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The Establishment of Hybrid Cell Lines from Human Pancreas.

A Thesis submitted for the Degree of Doctor of Philosophy (Ph.D.), at the University of Dublin, Trinity College.

By

George W. Reid

Department of Biochemistry,
Trinity College, Dublin.

September, 2000.
Declaration.

I declare that, except where otherwise stated in the acknowledgements, this thesis is entirely my own work and it has not been submitted to this or any other university as an exercise for a degree. I give permission to the library to lend or copy this thesis.

Signed:

[Signature]

George W. Reid

September 2000.
Dedicated to
Prof. James G. Devlin

A man of medicine and science.
Publications from this thesis.

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Abbreviations.

ABC  ATP-binding cassette
ACTH  adrenocorticotrophic hormone
AGE  advanced glycation end-product
ADP  adenosine 5'-diphosphate
AMP  adenosine monophosphate
ANP  atrial natriuretic peptide
ATP  adenosine 5'-triphosphate
BCA  bicinchoninic acid.
BCIP  5-bromo-4-chloro-3-indoyl phosphate
BSA  bovine serum albumin

CaM Kinase II  Ca$^{2+}$/calmodulin-dependent protein kinase II

CAPS  Ca$^{2+}$-dependent activator protein for secretion
CCK  cholecystokinin
cDNA  complementary deoxyribonucleic acid
CGRP  calcitonin gene-related peptide
CoA  coenzyme A
cpm  counts per minute
CREB  cyclic AMP response element binding factor
CRF  corticotrophin-releasing factor
CSP  cysteine string protein
Cv  coefficient of variation
DAB  3,3-diaminobenzidine tetrahydrochloride
DAG  diacylglycerol
DBI  diazepam-binding inhibitor
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
EDTA  ethylenediaminetetraacetic acid
FCS  foetal calf serum
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G protein</td>
<td>guanosine triphosphate (GTP)-binding protein</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>gravitational force at sea level</td>
</tr>
<tr>
<td>Gab-1</td>
<td>Grb 2-associated binder 1</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
</tr>
<tr>
<td>GHRH</td>
<td>growth hormone-releasing hormone</td>
</tr>
<tr>
<td>GIP</td>
<td>gastrin inhibitory peptide</td>
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<tr>
<td>Grb 2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GRP</td>
<td>gastrin-releasing polypeptide</td>
</tr>
<tr>
<td>Gs</td>
<td>stimulatory GTP-binding protein</td>
</tr>
<tr>
<td>GSIS</td>
<td>glucose-stimulated insulin secretion</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H(G)PRT</td>
<td>hypoxanthine (guanine) phosphoribosyl transferase</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine &amp; aminopterin &amp; thymidine</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IA-2</td>
<td>protein tyrosine phosphatase-2</td>
</tr>
<tr>
<td>IAPP</td>
<td>islet amyloid polypeptide (or Amylin)</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell autoantibodies</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IEF1</td>
<td>insulin enhancer factor 1</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrates</td>
</tr>
<tr>
<td>IU</td>
<td>Internationally standardised units</td>
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</tbody>
</table>
KRBB: HEPES-balanced Krebs-Ringer bicarbonate buffer & 0.5% BSA.

l: litre

LADA: latent immune diabetes in adults

LDCV: large dense core vesicles

lin: linear

log: logarithmic

m (prefix): milli \((10^{-3} \text{ x})\)

μ (prefix): micro \((10^{-6} \text{ x})\)

m: meter

MAP kinases: mitogen-activated protein kinases

MHC: Major Histocompatibility Complex

min: minute

MIP: molluscan insulin-like peptide

MODY: maturity-onset diabetes of the young

mol: moles

MRDM: malnutrition-related diabetes mellitus

MW: molecular weight

n: number of observations

n (prefix): nano \((10^{-9} \text{ x})\)

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NBD: nucleotide-binding domains

NBT: Nitro Blue Tetrazolium

NEFA: non-esterified fatty acids

NIDDM: non-insulin-dependent diabetes mellitus

NIMGU: non-insulin-mediated glucose uptake

NPY: neuropeptide Y

NSF: N-ethylmaleimide-sensitive factor

P: significance value

p (prefix): pico \((10^{-12} \text{ x})\)

PACAP: pituitary adenylate cyclase-activating polypeptide
PAGE  polyacrylamide gel electrophoresis  
PBS  phosphate buffered saline  
PDH  pyruvate dehydrogenase  
PDX1  pancreatic duodenum Xenopus homeodomain factor-1'  
(or Pancreatic/duodenal homeobox-1)  
PEG  polyethylene glycol  
PH  pleckstrin homology  
PHAS-I  phosphorylated heat- and acid- stable protein regulated by insulin  
PHHI  persistent hyperinsulinaemic hypoglycaemia of infancy  
PI4P5K  phosphatidylinositol-4-phosphate 5-kinase  
PKA  protein kinase A  
PKC  protein kinase C  
PMA  phorbol myristate acetate  
PMCA  plasma membrane Ca^{2+}-ATPase  
PMSF  phenylmethylsulfonyl fluoride  
PP  pancreatic polypeptide  
PTB  phosphotyrosine binding  
PtdIns 3-kinase  phosphatidylinositol 3-kinase  
PYY  peptide YY  
RAGE  cell surface receptor for AGE  
RIA  radio-immunoassay  
RNA  ribonucleic acid  
rpm  revolutions per minute  
SAPK2  stress-activated protein kinase 2  
SDS  sodium dodecyl sulfate [lauryl sulfate]  
SEM  standard error of the mean  
SERCA  sarco-endoplasmic reticulum Ca^{2+}-ATPase  
SH2  Src homology 2  
Shc  Src and collagen-homologous protein  
SRP  signal recognition particle  
SSR  signal sequence receptor  

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<td>TGN</td>
<td>trans Golgi network</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domains</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>U</td>
<td>standardised units</td>
</tr>
<tr>
<td>USF</td>
<td>upstream stimulating factor</td>
</tr>
<tr>
<td>v</td>
<td>versus</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume ratio</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>w/v</td>
<td>weight to volume ratio</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>%</td>
<td>percent</td>
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<td>&amp;</td>
<td>and</td>
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Summary.

The need for research into diabetes mellitus is ever increasing. Of specific interest are the mechanisms responsible for glucose-responsive insulin secretion and the autoimmune destruction of pancreatic β-cells. However, assessment of normal pancreatic β cell function at the molecular level continues to be hampered by the heterogeneity and limited yields of viable human experimental material. Pancreatic β cell lines have proven extremely useful in overcoming some of these problems, and thus the potential usefulness of a human pancreatic β cell line has long been recognised. At the onset of this project no functional human β cell line had been established, despite repeated attempts. It is only in the last year that such a cell line has at last been generated. Of all the reported attempts to establish a human β cell line, or a β cell line exhibiting human characteristics, none had employed the strategy of a cell fusion involving normal human β cells.

To this end human islet cells were isolated using two different protocols and fused to three different immortal cell lines. Slightly differing fusion protocols were employed and fused cells were cultured in a variety of selective media regimes. Fusions with CRI-G5 and RINm5F rat insulinoma cell lines resulted in 241 and 14 cell lines respectively. No stable cell lines were generated from fusions with the PDH-1 murine myeloma cell line. The use of glycerol during islet isolation or the presence of 16 μmol/l thymidine in the selective media resulted in failure to generate cell lines. The type of PEG and the batch of foetal calf serum did not present any detectable effects on the fusion success rate. The most successful fusion rate was observed with fusions of islet cells to CRI-G5 at a cell ratio of 1:3 and with a selective medium containing 11.1 mmol/l glucose, 2.31 μmol/l azaserine, and 0.2 mmol/l hypoxanthine.

Subcloning established fourteen HiCRI cell lines which were characterised with regard to: the species of secreted insulin; responsiveness of insulin secretion to glucose; insulin secretion levels; response to common insulin secretagogues; insulin
content; glucagon secretion; general morphology; growth rates; the presence of human antigens; and chromosome analysis.

The secretion of human insulin and other human β cell characteristics were not detected. However, the novelty of the cell lines was confirmed by the characterisation of the cell lines, and comparison to the CRI-G5 fusion partner cell line. The cell lines themselves displayed a wide and varied range of characteristics, with no two cell lines exactly similar. The cell lines therefore represent a panel of β cell lines which should prove useful models for future studies into β cell function and dysfunction.
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Chapter 1

Introduction.
1.1 The History of Diabetes.

"Diabetes is a dreadful affliction, not very frequent among men, being a melting down of the flesh and limbs into urine. The patients never stop making water and the flow is incessant, like the opening of aqueducts. Life is short, unpleasant and painful, thirst unquenchable, drinking excessive, and disproportionate to the large quantity of urine, for yet more urine is passed. One cannot stop them either from drinking or making water. If for a while they abstain from drinking, their mouths become parched and their bodies dry; the viscera seem scorched up, the patients are affected by nausea, restlessness and a burning thirst, and within a short time, they expire".

...... Aretaeus of Cappadocia 2nd century AD

(Papaspyros, 1964, adapted by MacFarlane et al. 1997)

The Ebers papyrus, dating from c. 1550 BC, testifies to the long history of diabetes. This papyrus contains descriptions of various diseases, including a polyuric state resembling diabetes mellitus. The first known clinical description of diabetes is credited to Aulus Cornelius Celsus (c. 30 BC – 50 AD), but it was Aretaeus of Cappadocia (c. 30 – 90 AD) who first used the term ‘diabetes’, which is from the Ionian Greek meaning ‘to pass through’ or ‘siphon’, as a generic description for conditions causing increased urine output. However, he concluded that diabetes was due to a fault in the kidneys and, despite the clarity of his account, Aretaeus would not have been able to distinguish it from the various non-diabetic disorders that present with polyuria. Likewise the Roman physician, Galen (131 – 201 AD) also emphasised the symptoms of excessive thirst and polyuria.

The association of polyuria with a sweet-tasting substance in the urine was first reported in Sanskrit literature dating from the 5th to 6th century AD at the time of two notable Indian physicians, Susruta and Charuka. The urine of certain polyuric patients was described as tasting like honey, being sticky to the touch and strongly attracting ants. During the same era, Chinese and Japanese physicians also described and drew attention to the sweetness of diabetic urine. Indian descriptions of this time appear to distinguish between two forms of diabetes, one affecting older, fatter people and the other affecting thin people who did not survive long.
The fact that diabetic urine tasted sweet was subsequently emphasised by Arabic physicians during the 9th to 11th centuries AD. Most notable of whom was Avicenna (960 – 1037 AD), who recommended treatment with lupin, fenugreek and zedoary seeds. To his credit, this mixture does indeed possess some mild hypoglycaemic activity.

As for so many other subjects diabetes was a victim of the historical period referred to as the ‘Dark Ages’. It was not until the 16th century that European physicians began to regain some of the lost knowledge of diabetes. But it was not until Thomas Willis made reference to the sweet taste of diabetic urine (Willis, 1674-75) that West European physicians finally caught up with their historical contemporaries of almost 1000 years earlier.

The eighteenth century saw the understanding of diabetes advance for the first time in 1000 years, most notably by Matthew Dobson who showed that the serum and urine of a diabetic patient contained a sweet-tasting substance (Dobson, 1776) and he proved that this material was sugar. He proposed that this was evidence that diabetes might be a systemic disorder rather than a disease of the kidneys. A few years after Dobson’s important paper, another English physician, John Rollo, published a paper in which he referred to the disease as diabetes mellitus. Using the adjective ‘mellitus’, (from the Latin and Greek roots for ‘honey’) to distinguish the disease from other polyuric diseases in which glycosuria does not occur (Rollo, 1797). Another important observation during this era was made by Thomas Cawley who noticed that diabetes may follow damage to the pancreas, for example through calculus formation (Cawley, 1788).

The endocrine, blood glucose-lowering properties of the pancreas began to be clarified in the second half of the 19th century. When Oscar Minkowski and Josef Von Mering removed the pancreas from a dog to determine the role of the organ in fat digestion, the dog unexpectedly displayed the typical signs of diabetes, with polyuria and incontinence, insatiable thirst, hyperphagia and wasting. Minkowski
showed that the dog was glycosuric and hypoglycaemic (Von Mering and Minowski, 1890). Thus the role of pancreatic disorders in causing diabetes was firmly established, and the attention of many workers was focused on the isolation of a hypothetical pancreatic product responsible for lowering blood glucose levels.

Paul Langerhans had identified clusters of cells within the pancreas (Langerhans, 1869), but it was Edouard Laguesse who suggested that these clusters might constitute the endocrine tissue of the pancreas. He named these clusters the ‘islets of Langerhans’ (Laguesse, 1893), which prompted Jean De Meyer to name the still hypothetical pancreatic product ‘insuline’ (from the Latin ‘insula’, and island), which he suggested was produced by the islets of Langerhans (De Meyer, 1904).

1.1.1 Discovery of Insulin.

On the 3rd May 1922 a group of workers in Toronto, Canada, presented their findings to the Association of American Physicians in Washington DC, in a paper entitled ‘The effect produced on diabetes of extracts of pancreas’. They were given a standing ovation for what the audience acclaimed as one of the greatest achievements of modern medicine, and all this within one year of the start of their experiments. Frederick Grant Banting and Charles H. Best performed much of the initial work of this group under the supervision of J.J.R. Macleod, who made the important suggestion to use acidified ethanol in the purification process. In truth, however, their work had been overshadowed by earlier reports (Zuelzer, 1907; Kleiner 1919; Paulesco, 1921), and it was not until James B. Collip joined the team that their landmark findings were made. Collip improved the extraction and purification technique of the active principle, which was used successfully for the first time in January 1922 in Toronto General Hospital. The subject Leonard Thompson, a 14-year old boy dying of diabetes, was injected with Collip’s preparation upon which his blood glucose returned to normal and his glycosuria and ketonuria were abolished. Thus this simple therapeutic trial inaugurated the use of insulin in the treatment of diabetes mellitus (Banting and Best, 1922). The group initially called the active principle ‘isletin’, but later accepted Macleod’s suggestion of ‘insulin’. It was only later that they discovered Jean De Meyer had already given
this title to the putative product of the islets of Langerhans. For this discovery Banting and Macleod received the Nobel prize in 1923, and announced that they would share the prize money with Best and Collip. After collaboration between the Toronto workers, Eli Lilly and Company, and various European scientists, insulin was widely available by late 1923. Suddenly, the question of the speed of death in diabetes was replaced by that of the quality of life with insulin and the central role of insulin in diabetes mellitus was firmly established.

1.1.2 Diabetes Mellitus after the discovery of insulin.

No longer a hypothetical active pancreatic product, the discovery of insulin allowed for research into diabetes to be focused, (around insulin), and the pace of discoveries quickened as a result. Many of these discoveries involved the first use of certain techniques and as such were landmarks not just for diabetes research but medical research as a whole. As a result, researchers into diabetes mellitus have received an unprecedented number of Nobel prizes for their work. Some, but by no means all, of these important landmarks are described below.

Shortly after it was established that insulin was a protein the first crystalline insulin was obtained and that these crystals were the zinc salt of the protein (Scott, 1934). Elucidation of the structure of insulin has had far-reaching consequences for many aspects of diabetes, ranging from the mechanism of the hormone’s action to the pharmokinetics of injected insulin and the design of novel insulin analogues. In 1955 Frederick Sanger and colleagues determined the sequence of amino acids in insulin, the first time that a protein had been completely sequenced. (Brown et al. 1955). This enabled insulin to be chemically synthesized, the first protein to be artificially synthesized (Katsoyannis et al. 1963). The insulin precursor, proinsulin, was identified in 1967 (Steiner and Oyer, 1967).

Between 1942 and 1944, Auguste Loubatières demonstrated the insulin-releasing ability of certain sulphonamides and led to the development of the clinically useful sulphonylurea drugs which are still used today to treat non-insulin-dependent
diabetes. A notable achievement by its own right, it was all the more remarkable for having been performed despite the occupation by German armed forces.

The technique of radioimmunoassay was developed in 1958 to measure insulin (Yalow and Berson, 1958). This achievement has had huge implications, as it not only allowed for the accurate measurement of insulin and hence was the cornerstone of much of the following diabetes research, but the technique has since been adapted to many other compounds. It may be an exaggeration to state that the entire field of endocrinology, clinical and research, has been based around this achievement of Yalow and Berson.

The sequence of rat insulin genes was reported in 1977 (Ullrich et al. 1977), and the insulin gene was one of the first human genes to be cloned and sequenced (Bell et al. 1980). Human insulin was also the first protein to be produced commercially by recombinant DNA techniques in 1979 (Goeddel et al. 1979), and the first primary sequence of a peptide hormone receptor was that of the insulin receptor, which was cloned and sequenced in 1985 (Ullrich et al. 1985; Ebina et al. 1985).

As a clear indication of the impact that the discovery of insulin has had, the long-term complications of diabetes have been a particular research priority for quite some time. Specifically the inability of insulin treatment to prevent chronic damage to the eyes, kidneys, nerves and blood vessels. Among the many achievements in this field, two of particular note were the development of a highly sensitive radioimmunoassay to measure albumin in urine has allowed the natural history of diabetic nephropathy to be defined (Keen and Chlouverakis, 1963) and the implicating of hyperglycaemia-induced overactivity of the polyol pathway in cataract formation (Kinoshita, 1974).

Research into diabetes mellitus has made great strides and gained much knowledge since the discovery of insulin. The advent of molecular biology only served to fuel this progress even more and recent years have seen our understanding of diabetes advance dramatically. Diabetes mellitus is now a major global health problem. The
incidence and prevalence of diabetes is escalating around the world, especially in developing and newly industrialised nations. The estimated number of 80 million sufferers in 1990 with projections to 300 million by 2025 (Zimmet, 1991; Betteridge, 2000). Our knowledge of diabetes is, however, still not total, but hopefully this will be rectified in the near future!

1.2 Insulin.

Diabetes mellitus is the name given to the clinical description of patients with a number of symptoms arising from raised glucose levels due to a malfunction in the body’s normal control of glucose homeostasis by insulin. Insulin is a polypeptide hormone which (as is discussed in greater detail in later Section 1.5) is synthesized in, and released into the blood circulation from, the β cells of the islets of Langerhans, which represent the endocrine component of the pancreas. The vast bulk of the pancreas organ is of exocrine nature, with endocrine islets of varying sizes embedded through out it. The islets of Langerhans consist of four different cell types:-

- A (or α) cells which secrete glucagon.
- β cells which secrete insulin.
- D (or δ) cells which secrete somatostatin.
- pancreatic polypeptide (PP) secreting cells.

The β cells can compose more than 60 per cent of the adult islet volume (Rahier et al. 1983), organised as a central core of the islet with the other cells arranged around them. As shown in Figure 1.2, the insulin molecule consists of two polypeptide chains, designated A and B, which consist respectively of 21 and 30 amino acid residues. The chains are linked by two disulphide bridges between residues (A)7 and (B)7, and (A)20 and (B)19. The A chain also contains an intrachain disulphide bridge linking residues 6 and 11. The A chain contains two helical regions involving the residues 1-18 and 13-19, whose axes are almost antiparallel. These are connected by an extended loop, which is bridged by the intrachain disulphide bond. The dominant secondary structural element of the B chain is the α-helical section.
between residues 9 and 19, followed by a sharp turn to the residue at position 23 (Wood and Gill 1997).

Figure 1.2 The primary structure of human insulin.

<table>
<thead>
<tr>
<th>A chain</th>
<th>B chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal</td>
<td>N-terminal</td>
</tr>
<tr>
<td>1 Gly</td>
<td>1 Phe</td>
</tr>
<tr>
<td>2 Ile</td>
<td>2 Val</td>
</tr>
<tr>
<td>3 Val</td>
<td>3 Asn</td>
</tr>
<tr>
<td>4 Glu</td>
<td>4 Gln</td>
</tr>
<tr>
<td>5 Gln</td>
<td>5 His</td>
</tr>
<tr>
<td>6 Cys --- S --- 7 Cys</td>
<td>6 Leu</td>
</tr>
<tr>
<td>7 Cys</td>
<td>7 Cys --- S --- 8 Gly</td>
</tr>
<tr>
<td>8 Thr</td>
<td>8 Gly</td>
</tr>
<tr>
<td>9 Ser</td>
<td>9 Ser</td>
</tr>
<tr>
<td>10 Ile</td>
<td>10 His</td>
</tr>
<tr>
<td>11 Cys</td>
<td>11 Leu</td>
</tr>
<tr>
<td>12 Ser</td>
<td>12 Val</td>
</tr>
<tr>
<td>13 Leu</td>
<td>13 Glu</td>
</tr>
<tr>
<td>14 Tyr</td>
<td>14 Ala</td>
</tr>
<tr>
<td>15 Gln</td>
<td>15 Leu</td>
</tr>
<tr>
<td>16 Leu</td>
<td>16 Tyr</td>
</tr>
<tr>
<td>17 Glu</td>
<td>17 Leu</td>
</tr>
<tr>
<td>18 Asn</td>
<td>18 Val</td>
</tr>
<tr>
<td>19 Tyr</td>
<td>19 Cys</td>
</tr>
<tr>
<td>20 Cys --- S --- 20 Gly</td>
<td>20 Gly</td>
</tr>
<tr>
<td>21 Asn</td>
<td>21 Glu</td>
</tr>
<tr>
<td>22 Arg</td>
<td>22 Arg</td>
</tr>
<tr>
<td>23 Gly</td>
<td>23 Gly</td>
</tr>
<tr>
<td>24 Phe</td>
<td>24 Phe</td>
</tr>
<tr>
<td>25 Phe</td>
<td>25 Phe</td>
</tr>
<tr>
<td>26 Tyr</td>
<td>26 Tyr</td>
</tr>
<tr>
<td>27 Thr</td>
<td>27 Thr</td>
</tr>
<tr>
<td>28 Pro</td>
<td>28 Pro</td>
</tr>
<tr>
<td>29 Lys</td>
<td>29 Lys</td>
</tr>
<tr>
<td>30 Thr</td>
<td>30 Thr</td>
</tr>
</tbody>
</table>

The amino acid sequence of human insulin. Amino acids are represented as standard three letter abbreviations, with each amino acid numbered according to its position from the N-terminal end of each peptide chain. Also shown are the three disulphide (S-S) bonds. (Adapted from Granner DK, 1985).
The residues 1-8 and 23-30 are in extended conformation when the insulin is not in crystalline form, and these regions are considered to be somewhat mobile, its exact conformation being determined by forces of aggregation (Derewenda et al. 1989). This latter characteristic would appear to be important for its biological activity (Dodson et al. 1983).

Insulin is phylogenetically ancient, being found not only in mammals, but also in birds, reptiles, both teleost and elasmobranch fish, and the very ancient hagfish. The structure of insulin and its gene have been highly conserved through evolution, evidently reflecting its crucial importance in the regulation of metabolism. Although variations are seen between species, structurally important residues of the core are generally conserved. Most notable of which are the disulphide bridges and glycine residues at bends in the polypeptide chains (Wood and Gill 1997). Porcine insulin is the closest to human insulin, with the only difference being the residue at position 30 on the B chain being alanine as opposed to threonine for human insulin. Rat I insulin is as for human insulin with the exception of residue A4 being aspartic acid (Asp) and B30 being serine (Ser). Rat II insulin has these same different residues but also has methionine (Met) at position B29.

The insulins are members of a large superfamily of molecules which all have some degree of homology in their sequence and probably in their tertiary structure (Wood and Gill, 1997). The insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), which are responsible for most of the insulin-like activity of serum, are single-chain proteins which contain sequences analogous to the C peptide and B chain moieties of the insulin precursor, proinsulin, and the A chain (extended at its C terminal by a D-peptide chain). IGF-1 and IGF-2 also exhibit close homology with insulin in the structurally important core regions. Bombyxin and molluscan insulin-like peptide (MIP), from the silkworm and mollusc respectively, have been described as distant members of the insulin superfamily. These peptides would appear to both play important roles in growth and development (Smit et al. 1988; Nagasawa et al. 1986). They both have the essential distribution of disulphide bridges and non-polar
residues capable of packing a hydrophobic core of similar volume to that of insulin (Jhoti et al. 1987).

The basic architecture of insulin seems, therefore, to be of ancient design, from which evolution has generated a family of molecules with a wide range of functions. Indeed insulin displays a range of functions in humans, including protein synthesis, mitogenesis and growth (Virkamäki et al. 1999). The primary role of insulin is undoubtedly in maintaining glucose (or fuel) homeostasis, and the definition of diabetes mellitus is chronic hyperglycaemia due to a malfunction in the body's normal control of glucose homeostasis by insulin. As such, much of the discussion contained herein is limited to glucose and fuel homeostasis.

1.3 Fuel homeostasis.

The energy requirements of the various tissues, and the metabolic substrates they use as fuels, differ considerably. The ways in which these substrates are provided also vary according to whether nutrients are entering the circulation from a meal, or being produced by the body's own energy stores. Energy needs are met by the oxidation of carbohydrate, fat and protein. Although these are ultimately derived from the diet, if they are not immediately required they must be stored so that they can subsequently be metabolised in a controlled fashion during fasting or starvation.

Carbohydrates, and glucose in particular, are an important source of energy for most living organisms. Some tissues, including the brain, blood cells and the renal medulla, have an obligatory requirement for glucose. The most prominent of which is the brain, which consumes up to 80% of the glucose utilized at rest after an overnight fast (Ferrannini and Groop, 1989). With prolonged fasting, the availability of ketone bodies increases markedly, and ketones become the major oxidative fuel of the brain. As a result the obligatory glucose requirement for the brain decreases, but is always an obligatory requirement (Kruszynska, 1997).

Because the lipid bilayers that make up cell membranes are impermeable to carbohydrates, transport systems are required for these compounds. For hexoses such
as glucose, fructose and lactose two different groups of transporters have been so far described. Sodium-linked glucose transporters, which actively transport glucose against a glucose-concentration gradient by using sodium co-transport as an energy source (Wright et al. 1991). The other group of transporters convey glucose by facilitated diffusion down glucose-concentration gradients in a process that does not require energy (Pedersen, 1993; Gould and Holman, 1993). This group consists of five homologous trans-membrane proteins, GLUT-1, 2, 3, 4, and 5, that are encoded by distinct genes. The GLUT proteins [see Table 1.3] have distinct substrate specificities, kinetic properties, and tissue distributions that dictate their functional roles, all with the common basic structure of 12 helical transmembrane domains. The transport of glucose across the cell membranes into muscle and fat cells is the rate-limiting step of glucose utilization in mammals (Owen et al. 1969; Owen and Reichard, 1971; McGuinness et al. 1993).

GLUT-1 is very widely distributed throughout the tissues, and probably mediates much of the body’s basal glucose transport and non-insulin-mediated glucose uptake (NIMGU). GLUT-2 is of particular interest in the islet of Langerhans β cell, where its high $K_m$ allows glucose entry into the β cell to increase in direct proportion to extracellular glucose levels. GLUT-3 is expressed by neurons and, together with GLUT-1 in the blood-brain barrier, permits glucose entry to the brain. GLUT-4 is expressed by insulin-sensitive tissues, such as skeletal muscle, cardiac muscle and fat cells. It is regulated by insulin, which has little effect on the other GLUT proteins, and unlike the other GLUT proteins, which lie in the membrane, GLUT-4 is normally located in vesicles in the cytoplasm. Insulin stimulates glucose transport in various ways. Firstly, it recruits GLUT-4 units from the intracellular pool, causing the vesicles that contain them to be translocated to the cell membrane, with which they fuse; in this situation the GLUT-4 units become able to function as pores through the membrane (Pedersen, 1993; Gould and Holman, 1993). Insulin may also enhance the activity of individual GLUT-4 units (Holman et al. 1990; Shepherd and Kahn, 1993) and is important in maintaining normal levels of the GLUT-4 protein in muscle and fat (Bourey et al. 1990). There would also appear to be an intracellular pool of GLUT-4 units that is not sensitive to insulin, but physical exercise can result
in this pool being drawn upon resulting in increased expression of GLUT-4 in muscle and fat cells (Coderre et al. 1995; Shepherd and Kahn, 1993).

Table 1.3 Characteristics of the five facilitated-diffusion glucose transporters.

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Approximate K&lt;sub&gt;m&lt;/sub&gt; for glucose (mmol/litre)</th>
<th>Tissue distribution</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT 1</td>
<td>20</td>
<td>Widely expressed: high concentrations in brain, erythrocytes and endothelial cells.</td>
<td>Constitutive glucose transporter.</td>
</tr>
<tr>
<td>GLUT 2</td>
<td>42</td>
<td>Kidney, small intestine epithelia, liver and pancreatic β cells.</td>
<td>Low-affinity glucose transporter: with a role in sensing glucose concentrations in pancreatic islets.</td>
</tr>
<tr>
<td>GLUT 3</td>
<td>10</td>
<td>Neurons and placenta.</td>
<td>High-affinity glucose transporter.</td>
</tr>
<tr>
<td>GLUT 4</td>
<td>2-10</td>
<td>Skeletal muscle, cardiac muscle and adipose cells.</td>
<td>Insulin-responsive glucose transporter</td>
</tr>
<tr>
<td>GLUT 5</td>
<td>NA</td>
<td>Small intestine, sperm, kidney, brain, adipose cells and muscle.</td>
<td>Fructose transporter with low affinity for glucose.</td>
</tr>
</tbody>
</table>

Where K<sub>m</sub> denotes the Michaelis-Menten constant, and NA not applicable (From Shepherd and Kahn, 1999).

