermore, neither glucagon nor somatostatin could be detected in RINm5F-MRC cell extracts in the above immunoblots and there was strong non-specific staining to

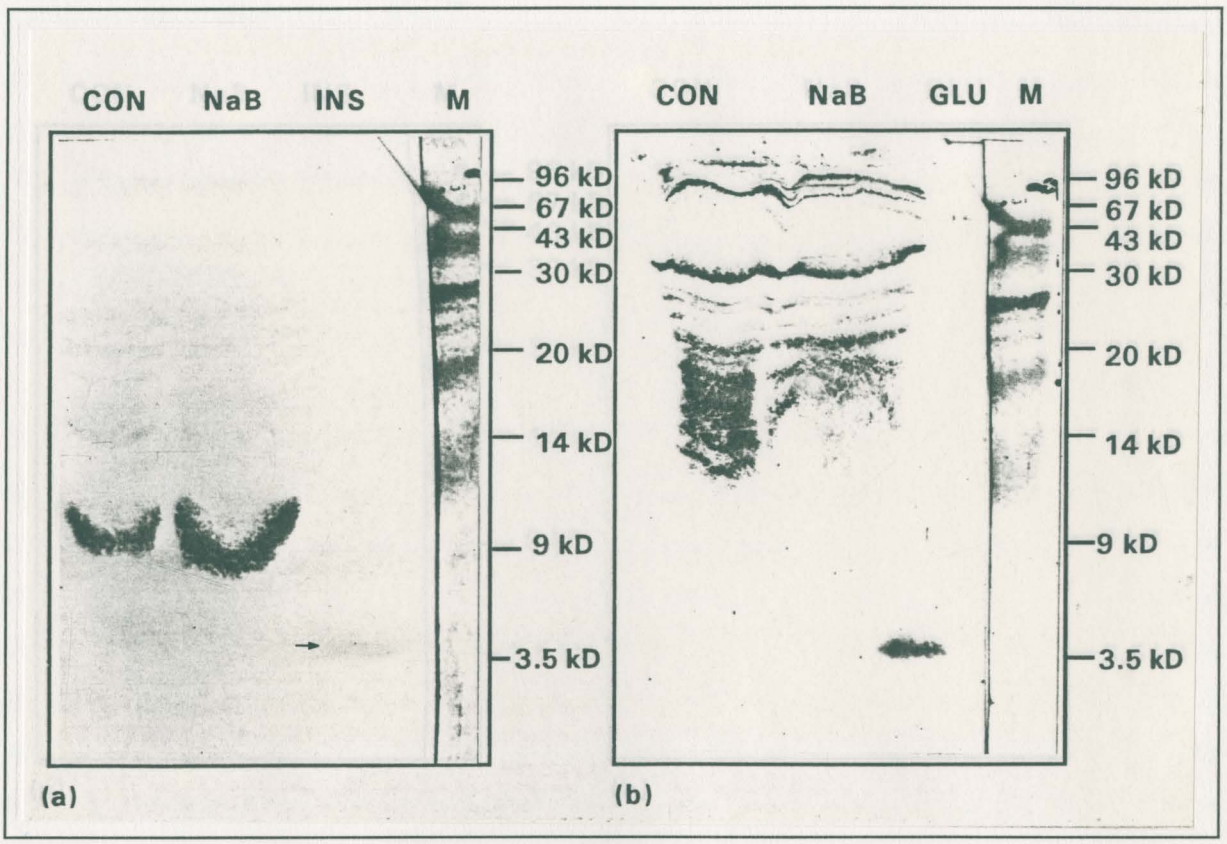


Fig 38. Immunoblot of crude cell extracts of RINm5F-MRC cells from untreated (CON) and sodium butyrate-treated (NaB) cultures immunolabelled to reveal (a) insulin or (b) glucagon.
(M) = the molecular weight marker stained with amido black. (kD = kilodaltons)

(a) A band showing strong insulin immunoreactivity that has a corresponding molecular weight of 9000 daltons (possibly proinsulin) is present in both cultures. The insulin standard (INS) that has a corresponding a molecular weight of approximately 3 500 daltons is faintly stained.
(b) An immunoblot stained for glucagon, demonstrates numerous intensely stained bands caused by cross-reactivity of the antiserum with non-specific proteins in both cultures (CON and NaB). The absence of immunoreactivity in regions corresponding to the glucagon standard (GLU) that has a molecular weight of 3 500 daltons, supports findings from whole-mount immunolabelling studies and from flow cytometry that glucagon-like immunoreactivity is non-specific.

numerous other proteins in RINm5F-MRC cells. Reasons for the lack of detection of these peptides in RINm5F-MRC are further discussed in Chapter 4. Since glucagon and somatostatin-like immunoreactivity seen in immunostained whole-mounts appeared to be caused by components other than antibodies to glucagon or somatostatin, and that neither peptides were detected by immunoblotting, it was unnecessary to perform the staining controls in this study. However, the specificity of the insulin, glucagon and somatostatin antisera had been previously established on dot blots (by other workers in our laboratory) by absorption with either insulin, glucagon or somatostatin. These same peptides were used for the standards on the above immunoblots.

4. DISCUSSION

In the adult mammalian pancreas, islet cells that produce insulin, glucagon, somatostatin or pancreatic polypeptide, have distinct functional and morphological characteristics. Current theories suggest that all hormone-producing cells are derived from multipotential stem cells (progenitor cells) that are found in the epithelium of the pancreatic duct and ductules. Although numerous studies have investigated islet cell differentiation in vivo, it is not clear whether individual mature islet cells are committed to expressing a pre-determined hormone-producing phenotype (i.e. lineage-restricted) or whether they retain the potential to express all islet cell phenotypes (i.e. are phenotypically plastic). Several clonal cell lines that have their origin in a transplantable islet cell tumour induced by X-irradiation (Chick et al., 1977), are heterogeneous (Gazdar et al., 1980; Madsen et al., 1986; Phillipe et al., 1987a). This heterogeneity strongly suggests that the rat islet cell tumour arose from transformed pluripotent islet stem cells. Thus these cell lines may potentially be of use for studying the differentiation pathways of the different hormone-producing cell types and to investigate the possibility that islet cells are inherently phenotypically plastic.