With reference to the brain, GLUT-3 expression in neurons and GLUT-1 in the blood-brain barrier permit the entry of glucose to the brain. Neither of these transporters are insulin sensitive. Glucose entry to most of the brain cannot be regulated by insulin or changes in blood flow, as it is in other tissues, and so the transport of glucose into the brain is dependent on blood-glucose concentration. In the brain and other tissues, glucose uptake increases as blood glucose levels rise, through a mass-action effect, provided that glucose is metabolised and low
concentrations are maintained intracellularly. The process is self-regulated to some extent, in that excessive glucose entry will lead to the accumulation of glucose-6-phosphate, which in turn inhibits the hexokinase that catalyses its formation. As a result intracellular free glucose levels rise and the concentration gradient falls, and glucose entry decreases (Kruszynska, 1997).

Prolonged elevation of blood glucose concentrations can result in blindness, renal failure, cardiac and peripheral vascular disease, and neuropathy. In the short term elevated blood glucose concentrations can impair cerebral function by causing osmotic diuresis, dehydration and loss of electrolytes. Perhaps more critical, however, are the effects on the brain of low blood concentrations of glucose, which can be to cause seizures, loss of consciousness, and death. Cerebral function is therefore critically dependent upon maintaining blood glucose concentrations within tight limits, to ensure a steady supply of glucose to the brain (Kruszynska, 1997).

Blood glucose levels rapidly rise after a meal. To counteract this, glucose is removed from the blood and stored in skeletal muscle and the liver as glycogen in the process of glycogenesis. This storage continues until blood glucose levels reach normal physiological limits. In this post-absorption stage the glucose level depends on the rates of entry of glucose into the circulation and of its uptake into the tissues, and under basal conditions these rates remain equal. As glucose is utilized and the blood concentration falls the glycogen stores are remobilized (Cherrington et al. 1987). In this remobilization glycogen is broken down by the process of glycogenolysis to yield glucose-6-phosphate, which is used in skeletal muscle to meet that tissue’s own needs. Even then, muscle glycogen is generally conserved during fasting, in order to meet any sudden demands for muscular activity. By contrast the glucose-6-phosphate produced in the liver can give rise to glucose which can be released for use by other tissues. The hepatic glycogen store, is one of the first stores to be called upon to keep the blood glucose levels within a tight range. Biopsy studies on humans have shown that the hepatic glycogen store is largely depleted after 24 h starvation (Hultman, 1978), but despite this blood glucose concentration remains constant (Cahill, 1970). This is due to the mobilization of fatty acids from the adipose tissue.
triacylglycerol store to supply gluconeogenic precursors and some tissues, notably skeletal muscle, changing their fuel of choice to fatty acids. Calculations based on the increase in the concentration of fatty acids in starvation and their turnover rate indicate that fatty acid oxidation can account for most the energy requirements of the tissues after 24 h starvation (Newsholme and Leech, 1983).

As well as by glycogenolysis, the liver can synthesize glucose via gluconeogenesis. The kidneys can also perform this de novo synthesis, indeed the liver and kidney are the only two organs able to release glucose for use by other tissues. During fasting, most of the glucose in the blood is supplied by the liver via glycogenolysis and gluconeogenesis, and most of this is used by the brain. Although quantitatively lactate is the most important gluconeogenic precursor, as up to 80% of lactate carbon is itself derived from glucose, the conversion of lactate does not represent net glucose synthesis (Kruszynska, 1997). 'Novo’ or ‘new’ glucose is derived from glycerol or amino acids, which are derived from the stores of fat and from protein, respectively.

Fat mass represents by far the major form of energy stores in the body. Triglyceride can be broken down in the process of lipolysis into glycerol and three molecules of non-esterified fatty acids (NEFA). The glycerol can be converted into glucose through gluconeogenesis in the liver, and the fatty acids may be oxidized. Partial oxidation of the fatty acids in the liver yields ketone bodies, which are important fuel substrates for the brain. Although there are no specific depots of storage proteins or amino acids, many body proteins are continually turned over yielding amino acids that are reincorporated into protein or metabolised. Protein turnover far exceeds normal protein intake and amino acids released from endogenous proteins are therefore crucial in protein metabolism. The nitrogen content of amino acids is removed by by deamination or transamination in the synthesis of various nitrogen-containing compounds, though their nitrogen content is primarily removed through the synthesis and excretion of urea. The carbon skeletons of surplus amino acids are either metabolised to carbohydrate (such as glucose), or fat, or oxidized as fuel. The carbon skeletons of most amino acids are oxidized in the liver, while the branch-
chain amino acids leucine, isoleucine and valine are metabolised mainly in muscle kidney fat and brain (May and Buse, 1989). Whilst total body protein does represent are large store of potential energy, depletion of body protein by more than 40% is not compatible with life, and as such is not an ideal energy store.

The regulation of all the processes involved in maintaining circulating fuels, of which glucose is arguably the most important, is not surprisingly a complex mechanism. It is accomplished by a combination of substrate availability, several hormones, and also by the activity of the sympathetic and parasympathetic divisions of the autonomic nervous system. Among these factors, of particular note is the secretion of insulin and glucagon from the islets of Langerhans. Both of these hormones play a pivotal role in the regulation of metabolism and the maintenance of blood glucose concentrations within normal physiological limits. Secretion of glucagon makes a notable contribution to the metabolic adaptation to stress, exercise and food deprivation. However, moment-to-moment adjustments in the rate of insulin secretion are the predominant mechanism through which carbohydrate, protein and lipid metabolism are normally regulated in the body. The other neural and hormonal pathways play physiological roles in the fine-tuning of insulin secretion, particularly following feeding and in situations associated with stress and hypoglycaemia (Havel et al. 1994).

1.3.1 Insulin and glucose homeostasis.
Insulin can be described as an anabolic hormone, in that it encourages the laying down of tissue and energy stores. Insulin encourages the formation of glycogen and fuel stores by suppressing glycogenolysis and gluconeogenesis in the liver and thereby decreasing hepatic glucose output, and by stimulating the uptake of glucose into certain peripheral tissues, especially skeletal muscle and fat. Basal insulin concentrations after an overnight fast are sufficient to inhibit glycogenolysis and restrain gluconeogenesis (Insel et al. 1975), while much higher levels are needed to stimulate glucose uptake into skeletal muscle and fat cells. Insulin is essential for the activation of hepatic glycogen synthase after feeding, but this role may be permissive, as glucose itself is the immediate effector of increased glycogen synthase.
activity and of the switch from net glucose output to net uptake in the liver (Newgard et al. 1983). In insulin-sensitive cells insulin also helps to maintain the glucose gradient across the cell membrane by stimulating the enzymes of both glycogen synthesis and glucose oxidation, which consume glucose-6-phosphate and glucose. Interestingly insulin does not cause net hepatic glucose uptake, or stimulation of liver glycogen deposition, unless the portal venous glucose concentration also rises. The extent of direct hepatic glucose uptake after a glucose load depends on the portal glucose level and an inward-directed glucose gradient across the hepatocyte membrane (Niewoehner and Nuttall, 1988; Wals and Katz, 1993). Both these factors are relatively small, which may explain the limited hepatic uptake of orally administered glucose of approximately 10% on the first pass (Pagliassoti et al. 1991; Kruszynska et al. 1993; Radziuk et al. 1978). This is because glucose coming directly from the gut comprises only about one-third of the total glucose delivered to the liver, the remainder being recirculated (Radziuk et al. 1978).

Glucose concentrations in the hepatocyte cytosol are higher than in plasma, which ensures that glucose leaves the cells in the fasting state. Passage of glucose across the hepatocyte membrane is facilitated by the GLUT-2 glucose transporter, and is rapid and not regulated by insulin (Pedersen, 1993; Gould and Holman, 1993). As hepatic glycogen stores begin to be consumed early in the postabsorptive period, priority is given to replenishing these hepatic glycogen stores when glucose enters the bloodstream after eating. Only when the glycogen stores have been expanded again can excess carbohydrate be channelled into the formation of triglyceride and fat storage. As liver glycogen content increases, glycogenolysis is enhanced even when plasma insulin and glucose levels are high (Magnusson et al. 1994). This may divert glucose and glucose-6-phosphate into glycolysis for lipogenesis, and into the pentose phosphate pathway to provide NADPH required for lipogenesis.

The liver will eventually take up between 25-50% of an oral glucose load, the amount depending on the extent of the glucose load (Ferrannini et al. 1985; Katz et al. 1983; Bratusch-Marrain et al. 1980; Kruszynska et al. 1993). Muscle can account
for up to 85% of the remaining glucose load (Katz et al. 1983) with fat cells accounting for the remainder, though this value can be higher in obese subjects (Marin et al. 1987). It would appear that gluconeogenesis is never completely inoperative, and that rises in insulin concentration following glucose ingestion exhibit only a small additional inhibitory effect on this low rates of gluconeogenesis (Magnusson et al. 1989; Radziuk, 1989; Schulman et al. 1990). However, the rise in insulin concentrations postprandially stimulates glycogen synthase and inhibits glycogen phosphorylase, thus ensuring that the glucose-6-phosphate produced by gluconeogenesis is channelled into glycogen rather than being released as glucose. As well as control by insulin, glucose itself regulates the activities of glycogen synthase and phosphorylase (Stalmans et al. 1974).

1.3.2 The role of the ‘counter-regulatory’ hormones to glucose homeostasis.

The actions of insulin are opposed by the ‘counter-regulatory’ hormones, which although they are continually secreted their release can be increased under conditions of physiological stress such as hypoglycaemia. Glucagon is one of these ‘counter-regulatory’ hormones, and it is the major factor that increases hepatic glucose output, and opposes the key metabolic effects of insulin (Lefebvre, 1995). As well as rapidly stimulating hepatic glycogenolysis it also increases the hepatic uptake of gluconeogenic precursors, in particular amino acids, and promotes their conversion to glucose by hepatic gluconeogenesis (Chiasson et al. 1975; Cherrington et al. 1987; Hue, 1987). As such glucagon can be described as a catabolic hormone. Reinforcing or complementing the catabolic actions of glucagon are the catecholamines adrenaline and noradrenaline, which are released by sympathetic nerves in the liver (Lager et al. 1986).

Hepatic gluconeogenesis produces one molecule of glucose-6-phosphate from two molecules of pyruvate, and the process shares most of its enzymes with the glycolytic pathway which converts glucose-6-phosphate to pyruvate. Those enzymes which catalyse the non-equilibrium reactions unique to either glycolysis or gluconeogenesis are key regulatory sites, whose activities determine the magnitude and direction of carbon flux. The fructose-6-phosphate – fructose-1,6-bisphosphate
cycle controls the first committed step of glycolysis, and the pyruvate – phosphoenolpyruvate cycle that of gluconeogenesis. Important enzymes involved include: Phosphofructokinase-1, fructose-1,6-bisphosphatase, pyruvate kinase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Regulation of these enzymes in the short-term involves modulation of their activities by substrates and hormones. Raised glucagon and decreased insulin levels reduce the activity of pyruvate kinase and stimulate phosphoenolpyruvate carboxykinase. Low insulin and high non-esterified fatty acids (NEFA) levels during the fasting state inhibit pyruvate dehydrogenase. These changes favour pyruvate formation from lactate and alanine, and the channelling of this pyruvate to phosphoenolpyruvate (PEP) via oxaloacetate, and thus an increase in the conversion of PEP to glucose and less to pyruvate. Glucagon also reduces fructose-2,6-bisphosphate, an allosteric effector of the fructose-6-phosphate – fructose-1,6-bisphosphate cycle. Reduced fructose-2,6-bisphosphate levels decrease the activity of phosphofructokinase-1 and enhance that of fructose-1,6-bisphosphate, thereby decreasing glycolysis and favouring gluconeogenesis (Kruszynska, 1997).

In the fasted state when insulin concentrations are normally low, glucagon secretion rises and the levels of the other counter-regulatory hormones are increased relative to insulin. This results in a decrease in the glucose oxidation by extrahepatic tissues and enhances muscle protein breakdown, causing increased release of lactate, pyruvate and alanine into the circulation. Lipolysis also increases and so increased levels of glycerol are delivered to the liver. These processes are held in check by basal insulin levels, which prevent unrestrained mobilization of fatty acids and gluconeogenic precursors from extrahepatic tissues. The supply of substrates for gluconeogenesis is further enhanced by their fractional extraction by the liver. The catabolism of alanine and other gluconeogenic amino acids is rate-limited by their carrier-mediated transport into hepatocytes. Several transport proteins are involved. The alanine transport system A is markedly stimulated by glucagon and catecholamines, while glucocorticoids play an important permissive role (Pozefsky et al. 1976). Hepatic extraction of lactate, pyruvate and glycerol is also enhanced when gluconeogenesis is increased, probably because this decreases their
intracellular concentrations and increases their concentration gradients across the cell membrane, thus facilitating uptake. As physiological circulating levels of lactate, pyruvate, glycerol and alanine lie below those needed for half-maximal stimulation of gluconeogenesis, an increased supply of these precursors stimulates their incorporation into glucose (Hue, 1987).

Insulin inhibits proteolysis and may stimulate protein synthesis under certain conditions. Insulin deficiency, glucagon excess both accelerate hepatic proteolysis via the lysosomal pathway (Mortimore et al. 1989), but the prime regulators are amino acids themselves and in particular leucine, which is thought to be mediated by a plasma membrane receptor (Miotto et al. 1992).

The supply of NEFA to the liver is important in regulating gluconeogenesis; their oxidation provides energy for the process and also increases intramitochondrial acetyl CoA, which stimulates the key gluconeogenic enzyme, pyruvate carboxylase. Inhibition of fatty acid oxidation leads to a marked decrease in hepatic gluconeogenesis, thus the inhibition of lipolysis by insulin after meals may contribute to insulin’s suppression of hepatic glucose output.

1.3.3 Insulin and fat metabolism.

Adipocytes and liver synthesize triglyceride from NEFA, derived from the circulation and glucose, and glycerol-3-phosphate, derived from glucose. Insulin stimulates lipogenesis by up to 10-fold by increasing the activities of several lipogenic enzymes pyruvate dehydrogenase (PDH), acetyl CoA carboxylase, and fatty acid synthetase, and partly by stimulating glucose uptake and therefore increasing the availability of pyruvate for fatty acid synthesis, and of glycerol-3-phosphate for their esterification. Insulin inhibits lipolysis by reducing the circulating levels of NEFA (Bonadonna et al. 1990), but crucially for diabetic subjects hyperglycaemia in the absence of a rise in insulin does not inhibit lipolysis (Caruso et al. 1990). Lipolysis is mediated by triglyceride lipase, whose activity is increased when it is phosphorylated by cAMP-dependent protein kinase. Thus triglyceride lipase is stimulated especially by catecholamines which act on β-
receptors to increase intracellular cAMP levels (Johnston and Alberti, 1982). Triglyceride accumulates through the esterification of NEFA with glycerol-3-phosphate. Human adipocytes lack the enzyme glycerokinase (Steinberg and Vaughan, 1965), which converts glycerol to glycerol-3-phosphate. And therefore glycerol cannot be used for triglyceride synthesis in the adipocyte. The glycerol-3-phosphate is instead taken up from circulation, having been derived from glucose via glycolysis. NEFA are also taken up by adipocytes and these can also be derived from glucose.

1.3.4 Ketone Bodies.

In the mitochondria of hepatocytes partial oxidation of NEFA yields ketone bodies. Long-chain fatty acids are converted to their coenzyme A (CoA) derivatives in the cytosol and transported across the mitochondrial membranes by the two carnitine shuttle enzymes. These enzymes, carnitine palmitoyltransferase I and II, are located on the outer and inner mitochondrial membranes respectively. Carnitine palmitoyltransferase I is the major site of control of hepatic fatty acid oxidation (McGarry et al. 1989), and acting upon this site insulin inhibits the carnitine shuttle while glucagon stimulates this shuttle.

The ketone bodies acetoacetate and 3-hydroxybutyrate are important fuels when carbohydrate is in short supply, or cannot be used efficiently. After a three day fast ketones can supply 30-40% of the total energy needs. They are particularly important to the central nervous system, which cannot use fatty acids for energy. The utilization of ketone bodies by the brain is concentration-dependent, and it primarily uses 3-hydroxybutyrate (Owen et al. 1967). Ketogenesis is regulated by the supply of NEFA reaching the liver and is therefore enhanced by insulin deficiency and excess of the counter-regulatory hormones that stimulate lipolysis. Hepatic NEFA supply is an important determinant of the rate of ketogenesis. Small increases in plasma insulin levels produce an abrupt fall in plasma NEFA levels (Keller et al. 1989; Johnston and Alberti, 1982). Physiological increases in plasma catecholamine levels increase plasma NEFA and ketone-body concentrations, even at basal insulin levels,
and suppression of insulin secretion with somatostatin potentiates this effect (Keller et al. 1989; Johnston and Alberti, 1982).

1.3.5 Interaction with insulin of islet hormones and peptides.

The release of glucagon is inhibited by hyperglycaemia, fatty acids, insulin and somatostatin, but glucagon is also a powerful stimulator of insulin and somatostatin secretion. Somatostatin, released from the D cells of the islets of Langerhans, is considered to be a potentially important local inhibitor of insulin, glucagon and PP secretion from adjacent β, A and PP cells respectively (Bonner-Weir, 1991; Marks et al. 1992). The inhibitory effect of somatostatin on insulin secretion is associated with decreased formation of cAMP (Pipeleers, 1987), coupled with G-protein mediated actions on ion channels that result in membrane hyperpolarization and a decrease in cytosolic Ca\(^{2+}\) concentration, (Nilsson et al. 1989; Kakei et al. 1994).

Although it possibly also interferes with an unidentified late step of exocytosis in insulin secretion (Nilsson et al. 1989). Somatostatin secretion is itself regulated by insulin, which tends to decrease somatostatin release. Somatostatin secretion is stimulated by a multitude of factors including increased levels of glucose, fatty acids, ketone bodies and glucagon.

Pancreastatin is a 49-amino-acid peptide, which is found in nervous tissue and a number of endocrine cells including the β, A and D cells of the islets of Langerhans. Studies using the perfused porcine pancreas indicated that pancreastatin was released in parallel with insulin in response to glucose and a number of other secretagogues (Ostenson et al. 1989). In the pig, porcine pancreastatin has no obvious effects on insulin secretion (Holst et al. 1990). In the rat, however, pancreastatin inhibits insulin secretion induced by glucose, arginine, and gastrin inhibitory peptide (GIP) and other insulinosuppressive agents (Efendic et al. 1987; Ishizuka et al. 1988; Silvestre et al. 1988). These effects can be aborted by pretreatment of insulin-secreting cells with pertussis toxin, suggesting that an inhibitory G protein is involved in its mechanism of action (Lorinet et al. 1989). Its ability to suppress glucose-induced insulin secretion is apparently mediated by an inhibitory effect on voltage-dependent calcium channels (Lindskog et al. 1992). The effects of the peptide on islet cell
secretions are presumably mediated by local effects, either on its cell of origin (autocrine) or adjacent cells (paracrine).

Amylin, or islet amyloid polypeptide (IAPP) is co-localized with insulin in β cell secretory granules. IAPP has been reported to inhibit secretagogue-induced release of insulin, indicating a possible autocrine role on the control of β cell function (Degano et al. 1993; Wang et al. 1993; Silvestre et al. 1994).

Diazepam-binding inhibitor (DBI), originally isolated from pig intestine (Chen et al. 1988), has been localized to the somatostatin-containing D cells of the human and porcine pancreas (Berggren et al. 1992). This peptide appears to inhibit insulin and glucagon secretion induced by a variety of secretagogues (Chen et al. 1988; Ostenson et al. 1990; Ostenson et al. 1994). However, the precise role of this peptide has yet to be determined.

C peptide was long thought to be little more than a waste by-product of the synthesis of insulin, but it has been shown to exert significant extra-pancreatic effects (Wahren, 1994). It has been shown to increase the glucose uptake into skeletal muscle by a mechanism not involving the insulin receptor (Zierath et al. 1996). But its effects may also include the islets themselves, as specific receptors for rat C peptide have been demonstrated on rat insulinoma cells (Flatt et al. 1986), and C peptide has been reported to inhibit the secretion of both insulin and glucagon (Toyota et al. 1975; Wojcikowski et al. 1977; Wojcikowski et al. 1983).

Thyrotropin-releasing hormone (TRH), endorphins and adrenocorticotropic hormone (ACTH) are all found in A and β cells and growth hormone-releasing hormone (GHRH) is present in PP cells. All of these factors enhance insulin release.

Inhibitors of insulin secretion include peptide YY (PYY), corticotrophin-releasing factor (CRF), atrial natriuretic peptide (ANP), dopamine and serotonin (5-HT). With the exception of the later two which are found in β cells, the rest seem to be restricted to the A cells. Though they clearly posses potential regulatory roles, their
significance in the physiological modulation of insulin secretion is uncertain (Flatt PR, 1997).

It was long postulated that there may be a feed-back loop, where insulin secretion caused a decrease in further insulin secretion, unless stimuli were still present. Recent studies have not only gone some way to discrediting this theory, but suggesting that the opposite occurs, namely an autocrine feed-forward loop. In vitro studies have shown that exogenous insulin added directly to normal islets causes transcriptional up-regulation of the preproinsulin gene, activation of protein translational machinery and insulin secretion (Leibiger et al. 1998; Xu et al. 1998; Aspinwall et al. 1999).

1.3.6 The role of islet neurotransmitters.

Islets receive a rich innervation of autonomic nerves containing classical and peptidergic neurotransmitters, which play an important role in the modulation of islet hormone release (Havel et al. 1994; Porte and Woods, 1990; Sundler and Bottcher, 1991). Cholinergic neurotransmitters that stimulate insulin secretion include acetylcholine, vasoactive intestinal polypeptide (VIP), and gastrin-releasing polypeptide (GRP) (Ahren and Karlsson, 1994). Whereas sympathetic neurotransmitters that inhibit insulin release include, among others, noradrenaline, galanin and neuropeptide Y (NPY) (Havel et al. 1994; Porte and Woods, 1990; Berggren et al. 1992; Ahren et al. 1991).

Activation of parasympathetic nerves during feeding is instrumental for the cephalic phase of insulin release, while increased sympathetic activity partly mediates inhibition of insulin secretion in situations of stress, including exercise and trauma.

The glucagon and PP responses to hypoglycaemia are partly mediated by parasympathetic nerve activation. Acetylcholine stimulates insulin, glucagon and PP release (Porte and Woods, 1990), by binding to muscarinic receptors (Grill and Ostensen, 1983) which are linked to the activation of phospholipase C (Morgan and
Montague, 1992). It also acts by activation of protein kinase C (Wollheim and Biden, 1986; Theler et al. 1992; Wang et al. 1993), and it may also promote calcium-ion entry into the β cells by an unknown pathway (Sanchez-Andres et al. 1994). The precise mechanisms by which vasoactive intestinal polypeptide (VIP) stimulates insulin secretion are not clear (Schebalin et al. 1977; Szecowka et al. 1980; Wahl et al. 1993; Kato et al. 1994). Gastrin-related polypeptide (GRP) is released by electrical stimulation of the vagus nerve (Knuthsen et al. 1985), and is a powerful stimulus to the secretion of insulin, glucagon, somatostatin and PP (Holst, 1992; Porte and Woods, 1990; Ahren and Karlsson, 1994; Wahl et al. 1994). GRP binds to specific β cell receptors (Wahl et al. 1992) leading to activation of phospholipase C and the generation of inositol-1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DAG) (Berggren et al. 1989). Additionally it inhibits the membrane potassium permeability, leading to depolarization and an increase of calcium-ion flux and hence stimulates insulin secretion.

Noradrenaline inhibits the secretion of insulin by activation of α$_2$-adrenergic receptors in the islet β cell membrane (Berggren et al. 1989), thereby lowering the cAMP and cytosolic Ca$^{2+}$ concentrations (Katada and Ui, 1979; Yamazaki et al. 1982; Sussman et al. 1987). It may also act by the closure of ATP-sensitive potassium channels via a pertussis toxin-sensitive inhibitory G protein (Havel et al. 1994; Porte and Woods, 1990).

Galanin inhibits both basal and stimulated insulin secretion via specific galanin receptors coupled by inhibitory G proteins to various mechanisms:- ATP-sensitive potassium channels, inhibition of adenylate cyclase and blockade of exocytosis (Amiranoff et al. 1987; Nilsson et al. 1989; Sharp et al. 1989; Cormont et al. 1991; Lang et al. 1994; McDermott and Sharp, 1995). Neuropeptide Y (NPY) has been reported to inhibit basal and glucose-stimulated insulin secretion (Moltz and McDonald, 1985; Pettersson et al. 1987), but the mechanisms involved are as yet unclear.
Various other peptides have been considered as possible physiological modulators of insulin secretion. These include cholecystokinin (CCK) and neurotensin which stimulate insulin secretion. Also pituitary adenylate cyclase-activating polypeptide (PACAP) which is inhibitory, and calcitonin gene-related peptide (CGRP) and substance P, both of whose actions are not elucidated as yet. Though PACAP is thought to raise cAMP levels and activate cAMP-dependent protein kinase (PKA) in manner similar to the actions of GIP (Thorens, 1995; Komatsu et al. 1996; Ding and Gromada, 1997).

1.3.7 Extra-islet and the incretin hormones.
The insulin secretory response to feeding is the culmination of both the direct effects of glucose and other nutrients upon β cells, together with indirect effects mediated through the enteroinsular axis. This latter mechanism comprises neural and hormonal pathways that are activated by feeding and thus plays an important role in augmenting the insulin response to feeding. The hormonal aspect of this axis is mediated by ‘incretin’ hormones. Most gastrointestinal hormones have been considered as candidates for the putative ‘incretin’ hormones, which are secreted during meals and augment the insulin response to levels above that when nutrients are administered parenterally. Present thinking has identified the most likely candidates to be GIP, GLP-1 (7-36) amide and possibly cholecystokinin CCK (Ebert and Creutzfeldt, 1987; Dupré, 1991; Morgan, 1992; Orskov, 1992; Thorens and Waeber, 1993). Both of the first two of these candidates are powerful glucose-dependent stimulators of insulin secretion. They act via specific receptors in the β cell plasma membrane which activates adenylate cyclase, leading to the generation of cAMP (Szecowka et al. 1982; Kreymann et al. 1987; Ebert and Creutzfeldt, 1987; Altman et al. 1987; Drucker et al. 1987; Orskov and Nielsen, 1988; Goke and Conlon, 1988; Flatt et al. 1990; Morgan, 1992; Thorens, 1992). This in turn affects the sensitivity of the β cell to Ca^{2+} through PKA activation and phosphorylation of associated proteins.

The physiological significance of CCK as a neural or hormonal mediator of enteroinsular activity is unproven, but there are CCK-A receptors on β cells, and at
high concentrations CCK can stimulate insulin secretion (Verspohl et al. 1986) via activation of phospholipase C, generation of IP$_3$ and stimulation of PKC activity (Zawalich et al. 1987).

1.4 Nutrient-insulin secretion coupling.
As has been discussed in previous sections a host of different physiological compounds alter the rate of release of insulin from pancreatic β cells, including neurotransmitters, neuropeptides, hormones, amino acids, and carbohydrate metabolic substrates such as glucose. Controlling the co-ordinated release of insulin from β cells in response to these physiological agents is therefore a highly complex but ordered process. In general physiological terms, however, the major physical determinant of insulin secretion in mammals is the blood glucose concentration, and the other physiological regulators act by modifying the prevailing influence of glucose on insulin secretion. However, it has been demonstrated *in vitro* that leucine and arginine are effective insulin secretagogues in the absence of glucose, and therefore qualify as initiators of insulin secretion. It is not the mere presence of glucose that initiates increases in insulin secretion, rather the metabolism of glucose by the β cell (Ashcroft et al. 1978). Similarly with other fuel substrates which can initiate insulin secretion it is not there mere presence but their metabolism (Figure 1.4) which is required for the stimulation of insulin secretion.
Figure 1.4 The main classes of substrates that can be metabolized by the pancreatic β cell.

Dashed arrows represent metabolic pathways containing several steps. KIC represents α-ketoisocaproate. (From Ashcroft and Ashcroft, 1992).
Isolated islets release insulin in response to glucose in a sigmoidal fashion. Concentrations of glucose below 5 mmol/l do not affect the basal rate of release insulin, and the largest increases in secretory rates occur at extracellular glucose levels of between 5.5 and 17 mmol/l, with half-maximal stimulation at 8 mmol/l (Figure 1.4b).

**Figure 1.4b** General representation of the insulin secretory responses of isolated islets of Langerhans to increasing glucose concentrations.

No stimulation is seen below a threshold of 5 mmol/l glucose concentration. Addition of a potentiator enhances secretion at all glucose concentrations, shifting the dose-response curve to the left (Adapted from Howell, 1997).

The shape of this characteristic dose-response curve is determined primarily by the regulatory activity of glucokinase, which is the rate-limiting factor for glucose phosphorylation in β cells and apparently acts as the 'glucose sensor', which couples insulin secretion to the prevailing glucose level. While glucose transport into the β cell via the GLUT-2 transporter does not appear to rate-limiting under normal circumstances (Van Schaftingen, 1994).
1.4.1 The electrical activity of islet β cells.