The cell line used in the present study is the RINm5F cell line, a subclone of the RINm parent culture (Gazdar et al., 1980) that has its origin in the rat insulinoma mentioned above. Initially, the RINm5F cell line secreted high levels of insulin and more than 80 % of the RINm5F cultures consisted of insulin-producing cells (Gazdar et al., 1980). After extensive passaging in vitro, insulin secretion declined. This correlated with a loss of differentiated characteristics of the B-cell phenotype (see Introduction). Furthermore, others had reported that RINm5F cells express nerve growth factor (Polak et al., 1993) and somatostatin in a subclone of RINm5F (Saulnier-Michel et al., 1992). From these reports, it seemed likely that the clone that gave rise to the RINm5F cell line originated from a transformed cell precursor. It had been previously reported that RINm5F cells obtained from different sources had different secretory and growth characteristics when grown under identical conditions (McEvoy et al., 1982). RINm5F-MRC cells had suffered suboptimal culture conditions during transportation to our laboratory. Therefore, growth and morphological characteristics and hormone-producing phenotype were examined to determine whether these cells still expressed the RINm5F phenotype and whether or not other islet-specific hormones were present.

4.1 Growth and morphology in culture:

The growth and morphology of cultures and expression of phenotype can be affected by numerous factors. These can arise from changes in the culture environment such as composition of the medium, concentration of gases, temperature and presence of micro-organisms. Changes in cellular composition such as altered gene expression, can also affect growth and cell morphology (Freshnay, 1987; Phillipe

et al., 1987b; Bartholomeuz et al., 1989; Karlsen et al., 1991). Shortly after the RINm5F-MRC cultures were established in our laboratory, a preliminary investigation was done to determine their growth rate, cell morphology and whether or not they produced peptide hormones. This served to ensure that these cultures were indeed of the RINm5F lineage and to serve as a reference for monitoring the stability of the cell line at later passages (Freshnay, 1987).

4.1.1 Cell growth:

Doubling times of the RINm5F-MRC cell line (27 to 31 hours) were markedly less than those of the RINm parent cell line (60 to 80 hours) (Gazdar et al., 1980) or of RINm5F clones in other laboratories (38 to 72 hours) (Praz et al., 1983; Muschel et al., 1986; Flatt et al., 1987;). However, the doubling time of RINm5F-MRC cells at a lower passage number was similar to that of faster growing clones reported previously (McEvoy et al., 1986). Increasing growth rate at later passages has been reported in RINm and RINr parent clones (Gazdar et al., 1980) and this trend was observed in RINm5F-MRC cultures.

Despite their common origin (Gazdar et al., 1980; Oie et al., 1983), different rates of proliferation and insulin secretion have been reported in RINm5F cell lines obtained from different laboratories, even when they are grown in the same laboratory under the identical conditions (McEvoy et al., 1986). These authors attribute these differences to previous culture conditions that may have selected for cells showing the growth and secretory characteristics that they observed. It is has been previously shown that insulin-secreting cell lines such as the RINm, RINr and HIT lines maintained in vitro under identical conditions in the same laboratory change their biological characteristics with time (Gazdar et al., 1980; Santerre et al., 1981). In the RINm parent cell line, cultures at later passages demonstrated higher growth rates and reduced their insulin secretory rates (Gazdar et al., 1980). RINr subclones that do not produce peptide hormones are reported to have higher growth rates than those of peptide-producing clones (Phillipe et al., 1987a). This indicates that growth rate is linked to the level of differentiation in RIN cell lines. Furthermore, the declining insulin secretion by RINm5F cells with time in culture has been previously linked with a state of dedifferentiation (Flatt et al., 1987).

In contrast, while insulin secretory rates declined in HIT-T15 cultures over time, the growth rate after almost two years in culture appeared to remain unchanged (Santerre et al., 1981). Recently, the decline in insulin secretion rate in this cell line has been shown to be due to constant exposure to high glucose concentrations and not to dedifferentiation (Robertson et al., 1992; Olson et al., 1993). An observation that is possibly relevant to the weak staining for insulin seen in RINm5F-MRC cells, is that insulin secretion was substantially lower in a RINm5F cell line that had been obtained from the

same source as the RINm5F-MRC cells, than insulin secretion in RINm5F cell lines obtained from other sources (McEvoy et al., 1986). The latter report, coupled with the low basal insulin secretion by RINm5F-MRC cultures, as well as the high growth rate observed in these cells, suggest that RINm5F-MRC cells have originated from RINm5F cultures of a higher passage number to those used by other authors. Changes in the composition of the medium, particularly that of foetal calf serum, can account for differences in growth and morphology. In order to ensure that changes in growth could reflect possible changes in phenotype, RINm5F-MRC cells were routinely grown in a standardised medium (RPMI 1640) containing serum from the same batch throughout this project.

Gazdar et al., 1980 reported that insulin secretion declined in later passages of RINm cultures. This and other reports of reduced insulin production in RINm5F cells with increasing time in culture (Flatt et al., 1987), suggests that later passages of RINm and RINm5F cultures contain less differentiated cells than those from earlier passages. The reduction of detectable insulin in immunostained RINm5F-MRC cultures at later passages is associated with an increase in growth rate compared to earlier passages that demonstrated insulin-like staining and slower growth rates. This suggests that RINm5F-MRC cultures have further dedifferentiated during the course of this study. Further support for a link between growth rate and state of differentiation is provided by the finding that the number of detectable insulin-containing cells from RINm5F-MRC cultures exposed to sodium butyrate was markedly raised above that of untreated cultures. These changes in insulin expression were accompanied by growth arrest. Effective growth arrest in RINm5F-MRC cells induced by 2mM sodium butyrate has been shown previously in RINm5F cells and other subclones of the RIN lineage (Green and Shields, 1984; Powers et al., 1988; Bartholomeusz et al., 1989; Karlsen et al., 1991). Increased expression of peptides accompanied by growth arrest in all of the studies mentioned above, is consistent with the increase in insulin expression in RINm5F-MRC cells.

Growth of RINm5F-MRC cultures was also arrested by mitomycin C. As there are no reports concerning the use of this substance on RIN cell lines, the significance of these preliminary results cannot be discussed appropriately until further experiments using this substance on RINm5F-MRC cells have been performed. However, the concentration of 0.1μg/ml of mitomycin C that effectively arrested growth without excessive toxicity, is less than that required to arrest cell division in fibroblasts (Freshnay, 1987). Although neither whole-mount immunolabelling nor flow cytometric studies were done on mitomycin C-treated cultures, immunolabelled sections of such cultures indicated that cells contained more insulin than those from untreated cultures. As the primary function of mitomycin C is to interrupt DNA synthesis thereby inducing growth arrest, these preliminary findings further support the association of growth and differentiation in these cells.

4.1.2 Morphology:

RINm5F-MRC cells, grown under routine culture conditions (untreated cultures) or in the presence of sodium butyrate, formed semi-compact colonies that contained elongated epithelial-like cells. Cytoplasmic processes extending from free cell surfaces were sparse in untreated cultures but were more abundant in cultures exposed to sodium butyrate or mitomycin C.

Although all RIN cell lines contain epithelial-like cells, not all the cell lines have the same morphology in culture. Sublines arising from the parent RINr line, that produced insulin or somatostatin tended to grow in clusters that had well-defined margins (Phillipe et al., 1987a). Cytoplasmic processes extending from free cell surfaces were rare in these cultures. On the other hand, the parent RINr and RINm cell lines formed semi-compact colonies containing elongated cells and abundant cytoplasmic processes (Gazdar et al., 1980). Similar morphology has been reported in another parent cell line (RIN 1056a) that was derived from a tumour formed in vivo from inoculations of a somatostatin-secreting RIN clone (Phillipe et al., 1987a). Semi-thin and thin sections of RINm5F-MRC cultures examined by light and electron microscopy and whole-mounts examined by fluorescence microscopy confirmed that RINm5F-MRC cells formed multilayered colonies in culture. This finding is in contrast to the report that RINm5F cells form a monolayer in culture (Flatt et al., 1987).

The homogeneous HIT-T15 β-cell line that arose from transformed terminally differentiated β cells tends to grow in compact clusters that have well-defined margins (Santerre et al., 1981). When grown in vitro, isolated native islet cells generally form compact islet-like aggregates (Halban et al., 1987, 1988). Cytoplasmic processes are absent from HIT-T15 cultures, from the differentiated BTC β-cell line and from native islet cell aggregates (Santerre et al., 1981; Polak et al., 1993). Only when islet cells are grown at low culture density, do they form neurite-like processes (Teitelman, 1990). From the above-mentioned reports, it appears that islet cell lines containing a large proportion of a "differentiated islet-cell phenotype" form compact colonies or cell clusters in vitro. At the other end of the scale, islet cell lines that do not produce peptides tend to grow as isolated cells until cultures become confluent (Phillipe et al., 1987a). This suggests that morphology in vitro can reflect the level of differentiation of the culture as a whole. The parent cell lines RINr, RINm and RIN 1056A, that contain differentiated and undifferentiated cells (Gazdar et al., 1980; Phillipe et al., 1987a), form semi-compact colonies. If this criterion is applied to RINm5F-MRC cells then it can be assumed that these cultures are populated by differentiated and undifferentiated phenotypes. The finding from immunofluorescence studies where only a subpopulation of cells contained detectable insulin is compatible with this idea.

Cell morphology and growth pattern of RINm5F-MRC cells from untreated cultures and from those

exposed to sodium butyrate agree with previous reports of growth and morphology for RINm5F cells (Bartholemeusz et al., 1989): viz. in untreated cultures, cells form semi-compact colonies with few cytoplasmic processes extending from free cell surfaces, whilst colony growth was sparse and cytoplasmic processes were more abundant in sodium butyrate-treated cultures. In sodium butyrate-treated cultures, these features were accompanied by an increase in the number of cells expressing the 3G5 and A2B5 gangliosides known to be markers of differentiated ß cells (Bartholomeusz et al., 1989, 1990a) and by an increase in insulin secretion in RINm5F-MRC cultures. However, the value of cytoplasmic processes as a morphological marker of a more differentiated insulin-producing phenotype has not been established, neither previously (Bartholomeusz et al., 1989) nor in the present study. The finding that cell size increased in a portion of RINm5F-MRC cultures exposed to sodium butyrate is consistent with previous reports (Bartholomeusz et al., 1989; Karlsen et al., 1991). As the size of native β cells is larger that of non-β cells (Pipeleers et al., 1985), it is possible that the larger cells in sodium butyrate-treated cultures represent the more differentiated β-cell phenotype.

4.1.3 Ultrastructure.

RINm5F-MRC cultures contained morphologically distinct cell types. Whilst the majority of cells were agranular or had sparse numbers of granules, those containing numerous granules were rare. None of the cells displayed morphological features that were similar to any of the four major hormone-producing cell types of the rat pancreas. This and the atypical granule morphologies of RINm5F-MRC cells are consistent with findings in subclones of the RINm and RINr lineages (Oie et al., 1983) and in clonal descendants of the RINm5F cell line (Saulnier-Michel et al., 1992). Likewise, agranular and sparsely granulated cells have been observed in other RINm5F cultures (Lombardi et al., 1986; Halban et al., 1988). In contrast, the rat insulinoma from which all RIN cell lines originated contained differentiated β and δ cells that were filled with characteristic membrane-bound secretory granules (Chick et al., 1977). The morphology of these insulinoma cells was similar to β and δ cells observed in sections of the NEDH rat pancreas prepared for in the present study and is consistent with that reported in other rat species (Pictet et al., 1972; Kaung, 1985). Gazdar et al., 1980 reported that cells from RINm and RINr parent cultures contained membrane-bounded granules. However, as these authors did not comment further on the morphology of the granules, it is not known whether the parent cultures also contained morphologically differentiated islet cell types.