Ion channels are membrane bound proteins that act as ‘ion tunnels’ by facilitating the influx of ions across the membrane systems of cells. The exchange of K\(^+\), Na\(^+\), Cl\(^-\) and Ca\(^{2+}\) ions across the internal membrane and plasma membranes of β cells is key to understanding how glucose, (or rather the metabolism of glucose), induces a complex pattern of electrophysiological events. Stimulation by glucose triggers a pattern of electrical activity of the β cell membrane which has two components. The first is composed of continuous spike activity, followed by a partial repolarization with spikes, after which slow waves develop. The current carrier during the electrical bursts consists of calcium ions entering the β cell through voltage-dependent channels in its membrane (Henquin et al. 1992).

As in all cell systems Ca\(^{2+}\) is a major signalling molecule. Control of cytosolic Ca\(^{2+}\) homeostasis is a complex matter in any cell, but perhaps in no other cell is it as critical as in the pancreatic β cell. It is therefore a highly integrated and dynamic process (Wollheim and Sharp, 1981; Gilon et al. 1999). Within β cells Ca\(^{2+}\) is buffered to diminishingly small quantities, by the maintained energy-requiring extrusion of Ca\(^{2+}\) across the plasma membrane by plasma membrane Ca\(^{2+}\)-ATPase (PMCA), and sequestration of Ca\(^{2+}\) into internal Ca\(^{2+}\) storage organelles by sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). The initiation of insulin release is critically dependent upon the influx of Ca\(^{2+}\) across the membrane (Matthews and Sakamoto, 1975; Wollheim and Sharp, 1981; Henquin and Meissner, 1984). Ionophores, such as A23187, which transport calcium ions across biological membranes, have been shown to evoke sustained insulin release in the presence of non-stimulatory concentrations of glucose. Moreover, islets or β cells which have been loaded with the calcium-sensitive fluorescent compounds fura-2 or quin-2, change their fluorescence within one minute of exposure to glucose (Hellman et al. 1992), during which insulin secretion is activated.

One of the first, and possibly the most important, ion channels studied in pancreatic β cells was the ATP-sensitive potassium (K\(_{ATP}\)) channels, which were shown to be
regulated by the internal cellular concentrations of ATP and ADP (Cook and Hales, 1984; Ashcroft et al. 1984; Kakei et al. 1986; Dunne and Petersen, 1986). It is now widely accepted that under resting conditions the membrane potential in β cells is determined by the Na⁺-K⁺ ATPase pump and K⁺ leakage through open $K_{\text{ATP}}$ channels. Following glucose uptake and metabolism by both glucokinase, ‘the glucose sensor’, and mitochondrial events, $K_{\text{ATP}}$ channels are closed as a consequence of the increased intracellular ATP-ADP ratio. $K_{\text{ATP}}$ channel closure leads to depolarization of the cell and the opening of voltage-dependent Ca²⁺ channels. The subsequent influx of Ca²⁺ leads to a sharp rise Ca²⁺ concentration in the cytosol close to the membrane (Ashcroft et al. 1992; Bokvist et al. 1995), which then initiates the release of insulin by the process of exocytosis. This latter action being mediated by different Ca²⁺ sensing proteins such as synaptotagmins, and involves the SNARE complex and its chaperones, such as N-ethylmaleimide-sensitive factor (NSF) and cysteine string proteins (CSPs), (Lang, 1999).

β cells express a $K_{\text{ATP}}$ channel complex formed by subunits belonging to at least two distinct families of proteins (see Figure 1.4.1). The K⁺ selective pore is formed by a member of the inward rectifier K⁺ channel family, Kir6.2. This protein comprises 390 amino acids and has a predicted membrane topology with two α-helical transmembrane domains linked by a sequence of conserved amino acids. The linking region shares sequence homology with the P region of voltage-gated K⁺ channels, and therefore is thought to be important for the control of K⁺ selectivity through the pore. The other subunit, SUR1, is a receptor with high affinity for sulphonylureas (Aguilar-Bryan et al. 1995). Human SUR1 is a member of the superfamily of ATP-binding cassette (ABC) proteins. Consisting of 1581 amino acids in length, it has two predicted intracellularly disposed nucleotide-binding domains (NBD), and 17 predicted transmembrane domains (TMD) organized into three regions designated TMDO, TMDI, and TMDII (Tusnady et al. 1997; Ashcroft and Gribble, 1998; Aguilar-Bryan et al. 1998). Currently it is thought that the $K_{\text{ATP}}$ channel of β cells is an octameric complex formed by four Kir6.2 subunits lining the pore coupled to four SUR1 subunits (Shyng and Nichols, 1997).
Figure 1.4.1 Components of and predicted topology of the \text{K}_{\text{ATP}} channel

(a) Shown are the two components of the \text{K}_{\text{ATP}} channel, SUR1 and Kir6.2. Also shown are the predicted sites for potassium channel opener binding (KCOI and KCOII) and sulphonylurea binding (SURB). The transmembrane domains of Kir6.2 are shown as M1 and M2, with the P domain thought to be important for control of K$^+$ selectivity through the pore.

(b) The \text{K}_{\text{ATP}} channel is formed by four SUR1 subunits surrounding four Kir6.2 subunits, to form an octameric structure with a central pore (From Dunne, 2000).

The Kir6.2 is the major site in the complex for ATP binding and ATP-induced channel closure, whilst ADP interacts with the SUR1 subunits. It is also known that neither subunit alone will form operational K$^+$ channels, but both must be co-expressed (Sakura \textit{et al.} 1995). This suggests that the both subunits are important for the complex’s ability to regulate ATP:ADP-mediated \text{K}_{\text{ATP}} channel activity, with the actions of ADP on SUR1 antagonizing the effects of ATP on Kir6.2 (Ashcroft and Gribble, 1998; Sharma \textit{et al.} 1999; Aguilar-Bryan and Bryan, 1999). The high
affinity of SUR1 for sulphonylureas may explain the effectiveness of sulphonylurea drugs in non-insulin-dependent diabetes mellitus (NIDDM) patients whose β cells no longer respond adequately to glucose, as the usual pathway for coupling insulin release to glucose concentration can be bypassed, the sulphonylurea drugs acting by closing the $K_{ATP}$ channels (Sturgess et al. 1985). Conversely $K_{ATP}$ channel openers, such as diazoxide, over-ride stimulus driven insulin secretion by preventing membrane depolarization and thus also preventing voltage-gated $Ca^{2+}$ entry to the β cell. Such a drug is of clinical use in the treatment of persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), indicating that defects in the stimulus-response coupling prevail in β cells of these patients (Dunne and Petersen, 1991).

PHHI arises from defects in either the Kir6.2 or SUR1 genes. Since these β cells lack operational $K_{ATP}$ channels, they are continually closed, the membrane potential is spontaneously depolarized in the absence of glucose metabolism. This leads to persistent activation of $Ca^{2+}$channels causing unregulated entry of $Ca^{2+}$ and persistent release of insulin as a consequence (Kane et al. 1996; Otonkoski et al. 1999). The gene for the ATP-sensitive potassium channel has been cloned (Bond et al. 1995), but despite being an obvious candidate to explain β cell dysfunction of NIDDM, no mutations with functional defects have has yet been found in patients with common type NIDDM, maturity-onset diabetes of the young (MODY) or gestational diabetes (Zhang et al. 1995; Inoue et al. 1997).

In β cells, whilst closure of $K_{ATP}$ channels critically determine the initial depolarization of the cell membrane and so govern first phase insulin release, several other types of $K^+$ channels are known to contribute to the complex electrophysiological profile that is generated in the presence of stimulatory concentrations of glucose. The delayed rectifier, $Ca^{2+}$-and voltage gated $K^+$ channels are involved in the subsequent generation of action potentials and establishing oscillations of electrical activity (Dunne and Petersen, 1991).
A second important control mechanism in the control of glucose-dependent insulin secretion is the ‘K\textsubscript{ATP} channel-independent pathway’ (Aizawa et al. 1998), which is thought to augment insulin secretion in the presence of raised cytosolic Ca\textsuperscript{2+} levels and be largely responsible for second-phase insulin secretion. Although the mechanisms are not completely resolved it is thought to cause Ca\textsuperscript{2+} entry through a KCl-induced depolarization (Gembal et al. 1992; Straub et al. 1998), but without K\textsubscript{ATP} channel operation. However, it has also been demonstrated to occur without raised cytosolic Ca\textsuperscript{2+} concentrations, via the simultaneous activation of protein kinases A and C (Yajima et al. 1999).

**Figure 1.4.1.2 The biphasic nature of normal insulin secretion.**

![Figure 1.4.1.2 The biphasic nature of normal insulin secretion.](image)

The *in vitro* release of insulin from isolated perfused pancreas in response to glucose is a biphasic process. An acute first phase of secretion which lasts only a few minutes is followed by a sustained second phase of secretion which persists for the duration of the high-glucose stimulus (From Howell, 1997).

The mechanism by which some amino acids stimulate the release of insulin is not clear; some have to be metabolised, while others only need to be transported into the \(\beta\) cell. In the case of arginine, it has been suggested that the inward movement of its cationic charge may depolarize the \(\beta\)-cell membrane and open voltage-gated calcium channels (Hermans et al. 1987). Leucine may generate ATP through its metabolism,
which in turn may close ATP-sensitive potassium channels in a similar way to glucose (Malaisse, 1992). Other amino acids also stimulate insulin secretion by elevating cytosolic Ca\(^{2+}\) concentration. However, in these instances, the effect is mediated by activation of glutamate dehydrogenase and either the generation of metabolic flux, in the case of L-glutamine, or membrane depolarization due to the inward transport of positive charge carried by the amino acids L-arginine, L-lysine and L-histidine, or by its cotransport with Na\(^+\) as is the case for L-alanine (Hellman et al. 1992; Berggren et al. 1994).

1.4.2 Other factors involved in stimulus-response coupling in β cells.

Cyclic AMP (cAMP) is a second messenger and acts as a potentiator of nutrient-induced insulin secretion. As such, cAMP does not initiate but potentiates insulin secretion following elevation of intracellular cAMP. (Howell et al. 1994). The site of action of cAMP is likely to be distal to the site of nutrient recognition. The major effects of cAMP are thought to be exerted through the activation of protein kinases. The phosphorylation of a number of cellular proteins by the activated cAMP-dependent protein kinase has been demonstrated (Malaisse, 1994).

The calcium-dependent regulatory protein, calmodulin, has been detected in islet cells and has been indirectly implicated in glucose-induced insulin release. The major effects of calmodulin in β cells are probably exerted through activation of calmodulin-dependent protein kinase activity, following which several proteins are phosphorylated (Persaud et al. 1992). Putative target proteins include tubulin and myosin light chains; it is therefore possible that calmodulin-dependent phosphorylation may regulate granule movement, although none of these proteins appears to be a major endogenous substrate for calcium-calmodulin-dependent protein kinase in β cells (Malaisse, 1994). Other calcium-dependent proteins have also been implicated in involvement in regulation of insulin secretion, specifically on exocytosis. These include Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM Kinase II), annexin, Ca\(^{2+}\)-dependent activator protein for secretion (CAPS) and the synaptotagmins (Sudhof and Rizo, 1988; Matsumoto et al. 1995; Marqueze et al. 1995; Tandon et al. 1998).
Protein kinase C (PKC) is stimulated by calcium and phosphatidylserine, and is further enhanced by diacylglycerol, which lowers its requirement for calcium to within the physiological range. A possible role for PKC activation in the control of insulin secretion has been demonstrated using phorbol myristate acetate (PMA), a specific activator of PKC, which stimulates insulin secretion. It sensitizes the system to calcium, stimulating insulin release at calcium concentrations that do not normally evoke release, and augmenting secretory responses to higher calcium levels (Persaud et al. 1992). However glucose-stimulated insulin secretion was completely unaffected in PKC-depleted islets, indicating that this enzyme is not involved in the secretagogue effect of glucose (Hii et al. 1987). Thus, these protein kinase pathways all appear to exert their effects by phosphorylating substrates in the β cell. So far, however, no common substrate has been identified that produces a single phosphorylated intermediate that might trigger granule translocation and exocytosis (Howell et al. 1994). Activation of protein kinase A (PKA) results in phosphorylation of intracellular proteins that mediate Ca\(^{2+}\) sensitization. PKA also promotes phosphorylation of voltage-dependent calcium channels and K\(_{ATP}\) channels, thereby increasing calcium-ion influx (Berggren et al. 1994). The protein kinase A-induced phosphorylation of K\(_{ATP}\) channels appear to involve both SUR1 and Kir6.2. (Begiun et al. 1999). PKC and PKA exert some of their effects on early stages of insulin secretion, by the generation of second messengers (Arkhammar et al. 1994; Ding and Gromada, 1997), but in addition PKA and PKC also effect late steps in the exocytotic process itself (Ämmälä et al. 1993; Ämmälä et al. 1994).

Cell membranes contain phosphatidylinositol bisphosphate which can be hydrolysed by the calcium-dependent enzyme phospholipase C, which is also membrane bound. Hydrolysis yields inositol-1,4,5-trisphosphate (IP\(_3\)), which has been found in many tissues to stimulate the release of calcium stored within the endoplasmic reticulum, so raising cytosolic calcium concentrations. Inositol metabolism is stimulated during glucose-induced insulin secretion and inositol depletion of β cells prevents this secretory response. In particular, glucose increases hydrolysis of phosphatidylinositol bisphosphate, probably as a result of activating the phospholipase C (Morgan et al. 1992), so generating IP\(_3\). Whether this activation is a direct effect of glucose, or
whether it results from a glucose-induced calcium rise, which in turn triggers activation of the calcium-dependent enzyme, remains an open question (Morgan et al. 1992); other stimuli act via a receptor coupled through a membrane-associated guanosine triphosphate (GTP)-binding protein (G protein) to phospholipase C (Jones et al. 1992). The quantitative importance of the intracellular calcium mobilization induced by IP$_3$ for the activation of insulin secretion remains unclear. Other products of phosphoinositol hydrolysis may play a significant role in the stimulus-secretion coupling in β cells, notably diacylglycerol, another product of phospholipase C-catalysed phospholipid breakdown. Diacylglycerol activates the enzyme PKC, and also stimulates insulin secretion by causing calcium ion sensitization through protein phosphorylation (Berggren et al. 1992). The production of diacylglycerol is accelerated by glucose and other stimuli, although, as discussed previously, this does not produce the actual signal that evokes insulin secretion in response to glucose.

Arachidonic acid is a major component of islet phospholipids, comprising about 17% of total β cell lipid content. It can stimulate insulin secretion in vitro and may be a second messenger in insulin secretion (Jones and Persaud, 1993).

The effects of hormones, islet peptides, and neurotransmitters on insulin secretion have been discussed previously. In general terms they are seen to modulate the primary effects of glucose and other nutrients on insulin secretion. There are at least three potential sites for the modulation of insulin secretion by hormones, peptides and neurotransmitters. Firstly, they may affect the ion channels that influence membrane potential and calcium-ion influx. Secondly, they may exert effects on the mobilization of intracellular calcium stores, notably the endoplasmic reticulum, which can influence cytosolic Ca$^{2+}$ concentration. Thirdly, they may modify Ca$^{2+}$ sensitivity, which can amplify or dampen the stimulatory action of calcium ions on the release of insulin secretory granules. Stimulatory or inhibitory guanosine triphosphate (GTP)-binding proteins mediate receptor binding effects by altering the activity of adenylate cyclase, phospholipase C, ion channels or other events that are involved in the release of insulin (Hellman et al. 1992; Berggren et al. 1992; Morgan and Montague, 1992; McDermott and Sharp, 1994; Persaud et al. 1994; Berggren et
al. 1994). Indeed receptor-linked G proteins may also be involved in the effects on membrane potassium channels (Gillison and Sharp, 1994). The primary counter-regulatory hormone, (of insulin secretion), is glucagon. Glucagon is a powerful stimulus to insulin secretion and operates by activating adenylate cyclase and generating cAMP within the β cell (Schuit and Pipeleers, 1986). This effect is mediated by specific glucagon receptors (Van Schravendijk et al. 1985; Kawai et al. 1995), which are linked to the activation of adenylate cyclase through a stimulatory GTP-binding protein (Gs) (McDermott and Sharp, 1994). cAMP in turn activates PKA, which sensitizes the β cell secretory machinery to calcium ions through the phosphorylation of intracellular proteins.

It has also been demonstrated that insulin secretion can be induced by glucose through a mechanism distinct from the closure of $K_{ATP}$ channels, alterations in Ca$^{2+}$ concentration, or activation of protein kinases A or C (Henquin et al. 1994). Such a mechanism may imply metabolic coupling factors other than ATP (Maechler et al. 1997). Malonyl Coenzyme A (CoA) and long chain fatty acid Co A have been proposed as alternative metabolic coupling factors in β cells, linking fuel metabolism to insulin secretion (Prentki, 1996). Fatty acyl-CoA is required for budding of coated transport vesicles from Golgi cisternae (Pfanner et al. 1989) and may thus be also involved in other transport steps. Insulin secretion can also be induced independently from Ca$^{2+}$ by intracellular application of guanine nucleotides such as GTP, GppNHp and GTPγS (Vallar et al. 1987; Proks et al. 1996).

1.5 Biosynthesis and exocytosis of insulin.

In humans, the gene encoding preproinsulin, the ultimate precursor of insulin, is located on the short arm of chromosome 11, close to that for insulin-like growth factor 2 (IGF-2) (Bell et al. 1980). It is 1355 base pairs in length and its coding region consists of three exons: the first encodes the signal peptide and the N-terminus of the preproinsulin, the second the B chain and part of the C peptide, and the third the rest of the C peptide and the A chain. Transcription and splicing to remove the introns yields a messenger RNA of 600 nucleotides, translation of which gives rise to preproinsulin, an 11.5-kDa polypeptide. Translation of preproinsulin
mRNA begins in the cytosol with the binding of free ribosomes to the molecule. Transfer to the endoplasmic reticulum membrane occurs co-translationally and is initiated by the binding of the merging signal sequence to the 54 kda subunit of the signal recognition particle (SRP), an 11S ribonucleoprotein complex (Eskridge and Shields, 1983; Kurzchalia et al. 1986). This retards further elongation of the nascent polypeptide chain and further translation does not continue until the SRP interacts with the SRP receptor on the endoplasmic reticulum membrane (Meyer et al. 1982). The nascent peptide chain is then transferred to the signal sequence receptor (SSR) where translation is completed, and is discharged into the cisternal space of the rough endoplasmic reticulum, where signal peptidase immediately cleaves off the N-terminal signal sequence consisting of 24 amino acids, to leave proinsulin. Proinsulin is a 9-kDa peptide, containing the A and B chains of insulin joined by the C peptide. The structural conformations of proinsulin and insulin are very similar, and a major function of the C peptide is to align the disulphide bridges that link the A and B chains so that the molecule is correctly folded for cleavage.

As shown in Figure 1.5, proinsulin is then transported by transfer vesicles to the Golgi apparatus (Orci, 1984). There the proinsulin passes through the cis, medial and trans Golgi cisternae by repeated cycles of vesicle budding and fusion in processes involving the sequential formation of vesicles with a cytoplasmic protein coat, followed by removal of the coat by a GTP-binding protein and GTP hydrolysis (Bourne, 1988; Tooze et al. 1990). Evidence indicates that not only monomeric GTPases, but also heterotrimeric G-proteins are involved in vesicle transport steps throughout the secretory pathway. The heterotrimeric GTPases consist of an α-subunit and a βγ dimer, both of which influence a number of intracellular effectors such as ion channels, adenylate cyclase, phospholipases and kinases (Hamm, 1998; Clapham and Neer, 1997).
The vesicle transfer continues to the trans Golgi network (TGN) where the secretory granules are formed, the function of the TGN being to sort proteins into different vesicles depending on their final destination (Griffiths and Simons, 1986). Partially coated with clathrin when they bud from the trans face of the TGN, these vesicles are referred to as immature granules, as they still contain predominately proinsulin. As the granules 'mature', insulin is formed through the sequential action of two endopeptidases (prohormone convertases 2 and 3) and carboxypeptidase H (Hutton,
Together these enzymes act to remove the C-peptide chain, finally yielding insulin. An important part of this maturing process is the acidification of the immature granule by a membrane bound ATP-dependent proton pump. Whilst the TGN and newly formed immature granules are near neutral pH, that of the insulin secretory granules is 5.0 - 5.5 (Hutton, 1982; Davidson et al. 1988). This acidification is aided by the excision and loss of basic amino acids during the proteolytic conversion of proinsulin to insulin. The mature secretory granule, or large dense core vesicles (LDCV) can be formed approximately one hour after translation of the insulin mRNA was started (Bailyes et al. 1992), and during this last maturation stage the shedding of the clathrin partial coat is an important factor in achieving full maturity (Arvan and Castle, 1998).

Insulin has a lower solubility that both C-peptide and proinsulin (Hutton, 1994) and coprecipitates with zinc ions to form microcrystals within the secretory granule (Orci et al. 1986; Howell et al. 1969), with this precipitation facilitated by the low pH within the granule. Insulin and C peptide are stored together in the granule sac and are ultimately released in equimolar amounts. Under normal conditions 95% of the hormone product is secreted as insulin and less than 5% as unconverted proinsulin (Bell, 1980).

The secretory granules also contain a whole range of other polypeptides, in relatively low abundance, two of which have been well characterised. β-granin is an analogue of chromogranin A, which is a component of neuroendocrine secretion vesicles originally identified in adrenal medullary tissue. Its abundance is 0.1-0.3% of that of insulin (Guest and Hutton, 1992) and its role is probably to facilitate storage of hormone in the granule. Islet amyloid polypeptide (IAPP; amylin) is present at a level of about 3% of the insulin concentration, and is co-localized with the insulin (Clark et al. 1989). It is a 37-residue peptide. The possible role of IAPP in the aetiology of diabetes is as yet unproven.

In normal β cells, insulin is released by the process of exocytosis, in which the granules first move close to the cell membrane, and the granule membrane and the
cell membrane then fuse together, releasing the granule contents. Following the incorporation of the granule membrane, the newly expanded plasma membrane is partly reabsorbed into the cell by endocytosis and recycled back to the Golgi complex for re-use; irregularities left in the plasma membrane after this process are called microvilli (Lacy, 1970).

1.5.1 Stimulus-coupling in insulin synthesis and exocytosis.

As has been discussed earlier glucose and other nutrients are able to increase insulin secretion. Acute (<2 h) increases in the extracellular concentration of glucose and certain other sugars results in a rapid and dramatic increase in proinsulin synthesis (Ashcroft et al. 1978). Insulin biosynthesis and secretion are not obligatorily coupled, as glucose-stimulated insulin secretion is inhibited in Ca^{2+} free medium, but biosynthesis is still activated (Pipeleers et al. 1973a). There is a sigmoidal relationship between glucose concentrations and biosynthetic activity, with a threshold glucose level of 2-4 mmol/l. This is lower than the threshold for the stimulation of insulin secretion, and probably ensures an adequate reserve of insulin within the β cell. Indeed, in cases where this leads to overproduction of insulin by the β cells, secretory granules may fuse with lysosomes and, in a process termed crinophagy, the insulin and other secretory granule contents are destroyed by proteolytic action (Halban and Wollheim, 1980).

Glucose has been shown to not significantly increase islet preproinsulin mRNA levels until after approximately two hours (Itoh and Okamoto, 1980). The effects on insulin mRNA levels result from a combination of a stabilizing effect on pre-existing mRNA (Welsh et al. 1985), increased processing of pre-RNA (Wang et al. 1997), and an increased rate of insulin gene transcription (Efrat et al. 1991). Translation of insulin mRNA however does increase within 20 min of exposure to glucose, and so must involve pre-existing insulin mRNA (Permutt and Kipnis, 1972; Ashcroft et al. 1978). Physiologically therefore glucose stimulates the replenishment of insulin reserves, and this is important to control of the second-phase insulin secretion. In the longer term (>2 h), glucose does increase islet preproinsulin mRNA levels by increasing the transcription of the insulin gene.
Transcription of the human insulin gene is dependent on sequences located within a region up to 350 base pairs from the transcription site and an adjacent hypervariable region, although further upstream sequences may also be important. Within the promoter proximal region of the human insulin gene, a number of cis-acting regulatory sequences have been identified (German et al. 1995), of which the most important appear to be the E1 and E2 boxes located at -104 and -232 respectively, and the A boxes (A1, A2, A3 and A5) located at -76, -130, -210 and -312, respectively. The E1 box (-104) has been demonstrated to bind the factor IEF1 (insulin enhancer factor 1), which is a heterodimer of the proteins Beta2/NeuroD and E47 (Lee et al. 1995; Naya et al. 1995).

At least four transcription factors are thought to bind to the E2 box. These include the upstream stimulatory factor (USF) and a RIPE3B1-like factor (Read et al. 1993; Read et al. 1995; Read et al. 1997).

Although the A boxes bind a number of homeodomain transcription factors (Rudnick et al. 1994), most recent interest has centred on one of these factors, PDX1. ‘Pancreatic duodenum Xenopus homeodomain factor-1’ or ‘pancreatic/duodenal homeobox-1’ (PDX1), has previously been referred to as insulin promoter factor 1, IDX1, STF1 and IUF1.

When activated PDX1 has been demonstrated to move from the cytoplasm to the nucleus. The first stage of this activation is the phosphorylation of PDX1, which has been shown to occur as a result of glucose metabolism. Other stages of modification are most probably involved in the activation of PDX1, as phosphorylation alone would struggle to account for the increase in molecular mass of PDX1 from 31 kDa in the inactivated form to 46 kDa in the activated form (Macfarlane et al. 1999). A similar control of transcription factor binding by its phosphorylation status is seen with the cyclic AMP response element binding factor (CREB), which also binds to the human insulin gene promoter (Marshak et al. 1996; Macfarlane et al. 1997).
Upon translocation of the transcription factor to the nucleus, activated PDX1 is known to bind to the promoter regions of not only the insulin gene, but a number of genes preferentially expressed in the β cell. These include those coding for, glucokinase, islet amyloid polypeptide and the glucose transporter GLUT-2 (Melloul et al. 1993). Within the human insulin promoter regions PDX1 has been shown to bind to the four A box sites A1, A2, A3, and A5, with all of these sites displaying the base sequence C(C/T)TAATG (Clark et al. 1993).

Current evidence suggests that the phosphorylation of PDX1 is mediated by the stress-activated protein kinase 2 (SAPK2) in a signal pathway that involves phosphatidylinositol 3-kinase (PtdIns 3-kinase) (Macfarlane et al. 1997). Unusually, despite being central to many of the pathways involved in nutrient-induced insulin secretion, it would appear that neither cAMP-dependent protein kinase nor protein kinase C mediates the glucose-induced activation of PDX1 (Macfarlane et al. 1997).

The presence alone of glucose is not enough to cause the activation of PDX1, but rather the requirement is glucose metabolism (Macfarlane et al. 1994). Indeed it is the requirement of nutrient metabolism as witnessed by the activation of PDX1 in the absence of glucose and presence of other metabolisable nutrients such as pyruvate, glyceraldehyde, fructose, mannose, certain amino acids and α-ketoisocaproate (Wu et al. 1999).

It would appear that there are a number of metabolic signals for glucose-induced and nutrient-induced transcription of the insulin gene. The role of glucose-6-phosphate has been demonstrated by 2-deoxyglucose which enters the cell and is phosphorylated but not metabolised any further. Despite this 2-deoxyglucose stimulated both PDX1 DNA-binding activity and insulin promoter activity (Wu et al. 1999). Glucose-6-phosphate is also thought to be a metabolic signal for the gene transcription of other anabolic peptides (Mourieras et al. 1997). The effective stimulation by other nutrients which do not result in the formation of glucose-6-
phosphate, such as xylitol point to other metabolic signals, perhaps xylulose 5-phosphate (Doiron et al. 1994).

Supporting the reports of the feed-forward loop effect of insulin on the secretion of insulin in human and rat β cells (Section 1.3.5) (Leibiger et al. 1998; Xu et al. 1998; Aspinwall et al. 1999), it has been demonstrated on human islets in vitro that insulin has a similar effect as glucose metabolism on the DNA binding of PDX1 (Wu et al. 1999). Insulin may also perform this autoregulatory role via the phosphorylated heat- and acid-stable protein regulated by insulin (PHAS-I), a phosphoprotein known to regulate initiation of mRNA translation. The phosphorylation state of this protein is increased by exposure to glucose or exogenous insulin, resulting in increased translation of mRNA (Xu et al. 1998).

It is now widely accepted that PDX1 is a key element in linking cytosolic events to nuclear signalling (Rafiq et al. 1998). The exact role of PDX1 in basal insulin transcription, however, is unclear since insulin gene transcription can occur in the absence of PDX1 (Macfarlane et al. 1997b), but it undeniably performs a role in glucose-responsive insulin secretion (Macfarlane et al. 1994). PDX1 is an essential component of the mechanisms by which glucose modulates both insulin promoter activity and endogenous insulin mRNA levels, thus at the very least it is required to provide the capacity to replenish insulin stores following secretory stimuli.