The atypical granule morphology in cells from sublines of RINm and RINr parent cultures and in RINm5F-MRC cells is consistent with that described in human insulinomas (Creutzveldt, 1985). Similarly, agranular and sparsely granulated cells are also found in human insulinomas. The low insulin content in human tumours populated by agranular cells or cells with atypical secretory granules has been attributed to ineffective storage of insulin in these cells or to an immature islet cell phenotype

(Creutzveldt, 1985). Cells of the embryonic pancreas are reported to contain atypical granules before the onset of cytodifferentiation (Pearse et al., 1973). An intriguing finding in a few well-granulated RINm5F-MRC cells, was the presence of pale zymogen-like granules. The endocrine and exocrine pancreas are thought to arise from a common ancestral cell (Rutter, 1980). The finding that cells in RINm5F-MRC cultures express both exocrine- and early endocrine-like characteristics suggests that these cells could be pancreatic stem cells. Furthermore, the similarity of granule morphology of RINm5F-MRC cells to that of other RIN cell lines or human insulinomas, confirms that cells from RINm5F-MRC cell line belong to a lineage derived from the rat insulinoma and that RINm5F-MRC cells do not contain any morphologically distinct islet-cell phenotypes

Canaliculi are generally found between islet cells in vivo (Weir and Bonner-Weir, 1990) and were observed between islet cells in sections of rat pancreas in this study. Microvilli arising from islet cells are frequently found in these spaces. Canalicular-like spaces also occur between HIT-T15 cells in culture but were not noted in other RINm5F cultures (Lombardi et al., 1986). Furthermore, the presence of canaliculi has not been reported in other RIN cell lines. As these prominent intercellular spaces between RINm5F-MRC cells did not appear to be due to a processing artefact, it was concluded that they provided passage for nutrients to cells in the deeper layers of the colonies. Occasional clusters of RINm5F-MRC cells possessed numerous microvilli, a morphological feature common to β cells (Pipeleers et al., 1985). Similarly, microvilli are present on HIT-T15 cells (Lombardi et al., 1986). However, microvilli were always associated with agranular cells in RINm5F-MRC cultures and thus may not indicate a specific hormone-producing cell type.

4.2 Immunolabelling studies of RINm5F-MRC cells:

Since reports of immunocytochemical studies on RINm5F cultures do not appear to be available in the literature, the results from labelling experiments in this study are compared with those from other RIN-derived cell lines. Analysis by microscopy revealed insulin-immunoreactive cells only in later passages of RINm5F-MRC cultures that had been exposed to sodium butyrate. In contrast, proinsulin in cell extracts and insulin-immunoreactive cells were detected by immunoblots and flow cytometry respectively from untreated and sodium butyrate-treated cultures. Furthermore, flow cytometry and immunostained whole-mounts confirmed that insulin immunoreactivity was specific and immunoblots indicated that immunoreactivity was due to proinsulin rather than insulin. On the other hand, specificity of glucagon and somatostatin immunoreactivity could not be established in RINm5F-MRC cells. Furthermore, staining was thought to be caused by non-specific antibodies in rabbit serum (see **4.5.2**). The finding that only a subpopulation of RINm5F-MRC cells stained for insulin is consistent with immunocytochemical studies done on other RIN-derived cell lines (Madsen et al., 1986; Phillipe et al., 1987a, Powers et al., 1988; Saulnier-Michel et al., 1992). Even in a subline of RINm5F that

was selected for its high insulin gene transcription, only 56% of the cell population contained detectable insulin (Saulnier-Michel et al., 1992). In contrast, insulin has been detected in all cells from the more terminally differentiated insulin-secreting HIT-T15 cell line (Santerre et al., 1981) and was present in 85% of the cells from the RINm5F cell line shortly after it had been established (Oie et al., 1983).

A major challenge presented in this study, was the difficulty in detecting insulin immunoreactivity in RINm5F-MRC cultures by light and fluorescence microscopy. Insulin positive cells in peroxidase- and fluorescence-stained whole-mounts of other RIN cell lines are reported to be strongly stained (Madsen et al., 1986; Phillipe et al., 1987a; Powers et al., 1988; Saulnier-Michel et al., 1992). This is in contrast to the findings of all whole-mount immunolabelling studies on RINm5F-MRC cultures, where insulin-like staining was weak, following development by both peroxidase and fluorescence procedures. Considering that RINm5F cultures were noted for high levels of insulin secretion and for the high number of detectable insulin-containing cells (Oie et al., 1983), the substantially weaker insulin staining in RINm5F-MRC cells compared with other RIN cell lines is surprising. This raises the question as to why detection of insulin in RINm5F-MRC cells was difficult in the present study.

Peroxidase-based staining protocols used in the present study were different to those used to detect hormones in other RIN cell lines (Madsen et al., 1986; Phillipe et al., 1987a; Powers et al., 1988; Saulnier-Michel et al., 1992). In RINm5F-MRC whole-mounts, antigen/antibody complexes were stained with avidin D biotinylated horse-radish peroxidase complex (ABC-HRP) and DAB. Immunocytochemical studies on other RIN cell lines have used ABC-HRP with 3-amino-9-ethylcarbazole (Phillipe et al., 1987a; Powers et al., 1988), peroxidase-labelled antibodies followed by 4-chloro-1-naphthol (Saulnier-Michel et al., 1992) or the peroxidase-anti-peroxidase technique of Sternberger, 1979 (Madsen et al., 1986). Since the ABC-HRP system is regarded as the most sensitive detection method (Larsson, 1988), it is unlikely that the staining system used in the present study is responsible for the poor staining signal. Furthermore, the strong insulin staining observed in frozen sections of intact rat pancreas, stained in parallel with whole-mounts of cultures, provides further support that poor staining in whole-mounts was not due to an ineffective detection system. Fluorescence-staining protocols were also different for RINm5F-MRC cells and other RIN cell lines (Madsen et al., 1986; Saulnier-Michel et al., 1992). Both the reported studies on other RIN cell lines used fluorescein-coupled antibodies whereas SA-FITC was used to stain RINm5F-MRC cells. The latter method is a three-step indirect labelling protocol that has the advantage over the former of amplifying the fluorescent signal. Since SA-FITC would maximally increase signal detection, it is probable that low fluorescence observed in insulin-containing RINm5F-MRC is due to low peptide content in these cells.