PDX1 is, however, only one component of a complex metabolic signalling system. As previously mentioned several other factors, such as E1 and E2 site binding factors are required for transcription of the insulin gene, and increased PDX1 binding activity will only exert an effect in the presence of the many other factors. At least one factor is also known to bind to a negative regulatory element of the human insulin gene (Sander et al. 1998). Indeed the effects of nutrients and hormones on the insulin promoter are dependent on the combined activity of a number of DNA sequences (Wu et al. 1999; German and Wang, 1994). These elements bind factors that, depending on the context, act in either a positive or negative manner (Clark and
Docherty, 1993). The net effect on the insulin promoter and insulin transcription may be the consequence of several signalling pathways acting on different factors.

It has been established that ATP is an absolute necessity at a stage prior to the release of vesicle contents, termed the priming stage (Vallar et al. 1987; Hay and Martin, 1992). This process requires submicromolar concentrations of Ca$^{2+}$ (Heinemann et al. 1993) and involves alterations in membrane lipids by phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) (Martin, 1997).

An hypothesis has been proposed to account in molecular terms for membrane docking and/or fusion of transport vesicles throughout the secretory pathway from the endoplasmic reticulum to the plasma membrane (Rothman, 1994). This process is thought to be induced by the pairing of specific proteins on the vesicle membrane, termed v-SNAREs, with cognate proteins on the target membrane, the t-SNAREs. These proteins when they pair are capable of forming extremely stable ring-like structures (Sutton et al. 1998). v-SNAREs already identified include VAMP, synaptobrevin and cellubrevin, whilst syntaxin and SNAP-25 have been identified as t-SNAREs. All of these are seen to be essential for Ca$^{2+}$ evoked insulin exocytosis (Niemann et al. 1994; Regazzi et al. 1995; Sadoul et al. 1995; Williamson et al. 1996; Wheeler et al. 1996). Additional proteins, such as exocyst, munc18/sec1 and munc13, to name but a few, are thought to regulate the functioning of the SNARE proteins (Kee et al. 1997; Orita et al. 1997; Wu et al. 1998).

The role of cytoskeletal elements, notably microtubules and microfilaments, in the intracellular translocation of the insulin storage granules has been extensively studied. However, the precise ways in which the metabolic signals developed during the stimulation of insulin secretion are translated into granule movement and exocytosis remain unknown. Microtubules are formed by the polymerization of tubulin subunits (54 kDa). They were first implicated in the mechanism of insulin secretion by the observation that colchicine and various other ‘microtubule (spindle) poisons’ all effectively inhibit insulin release (Howell, 1984). These agents have quite different effects on microtubule function. Colchicine disaggregates
microtubules by binding to their tubulin subunits, whereas vinblastine causes the formation of paracrystals of microtubular protein and nocodazole produces microtubule disaggregation through a different mechanism. As all these compounds inhibit glucose-stimulated insulin secretion, the dynamic turnover of tubulin to microtubules would appear to be important for the mechanism of secretion, rather than the total number of polymerized microtubules within the β cell. The localization of microtubules within the β cell has been studied in monolayer cultures (Boyd et al. 1982). Microtubules normally form a network radiating outwards from the perinuclear region, which is disrupted by prior exposure of the cells to colchicine. This microtubule framework may provide the mechanical pathway along which secretory granules move in an orientated fashion, but it is now clear that the microtubules themselves could not provide the motive force to propel the granules through the cytoplasm (Howell and Tyhurst, 1986). Other contractile proteins, especially actin and myosin, seem likely to participate in the secretory process.

Actin, the constituent protein of microfilaments, exists in cells in two forms, a globular form of 43 kDa and a filamentous form, which associates to form microfilaments. Evidence for the involvement of microfilaments in insulin release derives partly from the observation that insulin secretion is inhibited by cytochalasin B and phalloidin, both of which interfere with microfilament formation. In addition, the regulation of microfilament polymerization is governed by various factors similar to those which alter rates of insulin secretion (Howell, 1984). Myosin light and heavy chains have also been detected in rat islets at concentrations that are considerably higher than those found in liver, and have been localized by immunofluorescence to both β and A cells. It is possible that actin (microfilaments), tubulin (microtubules) and myosin might act together to transport granules. The microtubules acting as a network along which the granules are propelled by the interaction of actin and myosin, analogous to that in muscle.

The pathway of secretion via the storage granules is termed the 'regulated pathway', and in normal β cells probably carries approximately 95% of the insulin released from the cell. However, in some types of insulinoma cells and cell lines, an
alternative ‘constitutive pathway’ operates, which omits the conversion and packaging stages and allows the release of insulin from vesicles derived directly from the endoplasmic reticulum. Since the organelles involved in proinsulin processing are bypassed, the major product of the constitutive pathway is proinsulin (Halban, 1994). It has been proposed that the sorting of secretory proteins into the regulated pathway requires a positive signal and that the constitutive release occurs by default (Burgess and Kelly, 1987). The signal must be relatively independent of cell type and species, since many secretory cells can target transfected foreign secretory proteins to their own secretory granules. The ‘regulated pathway’ is remarkably efficient in normal tissue, with less that one percent of insulin released constitutively (Rhodes and Halban, 1987). However, a greater proportion of insulin is released constitutively in transplantable rat insulinoma cells (Sopwith et al. 1981), and this pathway is also disproportionatly increased in certain patients with NIDDM.

1.6 Insulin receptor

The many actions of insulin discussed previously are mediated on target tissues by a receptor. The insulin receptor is a large transmembrane glycoprotein complex which belongs to the large family of growth factor receptors with intrinsic tyrosine kinase activity. The receptor is a heterotetramer consisting of two ligand-binding 135 kDa α subunits and two tyrosine kinase 95 kDa β subunits. Whereas the α subunits are entirely extracellular, the β subunits span the plasma membrane and so have an intracellular domain, a transmembrane domain and an extracellular domain (Massague et al. 1982; Olson et al. 1988; Mosthaf et al. 1991). Insulin-like growth factor-1 (IGF-1) receptor is known to have a similar structure to the insulin receptor, to the extent that it will form chimeric hybrids with the insulin receptor (Frattali et al. 1992). Indeed insulin and IGF-1 have evolved from common ancestral precursors, as have the receptors for the peptides aswell. The insulin-like growth factors (IGF-I and IGF-II) are structurally related to insulin, and both the IGFs and insulin play important roles in the regulation of growth and metabolism in vertebrates (Navarro et al. 1999).
Insulin binding leads to activation of the tyrosine kinase of one of the two \( \beta \) subunits and a rapid cascade of autophosphorylation of the receptor, which in turn causes the phosphorylation of insulin receptor substrates (IRS). IRS that have been identified are IRS-1, IRS-2, IRS-3, IRS-4, Gab-1 (Grb 2 [growth factor receptor-bound protein 2]-associated binder 1), three isoforms of Shc (Src and collagen-homologous protein), and p62\(^{dok}\) (Sun et al. 1991; Rothenberg et al. 1991; Pelicci et al. 1992; Sun et al. 1995; Holgado-Madruga et al. 1996; Lavan et al. 1997\(^a\); Lavan et al. 1997\(^b\); Carpino et al. 1997). The phosphorylated IRS molecules form complexes with docking proteins such as phosphoinositide-3 kinase which is itself then activated and able to mediate many of the actions of insulin such as glucose transport, glycogen synthesis, protein synthesis and mitogenesis (Virkamäki et al. 1999).

Recent research has highlighted the importance of protein-interaction domains within this 'transfer of activation'. Such domains include pleckstrin homology (PH) domains, phosphotyrosine (PTB) domains, Src homology 2 (SH2) and SH3 domains (White, 1998).

Insulin receptors are in a constant state of turnover as it shuttles from the cell surface to an intracellular membrane pool (Carpentier et al. 1979; Goldfine et al. 1979; Schlessinger et al. 1980). Although insulin receptors do recycle spontaneously with a half-time of approximately 7-12 h, most are internalized by a process of ligand-mediated endocytosis, whereby insulin binding leads to internalization of the hormone-receptor complexes. Under the influence of ligand-mediated endocytosis the half-life of insulin receptors is reduced to 2-3 h (Kasuga et al. 1981). The prolonged presence of high levels of insulin can therefore lead to a 'down-regulation' in the numbers of insulin receptors on a cell's surface (Gavin et al. 1974; Knutson et al. 1983), which is of relevance to certain sub-groups of NIDDM sufferers (Kahn, 1980). Internalized receptors are degraded and replaced, though often some escape degradation and recycling, to make several cycles to and from the cell membrane. (Marshall and Olefsky, 1983). In a process called transcytosis insulin receptors can also shuttle from one side of a polarized cell to the other and back. Transcytosis and the general internalization of insulin with the insulin receptor may
play a role in the compartmentalization of some insulin signals (Virkamäki et al. 1999).

Although it could not be said to be a tailored drug, since its discovery came after 50000 substances were screened, L-783.281 is the first drug to mimic the actions of insulin binding to its receptor. Derived from a *Pseudomassaria* fungal extract it is a small non-peptidyl molecule which appears to bind directly to the intracellular β subunit of the insulin receptor and thus initiating a phosphorylation cascade (Zhang et al. 1999).

### 1.7 The causes of diabetes mellitus.

The defining features of IDDM is chronic hyperglycaemia caused by a complete or near complete deficiency in insulin, and will often present with severe weight loss. So severe is this insulin deficiency that the condition is life threatening and without insulin replacement therapy the patient will spontaneously develop ketoacidosis (see Section 1.8). The most common cause of IDDM is the 'type I' process, namely the autoimmune destruction of the islet β cells (Atkinson and Maclaren, 1994). It presents most commonly in children and young people with peaks before school age and around puberty (Bowen Jones and Gill, 1997), hence why it has been referred to as 'juvenile-onset diabetes'. It can afflict adults, but after the age of 20 years the incidence is thought to be a constant low level (Kilvert et al. 1984). Familial clustering of IDDM emphasises the role of genetic factors in susceptibility to the disease. A major portion of this clustering is due to sharing of alleles at susceptibility loci in the major histocompatibility complex on chromosome 6, most notably DQB1 and HLA-DRB1, as well as at least 13 other loci on nine chromosomes (Todd, 1995). As the genetic component is estimated to account for only 30 – 40% of the risk of developing IDDM (Kyvik et al. 1995), individual susceptibility depends more on the non-genetic factors operating on genetically susceptible subjects. These factors are probably environmental and include viral infections, diet (Monte et al. 1994), toxins and emotional stress (Dahlquist, 1993). The clearest contribution is seen by the seasonal variation in the incidence of IDDM, pointing to a strong role of viral infections (Atkinson and Maclaren, 1994) such as Coxsackie B (Fohlman and
Friman, 1993). The autoimmune attack of islet β cells is highly specific and the non-normal response can be seen in both the cellular and humoral aspects of the immune response over a prolonged period up to diagnosis of the disease (Tun et al. 1994). The targets of the immune attack are termed autoantigens and several autoantigens have been identified. Most prominent of these are glutamic acid decarboxylase (GAD) (Baekkeskov et al. 1990) and protein tyrosine phosphatase-2 (IA-2) (Hawa et al. 1997). Quite how normal proteins become autoantigens, the involvement of antigen presentation, and molecular mimicry by dietary or viral antigens all need further clarification. This field of research does offer enormous potential benefit to diabetes mellitus, with the potential to provide pre-diabetic screening and even disease prevention via immuno-intervention of the autoimmune destruction of the islet β cells (Leslie et al. 1999).

NIDDM is by far the most common type of diabetes, so fortunately it is a less severe form of diabetes than IDDM. Hyperglycaemia is less intense than in IDDM and spontaneous ketoacidosis does not occur. By the nature of its name, subjects do not have an essential requirement for glucose, and are often on therapy regimes involving diet and/or oral drugs such as sulphonylureas, metformin or biguanide. It is characteristically of increasing prevalence with advancing age (Harris et al. 1987), hence why it has in the past been referred to as maturity-onset diabetes, and it has a much stronger familial component than seen with IDDM (Barnett et al. 1981; Newman et al. 1987). When this condition does afflict the young it is also referred to as maturity-onset diabetes of the young (MODY). In the obese type of NIDDM the obesity itself, or cause of the obesity, is often the cause of the disease. Levels of insulin may be normal, or even raised, but due to a developed tissue resistance to insulin there is an ‘effective insulin’ deficiency (Golay et al. 1988). Eventually the overproduction of insulin to compensate for insulin resistance usually results in β cell exhaustion or ‘burn-out’ and an insulin deficiency (Bowen Jones and Gill, 1997b). In the non-obese subtype of NIDDM insulin is deficient, though not as severely as in IDDM (Reaven et al. 1989) and is particularly prevalent in Asia. Some NIDDM patients also present with weight loss and show signs of the autoimmune attack seen in IDDM (Atkinson and Maclaren, 1994; Rowky et al. 1992), common
terms for this sub-type of diabetes are latent immune diabetes in adults (LADA) and type I½. (Harris and Zimmet, 1997).

1.7.1 Classification of diabetes mellitus.

Several types of diabetes mellitus have already been mentioned in previous sections, though not all conform to the World Health Organization’s (WHO) classification system. The names given to types of diabetes mellitus have changed and evolved as our understanding of the condition have improved and the clinical tests for it have similarly improved. Also many of the terms used or still in use were derived from our understanding of how the condition came about in that patient. There is a reluctance by many to discard their terms for different types of diabetes when the supposedly improved terms are regarded as not as suitable. The current WHO classification system lists the clinical classes of diabetes as shown in Table 1.7.

Table 1.7.1 WHO classification of clinical classes of diabetes mellitus.

| Insulin-dependent diabetes mellitus (IDDM). |
| Non-insulin dependent diabetes mellitus, of the non-obese (NIDDM). |
| Non-insulin dependent diabetes mellitus, of the obese (NIDDM). |
| Malnutrition-related diabetes mellitus (MRDM). |
| Gestational diabetes mellitus (GDM). |
| Other types of diabetes mellitus associated with other specific conditions and syndromes. |

(Adapted from Keen and Barnes, 1997)

It is clear that due to the unsatisfactory features of this present classification system, it has not nor will it be accepted by the widespread clinical and scientific community. The WHO Study group has served notice that revision and refinement are now needed (World health Organization, 1994), and hopefully this matter will be resolved.
1.8 Metabolic disturbances of diabetes mellitus.
The metabolic disturbances experienced with diabetes depends primarily, among other factors, on whether it is non-insulin-dependent diabetes (NIDDM) or insulin-dependent diabetes (IDDM). The crucial difference between these two types of diabetes is the severity of insulin deficiency, or the effective deficiency. In IDDM this deficiency is profound enough to allow spontaneous, unrestrained lipolysis and enhanced ketogenesis, leading ultimately to diabetic ketoacidosis (Miles et al. 1980).
Hence IDDM is the more severe and life threatening of the two types of diabetes and correspondingly this coverage will centre on IDDM. The many metabolic roles of insulin have been discussed previously, where it can be seen that among the regulatory systems insulin has a dominant role. A rise in insulin concentration is seen to inhibit the catabolic counter-regulatory hormones, and even basal secretions of insulin keep this catabolism under control. In effect IDDM subjects suffer a double blow, as not only is anabolic nutrient-responsive insulin secretion missing, but the lack of even basal insulin secretion means that the catabolic counter-regulatory hormones go unchecked.

In IDDM fasting hyperglycaemia is due primarily to an increase in hepatic glucose production resulting from enhanced and unsuppressed gluconeogenesis (Wahren et al. 1972), due to hypoinsulinaemia (Hall et al. 1979). Fasting plasma levels of major gluconeogenic precursors lactate, pyruvate and glycerol are increased, although plasma concentrations of alanine may be reduced, due to increased extraction by the liver to fuel gluconeogenesis (Wahren et al. 1972; Wahren et al. 1976). Interestingly even in insulin-treated IDDM patients, blood lactate and pyruvate levels are often high and remain so when normoglycaemia is achieved by means of a feedback-controlled intravenous insulin infusion by the ‘artificial pancreas (Capaldo et al. 1984).

In the healthy basal state, when insulin levels are low, most glucose uptake (75-85%) is facilitated by GLUT-1 and GLUT-3 and is non-insulin-mediated (Gottesman et al. 1983; Baron et al. 1985), so even with IDDM patients brain glucose uptake and the ability of hyperglycaemia to promote glucose uptake by tissues other than the brain
by a mass-action effect, are both normal in IDDM (Hansen et al. 1985). Also brain glucose oxidation is normal or only slightly decreased in IDDM (Grill et al. 1990). Due to the mass-action effect and urinary excretion of glucose, subjects with IDDM show an increased rate of glucose disappearance from plasma, and yet still maintain hyperglycaemia (Hall et al. 1979; Pehling et al. 1984). This is partly due to failure to suppress hepatic glucose production, and partly due to diminished insulin-mediated peripheral uptake. The precise nature of the later is unknown, but storage of glucose as glycogen in muscle is decreased. In healthy subjects this insulin-sensitive glucose uptake by peripheral tissues especially in skeletal muscle, may account for 50-60% of total glucose disposal (Andres et al. 1956).

Lipolysis is stimulated by the combination of very low insulin levels and increased concentrations of the counter-regulatory hormones. Consequently, plasma NEFA levels are raised (Hall et al. 1979); these may worsen insulin sensitivity through the Randle cycle, and also fuel ketogenesis. Proteolysis is also unrestrained, resulting in a negative nitrogen balance and muscle wasting (Wahren et al. 1972; Bennet et al. 1991). Increased proteolysis might be expected to raise all plasma amino acid levels, but characteristically only the branched chain amino acids valine leucine isoleucine are increased (Wahren et al. 1972; Wahren et al. 1976; Luzi et al. 1990). Indeed plasma levels of several gluconeogenic amino acids, notably alanine, are decreased. This is probably due to increased use of the carbon skeletons of these amino acids for glucose synthesis through gluconeogenesis. The normal response to dietary protein of replacing proteolysis is also decreased in IDDM (Wahren et al. 1976), which only exacerbates the negative nitrogen balance and muscle wasting.

Ketone bodies are formed by partial β-oxidation of fatty acyl CoA derivatives in hepatic mitochondria. Ketogenesis is stimulated by the combination of insulin deficiency and excessive levels of glucagon and the other counter-regulatory hormones, especially of glucagon, through direct stimulatory effects on the carnitine shuttle (Chernick et al. 1972; Schade and Eaton, 1979). Also contributing is the rise in plasma NEFA levels which provides the substrate for ketogenesis. Ketone bodies (acetoacetate, 3-hydroxybutyrate and acetone) accumulate because they are produced
more rapidly than they can be metabolised (Hall et al. 1979; Miles et al. 1980; Johnston and Alberti, 1982), and they impair their own clearance at high concentrations (Miles et al. 1980; Fery and Balasse, 1985). The resultant accumulation leads to diabetic ketoacidosis. Acetoacetate and 3-hydroxybutyrate are osmotically active and strong organic acids, and lead to osmotic diuresis, dehydration, cardiovascular collapse and death.

1.8.1 Long-term complications of diabetes mellitus.
Even with insulin-replacement therapy, long-term complications and an increased mortality rate occur in diabetic patients (Kostraba et al. 1991). The most common complications are the development of diabetic nephropathy (Borch-Johnsen et al. 1985), retinal oedema leading to severe visual impairment, neural dysfunction, and atherosclerosis leading to cardiovascular disease and peripheral vascular disease, often resulting with limb amputation (Green and Hougaard, 1984; Waugh et al. 1989). The cause is generally attributed to prolonged exposure to hyperglycaemia. The damaging effects of glucose on cells, which contribute to the development of diabetic complications, are still not resolved. The three major hypotheses for this phenomenon are the sorbitol pathway (Dvornik D, 1987; Tomlinson, 1993; Yabe-Nishimura, 1998), the non-enzymatic glycosylation (glycation) of proteins (Brownlee, 1995; Hamada et al. 1996), and increased oxidative stress (Wallace, 1977). There are clear examples of inter-connections between the three hypotheses, for example oxidative stress clearly interacts with both the sorbitol pathway (Phillips et al. 1993; Rittner et al. 1999) and glycation (Soulis-Liparota et al. 1995; Lander et al. 1997). It has been suggested that these pathways can synergise by sharing the capacity to activate mitogen-activated protein kinases [MAP kinases] (Tomlinson, 1999). MAP kinases phosphorylate transcription factors, which in turn alter the balance of gene expression. In this way they can alter cellular phenotype, promote division or increase production of extracellular material. A specific isoform of protein kinase C (PKC) has been proposed as a MAP activator (Rowe-Rendleman and Eichberg, 1994).
Glycation is the non-enzymatic reaction between glucose and the amino groups of proteins. The two major factors in determining the extent of this reaction in vivo are the extent of hyperglycaemia and the duration. Although reversible, over prolonged periods these glycation products undergo further chemical rearrangements to form advanced glycation end-products (AGEs) (Brownlee et al. 1984). These are more stable and accumulate throughout the lifetime of the tissue, often altering and hindering the normal function of the tissue concerned (Lee and Cerami, 1987; Tariso et al. 1987; Tsilibary et al. 1988), altering the physical properties of a protein, and causing the production of damaging free radicals (Gillery et al. 1988; Hicks et al. 1988). Or they can react with RAGEs, cell surface receptors for AGEs. These receptors probably exist to facilitate the removal and clearance of AGEs, but it has been shown that ligand binding to RAGE activates MAP kinase (Lander et al. 1997; Thornalley, 1998).

May tissues contain an enzyme, aldose reductase which can convert hexose sugars to their respective sugar alcohols (polyols). These tissues include those which are susceptible to long-term damage in diabetes, notably pericytes in the retina, lens and kidney. This association has fuelled much speculation that excessive glucose flux through the polyol pathway in hyperglycaemic conditions may play a role in chronic diabetic complications (Kinoshita, 1974; Pugliese et al. 1991). Under the action of aldose reductase, glucose is converted into sorbitol, which can in turn be oxidized to fructose by sorbitol dehydrogenase. Overall these two reactions consume NADPH and generate NADH. Aldose reductase has a low affinity for glucose, and it only enters the polyol pathway when its intracellular concentrations are raised. Under normal conditions glucose is channelled preferentially to the glycolytic pathway whose initial step is catalysed by hexokinase, which has a much higher affinity for glucose. In insulin-independent tissues, the intracellular glucose concentration rises in proportion to the extracellular concentration, and glucose flux through the polyol pathway will increase.

The importance of diabetes mellitus as a major cause of mortality and morbidity and its claim on healthcare provision is increasingly recognised not only in developed
countries but also in developing countries (Betteridge, 2000). The need for research into diabetes mellitus is therefore ever increasing. Of specific interest is the need to gain a full understanding of the mechanisms responsible for glucose-responsive insulin secretion, and the immunology behind the autoimmune destruction of pancreatic β-cells seen in insulin-dependent diabetes mellitus [IDDM] (Baekkeskov et al. 1986; Eisenbarth, 1986). Central to this research is assessment at the molecular level of pancreatic β-cell function and dysfunction.

1.9 Hindrances to β cell functionality studies.

1.9.1 Anatomical considerations.
Assessment of pancreatic β-cell function and dysfunction at the molecular level has historically been limited by the availability of viable primary pancreatic β-cells. The anatomical organisation of the β-cells and the islets of Langerhans contributes to this problem. The islets are of varying sizes and differ from species to species but generally are under 800 μm. Though islets as small as 20 μm are numerous, the bulk of the total islet volume is accounted for by the islets greater than 50 μm (Volk and Wellmann, 1985; Henderson, 1969). They are embedded throughout the exocrine pancreas and represent less that 2% of the total pancreatic mass (McEvoy, 1981; Stefan et al. 1982).

1.9.2 Islet Isolation.
Islet isolation requires selective digestion of the exocrine tissue and purification of the endocrine islets (Moskalewski, 1965; Lacy and Kostianovsky, 1967). Relative to other cell isolations, the islets of Langerhans present a particularly difficult challenge. This has led to differing techniques being employed, though all based around selective digestion. These techniques are inherently time consuming and require highly skilled workers. The main difficulty is the need to remove the exocrine tissue from the islets without damaging the islets themselves. Also the generation of a viscous, gelatinous material is a common sight to workers during the
selective digestion process. This gel can be seen to entrap islets and thus decrease yield drastically (Burghen and Murrell, 1989).

The establishment of an automated method for the isolation of human pancreatic islets and its use in laboratories involved with islet banking for transplantation, has led to somewhat of a benchmark technique (Ricordi et al. 1988). This automated technique does allow for a less skilled work force and a decrease in the work load. It is however expensive and the isolation of intact islets free of exocrine tissue and the automation itself all come at the cost of decreased yields. Manual isolation is still best when yields are important, but there is no standardised or generally accepted manual isolation technique.

1.9.3 Donor Organs.

The donor organ is also a problem. Viable human pancreas organs available for research are rare. Researchers are dependant on the unknown timings of organ availability, and more and more the banking of human islets for transplantation is making the human organ unavailable for cellular research. The use of animals does overcome some of the problems encountered with human organs, specifically the ability to harvest the required number of organs when desired. The cost can be minimised by using animals routinely sacrificed in abattoirs. Porcine pancreas has not proved too useful due to unusually fragile islets (Ricordi et al. 1986) but bovine pancreas has proved to be of some use (Hering et al. 1989).

1.9.4 Heterogeneity of islets.

The use of organs from commercially slaughtered animals does however introduce the problem of ‘lack of consistency’, seen also with humans, where the islets isolated from two individual animals can never be guaranteed to function in exactly similar ways under the close scrutiny of laboratory experiments. In-bred laboratory rodents do minimise any lack of consistency between individuals and have become a mainstay for pancreatic islet research. The lack of consistency issue is compounded by the belief that not all islets within the same pancreas behave physiologically or biochemically in the same manner. The size of an islet and its location within the
pancreas are thought to be factors in this variation (Pipeleers, 1987; Schuit et al. 1988).

1.9.5 Heterogeneity of islet cells.
As discussed earlier the β cells are just one of at least four cell types present in the islet. To separate β cells from the other cell types requires the use of fluorescence-activated cell-sorting [FACS] (Van den Winkel and Pipeleers, 1983). This technique has also been the centre point of attempts to overcome the heterogeneity problem by standardisation of isolated rat β cells (Pipeleers et al. 1991). Access to FACS is limited and expensive. Hence to get large numbers of pure β cells isolated from a pancreas is at present financially impractical for most researchers.

1.9.6 Life span in culture.
Being differentiated endocrine cells, β cells do not proliferate in culture. Unless special techniques are used it is difficult to maintain them in culture for long periods (Kaiser et al. 1991; Beattie et al. 1996), during which unpurified β cells can suffer from fibroblast overgrowth (Yoshida et al. 1982). In summary, studies on pancreatic β cell function are hampered by the heterogeneity and limited yields of experimental material.

1.10 Advantages of a pancreatic β cell line.
To overcome this heterogeneity and limited yield of primary β cells, the usefulness of pancreatic β cell lines have long been recognised (Murray and Bradley, 1935), especially a human β cell line. As a result, numerous investigators have attempted to immortalise β cells and establish stable insulin-secreting cell lines. Being cell lines they are not only able to survive but grow and multiply in culture, whilst maintaining the ability to synthesise, process and secrete insulin. As such they provide a practically inexhaustible supply of pure viable cells which are inherently standardised, and can be harvested at will with a minimum of effort.
1.11 Previous attempts at establishment of a pancreatic β cell line.

1.11.1 Foetal and neonatal tissue.

The high proliferative characteristics of foetal tissue and yet fully differentiated islet cells continues to be an attractive hunting ground for investigators. Attempts have been made to use human and animal foetal islet cells to generate insulin-secreting cell lines; Human JHPI-1 (Matsuba et al. 1981); bovine (Sun et al. 1977); and neonatal rat (Ng et al. 1987). The common result, however, is that the insulin-secreting cells stop replicating or dedifferentiate rather rapidly in vitro, as witnessed by the loss of the ability to synthesize and secrete insulin (Beattie et al. 1996; Bouwens et al. 1997). The JHPI-1 cell line was reported to secrete insulin for more than 150 days. However this cell line does not appear to be acknowledged (Wollheim et al. 1990b; Poitout et al. 1996; Bouwens et al. 1997).

1.11.2 Tumour tissue.

In the quest for stable insulin-secreting cell lines the oldest and most tried technique has been to derive such a cell line from spontaneous human insulinomas. Reported cell lines include HIN D8 (Thivolet et al. 1986) and CM (Gueli et al. 1987). The HIN D8 ‘cell line’ was reported to secrete insulin after 7 months in culture (Thivolet et al. 1986), but the growth rate was so low that it could barely be referred to as a cell line. Several studies have been performed using these and other semi-permanent human insulinoma cell lines (Chick et al. 1973; Adcock et al. 1975; Cavallo et al. 1996), though the semi-permanence implies unsuitability to refer to these as cell lines (Poitout et al. 1996; Wollheim et al. 1990b).

1.11.3 Induced insulinomas

Animals do allow the luxury of not having to rely on chance discovery of an insulinoma. The insulinomas are induced chemically or by either virus or radiation. One and a half years after rats were irradiated with X-rays the RIN tumours developed. These were then transplanted under the skin of healthy rats (Chick et al. 1977). The secondary RIN tumours were then serially transplanted into NEDH rats or BALB/c mice. After further stages of culture and in vivo passaging, several RIN
and MSL cell lines were established. These represented the first true insulin secreting β cell lines. The most commonly used of these is the RINmSf subline (Gazdar et al. 1980). The cell line CRI-G5 was developed using the same serial transplantation technique of the original RIN tumour cells (Carrington et al. 1986). The In-111 hamster insulinoma cell lines were generated by (Uchida et al. 1979) using the oncogenic BK-virus. Dedifferentiation in culture was again pronounced and subcloning produced cells lines which secrete only islet hormones other than insulin (Takaki et al. 1986). Tumours induced chemically in rats by administration of nicotinamide and streptozotocin were propagated in culture, but the cell lines generated had almost no proliferative capacity (Masiello et al. 1983). More recently, the rat exocrine pancreatic AR42J cell line was established from a chemically induced tumour (Christophe, 1994). Culture of this cell line with betacellulin & activin A, or Hepatocyte Growth Factor, produced clones in which the exocrine function was lost in preference to the endocrine secretion of insulin, as a result of differential gene expression (Mashima et al. 1996a,b; Mashima et al. 1999).