Another cause for weakly stained RINm5F-MRC cells could be poor penetration of antibodies and staining complexes through the cell membrane and the cytosol. In this study, frozen sections of rat pancreas served as a positive method control since cell lines containing abundant pancreatic hormones were unavailable. However, in contrast to that seen in whole-mounts, peroxidase- and fluorescence-staining was intense in these method control sections. The accessibility of antibodies and reporter molecules to antigenic sites is fundamentally different between sectioned material and whole-mount preparations. Whilst antigenic sites are readily exposed on the surface of sections, antibodies and staining complexes must penetrate the cell membrane and cytosol of cells in whole-mounts in order to reach antigenic sites. In particular, the staining complexes of the ABC-HRP Vectastain staining system are substantially larger than antibody or SA-FITC molecules (Larsson, 1988) and may have difficulty penetrating the cross-linked cytosol. For this reason, whole-mounts of RINm5F-MRC cultures were lightly fixed in 2% paraformaldehyde and permeabilised by freeze-thawing following Larsson, 1988. Furthermore, as hormone peptides had been detected in other RIN cell lines permeabilised by freeze-thawing (Madsen et al., 1986; Phillipe et al., 1987a; Powers et al., 1988), this seemed to be the method of choice for the present study. In later studies, Triton X 100 was used in buffer solutions to aid the penetration of antibodies in whole-mounts that had been permeabilised by freeze-thawing. Under these conditions, insulin remained undetectable in untreated RINm5F-MRC cultures but was detectable once cultures had been exposed to sodium butyrate. These findings suggested that the lack of insulin detection in other labelling experiments in this study, was not caused by inadequate permeabilisation of the cells.

Thus the staining systems and permeabilisation did not appear to be the cause of low staining intensity in RINm5F-MRC cells. Therefore weak staining in the present study compared to strong staining reported in other studies discussed above, is most likely caused by an insulin content that is substantially lower in RINm5F-MRC cells than in other RIN cell lines. Furthermore the weak staining in only a few cells from frozen sections of untreated RINm5F-MRC cultures in which antigenic sites were readily exposed, suggests that insulin content in these cultures is below the lower limit of detection by immunocytochemistry. The findings from immunocytochemical studies on RINm5F-MRC cells thus suggest that the RINm5F cell line is less differentiated than other RIN cell lines. Moreover, the presence of positively stained cells for insulin detectable at a lower passage number but not at later passages suggests that RINm5F-MRC cultures had further dedifferentiated during the course of this study. This conclusion is further supported by the findings obtained from growth rate studies discussed above and by previous reports that insulin production of RINm cells declined over time in culture (Gazdar et al., 1980).

4.3 Detection of insulin, glucagon and somatostatin in whole-mounts stained by immunofluorescence or by immunoperoxidase staining:

The presence of insulin-containing cells in sodium butyrate-treated cultures detected by flow cytometry motivated a further investigation on whole-mounts from similarly treated cultures in order to determine whether insulin was detectable in immunolabelled preparations by peroxidase staining (ABC-HRP) and by immunofluorescence (SA-FITC). Both ABC-HRP and SA-FITC are reporter molecules that amplify signal detection of antigen-antibody complexes (Larsson, 1988). A single molecule of strepavidin is coupled to four molecules of fluorescein, whereas ABC-HRP is a substantially larger complex (Larsson, 1988). Evidence that antigen-antibody complexes are more efficiently detected by ABC-HRP than by SA-FITC (Larsson, 1988) was confirmed in the present study. Insulin staining was detectable at low magnification in peroxidase-stained preparations whereas insulin-containing cells were only detectable at high magnification by fluorescence. This was despite the higher concentrations of antiserum used to label preparations stained with SA-FITC (Hörsch et al., 1992). These authors reported that similar staining intensities in peroxidase-labelled and fluorescent preparations were obtained when higher concentrations of the primary antisera were used to label fluorescent preparations. In contrast to staining observed on RINm5F-MRC cells, insulin staining was detectable at low magnification in frozen sections from rat pancreas that had been labelled in parallel with whole-mounts. Furthermore, the similar staining intensity observed in frozen sections stained with peroxidase or by immunofluorescence, is consistent with that reported previously (Hörsch et al., 1992). The difference observed between staining intensity of peroxidase-labelled and of fluorescent RINm5F-MRC cultures has not been reported by others who have used these detection systems in other RIN cell lines (Madsen et al., 1986; Saulnier-Michel et al., 1992).

Quantifying the number of insulin-containing cells proved to be difficult in RINm5F-MRC cultures, the reasons being different for preparations stained with ABC-HRP or SA-FITC. Because the intensity of insulin-like staining was marginally above background in peroxidase-labelled preparations, insulin-positive cells could not be identified with certainty from background staining. Furthermore, because peroxidase-stained preparations are viewed under bright field illumination, the small differences between the intensity of transmitted light of positively stained cells and of background staining is not easily distinguished under these conditions (Sternberger, 1979). In contrast, fluorescence signals appear bright against a dark background and thus even faintly stained cells are well differentiated from background. The generalised background staining in peroxidase-stained, sodium butyrate-treated cultures not present in untreated cultures or in preparations stained by immunofluorescence, appear to be non-specific. It is thus possible that exposure to sodium butyrate may have induced expression of other proteins to which IgG molecules bind non-specifically and these in turn were detected by the more sensitive detection system of ABC-HRP.

On the other hand, insulin-stained cells were more easily differentiated from background in fluorescence-stained whole-mounts than in peroxidase-stained preparations. The depth of focus at high magnification is much less than that at lower magnifications. Because RINm5F-MRC cultures tend to grow as multilayered colonies, extensive optical sectioning through the cell layers was necessary to asses the number of immunopositive cells. Since immunonegative cells did not show any fluorescence, it was impossible to asses the percentage of positively labelled cells in the cultures. Another technical difficulty in determining the number of insulin-containing RINm5F-MRC cells by fluorescence microscopy was that fluorescent signals were photo bleached by the UV light. Others have reported similar problems with quantification of fluorescence-stained cells (Saulnier-Michel et al., 1992).

In contrast to the weakly stained insulin-labelled cells, glucagon- and somatostatin-like staining was intense. This was noted particularly in cultures that had been exposed to sodium butyrate and, as previously shown, was assumed to be non-specific (see **4.2**). In these preparations, peroxidase staining and fluorescence were detected at low magnification. Whilst stained cells could be easily resolved in peroxidase-stained preparations, individual cells from fluorescent-preparations were not clearly defined at lower magnifications. Non-specific staining appeared to be intracellular in peroxidase-stained whole-mounts, while optical sectioning of fluorescence-stained preparations showed that non-specific staining was associated with the cell surface. On the other hand, optical sections of cells stained for insulin revealed that fluorescent signals were distributed evenly within the cytoplasm.

The finding in this study that glucagon and somatostatin immunoreactivity were non-specific is in contrast to similarly labelled RIN-derived cell lines (Madsen et al., 1986; Phillipe et al., 1987a; Powers et al., 1988; Saulnier-Michel et al., 1992). Apparent staining in RINm5F-MRC cells, shown to be due to binding of non-specific antibodies in normal rabbit serum (see **4.6.2**), indicates that the serum in which antibodies to glucagon and somatostatin occur also contains antibodies that will bind non-specifically to RINm5F-MRC cells. Because glucagon and somatostatin antisera were raised in different rabbits, it is therefore likely that glucagon and somatostatin antisera will contain different non-specific antibodies. Non-specific antibodies in normal serum from rabbits may differ from those in glucagon or somatostatin antisera. Therefore, differences in non-specific antibodies in serum from different animals may demonstrate different staining patterns. The differential staining pattern seen between whole-mounts exposed to normal rabbit serum, absorption controls for glucagon or somatostatin, and the glucagon or somatostatin antisera, is consistent with this idea. As glucagon and somatostatin have been revealed in other RIN-derived cultures by antisera raised in rabbit (Madsen et al., 1986; Phillipe et al., 1987a; Powers et al., 1988; Saulnier-Michel et al., 1992), it is likely that these authors had absorbed out the non-specific antibodies before labelling their cultures. Furthermore, the marked increase in background staining observed in sodium butyrate-treated RINm5F-MRC

cultures has not been reported in other RIN-derived cell lines.

4.4 Immunoblotting:

Immunoblotting of electrophoresed cell extracts of RINm5F-MRC cells offered a means to confirm whether all glucagon or somatostatin-like immunoreactivity was non-specific. If glucagon or somatostatin were present, they would be identified by immunostained bands at sites corresponding to their molecular weights on the immunoblot. Neither glucagon nor somatostatin were detected by immunoblots performed on electrophoresed cell extracts of untreated or sodium-butyrate-treated RINm5F-MRC cultures. In addition, immunoblots labelled to reveal insulin indicated that insulin-like immunoreactivity was due to the presence of proinsulin in RINm5F-MRC cells.

Immunoblots of cell extracts labelled to reveal glucagon or somatostatin provided further support that immunoreactivity observed in peroxidase- and fluorescence-stained whole-mounts and detected by flow cytometry was non-specific. Furthermore, the finding that immunoblots indicated that proinsulin and not insulin was responsible for the insulin-like immunoreactivity detected by immunocytochemistry is consistent with previous reports that newly synthesised insulin is rapidly secreted in rat insulinoma cells (i.e. insulin secretion is constitutive and insulin is not stored)(Gold et al., 1984) and therefore cellular proinsulin content would be greater than insulin content (Creutzfeldt, 1985). Binding of anti-insulin to proinsulin has been shown before (Gold et al., 1984) and is thought to occur because insulin and its precursor molecule share a number of epitopes to which the antibodies can attach.

As neither insulin, glucagon nor somatostatin were detected on immunoblots, it is possible that insulin in RINm5F-MRC cell extracts was below the limit of detection afforded by immunoblotting. Another possible cause for not detecting insulin is that all the peptide in the gel had not transferred to the nitrocellulose. As nitrocellulose with a pore size of .45μm was used for immunoblotting in this study, it is possible that transfer of separated proteins from the gels did not occur under ideal conditions for low molecular weight proteins (Larsson 1988). Detection of peptide standards with molecular weights greater than 3 500 daltons such as glucagon or the B chain of insulin, but not of somatostatin or the A chain of insulin, strongly supports this conclusion.

An intriguing finding revealed by immunoblotting was the presence of a strongly stained band of proinsulin in cell extracts from untreated RINm5F-MRC cultures. The finding that insulin-like immunoreactivity was not observed by microscopy, was barely detectable by flow cytometry from untreated cultures, but was revealed by immunoblotting agrees with previous explanations stating that peptide concentrations that are below the limit of detection by microscopy in a relatively large portion of the cell population can be revealed by immunoblotting (Larsson, 1988). The more heavily stained

proinsulin band in cell extracts from sodium butyrate-treated RINm5F-MRC cultures is consistent with the presence of insulin-like immunoreactivity in similarly treated cultures by microscopy and flow cytometry. On the other hand, if few glucagon and somatostatin-producing cells are present in RINm5F-MRC cultures, then it is unlikely that these peptides would have been detected on the present immunoblots (Larsson 1988).

4.5 Radio-immunoassay:

Failure to detect intracellular insulin in later passages of RINm5F-MRC cells raised the question as to whether or not RINm5F-MRC cells produce insulin at all. Insulin secretion has been extensively studied in the RINm5F cell line (Praz et al., 1983; Nielsen et al., 1985; McEvoy et al., 1986; Flatt et al., 1987, 1988; Dereli et al., 1988; Lambert et al., 1989; Bartholomeusz et al., 1990a,b). Even in "dedifferentiated" cultures, insulin has been detected in the culture medium by radio-immunoassay (RIA) (Flatt et al., 1988). Therefore, a preliminary RIA study was done to determine whether RINm5F-MRC cells secreted insulin into the medium, and whether the positive response to sodium butyrate, indicated in peroxidase-stained sections of RINm5F-MRC cells, was coupled with increased insulin secretion. The results clearly showed that RINm5F-MRC cells secreted insulin over a 24 hour period and the concentration of insulin in the medium was markedly raised in cultures treated with sodium butyrate. Regrettably, further investigation by RIA to detect glucagon and somatostatin secretion was not possible because assays for these peptides were not available.