The use of induced insulinomas proved to be successful at generating cell lines that were relatively stable. That is to say the rapid dedifferentiation seen with cell lines from foetal or spontaneous insulinoma tissue was not witnessed with this technique, with the odd exception (Rae et al. 1979). The importance of RINmSf can not be over emphasised as it was relied upon heavily for over a decade and has led directly to the establishment of the more current cell lines INS-1 and INS-2. These were generated after culturing RINmSf in the presence of 2-mercaptoethanol (Asfari et al. 1992).

However, the technique of tumour induction followed by sequential in vivo-in vitro passaging is very arduous, time consuming, and could never be used to generate a human β cell line.

1.11.4 Induced tumours in transgenic mice.

A more recent technique was the induction of tumours in transgenic mice (Hanahan 1985). A recombinant oncogene was constructed by fusion of the regulatory region of the rat insulin II gene to the structural region of the SV40 T antigen gene. The
recombinant oncogene was then injected into fertilised mice embryos which were brought to term in pseudo-pregnant females. The offspring developed β cell tumours from which the β-TC insulin secreting cell lines were established (Efrat et al. 1988). Similar methodology was employed to establish the insulin-secreting cell lines MIN6 (Miyazaki et al. 1990), IgSV 195 (Gilligan et al. 1989), βHC (Radvanyi et al. 1993) and NIT-1 (Hamaguchi et al. 1991). The β-TC parental cell lines were later subcloned to produce a multitude of cell lines (Efrat et al. 1993; D’Ambra et al. 1990; Knaack et al. 1994). The use of transgenic mice has thus provided the current generation of β cell lines. They are stable, functional and in some cases exhibit glucose-responsive insulin secretion. Again, however, this technique could never be used to generate a human β cell line.

1.11.5 In vitro viral transformation.

A technique which could be tried on human islets was in vitro viral transformation. The HIT cell line was established by the in vitro simian virus SV-40 viral transformation of islets of Langerhans isolated from Syrian hamster, followed by serial cloning (Santerre et al. 1981). The subclone HIT-T15 is still one of the most widely used insulin cell lines, though it is somewhat unstable and a working life of only 20 passages is recommended (Wollheim et al. 1990). This dedifferentiation was even more evident in other cell lines generated by this technique, with the capacity to secrete insulin completely lost. (Niesor et al. 1976; Ide et al. 1988; Haukland et al. 1992). After the establishment of HIT in 1981 it seemed only a matter of time before a similar line of human origin was established. It was 10 years before any success was reported, albeit short lived. The HP62 cell line was established after the in vitro transformation of human islet cells by tissue-specific expression of SV-40 early region DNA, transfected using a recombinant vector (Soldevilla et al. 1991). The cloned cell line lost its capacity to secrete insulin after only 8 passages. Similar dedifferentiation was seen in the TRM cell lines created by in vitro transformation of human foetal islet cells (Levine et al. 1995; Wang et al. 1997).
1.11.6 Other genetic engineering techniques.
Transfection of mouse anterior pituitary cells with a viral promoter controlling the expression of the human pro-insulin cDNA, followed by the engineered expression of the GLUT-2 receptor has produced the AtT-20ins cell line which secretes human insulin with some degree of glucose-responsiveness (Moore et al. 1983; Hughes et al. 1992). This cell line is however not a pancreatic β cell line, rather a mouse pituitary cell line. Despite the earlier unsuccessful attempt on the MSL cell line (Madsen et al. 1998), the technique has been successfully applied to the RINr1046-38 cell line. In what has been termed ‘iterative engineering’, the resultant βG l/7 cell line secretes human insulin with glucose-responsiveness and is a pancreatic β cell line, albeit of rat origin (Clark et al. 1997; Hohmeier et al. 1997).

1.11.7 Neonatal PHHI cell lines.
In recent years, whilst studying the physiology of β cells from human patients suffering from persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), it was noticed that these β cells had the potential to spontaneously proliferate in vitro, and the cell lines Nesi B and NES2Y have been generated using these β cells.

Persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) is a potentially lethal disease of the newborn, characterized by unregulated and persistent hyperinsulinaemia resulting in profound hypoglycaemia. Treatment of this condition usually involves a near total removal of the organ. The primary cause of this condition has been identified as the absence of functional ATP-regulated potassium (K_{ATP}) channels in the β cells of sufferers (Kane et al. 1996; Dunne et al. 1997). The metabolic consequence of this loss of K_{ATP} functionality is primarily an unregulated entry of Ca^{2+} ions with a resultant stimulation of exocytosis, in short the insulin secretion stimulus is continually present (Macfarlane et al. 1999b).

This area did not seem overly promising when the first cell line Nesi B displayed all the unfavourable characteristics seen with foetal β cell ‘lines’. In that the cell line dedifferentiated rather rapidly in vitro, with the ability to synthesize and secrete...
insulin lost after only a few passages and, even despite this dedifferentiation, growth rates were too slow to be of practical use (Scott et al. 1992). Again displaying the apparent inverse relationship between the proliferation capacity and differentiation (Wollheim et al. 1990).

When the cell line NES2Y was first reported it appeared to offer no improvement on Nesi B, with poor unregulated insulin secretion and poor growth rates (Macfarlane et al. 1997). When detailed characteristics of this cell line were reported, they showed that the cells were subject to a short functional life (with cells earlier than passage 20 only of use), and had a limited ability to store insulin (Macfarlane et al. 1999; Wu et al. 1999; Macfarlane et al. 1999). Nevertheless, the fact that the cell line has survived and provided enough cells for thorough investigations is a major achievement. This cell line represents the first true human β cell line, albeit with some regulatory deficiencies.

The known deficiencies of NES2Y are a lack of PDX1 and operational K<sub>ATP</sub> channels (Macfarlane et al. 1997; Macfarlane et al. 1999). As a result, glucose has no effect on both insulin promoter activity and endogenous insulin mRNA levels, they have markedly impaired cytosolic Ca<sup>2+</sup> signalling mechanisms, they constitutively release insulin at an elevated rate in the absence of glucose. They do display a limited responsiveness to glucose, thought to be due to the K<sub>ATP</sub> channel-independent pathway of second phase glucose-induced insulin secretion (Aizawa et al. 1998; Straub et al. 1998; Macfarlane et al. 1999). Ironically in NES2Y it is thought to be the lack of functional K<sub>ATP</sub> channels resulting in an unregulated influx of Ca<sup>2+</sup> that fuels this small amount of glucose-induced insulin secretion (Macfarlane et al. 1999).

It is unclear as to why NES2Y cells proliferate in culture as these cells have impaired expression of the homeodomain transcription factor PDX1 (Macfarlane et al. 1997), which is very intriguing as PDX1 is involved in pancreatic β cell lineage determination (Jonsson et al. 1994). It has been hypothesized that this is the reason for the successful generation of NES2Y, in that NES2Y is ‘frozen’ at a stage of
differentiation at which they have retained the ability to replicate while expressing some of the characteristics of a fully differentiated β cell (Dunne, 2000).

1.11.4 Background to Iterative Human Technology

A shortcoming of 'iterative engineering' to produce a human β cell line (Section 1.11.6) was the lack of suitable human cell lines to engineer. NES2Y has answered this problem and has now also been subjected to 'iterative engineering' by transfection with the cDNA coding for human PDX1, and the two components of $K_{\text{ATP}}$ channels SUR1 and Kir6.2, with the resultant generation of the β cell line NISK9 (Macfarlane et al. 1999). This cell line does in many ways represent the 'holy grail', as it is a pure human β cell line with glucose-responsive insulin secretion. The properties of the $K_{\text{ATP}}$ channels expressed in the NISK9 β cells are identical to those in native tissue, and the cells display a marked decrease in insulin secretion under basal conditions.

Investigations involving NES2Y and NISK9 have already yielded much information about the promoter region of human insulin gene, and in particular the importance of the transcription factor PDX1. NES2Y cells transfected with PDX1 alone displayed an increase in insulin mRNA in response to glucose, indicating a clear role of PDX1 in regulating not only insulin promoter activity but also gene expression in response to glucose. Overexpression of PDX1 in these cells reinitiates a normal glucose-induced response of the promoter and of endogenous mRNA levels (Wu et al. 1999). Of further interest is the observation that the doubling time of PDX1 transfected cells was similar to NES2Y, indicating that PDX1 contributes only partially, if at all, to the ability of NES2Y to proliferate.

1.11.8 Cell Fusion.

At the onset of this project, there were only three reported attempts to establish an insulin-secreting pancreatic β cell line using the technique of cell fusion. A fourth attempt was reported during the completion of this current thesis. These reports are discussed in section 1.7.
1.12 Cell fusion generation of hybrid cell lines.

1.12.1 Background to Hybridoma technology.

Somatic cell fusion has been employed by researchers for quite some time (Barski et al. 1961), but the technique of cell fusion of a primary cell exhibiting the required characteristics and an enzyme-deficient myeloma cell line was first performed in 1975 (Köhler and Milstein, 1975). They fused primary B lymphocytes, some of which were known to be secreting antibodies to a particular antigen, with a myeloma cell line. The result was a hybridoma cell line with the prolonged growth capacity in culture of the parent myeloma cell line and the antibody secretion of the B lymphocyte. Clonal selection provided researchers for the first time with a monoclonal antibody. The technique was later improved by the use of immortal myeloma cell lines, called ‘fusion partners’, to provide a virtually everlasting source of a monoclonal antibody, and ‘immortality was imparted to a chosen lymphocyte’ (Goding, 1982). The usefulness of monoclonal antibodies has driven hybridoma technology to the point that cell fusion has become a classic technique. Hence much of our understanding of cell fusions comes from this field of technology.

Köhler and Milstein initially used Sendai virus as the fusogen but because of the difficulty of removing the virus from cell culture polyethylene glycol (PEG) soon became the fusogen of choice (Pontecorvo, 1976). Hydrophilic PEG removes water from around and even within cells resulting in increased contact between adjacent cell membranes, which in turn leads to agglutination, cell fusions and the formation of polykaryons (Knutton, 1979). It is also possible that the PEG provides the stimulus for the cell fusion. (Wojcieszin et al. 1983).

In most fusions a selective culture medium is employed to aid isolation of the fused cells from the non-fused cells. Since its first use (Littlefield, 1964) the selective medium of hypoxanthine & aminopterin & thymidine [HAT] has proven the most popular. Indeed, HAT has proven so successful that the use of any other selective media has generally been regarded as unnecessary.
Cultured mammalian cells can obtain purine nucleotides and thymidylic acid for DNA synthesis in two ways. Under normal circumstances cells can synthesize these molecules by the *de novo pathway*. The second mechanism is by the *salvage pathways* in which the cell utilizes exogenous purine bases and nucleosides to obtain the purine nucleotides and thymidylic acid.

When large numbers of cells of a myeloma cell line are cultured in the presence of 8-azaguanine or thioguanine, either of these is incorporated as guanine through the purine pathway via the enzyme hypoxanthine (guanine) phosphoribosyl transferase [H(G)PRT]. Azaguanine or thioguanine are cytotoxic and so only mutants which are deficient of the H(G)PRT enzyme will survive. A myeloma cell line cultured in this manner will thus consist of mutant cells deplete of the ability to synthesize DNA via the *salvage pathways* and are dependent on the *de novo pathway*.

The aminopterin in the HAT selective medium blocks the *de novo pathway* principally by inhibition of the enzyme dihydrofolate reductase. This blockage is lethal to the H(G)PRT deficient myeloma cells but is not lethal to normal cells with the *salvage pathway*, provided the required exogenous hypoxanthine and thymidine are present.

Thymidine was suspected of playing a major factor in the problems encountered in establishing hybridomas involving human T lymphocytes (Carson, 1979). This problem was over come by the use of selective media employing not aminopterin but azaserine (Foung *et al.* 1982). Azaserine is a diazo analogue of L-glutamine which irreversibly binds to L-glutamine amidotransferases and hence blocks the purine synthesis of the *de novo pathway*. It does however have minimal effect on the synthesis of thymidylic acid. Again this blockage is lethal to the H(G)PRT deficient myeloma cells but is not lethal to normal cells, provided the required exogenous hypoxanthine is present. Crucially however, exogenous thymidine is not necessary.

Following most PEG fusions to produce a monoclonal antibody-secreting cell line, a selective medium is employed to kill the myeloma cells. The primary B lymphocytes
do not proliferate in culture and eventually die naturally. Post-fusion cultures are visually checked for signs of cell proliferation, as witnessed by colony formation. The colonies are then screened for secretion of the desired antibody. Such a colony is termed as novel growth and is a hybridoma. The DNA from the B lymphocyte coding for the H(G)PRT enzyme and specificity of the antibody and the DNA coding for the continuous growth of the myeloma is all present in the same cell. Hence a hybridoma cell line secreting a monoclonal antibody is generated.

Studies on trans-species fusions have furthered our understanding of the processes involved. When human B cells were fused to mouse myeloma cells, human antibody secreting cell lines were established. When, however, the mouse myeloma cell line possessed some antibody secretion of its own before the fusion, the results were often more complicated. The immunoglobulins produced were often hybrids, produced as a result of mixed association between heavy and light chains of the two cells fused (Cote et al. 1983; Edwards et al. 1982). It is therefore preferable that the fusion partner cell line should not itself express the property sought for in the primary cell.

The hybridoma will tend to have a morphology similar to the parent myeloma cell line and will contain less than the sum of the chromosomes of both the parents. So almost immediately chromosomes are lost. This is particularly prevalent during the first hybrid cell replications. A single colony [monoclonal] of novel cell growth will often consist of cells displaying different characteristics (Campbell, 1984). Subcloning is therefore a necessary technique to separate these different cells and establish a true monoclonal cell line, or at least cells all secreting the same antibody. Although less prevalent, chromosome discharge does continue throughout the life span of a hybridoma cell line. A true monoclonal cell line consisting only of multiple copies of the same cell is probably never maintained but repeated subcloning is performed to limit this phenomenon (Weissman and Stanbridge, 1980). There is evidence that a subpopulation of cells which has lost the ability to synthesize and secrete antibodies gains a competitive growth advantage over secreting cells. Without early subcloning the secreting cells can become out-populated and
swamped, making any attempts to isolate secreting cells very difficult (Cote et al. 1983).

Whether the fusion is human-human or a trans-species human-mouse it has been demonstrated that over time in culture most of the chromosomes derived from the primary B lymphocyte are lost (Gravekampf et al. 1987). This of course is of little concern provided the cell line continues to secrete the desired antibody, as the genetic material required from the primary B lymphocyte is obviously still present. Clearly however, it is not so much a matter of ‘immortality imparted to a chosen lymphocyte’ (Goding, 1982) but rather some DNA from a lymphocyte being expressed in an immortal myeloma cell line.

This principle of hybridising a cell type of interest to a cell line in the hope of achieving a functional hybrid cell line with continuous growth capacity in culture led to cells other than B lymphocytes being employed (Widman et al. 1979). The need for a β cell line more functional than the RINm5f led researchers to try this technique.

1.13 Cell fusion to establish β cell lines.

[1] Boyd et al. (1982) used PEG to fuse a primary culture of human insulinoma cells with the mouse fibroblast cell line LMTKCI1D, using HAT as the selective medium. Although it was confirmed that this trans-species fusion could produce true hybrid cells, no cell line secreting insulin was established. Some 147 cell lines were obtained, of which 13 secreted what was determined to be proinsulin. Designated as L-1C and L-2C, these 13 cell lines were unstable as witnessed by the rapid decline in proinsulin secretion after only 1 month in culture. Secretion was minimal after 2 months and ceased completely after 7-8 months in culture.

[2] Takaki et al. (1984) were the first to report an attempt at establishing an insulin-secreting pancreatic β cell line using the cell fusion technique where the primary cell was a normal healthy cell. They attempted three different fusions utilizing PEG. These were the fusing of hamster islet cells to either: [i] 3T3 mouse fibroblast cell
line; [ii] a hamster liver cell line; or [iii] the hamster insulinoma cell line In-111-6TG\(^R\).

Fusions involving In-111-6TG\(^R\) did produce twenty hybrid cell lines, six of which secreted glucagon. These cell lines were designated HIP but no insulin-secreting cell lines were established. The other fusions produced unstable hybrid cell lines which quickly lost the capacity to secrete hormones. Where as Boyd \textit{et al.} had some success fusing human insulinoma cells to a mouse fibroblast cell line, Takaki \textit{et al.} did not have similar results when they employed their mouse fibroblast cell line. This could be put down to a multitude of reasons but their use of normal cells as opposed to insulinoma cells would have to be considered. The failure of the first two cell lines led the authors to deduce that when normal \(\beta\) cells were fused it was important that the fusion partner cell line should be a \(\beta\) cell line. However, they wrongly deduced that the cells should also be the same species.

\cite{3} Aponte \textit{et al.} (1991) fused a rat intestinal epithelial cell line to rat pancreatic islet cells. PEG was again the fusogen and HAT was used as the selective medium. The cell line hBRIE 291-i2 was established and secreted insulin for up to 3 months in culture.

\cite{4} Pancreatic islet cells from New England Deaconess Hospital (NEDH) rats were fused to the RINm5f cell line. The more modern technique of electrofusion was utilized and hybrid cells were isolated by cloning rather than a selective medium. The resultant BRIN cell lines displayed improved functionality compared to the parental RINm5f cell line (McClenaghan \textit{et al.} 1996\(^{a,b}\)).

1.14 Chromosome analysis of hybrid cells.

The loss of DNA witnessed in hybridomas is also seen in non-transformed insulinoma cell lines (Scott \textit{et al.} 1992). So it is not surprising that this should occur with islet hybrid cell lines. The discarding of chromosomes starts almost immediately and can continue throughout prolonged culture. In the HIP lines the secretion of glucagon was examined in respect to the loss of DNA over time (Takaki
et al. 1984). No correlation was found between this expression of cellular function and the stability of chromosome number.

Human chromosome 11 is the location of the insulin gene (Owerbach et al. 1982). In one hybrid cell line, L-1C-8h, the isozyme marker for chromosome 11 was detected and the cell line secreted human proinsulin. Chromosome analysis by banding techniques did not however reveal human chromosome 11. The authors concluded that in this cell line a break had occurred in chromosome 11, the remaining fragment of which allowed for proinsulin secretion but made it impossible to identify morphologically (Boyd et al. 1982).

1.14.1 Insulin expression in hybrid cells. Studies on insulin secretion using rat or mouse \( \beta \) cells are particularly complex due to the rodent cells possessing two nonallelic insulin genes (I and II), and two forms of insulin are secreted. (Lomedico et al. 1979). RIN cells, however, have been reported to express only the rat I gene (Fiedorek et al. 1990). Iterative engineering and hybrid \( \beta \) cell lines have shown that when the DNA coding for human insulin is inserted into rodent \( \beta \) cells various results can occur. The human insulin gene and the two rodent insulin genes can be present in a single cell and they can all be expressed in the same cell. Though present, some insulin genes can be non-expressed or expression can change over time in culture (Lund et al. 1993; Besnard et al. 1989). When multiple copies of the DNA coding for human insulin are inserted they and the rodent insulin genes can all be expressed at the same time (Clark et al. 1997).

1.15 Possible parameters in establishment of hybrid \( \beta \) cell line by PEG fusion Many factors can influence the success rate of hybridoma establishment from a PEG fusion. Particularly important is the source of PEG, the molecular weight (MW) of the PEG, the foetal calf serum (FCS), and the myeloma fusion partner cell line (de St. Groth SF and Scheidegger D, 1990; Cote et al. 1983). Factors that can lead to the complete failure to establish a hybridoma cell line are however less numerous. Mycoplasma contamination and thymidine concentrations are usually the cause.
Thymidine at the millimolar concentration is known to inhibit DNA synthesis in most mammalian cells and some human cells are susceptible at even lower concentrations. Paradoxically, thymidine deficiency can be responsible for fusion failures involving cells contaminated with mycoplasma (Campbell, 1984).

These factors are however from the field of hybridoma technology, and the factors that could effect the establishment of hybrid β cell lines are virtually unknown. Premium batches of FCS are graded according to their relative ability to support hybridoma establishment and growth, due primarily to their high concentrations of cytokines. This could be of importance as cytokines are known to be toxic to human islet cells in culture (Rabinovitch et al. 1990). Studies on the culture of islet cells and β cell lines identified RPMI-1640, with a glucose concentration of 11.1 mmol/l, as the culture medium of choice. It was superior to other media with regard to both glucose induced insulin release and biosynthesis (Andersson, 1978). The use of culture medium with an approximate 10 mmol/l glucose concentration was later confirmed as beneficial to the life span of islet cells in culture, and the use of glucagon and 3-isobutyl-1-methylxanthine (IBMX) culture supplements was also advised, (Ling et al. 1994). The same author also reported that glucose concentrations as high as 30 mmol/l were seen to improve the glucose-stimulated insulin secretion of islets, in comparison to 3 mmol/l. To balance this, however, it has been demonstrated that long exposure of β cells and cell lines to such glucose concentrations can diminish insulin secretion and glucose-responsiveness (Olson et al. 1993; Kaiser et al. 1991; Eizirik et al. 1992; Briaud et al. 1999).

Nicotinamide was reported to protect against β cell desensitisation to glucose after prolonged exposure to high glucose and to even improve the glucose responsiveness after desensitisation had occurred (Ohgawara et al. 1993).

The ratio of islet cells to fusion partner cells is another parameter to be considered. Cell ratios of 1:2 and 1:1 have been used previously (Aponte et al. 1989; Takaki et al. 1984). Takaki et al. used the incomprehensible, but most probably weak, centrifugation of 1000 rpm to fuse the cells after PEG treatment. The other previous
PEG fusions have employed forces of less than 1g, in that they allowed the cells to naturally sediment together in culture medium (Boyd et al. 1982; Aponte et al. 1989).

1.16 Desirable Functionality of a β cell line.

1.16.1 Glucose stimulated insulin secretion (GSIS)

Although it may be expected that a β cell line would desirably function like native β cells, this is not completely correct. The main desirable attribute of a β cell line is glucose-stimulated insulin secretion (GSIS). There is still some debate over whether or not this is a native attribute. Perifused islets will display a GSIS similar to the rise in insulin levels seen in vivo in response to increased glucose concentrations in the physiological range of 5 to 15 mmol/l (see Figure 1.4b), with 5 mmol/l the threshold glucose concentration (Lindström, 1984; Eizirik et al. 1988). Isolated β cells, however, do not display GSIS under the same conditions unless they are allowed to reaggregate into pseudo-islets. The infrastructure of the islets, the heterogeneity of islet cells therein, and cell to cell contact have all been proposed as possible explanations for this phenomenon (Pipeleers et al. 1982; Halban et al. 1982; Hopcroft et al. 1985; Pipeleers. 1987).

Pancreatic β cell lines have confused this issue even more. Although some β cell lines do not display GSIS, many of them display weak GSIS at glucose concentrations between 1 - 5 mmol/l, which is below the natural physiological range (Newgard, 1996; Hohmeier et al. 1997; Gilligan et al. 1989; Hamaguchi et al. 1991). As for the few cell lines which do display GSIS within the physiological range, the increase is again weak, in that it is not of the same magnitude seen with islets or reaggregated β cells. INS-1 displayed a 2.2-fold increase in GSIS over the 1 to 10 mmol/l glucose range but requires 2-mercaptoethanol to achieve this (Asfari et al. 1992). The cell line βTC-6F7 has been reported to offer very good GSIS but the presence of IBMX seems to be required to potentiate the effects of glucose (Knaack et al. 1994). βHC-9 displayed a 3-fold increase in GSIS over the 5 to 20 mmol/l
glucose range (Noda et al. 1996), and some BRIN lines have been reported to display up to a 1.9-fold increase in GSIS between 4.2 and 8.4 mmol/l glucose concentrations (McClenaghan et al. 1996). HIT-5B5 cells displayed a 2-fold increase in GSIS between 1.25 mmol/l and 7.5 mmol/l glucose concentrations, whereas native hamster islets displayed a 7-fold increase over the same glucose range (Santerre et al. 1981). Probably the best GSIS was reported in the MIN6 cell line, which showed a 7-fold increase in GSIS between 5 mmol/l and 25 mmol/l glucose concentrations, but even here reaggregated native mouse β cells display a 10 to 25-fold increase over the same glucose range (Ishihara et al. 1993).

The factors involved in the glucose-responsive insulin secretion are many and their interactions are complex, and so it is difficult to attribute GSIS to one key factor alone. Whilst much information has been obtained by the iterative engineering of β cell lines, care must be taken in oversimplifying the complexity of GSIS. When GSIS is obtained in a β cell line following transfection with a missing or deficient factor, it merely confirms that the factor is one of the many involved in GSIS. Any key quality it posses could merely be due to it being the only factor that was deficient. That said however, factors that are required for good GSIS include; predominance of the glucose transporter GLUT-2 as opposed to GLUT-1; a high glucokinase to hexokinase enzyme ratio; functional $K_{\text{ATP}}$ channels; and adequate PDX1 expression (Tal et al. 1992; Tiedge et al. 2000; Macfarlane et al. 2000).

Whether or not GSIS is a phenomenon displayed in native β cells in monolayer culture, it is a highly sought attribute as it enables the islet mini-organ to be studied at the molecular level.

### 1.16.2 Secretagogues, modulators of insulin secretion.

Characterisation of β cell lines often involves the use of secretagogues, where the responses are used to compare cell lines to native islets. The responses can help determine deficiencies in the cell line which may be responsible for poor GSIS. Much of this introduction has involved the modulation of insulin secretion by numerous factors. These have ranged from metabolic substrates, neurotransmitters,
drugs, hormones, and other peptides. The role of intracellular Ca\(^{2+}\) concentration, and its modulation by K\(^+\) channels and cAMP, has been central to stimulus-coupling of insulin secretion of most of these factors. Modulators of insulin secretion commonly tested on \(\beta\) cell lines are described below.

Glucagon raises cellular levels of cAMP, and IBMX is a potent inhibitor of cyclic nucleotide phosphodiesterases, hence it also elevates cellular levels of cAMP. The result of raised levels of cAMP is to potentiate glucose action (Henquin and Meissner, 1984; Praz et al. 1983). The effects of IBMX can be even further potentiated by glutamine (Gilligan et al. 1989). High extracellular concentrations of K\(^+\) ions have the result on normal \(\beta\) cells with functional K\(_{ATP}\) channels of causing the opening of the voltage-dependent Ca\(^{2+}\) channels, leading to influx of Ca\(^{2+}\) in increase in insulin secretion (Praz et al. 1983; Macfarlane et al. 1999\(^b\)). Thus KCl can be used to test for functional K\(_{ATP}\) channels, although KCl alone can not be used to test for dysfunctional K\(_{ATP}\) channels.

1.16.3 Cellular insulin content.
MIN6 is again probably the best cell line as it is the closest to mimicking native islets. This cell line contains about 20% of that seen in islets and can release 3.5% of this content in a 60 min incubation, as opposed to 10% for islets (Ishihara et al. 1993; Lindström, 1984). RINm5F has been reported to release 24% of its insulin content in similar conditions, but its content is less than 0.5% of the islet (Halban et al. 1983).

1.16.4 Lack of glucagon secretion.
Synthesis of glucagon by \(\beta\) cell lines might represent a feature of dedifferentiation since this co-expression of islet hormones is seen in progenitor cells during pancreas development (Hashimoto et al. 1988). Consequently glucagon secretion is desirable in its absence. RINr, RINm, CRI and MSL all resemble transformed islet stem cells, with the co-secretion of glucagon, insulin and often other islet hormones, which suggests a pluripotent nature of the original RIN tumour from which all these lines were derived (Madsen et al. 1989). The molar ratio of insulin release to glucagon...
release in the absence of glucose is approximately 500 in \( \beta \)TC cells (Poitout et al. 1995), and approximately 50 in HIT-T15 cells (Diem et al. 1990).

1.16.5 Antigens.

Even if the properties themselves are of little immediate use, the more of the native \( \beta \) cell properties expressed in the hybrid cell line, and hence the more closely it resembles the native cell, the better. The expression of native antigens can, however, be of immediate use, particularly if it is a diabetic autoantigen.

As discussed in Section 1.7 some forms of diabetes mellitus are caused by an autoimmune attack of antigens (autoantigens). Early studies centred on the search for a single responsible autoantigen, but it became clear that many different autoantigens existed and many different forms of the autoimmune attack could cause diabetes (Mandrup-Poulsen and Nerup, 1990). Some of these types of autoimmune attack were \( \beta \) cell specific, whilst others were accompanied by the autoimmune attack of other endocrine tissues. This latter case pointed to endocrine secretory vesicles as a probable location of the autoantigen (Fialkow et al. 1975). The \( \beta \) cell specific attack intrigued immunologists for some time, with the focus centering on a mysterious 64 000 MW autoantigen (Baekkeskov et al. 1982). It was assumed that the specificity of the attack indicated that the autoantigen was involved in insulin synthesis or secretion, but the \( \beta \) cell proved to have more unique features than just insulin secretion. It is uniquely targeted by some viruses which have been shown to be involved in some cases of diabetes caused by autoimmune attack (Yoon et al. 1987).