Basal insulin secretion over 24 hours in untreated RINm5F-MRC cultures was 2.7μU/10^7 cells whilst that in sodium butyrate-treated cultures was 4.55μU/10^7 cells. This is substantially less than insulin secretion in the RINm and RINr parent clones that was 250-900μU/10^6 cells and 100 μU /10^6 cells over 24 hours respectively (Gazdar et al., 1980). These authors reported that basal insulin secretion in RINm cultures that were maintained in culture for 400 days, remained at 150 - 250 μU/10^6 cells over 24 hours. When RINm5F clones were established , their insulin secretory rate was 218μU/10^6 cells/24 hours (Oie et al., 1983). Although there are many studies on insulin secretion from cultured RINm5F cells, most workers have investigated release over short periods and thus the data from these studies cannot be compared to the findings of the present study. However, insulin secretion in RINm5F-MRC cultures is clearly several orders of magnitude less than other cell lines of the same lineage (RINm) and these data indicate that insulin production and consequently cellular insulin content would follow suit. On the other hand, others have shown that insulin secretion in RINm5F cultures grown on plastic substrates in the presence of serum, is highly variable with secretory rates of 0-144μU/10^6 cells over 24 hours (Muschel et al., 1986). These findings suggest that insulin secretory rates cannot be directly correlated with other data such as growth or differentiation. Although the determination of basal insulin secretion rates in RINm5F-MRC cultures was a preliminary study, the

objective of this study was realised; viz. the results verified that RINm5F-MRC cells produced insulin albeit in small amounts, and that insulin production had increased in the presence of sodium butyrate. Furthermore, the low rate of insulin secretion by RINm5F-MRC cultures correlated with the findings which thus far had indicated that insulin content was close to the lower limit of detection by peroxidase-staining techniques.

4.6 Flow cytometry:

To my knowledge, the use of flow cytometry to detect pancreatic hormonal peptides has not been reported previously. Studies of intracellular antigens by flow cytometry are relatively few compared to those of cell surface antigens (Schroff et al., 1984; Jacobberger et al., 1986; Levitt and King, 1987; Slaper-Cortenbach et al., 1988; Srivastava et al., 1992; Hjelsteun and Davies, 1994). Furthermore, flow cytometric studies have investigated intracellular proteins that are present in abundance and that are substantially larger than hormonal peptides. There are no available reports that have used flow cytometry to detect low abundance antigens. Flow cytometry provides rapid and accurate quantifiable measurements of intracellular antigens. During sample analysis, cell suspensions are aspirated and individual cells pass a beam of UV light that excites the fluorochrome attached to antibodies or other reporter molecules. The resultant emission of fluorescence is electronically amplified via a photomultiplier tube and fluorescence histograms are generated from fluorescent signals obtained from each cell.

4.6.1 Fixation and permeabilisation:

As required for whole-mount immunolabelling, cells must be fixed and permeabilised in order to retain antigenicity and to ensure adequate penetration of the immunoreagents (Larsson, 1988). The choice of fixation and permeabilisation may affect intracellular antigens and background fluorescence (Jacobberger et al., 1986; Larsson, 1988). While fixation and permeabilisation in methanol may yield very low non-specific fluorescence (Levitt and King, 1987; Hjelsteun and Davies, 1994), it is generally not suitable for fixing low molecular weight proteins such as hormonal peptides (Larsson, 1988). On the other hand, the present study demonstrated that insulin, glucagon and somatostatin antigenicity had been preserved in immunolabelled sections of rat pancreas that were fixed in 2% paraformaldehyde. By using the same fixative for flow cytometry, it was possible to establish to what extent the same levels of antigenicity observed in peroxidase-stained whole-mounts were detectable by flow cytometry.

The low recovery of RINm5F-MRC cells that were simultaneously fixed and permeabilised in this study is in contrast to that reported for samples simultaneously fixed and permeabilised in paraformaldehyde and acetone by Slaper-Cortenbach et al., 1988: these authors reported at least 85%

cell recovery from such samples. However, it is not clear whether cell recovery had been determined at the end of the labelling procedure in their study. The markedly reduced cell yield in simultaneously fixed and permeabilised RINm5F-MRC samples compared to those that were either sequentially fixed and permeabilised or fixed without permeabilisation strongly suggests that integral cellular components were extracted by the detergent before they could be cross-linked by the fixative, thereby increasing cell fragility. This conclusion is supported by previous reports that up to 25% of total protein is extracted by Triton X 100 from unfixed cells (Felix, 1982). Of significance to the present study, is that proteins with a molecular weight less than 70 000 daltons are preferentially extracted by Triton X 100 (Felix et al, 1982) from unfixed cells. Had the simultaneous fixation and permeabilisation procedure been used for immunolabeling studies, it is most likely that the hormonal peptides investigated in this study would have been extracted before cells were immunolabelled.

4.6.2 Establishment of an immunolabelling protocol for flow cytometry:

The poor or lack of staining in sectioned RINm5F-MRC cells and in whole-mounts respectively, and the confirmation by RIA that these cells secreted insulin into the culture medium, suggested that the peptide content was below the limit of detection by light microscopy. The use of strepavidin to amplify fluorescence signals has been discussed in the previous section. The SA-FITC system has been used previously to detect large T antigen (Srivastava et al., 1992). This article was published after the present flow cytometry experiments were initiated and was the first to address the analysis of low levels of intracellular antigens by flow cytometry.

Because of the vast increase in sensitivity of detection offered by flow cytometry, optimisation of the concentrations of immunoreagents was necessary to obtain the greatest sensitivity of detection in RINm5F-MRC cells. This study systematically examined the effect of different concentrations of each of the antisera and of SA-FITC on the fluorescent signal. Increased non-specific fluorescence due to the secondary antibody (IgG) and to SA-FITC has been previously investigated (Srivastava et al., 1992). The latter authors reported that fluorescence increased proportionally with increasing concentrations of IgG or SA-FITC. The finding that fluorescence was minimally changed by different concentrations of SA-FITC, whereas it was significantly increased with higher concentrations of IgG (Srivastava et al., 1992) contrasts with results obtained in the present study. A possible cause for the dissimilarity in the findings of Srivastava et al., 1992 and those using RINm5F-MRC cells, is that the former authors had titrated the IgG and SA-FITC against a fixed concentration of primary antiserum and thus effectively examined saturation of IgG binding with primary antibody and saturation of SA-FITC binding with antigen-antibody complexes. In the present study, titrations of biotinylated goat anti-rabbit IgG (b-GAR-IGG) and SA-FITC were done on RINm5F-MRC cells that had not been exposed to primary antiserum in order to examine the effect of different concentrations of these

immunoreagents on non-specific fluorescence. The small variability in fluorescence between samples labelled with different concentrations of b-GAR-IgG confirms that b-GAR-IgG did not bind non-specifically to RINm5F-MRC cells and was most likely removed by the buffer rinses before staining with SA-FITC. In contrast, the marked increase in fluorescence with increasing concentrations of SA-FITC can be attributed to either non-specific binding of SA-FITC or to failure to adequately wash SA-FITC out of the cells after staining. In the present study, further titrations of SA-FITC against fixed concentrations of b-GAR-IgG and primary antiserum showed that 5μg/ml SA-FITC produced fluorescence closest to background (unstained samples) and that this concentration best separated background from immunolabelled samples. In contrast others have reported that similar results were obtained using 20μg/ml SA-FITC (Srivastava et al., 1992).