Certain protein tyrosine phosphatases, such as IA-2 are found, apart from the brain, only in the pancreatic \( \beta \) cell. IA-2 and the related cleavage product ICA-512 represent important autoantigens in diabetes (Rabin et al. 1994; Hawa et al. 1997; Hawkes et al. 2000). Although identified (Nayak et al. 1985; Atkinson and Maclaren, 1993), many other autoantigens have been overshadowed by the identification of glutamic acid decarboxylase (GAD) as an important islet cell autoantigen (Baekkeskov et al. 1990). Unfortunately GAD has a molecular weight
close to but not 64,000, and so fails to match the one characteristic known about the mysterious 64,000 MW autoantigen (Baekkeskov et al. 1990).

Further work is still required in this field. Specifically, is there an important human diabetic autoantigen which is normally involved in insulin synthesis or secretion? Also, just how and why native antigens become targeted for immune attack and the role of molecular mimicry by viruses (Yoon et al. 1987). The role of MHC molecules is of particular interest as it is thought that in cases of autoimmunity MHC molecules preferentially present peptides of other endogenous MHC proteins and these MHC-derived peptides might represent potential targets for autoreactive T cells (Durinovic-Bello I, 1998).

Work in this field has been historically hindered by the lack of suitable human β cells, and a reliance on animal cells. This has lead to conflicting results and some confusion, due to the general cross-species reactivity of human antibodies (Yamashiro et al. 1990). Obviously, when using human diabetic sera, antigen studies should ideally be performed not on rat cells and cell lines but on human cells, or at least on cells expressing the human antigens.

The βHC cell line has proven of some use in this field as it expresses GAD and functional class I MHC antigen-presenting molecules (Radvanyi et al. 1993). Immunofluorescence studies on HP62, derived from human islet cells (Soldevilla et al. 1991), did not display any antigens reactive to human islet cell autoantibodies [ICA] (Munoz et al. 1992). The semi-permanent human insulinoma cell line CM has been used with some success in the role of virus induced cytokine release and the subsequent alterations in the immune response (Cavallo et al. 1992). This cell line has also been reported to express GAD and GM2-1, a native islet ganglioside, and is reactive to human ICA (Cavallo et al. 1996). As such this cell line would seem to be a good model for investigations into human autoimmunity in type I diabetes. The permanence of this cell line is of question and, although all reviewers have subsequently discounted it, it is still providing a model for the occasional study (Schwingshackl et al. 1998). This does, however, reiterate a truly desirable attribute...
of a novel β cell line, that it should be human or at least part human. Of particular interest would be presence of all the human components required to synthesize and secrete human insulin. NES2Y does meet these requirements (Macfarlane et al. 1997), and data on the autoantigen status of this cell line are eagerly awaited.

1.17 PDH-1 fusion partner cell line.

PDH-1 is a mouse x human hybridoma cell line derived from the cell line P3-NS1/1-Ag4-1 [NS1], which in turn was originally derived from MOPC-21, a BALB/c murine myeloma cell line. The NS1 line was fused with human peripheral lymphocytes on six sequential occasions, the last two times by the author. All humans were either diabetic or pre-diabetic, and after each fusion hybridomas were selected for the non-production of antibody. These were in turn transformed into fusion partner cell lines and used for the next fusion. The subclone PDH-1 was selected because of its high success rate in generating hybridomas from human lymphocytes, while being a non-secretor of antibody itself. The development of autoimmunity in diabetes is known to involve, among others, a genetic factor (Todd, 1990). It was optimistically hoped that the content of human diabetic DNA within PDH-1, may contain genes involved in the expression of various antigens or autoantigens.

1.18 Summary of the relevance of this project.

At the onset of this project all attempts at establishing a permanent and insulin-secreting human β cell line had failed (Boyd et al. 1982; Wollheim et al. 1990b; Soldevilla et al. 1991; Poitout et al. 1996; Levine et al. 1995; Bouwens et al. 1997). Although the cell line NES2Y was generated in 1997 (Macfarlane et al. 1997), it was unclear whether this cell line’s secretion or growth rates suffered over time in vitro as with other primary β cell lines. That NES2Y was a permanent human β cell line was only recently resolved, when it’s characteristics were detailed and reported (Macfarlane et al. 1999c). The subsequent generation of the NISK9 (Macfarlane et al. 1999b) and NESK (Macfarlane et al. 2000) cell lines by the iterative engineering of NES2Y has fulfilled the need for a functional human β cell line. It has also underlined the fact that the older methods of cell fusion employed in this project
have, over a short period of time, been rendered virtually obsolete by the rapid advance in the technology of molecular engineering of cells.

At the onset of this work three reported attempts had been made at establishing a permanent and insulin secreting β cell line using PEG fusion. All but one of these attempts failed (Boyd et al. 1982; Takaki et al. 1984). The one reported success was the rat cell line hBRIE 291-i2 (Aponte et al. 1991), which was reported to secrete insulin for over 3 months in culture. The establishment of the rat BRIN cell lines by electrofusion was reported during the completion of this thesis (McClenaghan et al. 1996a,b). Of these reported studies only the unsuccessful attempt by Boyd et al. (1982) was on human cells. In that attempt they fused human insulinoma cells to a mouse cell line. To date no reported attempts have been made to establish a permanent and insulin secreting β cell line by cell fusion involving normal human islet cells.

1.19 Objectives

The objectives of the work presented in this thesis are as follows:

- To see if a trans-species cell fusion could produce a stable insulin-secreting hybrid cell line by fusing normal human islets to rodent cell lines.
- If any stable insulin-secreting hybrid cell lines are established, to identify and isolate any which secrete human insulin.
- To identify hybrid cell lines with vastly improved insulin secretion levels, compared to the parent fusion partner cell line.
- On the selection basis of human insulin secretion and/or insulin secretion rates, to select and characterise as many of the cell lines as possible. To be characterised in terms of glucose-stimulated insulin secretion; response to common secretagogues; insulin content; glucagon secretion; growth rates; and chromosome counts. All with reference and comparison to the parent fusion partner cell line.
- To check the selected cell lines for any detectable human diabetic antigens, and human DNA content.
Chapter 2

Materials and Methods.
2.1 Grade of chemicals and water

Chemicals were, unless otherwise stated, of analytical grade. Water when used was deionized on site using the Millipore resin purification system: Millipore, Milford, USA.

2.1.1 Fusion Partner Cell Lines

CRI-G5, RINm5f and PDH-1 were obtained with permission from Guildhay Antisera Ltd, Guildford, England. CRI-G5 was used with the kind permission of Prof. C.N. Hales.

2.1.2 Materials for the generation and culture of cell lines.

Sigma Chemical Company Ltd, Dorset, UK. [later to become Sigma-Aldrich Company Ltd, Dorset, UK.]:

NaCl, KCl, KH₂PO₄, MgSO₄·7H₂O, CaCl₂·2H₂O, Na₂HPO₄, NaHCO₃, MgCl₂·6H₂O, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), D(+)-glucose, NaOH, glucagon, nicotinamide, 3-isobutyl-1-methylxanthine (IBMX), L-glutamine, aprotinin, bovine serum albumin (BSA) [fraction V].

Hybri-max grade:

Dimethyl sulfoxide (DMSO), foetal calf serum, FCS, 8-azaguanine, azaserine, hypoxanthine, HAT supplement, polyethylene glycol (PEG) [MW 1450].

Cell culture grade:

RPMI-1640, ethylenediaminetetraacetic acid-disodium salt (EDTA) 0.02% solution, 200 mmol/l L-glutamine solution, collagenase type XI, penicillin & streptomycin solution, FCS, Trypan Blue 0.4% solution.

All sterile culture plastics were Falcon® products: vented culture flasks 25 cm², 75 cm² and 175 cm²; 24-well plates, 96-well plates; 1, 10 and 25 ml serological pipettes; Pasteur pipettes; 50 ml polypropylene centrifuge tubes; Petri dishes: Becton Dickinson and Company, NJ, USA.
RPMI-1640 (0 mmol/l glucose), thymidine, polyethylene glycol [PEG] (MW 4000), Myoclone Super Plus FCS, porcine pancreas trypsin (1000-1500 U/mg): GibcoBRL, Paisley, UK.

Ethanol, concentrated HCl, glycerol, 1L polypropylene wide-neck container : BDH, Dorset, UK.

0.2 μm syringe filters: Gelman, Ann Arbor, USA.

Dispase type I: Boehringer Mannheim, Mannheim, Germany.

Stainless steel meshes of 1000 μm and 50 μm aperture: Endecotts, London, UK.

14 - 25 gauge hypodermic syringe needles: Becton Dickinson and Company, Dun Laoghaire, Ireland.

Coomassie protein assay: Pierce & Warriner Ltd, Chester, UK.

2.1.3 Additional materials used for insulin measurement.

Sigma Chemical Company Ltd, Dorset, UK.

NaH$_2$PO$_4$, thiomersal [thimerosal], EDTA-dissodium·2H$_2$O.

RIA grade

BSA grade


Guinea pig anti-rat insulin: Biogenesis Ltd, Poole, UK.

Charcoal stripped human serum: Guildhay Antisera Ltd, Surrey, UK.

Rat insulin standard: Linco Research, St. Louis, USA.

Rat insulin standard. Novo Biolabs, Bagsvaerd, Denmark.

Human Actrapid insulin: Novo Nordisk, Bagsvaerd, Denmark.

1272 Clinigamma counter: LKB, Finland.

2.1.4 Additional materials used in HPLC.

HPLC grade water was obtained using the Milli-Q water purification system: Millipore, Milford, USA.

Acetonitrile, HPLC grade: Labscan Ltd, Dublin, Ireland.
Triethylammonium phosphate (TEAP) 1 mol/l, HPLC grade: Fluka BioChemika, Neu-Ulm, Switzerland.
LiChrosorb RP-18 (5 μm) 250 mm x 4 mm column: HiCrom, Reading, UK
HPLC equipment was the Beckman System Gold with Solvent module 125, Detector 166, and software GOLDV810: Beckman Instruments Inc, Brea, USA.

2.1.5 Additional materials used in the antigen studies.
Sigma Chemical Company Ltd, Dorset, UK.
Phenylmethylsulfonyl fluoride (PMSF), tween 20, Triton X-100, Sigma Fast™ DAB tablets.

Electrophoresis grade:
Tris-Cl [Trizma-Cl], DL-dithiothreitol, sodium dodecyl sulfate [lauryl sulfate] (SDS), glycerol, bromophenol blue, ammonium persulfate, glycine.
Sodium azide, (NH_4)_2SO_4, sodium acetate, methanol: BDH, Dorset, UK.
BCA protein assay: Pierce & Warriner Ltd, Chester, UK.
DEAE-Sephalcel, CNBr-activated Sepharose® 4B: Pharmacia LKB Biotechnology, Uppsala, Sweden.

Minicon™-B clinical sample concentrators: Amicon Ltd, MA, USA.
Sheep anti-CRI-G5: Guildhay Antisera Ltd, Guildford, UK.
Human diabetic sera: Beaumont Hospital, Dublin, Ireland.
Protogel™ acrylamide and methylenebisacrylamide stock: National Diagnostics, NJ, USA.
Cooled midget 2050 electrophoresis unit: LKB, Bromma, Sweden.
Trans-Blot® electrophoretic transfer cell: Bio-Rad Laboratories Inc., Richmond, USA.

Hybond™-C nitrocellulose membrane: Amersham International PLC, Amersham, UK.
Sheep anti-human IgG enzyme labelled with horseradish peroxidase: The Binding Site Ltd, Birmingham, UK.
2.1.6 Additional materials used for chromosome analysis of cell lines and in situ hybridisation.

Colcemid®, 75 mmol/l KCl solution: GibcoBRL, Paisley, UK.
Glacial acetic acid, DPX: BDH, Dorset, UK.
Total Human DNA probe reagent kit containing: fluorescein labelled avidin solution;
formamide; twenty fold strength SSC buffer; biotin labelled Total Human DNA probe; PBD buffer; propidium iodide; Antifade: Oncor, Gaithersburg, USA
Giemsa stain; Sigma Chemical Company Ltd, Dorset, UK.

2.2 General culture of cell lines.

Cell lines were routinely cultured in RPMI-1640 containing 10% Foetal calf serum (FCS). The glucose concentration of this culture medium, unless otherwise stated, was 11.1 mmol/l. All cultures were maintained in a Stericult CO$_2$ incubator at 37°C with 5% CO$_2$ in air.

2.2.1 Cell line harvesting and splitting.

PDH-1 required only gentle bumping to dislodge cells into the culture medium, which was then centrifuged 200g for 5 min. RINm5f and CRI-G5 both required the removal of the culture medium and immersion of the cell lines in EDTA 0.02% solution at 37°C for 10 min. Culture flasks were then bumped vigorously and the EDTA solution removed for centrifugation 200g for 5 min. In normal culture conditions the cell lines were split at a ratio of 1:20. That is to say that after splitting there was 5% of the cells left in the flask, and this is referred to as a ‘passage’.

2.3 Creation of Fusion Partner Cell Lines.

All cell lines were rendered H(G)PRT-deficient by sensitisation to 8-azaguanine. Each of the cell lines was grown in RPMI-1640 containing 10% FCS in 175cm$^2$ culture flasks until approximately 50% confluence was attained. FCS was then added to obtain 20% concentration and 8-azaguanine was added to obtain a 16.5 $\mu$mol/l concentration. After four days the flask was agitated gently and the culture medium replaced with RPMI-1640 containing 20% FCS and 33 $\mu$mol/l 8-
azaguanine. After a further four days more 8-azaguanine was added to obtain a concentration of 66 μmol/l. After four days the flask was agitated gently and the culture medium replaced with fresh medium containing a concentration of 0.132 mmol/l 8-azaguanine. Two days later each cell line was harvested and subcloned by limiting dilution in 96-well culture plates and cultured in the same medium as before the harvest. In under two weeks the plates were examined and the shape of the colonies was used to confirm a subclone. A single colony (subclone) was transferred to 24-well culture plate and with continual culture in the presence of 0.132 mmol/l 8-azaguanine expanded sequentially to vented culture flasks of 25cm², 75cm² and finally 175cm². In this final size flask the FCS was reduced to 10% in the culture medium and the 8-azaguanine was discontinued, unless the total time exposed to the 0.132 mmol/l concentration was less than five weeks.

When the cell line attained confluence in the culture flask it was split at a ratio of 1:5 to four further flasks and all fed with the normal culture medium of RPMI-1640 & 10% FCS. When these flasks reached confluence the cell line was harvested for freezing. Large stocks of the cell lines were cryogenically frozen at -70°C in sterile DMSO 10% and FCS 90%. After three weeks cell harvesting was ceased and all flasks were cultured in RPMI-1640 & 20% FCS containing 2.31 μmol/l azaserine and 0.2 mmol/l hypoxanthine. Thus the cell lines were checked for their inability to survive in this selective culture medium, and were then referred to as fusion partner cell lines.

Upon receipt of a pancreas, the cell lines required for fusion were thawed and washed three times in RPMI-1640 & 10% FCS by centrifugation at 200g for 5 min. Each fusion partner cell line was seeded into five vented 175cm² culture flasks and cultured. After four hours these flasks were decanted to another five flasks and re-fed with fresh RPMI-1640 & 10% FCS, and all flasks were again cultured. Within 1-2 days, when the cell lines were visibly recovered from the thawing process, fusion could proceed.
2.3.1 Preparation of fusion partner cell lines for fusion.
PDH-1 was harvested by replacing the culture medium in each flask with 50 ml RPMI-1640 and gently bumping the culture flasks. The collected media was centrifuged 200g for 5 min and cell line pellets were pooled. The pool was washed twice by resuspension in 50 ml RPMI-1640 and centrifugation 200g for 5 min. After a final resuspension in 50 ml RPMI-1640, the cell line was cell counted.

RINm5f and CRI-G5 were harvested by replacing the culture medium in each flask with 5ml EDTA 0.02% solution and incubating at 37°C for 10 min. Culture flasks were then bumped vigorously and the EDTA solution removed and centrifuged 200g for 5 min. The cell pellets were pooled and washed by resuspension in 50 ml RPMI-1640 followed by centrifugation 200g for 5 min. The cell line pellet was then resuspended in 25ml EDTA 0.02% solution and incubated at 37°C for 10 min. Gentle aspiration through a 10ml serological pipette was continued until cell clumping was removed. When this was attained 25 ml RPMI-1640 was added to counteract prolonged exposure to EDTA, and a sample of the cell suspension was taken off for cell counting.

2.3.2 Cell Counting.
All cell counts were performed on a standard Neubauer Haemocytometer using standard techniques, with the exception that Trypan Blue stain was deemed unhelpful and hence not used. Cells were counted in the centre and four corner squares of both chambers present. Total cells harvested were calculated using the formula:

\[
\text{Total Cell Number} = \text{Cells counted in ten squares} \times \text{Total volume of material sampled} \times 10^3
\]

2.4 Isolation of islet cells from human pancreas.
The isolation of islet cells was performed by enzymatic digestion of the pancreas, using an adaptation of published techniques (Burghen and Murrell, 1989; Gray et al. 1984).
2.4.1 Composition of Isolation Buffers.

All buffers were sterile filtered through 0.2 μm syringe filters.

Isolation buffer 1

Hank’s balanced salt solution & HEPES (HBSS) & glycerol, pH 7.8 at 39°C.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/l)</th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
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<tr>
<td>KCl</td>
<td>0.4</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.06</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
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</tr>
<tr>
<td>Na₂HPO₄</td>
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</tr>
<tr>
<td>NaHCO₃</td>
<td>0.35</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>HEPES</td>
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</tr>
<tr>
<td>glucose</td>
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</tr>
<tr>
<td>glycerol</td>
<td>62.5</td>
</tr>
</tbody>
</table>

Isolation buffer 2

Hank’s balanced salt solution & HEPES (HBSS), pH 7.8 at 39°C.

As for isolation buffer 1 but with no glycerol present.

Isolation buffer 3

Hank’s balanced salt solution & HEPES (HBSS), pH 7.8 at 39°C.

This was the same as isolation buffer 2 but with the omission of MgCl₂·6H₂O.

Puck’s Ca²⁺/Mg²⁺-free buffer, pH 7.4 at 4°C

<table>
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<tr>
<td>NaHCO₃</td>
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</tr>
<tr>
<td>Glucose</td>
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</table>
2.4.2 Organ procurement.

The pancreas organs were obtained by Mr. D. Hickey from human cadaver [necro] kidney donors. Permission was obtained from the next-of-kin with the express knowledge that the organ was to be used for research. Upon retrieval of the organ, the surgical team placed the pancreas into chilled sterile buffer and placed on ice. The buffer was either isolation buffer 2, for the first three organs, or isolation buffer 3, for the fourth and fifth pancreas. Isolation of the islets commenced as quickly as possible and on all occasions this was in under five hours. The five organs were harvested over a 15 month period.

<table>
<thead>
<tr>
<th>Pancreas</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Trimmed weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>F</td>
<td>92g</td>
</tr>
<tr>
<td>2</td>
<td>?</td>
<td>?</td>
<td>54g</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>F</td>
<td>52g</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>M</td>
<td>76g</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>M</td>
<td>29g</td>
</tr>
</tbody>
</table>

2.4.3 Islet cell Isolation Procedure.

Under sterile conditions the pancreas was trimmed by the removal of fat, peritoneum and superficial blood vessels. Using only fingers and surgical scissors the pancreas was then chopped and gently torn into pieces of approximately 5 mm$^3$ and placed into a sterile polypropylene 1000 ml wide necked container [the digestion container]. Fresh isolation buffer at 39°C was added to achieve 10 ml/g of pancreatic tissue, and placed on a magnetic stirring hot plate. For the first, second and third pancreas the isolation buffer 1 was used. For the fifth pancreas isolation buffer 3 was used. The fourth pancreas was split into two aliquots each receiving either isolation buffer 1 or 3.

Enzymatic digestion of the pancreas was started when the temperature reached 39°C by the addition of 0.2 mg/ml collagenase type XI and 2.5 mg/ml Dispase type I, and was further supplemented with 100U/ml Penicillin and 0.1 mg/ml streptomycin. With these additions the buffer was designated the digestion buffer. Digestion was
for three periods of 15-20 min on the stirring hot plate at 39°C, with gentle agitation provided with a sterile magnetic flea. The pH was monitored with a pH electrode and altered using sterile 1 mol/l NaOH and 0.3 mol/l HCl as needed to keep between pH 7.7-7.9.

2.4 Preparation of Islets from Pancreatic Tissue

After each digestion period the resultant brew was first passed sequentially through stainless steel meshes of 1000 μm and 50 μm aperture. The latter mesh had been soldered to form a cone. Pancreatic tissue collected on the 1000 μm mesh was placed back into the digestion container to which fresh digestion buffer was added. The same volume was used for each period of the digestion. Material collected on the smaller 50 μm mesh, designated as the islet enriched fraction, was washed off the mesh with Puck's Ca²⁺/Mg²⁺-free buffer, and kept at 4°C.

2.4.4 Dissociation of Islets

When the three digestion periods were over the three islet enriched fractions were centrifuged in 50 ml sterile centrifuge tubes at 200g for 5 min and pooled in 9 ml EDTA 0.02% solution at 39°C, to which 1 ml of freshly thawed trypsin 2.5% solution (w/v) in the above EDTA solution was then added. Following incubation at 39°C for 10 min the preparation was passed by syringe through reducing needle apertures of 16 gauge, 18 gauge, 21 gauge and finally 25 gauge. Samples were removed and observed in a Petri dish under microscope to confirm if dissociation was complete. Dissociation was stopped by the addition of chilled isolation buffer 2 containing 1% FCS, to obtain a total volume of 50ml. For the fourth and fifth pancreas isolation buffer 3 was used in place of isolation buffer 2.

The dissociated islets were then centrifuged 200g for 5 min. The supernatant was re-centrifuged 1200g for 10 min and the supernatant of this was again centrifuged 1200g for 30 min. Each of the three centrifugation pellets obtained were washed twice in RPMI-1640 & 10% FCS using the same centrifugation regime used to obtain that pellet. Finally the three pellets were pooled in a vented 175 cm² culture flask using 100 ml RPMI-1640 & 10% FCS and supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin. These cells were cultured for between one
and two days in 5% CO\textsubscript{2} at 37°C. These cells are henceforth referred to as islet cells, although it is acknowledged that they were actually islet cell enriched pancreatic cells.

2.5 Preparation of islet cells for fusion.

The culture flask containing the islet cells was gently bumped and the culture medium collected into 50 ml sterile centrifuge tubes for centrifugation at 200g for 5 min. The cell pellets were pooled in RPMI-1640 and the original supernatants were centrifuged 1200g 10 min. Again the cell pellets were pooled in RPMI-1640 and the supernatant centrifuged 1200g 30 min, and these cell pellets also pooled in RPMI-1640. The three islet cell pools were washed three times by resuspension in RPMI-1640 after centrifugation at the speed which produced each of the respective pellets. The final resuspension pooled the three fractions together.

2.5.1 Fusion protocol.

The washed islet cells were then cell counted on a haemocytometer, and divided into aliquots of 8.5x10\textsuperscript{6} cells in 50 ml sterile centrifuge tubes. The washed fusion partners cell lines were similarly divided into aliquots of 25.5x10\textsuperscript{6} cells, which were layered on top of the islet cell aliquots. Each centrifuge tube therefore contained 34x10\textsuperscript{6} cells, comprising of islet cells and fusion partners at a 1:3 ratio. For a few fusions with islet cells from the first four organs, islet cell to fusion partner ratios of 1:1 and 3:1 were also used. Firm cell pellets were obtained by centrifugation at 1200g for 30 min, and the supernatant was decanted to obtain as dry a cell pellet as possible. The cell pellet was disrupted by gentle bumping of the centrifuge tube and this was continued as 1 ml of 50% PEG solution 37°C was added drop by drop over 1 min. The PEG used was either of MW 1450 or MW 4000. The bumping was continued for 1 min, then 1 ml of RPMI-1640 at 37°C was added over the next minute, again with gentle bumping. 10ml RPMI-1640 at 37°C was added over the next 9 min, followed by 25 ml over the next 2 min and 14 ml over the final minute.

The cells were then centrifuged 1200g for 30 min. The supernatant was decanted and the dry cell pellet gently disrupted and 50 ml of a selective medium at 37°C was
added over 1 min. Aspiration through pipette ensured the full dispersion of the cell pellet. Where the pancreas islet cell to fusion partner ratios of 1:1 and 3:1 were used, the resultant fused cells were incorporated into cell suspensions from the normal fusions involving the same MW PEG, where the fusion ratio was 1:3. In all occasions the volume of selective media was altered as necessary to attain approximately $0.5 \times 10^6$ fusion partner cells /ml of selective medium.

2.5.2 Post-fusion culture in selective media.

For the polyethylene glycol fusions various different selective media regimes were used. Except where otherwise stated all were supplementations to RPMI-1640 & 20% FCS:

- 0.4 µmol/l aminopterin, 0.2 mmol/l hypoxanthine, 16 µmol/l thymidine (HAT).
- 2.31 µmol/l azaserine, 0.2 mmol/l hypoxanthine, 16 µmol/l thymidine.
- 2.31 µmol/l azaserine, 0.2 mmol/l hypoxanthine.
- 2.31 µmol/l azaserine, 0.2 mmol/l hypoxanthine, 50 µmol/l IBMX, 10 nmol/l glucagon.
- 2.31 µmol/l azaserine and 0.2 mmol/l hypoxanthine, the RPMI-1640 used had a 5.55 mmol/l glucose concentration.

Three different batches of FCS were used to form the base medium of RPMI-1640 & 20% FCS. These batches were:

- Myoclone Super Plus, Gibco.
- Hybrimax, Sigma.
- Standard lot 92H3375, Sigma.

The resultant 50 ml of post-fusion islet cells and fusion partner cells was dispensed into 96-well culture plates, with approximately 200 µl - 300 µl per well, and cultured in 5% CO$_2$ at 37°C. Plates were observed after two days for bacterial contamination, as no antibiotics were present. Most fusions were performed within two days of the start of islet cell culture, and all were performed in under seven days.
After the final fusions each fusion partner cell line was pooled into one flask and cultured in 5% CO$_2$ at 37°C until confluence was attained. The cells were counted and then subjected to mock fusions. Only one batch of FCS was used in the selective media and no islet cells were present, but all other parameters were kept constant. The viable fusion partners were cell counted again and dispensed in each of the selective mediums into 96-well plates and cultured in 5% CO$_2$ at 37°C. These were observed over the next four weeks to ensure that the fusion partner cell lines were still lethally susceptible to each selective medium.

The islet cells obtained from the first pancreas were fused only to the PDH-1 fusion partner cell line and only HAT was employed as the selective medium. The islet cells from the second and third organs were fused with PDH-1, RINm5f and CRI-G5, with HAT used as the selective medium. The islet cells from the last two organs were fused with PDH-1, RINm5f, and CRI-G5 and the full panel of selective media discussed earlier was used.

2.5.3 Expansion of novel cell growth.

Fused cells in 96-well culture plates were observed by microscope from two weeks onwards. Novel growth was identified by the formation of a colony. After three weeks all wells were topped up with fresh culture medium, using the appropriate selective medium used for the fusion. After five weeks each novel colony had the culture medium aspirated off and replaced with 300 µl EDTA 0.02% solution at 37°C. After 10 min at 37°C, vigorous aspiration was used to transfer the EDTA solution containing the cells to 24-well culture plates. Where more than one colony growth formation was observed in the same well of the 96-well plates, each colony was regarded as a different cell line. Efforts were made to transfer such colonies to separate wells on the 24-well plates. The selective medium used for each colony was added to the appropriate well in the 24-well plates, with the exception that neither IBMX nor glucagon was used from this point on.
2.6 Establishment of cell lines.

All cell lines were cultured continually in a selective medium for at least six weeks, after which they were re-fed with the same culture medium as before but with the aminopterin or azaserine removed. Thus the lethal content of the selective media was removed, indeed the medium ceased to be selective.

2.6.1 Selection of cell lines.

After seven days in culture the cell lines were rinsed with RPMI-1640 and re-fed with normal culture medium RPMI-1640 & 10% FCS (lot 92H3375), and returned to culture in 5% CO$_2$ at 37°C for two hours. Densely populated cultures of the CRI-G5 fusion partner cell line were similarly cultured to provide a negative control. The medium from each well was removed and centrifuged 250g for 5 min and the top 600 µl taken off for the human insulin screen. The cell lines were transferred, using EDTA 0.02% solution as before, to 25 cm$^2$ vented culture flasks and were fed with RPMI-1640 & 20% FCS. When growth was seen to occur, the next required feeding was performed with RPMI-1640 & 10% FCS.

Cell lines of no immediate interest, from the results of the human insulin screening assay, were harvested twice before culture was discontinued. Cell lines from the first harvest were cryogenically frozen in pools of 5 cell lines. Pools of approximately 15 cell lines were obtained from the second harvest. These were treated as described in Section 2.10.1 and kept at -70°C for antigen studies.