Having established the concentrations of IgG and SA-FITC that showed least non-specific fluorescence, insulin, glucagon and somatostatin antisera were titrated against a fixed concentration of IgG and SA-FITC. Titrations were performed on untreated (to act as a negative tissue control) and sodium butyrate-treated (to act as the positive tissue control) cells. The greatest shift in fluorescence in that of sodium butyrate-treated samples above that of untreated cultures, was seen in samples labelled with the highest concentration of antiserum (i.e. 1/50 dilution). Although Srivastava et al., 1992 used different cell lines and antisera, their findings are in general agreement with the results of the above experiment. The purpose of performing primary antibody titrations on cells expressing the specific antigen (positive control) and on those not expressing the specific antigen (negative control), is to obtain the dilution of antiserum that best separates fluorescence (peak separation) between the two samples (see Srivistava et al., 1992). Others have shown that separation of fluorescence peaks is poor when background is high (Hjelsteun and Davies, 1994). In the present study, fluorescence of untreated RINm5F-MRC cells should effectively be close to background. Although this was shown to be true for untreated cultures labelled to detect insulin, fluorescence was markedly raised above that of background in untreated cultures labelled to reveal glucagon and somatostatin. Therefore, lower concentrations of glucagon and somatostatin antisera were used in order to reduce "background" fluorescence.

Specificity controls analysed by flow cytometry confirmed that not more than 2% of the cell population was insulin positive whereas up to 38% of cells from sodium butyrate-treated cultures contained detectable levels of insulin. In contrast, the apparent glucagon- and somatostatin-like immunoreactivity was shown to be non-specific. This finding is consistent with data from immunostained RINm5F-MRC whole-mounts. An intriguing observation is the raised fluorescence in all specificity controls in sodium butyrate-treated samples labelled to detect glucagon and somatostatin compared to that in untreated samples. Possible causes for the observed increase in fluorescence are

discussed below.

A likely cause for the non-specific staining is the presence of non-specific antibodies in the antiserum. Examination of whole-mounts by fluorescence microscopy revealed that staining was associated with the cell surface of RINm5F-MRC cells. In addition, increased fluorescence, markedly above that of background, in samples incubated with normal rabbit serum suggested that the fluorescence in samples labelled to reveal glucagon or somatostatin was due to non-specific antibodies in rabbit serum. Non-specific staining seen in peroxidase-stained whole-mounts incubated with rabbit serum is consistent with these findings. Examination of cells in the culture dishes and in suspension showed that cell size had increased in the presence of sodium butyrate. Therefore it is likely that non-specific binding would occur over a greater cell surface area, and thereby increase fluorescence.

A second cause for the non-specific staining could be due to the presence of receptors to the antigens in the pre-absorbed antiserum on the cell surfaces. Others have established that RINm5F cells contain glucagon and somatostatin receptors on their cell surface (Korman et al., 1985; Sullivan and Schonbrunn, 1988). Excess antigen (such as glucagon or somatostatin) in pre-absorbed antiserum solutions may bind to cell surface receptors, whereupon antibodies in turn bind to the antigen (Larsson, 1988). In the present study, the fluorescence of glucagon and somatostatin absorption controls from untreated and sodium butyrate-treated cultures was greater than that of samples labelled with anti-glucagon serum or anti-somatostatin serum. This suggests that the difference between fluorescence of absorption controls and of glucagon- or somatostatin-stained preparations was indeed due to binding of antigens to cell surface receptors. Furthermore, due to the increased cell size in sodium butyrate-treated cultures, the increase in fluorescence is most likely caused by binding of antigens to a higher number of glucagon or somatostatin receptors on each cell. However, the extent to which increased fluorescence is solely caused by non-specific antibodies in rabbit serum or by antigens that have bound to their corresponding cell surface receptors, can only be resolved once cells are labelled with antiserum in which non-specific antibodies have been removed.

4.7 Conclusion:

Results from immunolabelling experiments analysed by flow cytometry, immunoblotting and RIA, established that RINm5F-MRC cells contained and secreted low levels of insulin or pro-insulin. At later passages, intracellular insulin content in individual cells was below the limit of detection by light and fluorescence immunocytochemistry, yet was revealed after cultures had been exposed to sodium butyrate for three days. Results from flow cytometry and immunofluorescence microscopy established that a portion of the cultures had increased their cellular insulin content in response to sodium butyrate. Nevertheless, cellular insulin content was substantially lower than that seen in islet cells from

frozen sections of rat pancreas. Flow cytometry, immunocytochemistry and immunoblotting also established that all glucagon- and somatostatin-like immunoreactivity was non-specific: evidence from fluorescence microscopy showed that non-specific binding was associated with the cell surface of RINm5F-MRC cells. From these results, it appeared that RINm5F-MRC cells had dedifferentiated with time in culture. Furthermore, this apparent dedifferentiation was associated with a faster growth rate. The ultrastructure of RINm5F-MRC cultures confirmed that morphologically differentiated hormone-producing phenotypes were not present and that the majority of the cell population consisted of agranular cells. The prominent number of agranular cells in RINm5F-MRC cultures and the detection of proinsulin and not insulin in immunoblots, is consistent with the report that agranular insulinomas contained higher levels of proinsulin than insulin (Creutzfeldt, 1985). Although proinsulin was the only hormone detected in RINm5F-MRC cultures, the present study cannot confirm that RIN cultures comprise a truly homogenous cell population of insulin-producing cells, as insulin-immunoreactivity was undetectable in the majority of the cells. Further studies would be required to unequivocally establish whether these cultures produce peptides other than insulin. Only then will it be possible to determine whether this cell line is suitable for the study of islet cell differentiation in vitro.

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