2.6.2 Screening of cell lines for human insulin release.

The screening for human insulin was performed using the developed 'parallel radioimmunoassay'. The reader is directed to Chapter 3 for the development of this assay, as well as the methods used and the results obtained on the novel cell lines. Cell lines were deemed 'of interest' on the basis of a different 'ratio of derived insulin values' compared to rat insulin. Subcloning was performed to isolate out the possible human insulin-secreting cell lines.
2.6.3 Subcloning of cell lines.

The cell lines of interest were subcloned by limiting dilution in RPMI-1640 & 20% FCS. Cells not used in the subcloning were seeded into 75 cm² culture flasks and cultured in 5% CO₂ at 37°C. These cells were eventually cryogenically frozen and the flask culture discontinued. Approximately four weeks later, subclones were identified as single colony growth formation and transferred to 24-well plates. After a further week the human insulin screening assay was repeated a before.

Logistics dictated that only three subclones of each cell line could be kept. Picked on the basis of the human insulin screening assay the subclones were cultured in normal medium and grown through 25 cm², 75 cm² and finally 175 cm² sized vented culture flasks. Cryogenic stores of the subcloned cell lines were established, and one subclone of each cell line was kept in continuous culture.

2.6.4 External measurement of human insulin.

Culture media from some subclones, which continually exhibited a high ‘ratio of determined insulin values’ in the human insulin screening assay, were sent for further analysis. The laboratory of Prof. C.N. Hales graciously ran the media samples on an immuno-assay specific for human insulin, with no measurable cross-reactivity to rat insulins.

2.7 Hormone release of cell lines.

Insulin secretion and content, and glucagon secretion were assessed on the subcloned cell lines HiCRI-D1c, HiCRI-D2c, HiCRI-D3c, HiCRI-D4c, HiCRI-D5c, HiCRI-D6c, HiCRI-D7c, HiCRI-D8c, HiCRI-E11c, HiCRI-E12c, HiCRI-E13c, HiCRI-E14c, HiCRI-E15c, HiCRI-E16c. All were at passage 19 and were at least 34 weeks in culture. Later passages of HiCRI-D3c (passage 57) and HiCRI-D4c (passage 66) were also assessed. Both of these lines had been in culture for approximately 73 weeks. These were designated as HiCRI-D3c-p57 and HiCRI-D4c-p66 to differentiate from those of the earlier passage.
2.7.1 Preparative culture for hormone release studies.

Each cell line, and the parental CRI-G5, were dispensed into 24-well culture plates at a constant cell concentration in RPMI-1640 & 10% FCS. The cell concentration varied according to each cell line but was always between 4-6 x 10^4 cells/ml, with 1 ml added to each well. A portion of the plates also received 25 µl of 0.41 mol/l nicotinamide, prepared in RPMI-1640 & 10% FCS and sterile filtered, to obtain a final 10 mmol/l concentration. All cell lines were cultured for 48 h in 5% CO₂ / 95% air at 37°C, before commencement of static incubations.

2.7.2 Composition of static incubation buffer.

The buffer was equilibrated in 5% CO₂ / 95% air, with pH 7.4 at 37°C, and sterile filtered using 0.2 µm syringe filters.

HEPES-balanced Krebs-Ringer bicarbonate buffer, pH 7.4

119 mmol/l NaCl
4.74 mmol/l KCl
2.54 mmol/l CaCl₂·2H₂O
1.19 mmol/l MgSO₄·7H₂O
1.19 mmol/l KH₂PO₄
25 mmol/l NaHCO₃
10 mmol/l HEPES
5 g/l BSA

2.7.2.1 Modulators of insulin secretion.

Various known modulators (secretagogues) of β cell function were prepared in the HEPES-balanced Krebs-Ringer bicarbonate buffer. The final concentrations of these were: 1, 2, 4, 8, 16, 32 mmol/l glucose, with or without 10 mmol/l nicotinamide. The 2 mmol/l and 16 mmol/l glucose concentrations were used to prepare: 20 mmol/l KCl, 1 µg/ml glucagon, and together and separately 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX) and 8 mmol/l glutamine.
2.7.3 Static incubations.
Cells were thoroughly washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer (KRBB) and pre-incubated in 0.5 ml of KRBB for one hour in 5% CO₂ at 37°C. The cells were then again thoroughly washed twice in KRBB. Each of the modulators in the KRBB was then applied, 1 ml to appropriate wells of all the cell lines. The glucose concentrations containing nicotinamide were only applied to cells which had been cultured with nicotinamide, and these cells were not subjected to the other modulators. The cells were then cultured in 5% CO₂ at 37°C for two hours, after which the culture buffer was collected. All culture buffer samples were centrifuged 200g for 5 min and the top 700 µl taken off for insulin measurement.

2.7.4 Insulin measurement.
The insulin concentration in collected samples was measured using the Phadeseph insulin RIA kit, which is discussed in greater detail in Chapter 3. Triplicate sample volumes of 50 µl were assayed. In all cases the kit primary antibody was replaced with the Biogenesis anti-rat insulin primary antibody and the assay was calibrated against rat insulin standards.

2.7.5 Measurement of cellular protein content.
The cells were then washed extensively in phosphate buffered saline, pH 7.4 (PBS): 134 mmol/l NaCl, 2.7 mmol/l KCl, 1.47 mmol/l KH₂PO₄, 24.17 mmol/l Na₂HPO₄. The cells were allowed to air dry, before 0.25 ml of 0.1 mol/l NaOH was added to each well, and left at 4°C for 4 hours before measured for protein content. Protein determination was then performed using a Coomassie protein assay against a standard of BSA prepared in 0.1 mol/l NaOH.

2.7.6 Measurement of cellular insulin content.
Twelve wells of each of the cell lines were treated as for the static incubations. After the pre-incubation step the cells were thoroughly washed twice in KRBB, and then extensively in PBS and allowed to air dry. Half the wells were assessed for protein content as before, while the other wells received 500 µl of acidified ethanol, (75% ethanol, 25% water containing HCl for final concentration of 0.18 mol/l). These
wells were kept in air tight conditions overnight at 4°C, followed by one week at -70°C. The acidified ethanol solution was removed from each well, while attempting to scrape off any cell remnants from the wells. The resultant solutions were vortexed vigorously and centrifuged 1000g for 5 min at 4°C. 50 µl samples were aliquoted and freeze-dried. Before insulin measurement by RIA, 50 µl RIA assay buffer was added and left for four hours at room temperature and finally vortexed.

### 2.7.7 Measurement of glucagon release from cell lines.

For each cell line separately, 400 µl aliquots from the static incubation culture samples containing 16 mmol/l glucose were pooled. A total volume of 54 µl of the PBS buffer containing the following supplements was added to 600 µl of pooled culture sample to attain the final concentrations of 926 IU/ml Aprotonin and 2.8 mmol/l EDTA. The samples were then mixed for 1 min and kept frozen at -20°C until measured. The N-terminal glucagon measurement was performed externally at the Dept. of Medicine, Royal Victoria Hospital, Belfast. The assay was a RIA calibrated against porcine glucagon.

### 2.7.8 HPLC analysis of insulin-like immunoreactive profile secreted from novel cell lines.

HPLC analysis was performed on the culture sample pools created for the measurement of glucagon. After aliquoting for glucagon, the remainder of each sample was filtered through 0.2 µm syringe filters and stored with static incubation buffer at -70°C until HPLC analysis was performed. Upon thawing the samples and the static incubation buffer were filtered through 0.2 µm syringe filters. Rat insulin standard was diluted in the static incubation buffer to attain concentrations of 10 ng/ml and 100 ng/ml. Reversed-phase HPLC analysis was performed using the method of Efrat et al. (1988), using a LiChrosorb RP-18 (5 µm) 250 mm x 4 mm column. The column was equilibrated with 25% (v/v) Acetonitrile & 75% (v/v) 0.125 mol/l triethylammonium phosphate (TEAP), pH 4.0. Flow rate was set at 1 ml/min and the optical absorbance was monitored at a wavelength of 210 nm. A 100 µl sample loop was used in the automatic sample injection, after which the
acetonitrile concentration was increased by linear gradient to 33% over 90 min. The column was then re-equilibrated to 25% acetonitrile for 2 h and the same sample repeated as before but monitored at a wavelength of 276 nm.

2.8 Morphologic observations.
Cell lines were photographed after normal culture in 75 cm² culture flasks. For the photography the culture medium was replaced with RPMI-1640. Within four hours all flasks were photographed through a phase contrast microscope (Olympus Optical Company, Tokyo, Japan) at x20 lens magnification. The passage number of each cell line was as for the static incubation plus one. All cell lines were then returned to normal culture conditions.

2.9 Measurement of cell line growth rate.
Cells of each cell line, at the same passage number as used for the morphologic observations, were harvested and centrifuged 100g for 3 min. Cells were then dispersed thoroughly in RPMI-1640 & 10% FCS and cell counted. Precise volumes were used to inoculate five 25 cm² flasks, for each cell line, and the total volume made up to 6 ml RPMI-1640 & 10% FCS. Inoculation was therefore a constant for each cell line and every cell line received between 30 000 and 40 000 cells. Care was given to the symmetrical placement of the flasks in the incubator, where the flasks were cultured as normal. Flasks were observed daily by microscope until cell growth was well established with approximately 5% confluence. One flask of each cell line was then harvested and this was regarded as zero hour of day 0. At 24 h intervals the remaining flasks were harvested. For each harvest the culture medium was completely aspirated off and replaced with 3 ml of 0.02% EDTA solution. Ensuring complete separation of the cells from the flasks, the cells were dispersed by repeated gentle pipette aspiration before a sample was taken for cell counting. Flasks which attained greater than 90% confluence, or where there was any sign of the cell monolayer detaching, were regarded as no longer in the exponential growth phase and were not cell counted. Cell counts were analysed by non-linear regression and manual semi-log₂ plots, to ensure that only the exponential growth phase was
covered. Hence, data points at the extremities of the analysis time, which flattened the semi-log plot, were removed from the analysis and classed as overgrown.

2.10 Antigen studies.

2.10.1 Preparation of cell line samples.

The novel non-subcloned cell lines, for which CRI-G5 was the fusion partner, were harvested and combined in pools of approximately 15 cell lines at 0.1g cell weight/ml H$_2$O & 0.1% sodium azide (w/v). Phenylmethylsulfonyl fluoride (PMSF) and EDTA were both added to attain final concentrations of 1 mmol/l and 2 mmol/l respectively. Cells were kept at -70°C until use. After thawing the cell suspensions were combined further into two pools, one pool for each pancreas of origin. PMSF and EDTA were added again as before, and the cells were disrupted with a hand homogenizer. Human islets and the subcloned cell lines were similarly treated, although larger harvests were involved and no more than four cell lines were combined per pool. An equal volume of 0.2 mol/l PBS (x2 concentrated stock: 16 mmol/l Na$_2$HPO$_4$, 4 mmol/l NaH$_2$PO$_4$, 1 mol/l NaCl and 0.2% sodium azide), pH 7.4 was added to each cell homogenate. Triton X-100 was added to attain 1% (v/v), PMSF and EDTA were both added to attain final concentrations of 1 mmol/l and 2 mmol/l, [ignoring that already present]. The cell homogenates were roller-mixed overnight at 4°C and centrifuged 20 000g for 4 h at 4°C, followed by protein estimation performed using a BCA protein assay against a BSA standard.

2.10.2 Preparation of affinity matrixes.

Two antiseraums were used to prepare affinity columns:-

- Sheep anti-CRI-G5, raised against solubilized CRI-G5 cells.
- Pool of human serums, consisting of:
  - Two pancreas transplant patients with failing glucose toleration tests (GTT) and high immunoglobulin levels despite immunosupression.
  - Over twenty recently-diagnosed type I diabetics.
Both pools of antiserum were purified by precipitation with (NH$_4$)$_2$SO$_4$ at a final concentration 35% (w/v). Precipitation was for 2 hours at room temperature followed by centrifugation at 20 000g 60 min. The pellet was reconstituted in water to the original volume of serum and dialysed thoroughly in 100 mmol/l NaHCO$_3$ & 0.15 mmol/l NaCl, pH 8.0. The semi-purified serums were processed by DEAE-cellulose chromatography in the bicarbonate buffer, using DEAE-Sephacel equilibrated with the same buffer. A flow rate of 1 ml/min was used through a bed volume at least 10 times the volume of the serum, and 3 ml samples were collected. The first eluted peak (IgG) was identified by optical absorbance at a wavelength of 280 nm, and quantified by BCA protein assay kit against a BSA standard. The purified antibodies were concentrated using Minicon™-B clinical sample concentrators and reconstituted with 100 mmol/l NaHCO$_3$ & 0.5 mol/l NaCl, pH 8.3 to obtain a protein concentration of 4 mg/ml. The purified antisera were bound to CNBr-activated Sepharose® 4B using the stated coupling procedure for this product. Protein determination of the non-bound fraction indicated that the binding efficiency was in the order of 2.9 - 3.2 mg IgG/ml swollen affinity gel.

2.10.3 Affinity chromatography.

40 mg protein of cell homogenate was applied to 30 ml of the sheep anti-CRI-G5 affinity matrix and roller-mixed in a glass container for 2 h at room temperature. The matrix was allowed to settle before the cell homogenate was decanted off and collected. The matrix was then poured to column and washed with 20 ml 0.1 mol/l PBS & 1% Triton X-100, with this wash also collected. Regeneration of the affinity matrix was performed using cycles of alternate high and low pH with 0.1 mol/l Tris·Cl & 0.5 mol/l NaCl, pH 8.5 and 0.1 mol/l sodium acetate & 0.5 mol/l NaCl, pH 4.5. The non-bound homogenate was reapplied to the matrix and the process repeated, with the exception that the wash step was not performed. The final non-bound cell homogenate was applied to the 3 ml of the human affinity matrix and roller mixed overnight at 4°C. The preparation was poured to column and allowed to drain. The affinity column was washed with 20 ml 0.1 mol/l PBS & 0.1% Triton X-100, followed by 20 ml 0.1 mol/l PBS, and finally 30 ml H$_2$O. Elution was
performed with 5 ml 20 mmol/l HCl (Guest et al. 1989), and washed through with 5 ml H₂O. The collected eluate was then freeze-dried.

2.10.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, (SDS-PAGE).

Samples were reconstituted in 100 µl of sample buffer, 0.05 mol/l Tris-Cl, pH 6.8 containing 100 mmol/l dithiothreitol, 2% (w/v) SDS, 10% (v/v) glycerol and 0.025% (w/v) Bromophenol Blue. Samples were heated at 100°C for 5 min and 20 µl loaded into sample wells. Electrophoresis was performed on slab gels polymerised from 10% (w/v) acrylamide and 0.3% (w/v) N,N'-methylenebisacrylamide, using the discontinuous buffer system of Laemmli (1970). The electrophoresis was performed on a LKB 2050 cooled midget unit. Transfer of proteins from the polyacrylamide gel to solid support was achieved by electro-blotting with a Trans-Blot® electrophoretic transfer cell onto Hybond™-C nitrocellulose membrane (Towbin et al. 1979).

2.10.5 Western Blotting.

Composition of the Western blot buffer, Tris buffered saline, pH 7.5 :

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
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<td>50 mmol/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mmol/l</td>
</tr>
<tr>
<td>Thiomersal</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.02% (v/v)</td>
</tr>
</tbody>
</table>

The nitrocellulose membrane was dried before thorough re-soaking in the blotting buffer & 3% BSA (w/v) for two hours at room temperature, after which the nitrocellulose membrane was dried again. After thorough soaking of the membrane in a minimal volume of the blotting buffer & 3% BSA, primary antibody was added at a dilution of 1:50. The primary antibody was the unpurified pool of human sera consisting of pancreas transplant failures and newly-diagnosed diabetics. The nitrocellulose membrane was incubated on a rocking platform at 4°C overnight. The membrane was then washed three times in blotting buffer & 3% BSA for 20 min on a rocking platform at room temperature, before the secondary antibody was applied at a dilution of 1:500 in the blotting buffer & BSA. The secondary antibody was
sheep anti-human IgG, enzyme labelled with horseradish peroxidase, and incubated for 2 hours on a rocking platform at room temperature. The membrane was then washed four times in blotting buffer for 20 min on a rocking platform at room temperature, before being transferred to a new container for the fifth and final wash in blotting buffer for 60 min on a rocking platform at room temperature. Development was achieved using Sigma Fast™ DAB tablets (3,3-diaminobenzidine tetrahydrochloride), [when dissolved in water these tablets supply the necessary buffer and peroxide for substrate development]. The end-point of development was determined visually, after which the membrane was immersed in tap water for 10 min and then finally allowed to dry thoroughly in air.

2.11 Chromosome analysis of cell lines

Karyotype analysis of the novel cell lines and the parental CRI-G5 was performed by conventional techniques. The cells used were those from the morphologic observations and as such the passage number of each cell line was as for the static incubation plus one. All 75 cm² culture flasks were allowed to grow to greater than 75% confluence. To arrest mitosis in metaphase Colcemid® was added to attain 0.1 μg/ml concentration in 40 ml of culture medium and returned to culture in 5% CO₂ for 2 hours. Flasks were then vigorously agitated until cells visibly parted from the growing surface. The culture medium was collected and centrifuged 200g for 10 min, and the cell pellet was gently disrupted during the gradual addition of 15 ml 75 mmol/l KCl, pre-warmed to 37°C. After a further 30 min at 37°C cells were centrifuged 200g for 5 min. The cell pellet was gently dispersed into 6 ml of fixative [75% methanol, 25% glacial acetic acid] and centrifuged 100g for 5 min. This addition of fixative was repeated twice more, with the final cell pellet resuspended in 1 ml of fixative and stored at -20°C for at least one week.

When proceeding, the cells were dispersed thoroughly into 1 ml of fresh fixative after centrifugation 100g for 3 min. Using a pasteur pipette, one drop of the cell suspension was allowed to fall from a height of about 5 cm onto one end of a glass microscope slide and the drop was encouraged by tilting to spread over the remainder of the slide. This was repeated for at least six microscope slides and all
were air dried for 60 min. All but two of the slides were then stained by immersion in 0.3% aqueous Giemsa stain for 7 min. Slides were then washed with tap water, drained and left to air dry for 2 h. When completely dry the slides were mounted in DPX and protected by cover slip, and were viewed on a Leica Leitz Diaplan microscope under a 100x oil immersion lens.

2.11.1 Chromosome *in situ* hybridisation.

Chromosome *in situ* hybridisation was performed using a reagent kit supplied by Oncor and following the prescribed protocol. Unstained chromosome spreads on microscope slides were incubated in double strength (2x) SSC buffer, pH 7.0, at 37°C for 30 min. Slides were then dehydrated by the sequential immersion for 2 min at room temperature in 70%, 80% and finally 95% ethanol in water, and then allowed to air dry. The slides were then immersed in a denaturation solution, pH 7.0: 70% formamide; 20% H2O; 10% twenty fold strength SSC buffer. Denaturation was for 2 min at 72°C. The slides were again dehydrated as before, but the ethanol solutions were pre-cooled to -20°C. On the same day 31.5 μl of the biotin labelled Total Human DNA probe was heated at 72°C for 5 min and then placed on ice until use within 1 hour. Slides were then heated to 37°C and the probe applied to the slide, and covered by glass coverslip. The edges of the coverslip were completely sealed with rubber cement and the slides left overnight in a humidified container at 37°C. The following day the coverslip was dislodged with no sideways motion, and the slide immersed without agitation in double strength SSC buffer, pH 7.0, for 8 min. This step was repeated using fresh buffer before 60 μl of the fluorescein labelled avidin solution was applied to the slide and covered with plastic coverslip. After incubation in darkness at 37°C for 5 min, the coverslip was carefully removed, again with no sideways motion, and rinsed three times in fresh PBD buffer for 2 min. Slides were then counter-stained with 20 μl of propidium iodide at a final concentration of 0.3 μg/ml in Antifade and a coverslip applied. Slides were viewed on a Leica Leitz Diaplan microscope, with an appropriate dual band pass filter, and viewed under 100x oil immersion lens. Excitation was provided by a fitted 50 watt mercury fluorescent bulb.
2.12 Statistical analysis.

Data were analysed using Prism™ (Graphpad Software Incorporated). Simple statistics were calculated using Column Statistics on Prism™. This gave the mean and standard error of the mean (SEM) of the data. The normal distribution of data was assumed under established criteria (Campbell and Machin, 1989; Huck and Cormier, 1995). These criteria were: the inherent nature of the data; the inability to check with under 12 samples; and previous studies of this nature making the same assumption.

ANOVA with appropriate post tests was used to assess significance. For the glucose concentration curves the post test of Bonferroni for selected pairs of columns was used. This selection was predetermined and was constant for each concentration curve. Selection always tested significance between two adjacent concentrations only. The post test of Tukey was used for the more speculative analysis of the glucose concentration curves. The post test of Dunnett was used to compare all values versus a control, which was always predetermined and a constant. Prism™ automatically checked during all ANOVA calculations for the ‘Bartlett’s test for equal variances’. When used, the Student’s t-test was also performed by Prism™. All data are presented as mean (x) ± SEM with the total number of observations (n), and the significance (P value). Significance was in all cases using the null hypothesis and regarded as significant if P < 0.05. It is acknowledged that the analysis regimes utilized could introduce Type II error, but given the nature of the study, this was preferable to the alternative Type I error.

2.12.1 Non-linear regression analysis.

Non-linear regression analysis was performed using Prism™ (Graphpad Software Incorporated). Doubling time was automatically calculated using the in built exponential growth equation: Doubling time = 0.69/k, where k is the exponential rate constant.
Chapter 3

Method Development:

Development of human insulin assay.
3.1 Introduction to the Phadeseph human insulin RIA kit.

The assay to measure insulin levels was based around the Phadeseph human insulin RIA kit, a competitive RIA which utilizes a double antibody solid phase technique. A fixed volume of sample or standard is added to a fixed volume of human insulin labelled with I\(^{125}\) (‘hot insulin’) and incubated with a limited amount of the primary antibody of guinea pig anti-human insulin. As there is not enough primary antibody to bind all the insulin present, the hot insulin and the sample insulin compete for the antibody and thus the more insulin present in the sample the less hot insulin is bound. The secondary antibody of sheep anti-guinea pig IgG is then added and incubated to allow it to bind the primary antibody. Supplied bound to sepharose, the secondary antibody facilitates the centrifugal pelleting of the immune complexes comprising of secondary antibodies bound to primary antibodies which are themselves bound to insulin. Thus bound insulin is separated from free insulin. The more insulin in the sample the less hot insulin is bound and then pelleted out, thus the concentration of sample insulin is inversely proportional to the amount of hot insulin pelleted out. Gamma counting of the pellets provides a quantitative measure of the amount of hot insulin pelleted out, in the form of radioactive counts per minute (cpm). The use of standards containing known amounts of insulin allows the amount of insulin in the samples to be quantified. This is performed by calculating the percentage of activity bound of the standards using the following formula:

\[
\text{% activity bound = } \frac{\text{cpm of sample or standard}}{\text{cpm of the zero standard}} \times 100
\]

The percentage of activity bound values are then plotted in linear scale against the concentration of the standards in \(\log_{10}\) scale to form a standard curve. The percentage of activity bound values are then calculated for control samples and unknown samples and the corresponding insulin values read off the standard curve.
3.1.1 Methods.

Composition of the RIA buffer, pH 7.4:

- Na₂HPO₄ 4.6 g/l
- NaH₂PO₄ 1.2 g/l
- Thiomersal 0.25 g/l
- EDTA disodium·2H₂O 3.72 g/l
- BSA fraction V [RIA grade] 5 g/l

A rat insulin standard curve was constructed by dilution with the assay buffer to attain concentrations of 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.150625 ng/ml. A human insulin control curve was obtained using the Phadeseph kit human insulin standards, with human insulin concentrations of 9.6, 4.0, 1.2, 0.4, 0.12 ng/ml. Assay buffer alone was used as the zero standard. All standards were dispensed (50 µl) in quadruplicate to LP4 tubes, to form two sets of duplicates. Only one set of duplicates was analysed in this assay, the other set was analysed as discussed in section. 50 µl of the Phadeseph kit I¹²⁵ labelled insulin solution was added to the tubes which were then centrifuged briefly 200g for 3 minutes, and then 50µl of the Phadeseph kit primary antibody guinea pig anti-human insulin solution was added. The tubes were again centrifuged briefly 200g for 3 minutes, and then vortexed. Samples were incubated at room temperature for 2 hours and then 1 ml of the Phadeseph kit secondary antibody solution and incubated for 30 minutes at room temperature. Tubes were then centrifuged 1500g 10 minutes, and then decanted and gently blotted dry while inverted. Tubes were gamma counted for 5 minutes on a 1272 Clinigamma counter, and the insulin concentration quantified using the rat insulin standard curve. In this laboratory this was performed automatically by RiaCalc software by Wallac. This software constructs a spline smoothed best fit standard curve and automatically calculates unknown sample values from this curve. This computer software programme has various in-built assay checks including duplicate sample cv values and degree of ‘goodness of fit’ of the spline curve. The software also checks for the presence of plateaux or secondary curves in the standard curve, which would both result in a single cpm value no longer corresponding to a
single insulin concentration. Such cases would be indicative of the 'hook effect' seen in many immunoassays, where the concentration limits of the assay are exceeded.

3.1.2 Results

Shown in Figure 3.1 are the plots of rat insulin standard curve and a human insulin control curve, where the zero value was 4928 cpm.

Fig. 3.1 Concentration curves of human and rat insulin standards using the Phadeseph RIA kit.

Rat ■ and human ▲ insulin standards were assayed using the Phadeseph insulin RIA kit as described in Section 3.1.2. Displayed are the radioactive counts per minute (cpm) values for the rat and human insulin standards at various known concentrations. Points represent mean of duplicate values. Also shown is δK, the difference in insulin concentrations between human and rat insulin at 2192 cpm.
3.1.3 Discussion

The reasons for the difference in the curves is due to the difference in affinity of the primary antibody for human insulin versus rat insulin. To be specific the kit primary antibody had a stronger affinity for human insulin. The cross-reactivities of this antibody were stated in the product specifications as: Human insulin 100%; bovine insulin 100%; human pro-insulin 41%; and C-peptide 0.1%, but the cross-reactivity with rat insulin was not provided.

Both curves could be used as a standard curve. In this case the assay was calibrated using the rat insulin curve. The rat insulin standard 1.25 ng/ml gave a count of 3966.0 cpm. The human insulin control sample 1.25 ng/ml human insulin gave a count of 2192.0 which when read on the rat insulin standard curve equated to a rat insulin concentration of 12.1544 ng/ml. This value was a pure artefact due to the horizontal difference in the curves at 2192.0 cpm, as represented in Fig. 3.1 by δK. All the human control samples equated to artefactually high concentrations when read from the rat insulin standard curve, though the value of δK varied according to the cpm value. Thus, in this assay if a pure insulin sample of unknown concentration produced a gamma count of 2192.0 cpm it would not be known if this sample contained 1.2 ng/ml of human insulin or 12.15 ng/ml of rat insulin. This assay therefore relied on the species of insulin being known to allow determination of the insulin concentration. This was not possible, as it was unable to determine the species of insulin.

3.2 Introduction to the Biogenesis adapted Phadeseph insulin RIA.

The previous assay discussed in Section 3.1 – 3.1.4 was adapted by the replacement of the Phadeseph kit guinea pig anti-insulin primary antibody by another primary antibody. Produced by Biogenesis, this different primary antibody was supplied as a guinea pig ‘anti-rat insulin’ antibody and hence referred to as that. Cross-reactivities were stated in the product specifications as: human insulin 100%; bovine insulin 100%; human proinsulin 56.25%; and rat insulin 62.5%. This assay is referred to as the Biogenesis RIA, to differentiate this assay from the previous assay discussed in Section 3.1.
3.2.1 Methods

The Biogenesis guinea pig antibody to rat insulin was diluted 1:500 (v/v) as recommended by the product guidelines. Dilution was performed with the RIA buffer (Section 3.1.2) containing charcoal stripped human serum 1:500 (v/v). 50 μl of Phadeseph kit I\textsuperscript{125} labelled insulin solution was added to the tubes which were then centrifuged briefly 200g for 3 minutes, and then 50μl of Biogenesis primary antibody solution was added. The tubes were again centrifuged briefly 200g for 3 minutes, and then vortexed. The assay was incubated, processed and analysed as discussed previously in Sections 3.1.2 – 3.1.4, again with the insulin concentrations quantified using the rat insulin standard curve.

3.2.2 Results.

Shown in Figure 3.2 are the plots of rat and human insulin standard curves, using the Biogenesis primary antibody. The zero insulin concentration gave a gamma count of 5367 cpm. As seen previously in Figure 3.1 the different species of insulin produce different standard curves, due to the difference in affinity of the primary antibody for human insulin versus rat insulin, with the Biogenesis primary antibody displaying a stronger affinity for human insulin. The human insulin controls were analysed as samples on the assay calibrated using the rat insulin curve. The rat insulin standard 1.25 ng/ml gave a count of 4270.3 cpm. The control sample 1.2 ng/ml human insulin gave a count of 3203.8 cpm which when read on the rat insulin standard curve equated to a rat insulin concentration of 2.99 ng/ml.
Rat ■ and human ▲ insulin standards were assayed using the Biogenesis insulin RIA as described in Section 3.2.2. Displayed are the radioactive counts per minute (cpm) values for the rat and human insulin standards at various known concentrations. Points represent mean of duplicate values. Also shown is $\delta B$, the difference in insulin concentrations between human and rat insulin at 3203.8 cpm.

3.2.3 Discussion.

The control sample 1.2 ng/ml human insulin equating to a rat insulin concentration of 2.99 ng/ml was due to the horizontal difference in the curves at 3203.8 cpm as represented in Figure 3.2 by $\delta B$. All the human control samples equated to artefactually high concentrations when read from the rat insulin standard curve, though the value of $\delta B$ varied according to the cpm value. In this assay, if an unknown sample produced a gamma count of 3203.8 cpm it would not be known if this sample contained 1.20 ng/ml of human insulin or 2.99 ng/ml of rat insulin. As seen with the Phadeseph kit assay, this assay also relied on the species of insulin.
being known to allow determination of the insulin concentration. This was not possible, as it was unable to determine the species of insulin.

3.3 Introduction to the ‘parallel RIA’ human insulin screening assay.

The main characteristic sought for in the novel cell lines was the secretion of human insulin. As both the RINm5f and CRI-G5 parent cell lines secrete rat insulin, any screening for novel cell lines secreting human insulin would be complicated by the probable presence of rat insulin, and the screening assay should be able to distinguish human from rat insulin.

Both the assays in Sections 3.1.4 and 3.2.4 were not suitable as human insulin screening assays, and both relied on the species of insulin being known to allow determination of the insulin concentration. They could only have been suitable if the primary antibody exhibited 0% cross-reactivity with rat insulin. Similarly, identification of a rat insulin sample would require a primary antibody with 0% cross-reactivity with human insulin. As no such antibodies were commercially available it was necessary to develop a screening assay for human insulin. With this aim, what is termed as a ‘parallel radioimmunoassay (RIA)’ was developed.

Shown in Table 3.3 are the results obtained for the rat insulin standard 10 ng/ml and the human control sample 1.2 ng/ml by the Phadeseph and Biogenesis assays in sections 3.1 and 3.2. Both these assays were calibrated against the rat insulin standard curve, therefore while the rat insulin values were accurate the human insulin sample produced an artefactually high result in each assay. Crucially the degree of this artefact differed according to which assay was used. That is to say the horizontal difference between the rat and human insulin curves was different for each assay. Represented at a single insulin concentration as $\delta K$ and $\delta B$ in Figures 3.1 and 3.2, for human insulin $\delta K \neq \delta B$ but for rat insulin $\delta K = \delta B = 0$. Also shown in Table 3.3 are the ‘ratio of derived insulin values’, which was obtained by dividing the insulin concentration value obtained from the Phadeseph kit assay (section 3.1.3) by the value for the same sample from the Biogenesis assay (section 3.2.3). As both assays were calibrated against the rat insulin standard curve, the rat insulin sample
10 ng/ml was reported accurately as approximately 10 ng/ml by both assays. The assays reported two very different results for the human insulin sample 1.2 ng/ml, and both of these results were artefacts. This provided the basis for a human insulin screening assay.

**Table 3.3 Description of the ‘Ratio of derived insulin values’**.

| Insulin concentration value derived using the named radioimmunoassays, calibrated against rat insulin standards. | Ratio of derived insulin values: Phadeseph value / Biogenesis value |
|---|---|---|
| **Phadeseph assay** | **Biogenesis assay** | **Biogenesis value** |
| Rat insulin 10 ng/ml | 10.0 | 10.0 | 1.0 |
| Human insulin 1.2 ng/ml | 12.15 | 2.99 | 4.06 |

When the an unknown sample produced an insulin value of 12.15 ng/ml on the kit assay it would not be known if this was human or rat insulin. If the result for the same sample on the biogenesis based assay was approximately 12.15 ng/ml, and the ratio of derived insulin values was therefore approximately 1, the sample could be assumed to be rat insulin. If however this result was approximately 2.99 ng/ml, and the ratio of derived insulin values therefore approximately 4.06, the sample could be assumed to be human insulin. For accuracy’s sake however, the ratio of derived insulin values for the rat insulin should have been calculated at 12.15 ng/ml and not 10.0 ng/ml as in Table 3.3. As δK and δB both differ according to the cpm count, the ratio of derived insulin values of human insulin sample also varies according to the concentration of human insulin. The fitting of the spline curve to create the standard rat insulin curve can also introduce small differences in the results obtained with rat insulin samples. Thus exact derived insulin values for rat insulin also vary according to insulin concentration. It was necessary therefore, to establish the ratio of derived insulin values over a range of concentrations of both human and rat insulin.
This procedure would allow for a rat insulin sample to be distinguished from a human insulin sample, when a sample is only either rat insulin or human insulin. In cell line culture media samples this is not often the case. Probably also present would be bovine insulin from the foetal calf serum, rat and/or human C-peptide and proinsulin. As these also cross-react with the primary antibodies they could be expected to alter the ratio of derived insulin values of samples. Also of concern is the existence of two types of rat insulin as discussed in Section 1.14.1, each of which may exhibit different affinities for one or both of the primary antibodies. The rat insulin standards used in this project possessed incomplete data as the manufacturers were unsure of the ratio of rat I : II insulin (personal communications). Still further, the proportions of rat insulins secreted by the CRI-G5 cells was uncertain, and so the ‘ratio of derived insulin values’ for rat insulin secreted by CRI-G5 cells may not match the rat insulin standards. As a final consideration was the possible presence of both human and rat insulin within the same sample. As discussed in Section 1.12.1 this could occur due to a mixed pre-subcloning population of novel cells, or the simultaneous secretion of both species of insulins by a novel cell.

3.3.1 Methods.

A top rat insulin standard of 38.4 ng/ml was constructed in assay buffer, which was then serially diluted in half with assay buffer. The top Phadeseph kit human insulin standard of 9.6 ng/ml was similarly diluted. Both the previous insulin samples were combined to form stock samples of either; Rat insulin 4.8 ng/ml & human insulin 4.8 ng/ml; or rat insulin 8.64 ng/ml & human insulin 0.96 ng/ml. Again these were serially diluted in half with assay buffer, as was also culture medium containing 10% FCS from a culture flask of CRI-G5 cells. A neat sample of culture medium containing 10% FCS was also included. All standards and samples were dispensed (50 μl) in quadruplicate to LP4 tubes, to form two sets of duplicates. 50 μl of the Phadeseph kit I125 labelled insulin solution was added to all tubes which were then centrifuged briefly 200g for 3 minutes, and then 50μl of the appropriate primary antibody solution was added. One set of duplicate tubes received the Phadeseph kit primary antibody solution, while the other set received the Biogenesis primary antibody solution as described previously in Section 3.2.2. The tubes were again
centrifuged briefly 200g for 3 minutes, and then vortexed. Samples were incubated at room temperature for 2 hours and then 1 ml of the secondary antibody and incubated for 30 minutes at room temperature. Tubes were then centrifuged 1500g 10 minutes, and then decanted and gently blotted dry while inverted. Tubes were then gamma counted for 5 minutes and the insulin concentration quantified using the rat insulin standard curve, as described in Section 3.1.2. The ratio of derived insulin values was calculated for each sample and standard and then plotted against the insulin concentration value from the Biogenesis assay.

3.3.2 Results.

Only trace amounts of insulin were detected in the control of RPMI-1640 & 10% FCS, and hence bovine insulin was removed from all considerations. The samples of culture medium from CRI-G5 cells closely mimicked the response seen for the rat insulin standards. As shown in Figure 3.3, the parallel RIA was able to differentiate between rat and human insulin. It could distinguish human insulin within samples which also contained rat insulin, even when the human insulin accounted for only one tenth of the total insulin present. The ability to distinguish human from rat insulin did suffer at levels below 0.5 ng/ml.
Serial dilutions of rat insulin standards (O), human insulin standards (▼), CRI-G5 (▲), rat insulin & human insulin standards combined at ratio 9:1 (●), and rat insulin & human insulin standards combined at ratio 1:1 (◆), were assayed using the parallel RIA as described in Sections 3.3 and 3.3.2. The value for the ratio of derived insulin values is plotted against an x-axis of the insulin value obtained from the Biogenesis assay, with both assays calibrated against rat insulin. All points represent one sample measured in duplicate, where the Coefficient of Variation was never greater than 7%.

3.3.3 Discussion.

The close similarity of the 'ratio of derived insulin values' of the rat insulin standards and the rat insulin secreted by CRI-G5 cells inferred that the proportion of rat insulin types in the rat standard was not of concern. Indeed the insulin secretory profile of CRI-G5 cells was not of concern, in that any rat C-peptide or any other co-secreted products with cross-reactivity with the primary antibodies did not cause any
dissimilarity in the ratio of derived insulin values between the rat insulin standards and culture media from CRI-G5 cells. This also applied to the bovine insulin present in the CRI-G5 culture medium, and combined with the trace level of this species of insulin it was disregarded as an interfering factor.

Although the assay was far from definitive and falsely positive results could not be ruled out, any cell lines secreting human insulin would also be identified, and as such the assay functioned as a moderately effective screening assay. At the very least it identified cell lines with a profile of secretory products which differed from the parental CRI-G5 cell line.

3.4 Working Parallel RIA protocol.

Rat insulin standard 9.6 ng/ml and the Phadeseph kit human insulin control 9.6 ng/ml were both serially diluted in half with the assay buffer to a lowest concentration of 0.15 ng/ml. The rat insulin standards were supplemented with a more concentrated insulin standard (Novo Biolabs) which was similarly serially diluted in half to form a concentration range from 200 ng/ml to 1.5625 ng/ml. These latter insulin standards are hence referred to as the ‘new Biolabs rat insulin standards’, to differentiate from the ‘original’ rat insulin standards used in sections 3.1 – 3.3.4. A more concentrated human insulin control (approximately 500 ng/ml) was derived from human Actrapid™ insulin (Novo Nordisk), and then serially diluted in half thirteen times. These new supplemental insulin standards and controls were previously validated against the original standards using the parallel RIA. In as such, both sets of rat insulin standards displayed similar ‘ratio of derived insulin values’, and likewise for the two sets of human insulin controls. Assay buffer alone was used as the zero standard. Two hour culture samples from the novel cell lines and a densely populated culture of CRI-G5 cells were gathered as described in sections 2.6.1. The media samples from the novel cell lines were assessed neat and diluted in half with RIA assay buffer. This was primarily to identify any ‘hook’ effects seen in immunoassays when a sample contains too high a concentration of the measured compound. The CRI-G5 culture media sample was serially diluted in
half to one sixteenth the original concentration, creating four dilutions and the neat sample for assessment.

Standards, controls and samples were analysed on both the Phadeseph and Biogenesis assays as described in section 3.3.2, and hence were screened for human insulin using the ‘parallel RIA’. The term ‘parallel RIA’ came from the necessity that the same sample was split for analysis on both the constituent Phadeseph and Biogenesis assays, which were both started and completed within thirty minutes of each other, to combat any interference caused by insulin decay. The centrifugation steps imposed small inter-assay differences in the incubation timings of the Phadeseph and Biogenesis assays. This inter-assay variation did not interfere with the parallel RIA provided that intra-assay variation was avoided. To combat this all samples, controls and standards in the Phadeseph assay were processed exactly the same, and likewise all samples, controls and standards in the Biogenesis assay were processed exactly the same.

The ‘original’ rat insulin standards 9.6 – 0.15 ng/ml were used to construct a preliminary standard curve from which the concentration of the ‘new Biolabs rat insulin standards’ were verified before using these values to supplement the values of the ‘original’ standards in order to extend the range of the final rat insulin standard curve. The insulin concentration results were read from this final rat insulin standard curve. The actual concentration of the human Actrapid™ insulin standards were very approximate, due to the nature of this suspension. This was not considered a concern as it was the insulin concentration values produced by the Biogenesis assay and the ratio of derived insulin values which were used, hence an accurate human insulin concentration was not required. For consistency’s sake, the new insulin standards and controls were only used to supplement the results of the ‘original’ rat insulin standards and human insulin controls.

3.4.1 Results and discussion.

For a full description of the results of screening of the novel cell lines for human insulin secretion, the reader is directed to Results-Sections 4.3-4.3.2. Reporting here
is limited to discussion concerning validation of the assay. In Figure 3.4 are shown the ratio of derived insulin values for various dilutions of the rat insulin standards, human insulin controls, and culture medium from CRI-G5 cells and the novel cell line HiCRI-D3. The ratio of derived insulin values of human and rat insulin were most clearly different over the concentration range of 0.92 ng/ml to 63 ng/ml (0.16 to 10.89 pmol), though differentiation could be made between 0.29 ng/ml to 124 ng/ml (0.05 to 21.44 pmol). The rat insulin/s secreted by the CRI-G5 cells, (and any other co-secreted products), closely matched the ratio of derived insulin values of the rat insulin standards. The ratio of derived insulin values for the HiCRI-D3 cell line matched neither the rat insulin standards nor the human insulin controls, and the respective concentrations of the neat sample and the half diluted sample confirmed that this was not a hook effect. Although these results did not confirm that human insulin was present, they did indicate that human insulin could be co-present with rat insulin. In as much, clearly the results of HiCRI-D3 did not match the rat insulin standards and neither did they match the parental CRI-G5 cell line, and the slightly elevated ratio of derived insulin values was previously seen in Section 3.3.3 when rat and human insulin were co-present.

As discussed in Section 2.6.3 this HiCRI-D3 cell line was then subcloned to produce a number of monoclonal cell lines which were then screened for human insulin secretion. This was expected to identify subclones which secreted rat insulin, but also at least one subclone which secreted human insulin, and thus was responsible for the elevated ratio of derived insulin values. The screening did identify subclones whose secretory products closely matched the ratio of derived insulin values of the rat insulin standards. It did not identify any subclones which matched the human insulin controls. Curiously some subclones exhibited slightly elevated ratio of derived insulin values, similar to the the non-subcloned cell line shown in Figure 3.4. As discussed previously in Section 1.14.1, a plausible explanation for this was that within the polyclonal cell line HiCRI-D3 a monoclonal cell line was co-secreting both rat and human insulin.
Fig. 3.4 Ratio of derived insulin values from the parallel RIA of the insulin secreted by the novel cell line HiCRI-D3.

Culture sample from novel cell line HiCRI-D3 (□), resulting from a 2 hour static incubation in RPMI-1640 & 10% FCS, was assayed neat and diluted 1:1 using the parallel RIA as described in Section 3.4. Also assayed were serial dilutions of rat insulin standards (■), human insulin standards (▲) and culture medium from CRI-G5 cells (▼). The value for the ratio of derived insulin values is plotted against an x-axis of the insulin value obtained from the Biogenesis assay, with both assays calibrated against rat insulin. All points represent one sample measured in duplicate, where the Coefficient of Variation was never greater than 4%.

Although the parallel RIA screening assay was able to identify cell lines co-secreting rat and human insulin, the assay was unable to confirm that this was the reason for the elevated results of the ratio of derived insulin values for the HiCRI-D3 cell line.
Thus, culture medium from this subcloned cell line was assessed externally for the presence of human insulin (Section 2.6.4). The key to this assessment was the use of a primary monoclonal antibody to human insulin which exhibited no cross-reactivity to rat insulin, proinsulin or C-peptide. The culture media of the HiCRI-D3 subcloned cell line was shown by the external assay to not contain human insulin.

As human insulin was absent it was unclear what had caused the elevated results of the ratio of derived insulin values of HiCRI-D3. The presence of human proinsulin as opposed to human insulin could not be ruled out as the cause. Perhaps a more plausible possibility was that the relative proportions of rat proinsulin and/or Rat I and II insulin in the culture medium of the HiCRI-D3 cell line were different than for the CRI-G5 cell line and the rat standards. The latter two were already seen to be similar in terms of the ratio of derived insulin values. This could also be true for other products co-secreted with the rat insulin HiCRI-D3 cell line, products which were not accounted for. Attempts to resolve this query by HPLC analysis of the products secreted by HiCRI-D3 were unsuccessful (Section 4.3.4), and hence the cause of the elevated results of the ratio of derived insulin values remained unclear.

3.5 Summary.
The parallel RIA assay developed to screen for human insulin was slightly flawed in that unknown factors mimicked the results seen when human insulin was co-present with rat insulin. However, the assay was still viable. Under the assumption that a novel cell colony which secreted human insulin was generated, but was co-present with cells which secreted rat insulin. The over-riding concern was that such a cell colony would not be overlooked and missed. Had such a cell colony been present it would have been identified, by the elevated ratio of derived insulin values, as a colony to be subcloned. So although some cell lines which did not secrete human insulin were also deemed worthy to be subcloned, a human insulin-secreting cell colony would not have been missed, without the need to subclone every novel cell growth which secreted insulin.
Had a human insulin secreting cell line been generated, subcloning would have produced at least one subclone whose insulin exhibited a ratio of derived insulin values which matched the human insulin controls. Indeed when no subclones were seen to match the human insulin controls in terms of the ratio of derived insulin values, the assay correctly identified that no cell lines secreting only the human species of insulin were present.

Despite the slight flaw in the parallel RIA, when unknown factors mimicked the results seen when human insulin was co-present with rat insulin, the assay still facilitated screening and the subsequent subcloning, by narrowing the search down to just fourteen cell lines out of a total of one hundred and ten insulin secreting cell lines. Ironically the flaw itself did identify cell lines whose secretory products were different from the parental CRI-G5 cell line, and hence helped to confirm their novelty.

Of course a better screening assay would have been one involving a primary antibody to human insulin which exhibited no cross-reactivity with rat insulin, but no such antibodies were commercially available. The primary antibody used during the external assessment (Section 2.6.4) would have been an ideal antibody, but its existence was not known to this author until after the cell lines had been screened. The limited access to this antibody meant that this too was unavailable for the screening of the large number of samples required. Hence the parallel RIA was developed out of necessity, and had a human insulin-secreting cell line been present the parallel RIA would have detected it and aided in its isolation.
4.1 Islet cell yield

In all five human pancreatic organs were obtained and isolated in chronological order in Table 4.1. After showing the resulting number of single pancreatic cells that were available for RNA isolation following the procedures of enzymatic digestions, and the cell isolation as described in Section 2.4 to 2.4.4.

Table 4.1 Approximate yields of isolated human pancreatic cells from the enzymatic digestion of human pancreas organs.

Chapter 4

Results.
4.1 Islet cell yields.

In all five human pancreas organs were obtained and are listed in chronological order in Table 4.1. Also shown are the resulting numbers of the single pancreatic cells that were available for PEG fusion following the enzymatic digestions and islet cell isolations as described in Sections 2.4 to 2.4.4.

Table 4.1 Approximate numbers of isolated single pancreatic cells from the enzymatic digestions of human pancreas organs.

<table>
<thead>
<tr>
<th>Pancreas</th>
<th>Total cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.7 x 10^8</td>
</tr>
<tr>
<td>2</td>
<td>4.1 x 10^8</td>
</tr>
<tr>
<td>3</td>
<td>3.2 x 10^8</td>
</tr>
<tr>
<td>4</td>
<td>4.9 x 10^8</td>
</tr>
<tr>
<td>5</td>
<td>2.5 x 10^8</td>
</tr>
</tbody>
</table>

4.2 Fusion parameters.

In all 216 fusions were performed on islet cells. The aim of the differing fusion procedures and environments (described in Sections 2.5-2.5.3) was primarily to obtain a novel cell line, not to assess the various parameters used. As such, no serious attempts were made to assess these parameters for statistical significance. However, no novel cell lines were established when either glycerol was present in the isolation buffer, or when 16 μmol/l thymidine was present in the selective medium.

The parent fusion partner cell line PDH-1 resulted in only 2 unstable cell growths, both from the fifth pancreas. These clonal cells survived in culture for less than 2 months before dying. Use of RINm5f as the fusion partner cell line did produce 14 novel cell lines, all from the fourth pancreas. CRI-G5 generated 147 and 94 cell lines from the fourth and fifth pancreas respectively. As similar numbers of islet cells from the fourth and fifth pancreas were involved for both the CRI-G5 and RINm5f
fusions, the results are directly comparable, indicating that CRI-G5 was over fifteen fold more successful as the fusion partner than RINm5f.

In the fusions involving CRI-G5 in which glycerol was not used in the isolation buffer and neither HAT nor 16 μmol/l thymidine were present in the selective medium, some further comparisons could be made. The use of 5.55 mmol/l or 11.1 mmol/l glucose concentration in the selective media resulted in success rates of 9 and 33.143 novel cells / 3.75 x 10^7 fused islet cells, respectively. Within this 11.1 mmol/l glucose concentration subset, the presence of IBMX and glucagon, the batch of FCS and the type of PEG used did not present any obvious differences on the fusion success rate.

Novel cell lines were generated from fusions in which the CRI-G5 to islet cell ratio was solely 3:1. Although it was not known how this compared to the other ratios of 1:1 or 1:3, the ratio of 3:1 was the only confirmed success. Therefore only this ratio was validated.

4.3 Human insulin screen of cell lines from the fourth pancreas.

The screening for human insulin was performed using the developed 'parallel radioimmunoassay' as described in Section 3.4. Of the 161 novel cell lines established from the fourth pancreas, insulin secretion was not detected in 98 of these cell lines. All cell lines derived from the RINm5f fusion partner cell line were in this group. Insulin secretion levels were comparable to CRI-G5 in 34 cell lines, and 29 cell lines secreted insulin in excess of that seen in the CRI-G5 control (2.68 ng/well/2h). Most of the insulin secreted by the cell lines were determined to be rat insulin by 'the ratio of derived insulin values' plot. However, seven cell lines secreted insulin that did not match the rat insulin curve in this plot. It was assumed that human insulin was present in the culture medium of these cell lines, albeit with rat insulin also present. These cell lines were designated as HiCRI-D1, HiCRI-D2, HiCRI-D3, HiCRI-D4, HiCRI-D5, HiCRI-D6, HiCRI-D7, and are represented in Fig. 4.3, along with one very high secretor of insulin HiCRI-D8.
Fig. 4.3 Parallel RIA screening assay for human insulin secretion from novel cell lines from fourth pancreas.

Rat (■) and human (▲) insulin standard curves were assayed as well as culture medium from novel cell lines and the parent cell line CRI-G5 (▼). Media from each of the cell lines from a 2 hour static incubation in RPMI-1640 & 10% FCS, were assayed neat and diluted x2 in assay buffer, except for CRI-G5 which was run neat and diluted x2, x4, x8 and x16 in assay buffer. The assay method used was the developed 'parallel radioimmunoassay' as described in Section 3.4. The cell lines represented are HiCRI-D1 (●), HiCRI-D2 (●), HiCRI-D3 (▲), HiCRI-D4 (△), HiCRI-D5 (★), HiCRI-D6 (●), HiCRI-D7 (○), HiCRI-D8 (×), and were assayed after the 2nd passage. All points represent one sample measured in duplicate. The Coefficient of Variation was never greater than 6%. The ratio of derived insulin values was determined by the following formula:

\[
\text{Ratio (y-axis ordinate) = } \frac{A}{B} \times (x-axis ordinate) \\
A = \text{Insulin concentration using the anti-insulin primary antibody and rat insulin standards.} \\
B = \text{Insulin concentration using the anti-rat insulin primary antibody and rat insulin standard}
\]
4.3.1 Human insulin screen on cell lines from the fifth pancreas

The screening for human insulin was performed as for cell lines from the fourth pancreas, using the developed ‘parallel radioimmunoassay’ as described in Section 3.4. Although the 2 unstable clonal cells derived from the PDH-1 fusion partner cell line did not survive long enough for the human insulin screening assay, their culture medium was assessed. Neither samples contained detectable levels of insulin. Insulin secretion was not detected in 51 of the 94 novel cell lines established from the fifth pancreas. Insulin secretion levels were comparable to CRI-G5 in 23 cell lines, and 20 cell lines secreted insulin in excess of that seen in the CRI-G5 control (2.56 ng/well/2h). Six of the novel cell lines secreted insulin that did not match the rat insulin curve in the ‘ratio of derived insulin values’ plot. These cell lines were designated as HiCRI-E11, HiCRI-E12, HiCRI-E13, HiCRI-E14, HiCRI-E15, HiCRI-E16, and are represented in Fig. 4.3.1.
Fig. 4.3.1 Parallel RIA screening assay for human insulin secretion from novel cell lines from fifth pancreas.

Rat (■) and human (▲) insulin standard curves were assayed as well as culture medium from novel cell lines and the parent cell line CRI-G5 (▼). Media from each of the cell lines resulting from a 2 hour static incubation in RPMI-1640 & 10% FCS, were assayed neat and diluted x2 in assay buffer, except for CRI-G5 which was run neat and diluted x2, x4, x8 and x16 in assay buffer. The assay method used was the developed 'parallel radioimmunoassay' as described in Section 3.4. The cell lines represented are HiCRI-E11 (●), HiCRI-E12 (●), HiCRI-E13 (●), HiCRI-E14 (▲), HiCRI-E15 (●), HiCRI-E16 (●), and were assayed after the 2nd passage. All points represent one sample measured in duplicate. The Coefficient of Variation was never greater than 7%. Plot as for Figure 4.3.
4.3.2 Human insulin screening assay of subcloned cell lines.
Subclones were seen to secrete insulin which matched the rat insulin control in terms of ‘the ratio of derived insulin values’. Some subclones, however, secreted insulin which gave similar results to those of the original cell lines (Figs. 4.3 & 4.3.1), in that the insulin did not match the rat insulin controls. Of the 14 cell lines subcloned, one subclone of each was selected. Each subclone was selected due to its secretion of an insulin which did not match the rat insulin controls, nor the parental CRI-G5 control. The subclone of HiCRI-D8 was selected for its continued high secretion of insulin and referred to as HiCRI-D8c. This terminal ‘c’ supplement was applied to all the designations to differentiate the subclones from the original cell lines, (clonal cell growths).

4.3.3 External measurement of human insulin.
External assessment by an immuno-assay specific for human insulin, with no measurable cross-reactivity to rat insulins, was performed on culture samples from HiCRI-D1c, HiCRI-D3c, HiCRI-E12c, HiCRI-E13c and HiCRI-E16c. All these cell lines tested, at passage 24, were chosen as they continually exhibited a high ‘ratio of determined insulin values’. The external assessment found them to be negative for the presence of human insulin. Hence the high ‘ratio of determined insulin values’ could not be attributed to the co-presence of a small amount of human insulin with larger amounts of rat insulin. As to what then was the cause of the high ‘ratio of determined insulin values’, it was a possibility that this was due to a difference in the immunoreactive profile of secretory products of these cells lines versus that of the parental CRI-G5 cell line.

4.3.4 HPLC analysis of insulin-like immunoreactive profile secreted from novel cell lines.
The rat insulin standard of 100 ng/ml introduced 10 ng of insulin to the column but no peak other than the BSA peak was detected at optical wavelengths of 210 nm or 276 nm, using the HPLC method described in Section 2.7.8. This standard was approximately three times the highest sample concentration, which was also applied to the column and failed to register any peaks other than the BSA. The control
sample of the static incubation buffer confirmed that the sole peak was due to BSA. The HPLC analysis was repeated externally using the same set up but with different equipment and column. The sample analysed was 100 ng/ml rat insulin standard prepared in static incubation buffer but with no BSA present. The failure to detect any insulin peaks at the optical wavelengths of either 210 nm or 276 nm was confirmed. As no insulin peaks were detected no results are shown.

4.4 Insulin release in response to glucose.

All novel cell lines were assayed at passage 19, with the exceptions of HiCRI-D3c-p57 (passage 57) and HiCRI-D4c-p66 (passage 66). The methods used are described in detail in Sections 2.7-2.7.5. Cell lines were plated at a constant cell concentration between 4-6 x 10^4 cells/well and cultured for 48 h in 5% CO_2 / 95% air at 37°C in both RPMI-1640 & 10% FCS and RPMI-1640 & 10% FCS containing 10 mmol/l nicotinamide. Cells were then washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.5% BSA (KRBB) and pre-incubated in 0.5 ml of KRBB (pre-equilibrated in 5% CO_2 / 95% air at 37°C) for one hour in 5% CO_2 at 37°C. Cells were then washed twice in KRBB before static incubation was performed for two hours in 5% CO_2 at 37°C. The static incubation buffer was KRBB (pre-equilibrated in 5% CO_2 / 95% air at 37°C) containing either: 1, 2, 4, 8, 16, 32 mmol/l glucose, with or without 10 mmol/l nicotinamide. The glucose concentrations containing nicotinamide were only applied to those cells which had been cultured with nicotinamide. After the static incubation the culture buffer from all cells was collected, centrifuged 200g for 5 min and the top 700 μl taken off for insulin RIA measurement. Triplicate sample volumes of 50 μl were assayed, using the Phadeseph insulin RIA kit and protocol, but in all cases the kit primary antibody was replaced with the Biogenesis anti-rat insulin primary antibody and the assay was calibrated against rat insulin standards. The cells were washed extensively in PBS pH 7.4, and allowed to air dry before 0.25 ml of 0.1 mol/l NaOH was applied to each well and left at 4°C for 4 hours. Protein content determination was then performed on the resultant cell lysates using a Coomassie protein assay against a standard of BSA prepared in 0.1 mol/l NaOH.
The effects of various glucose concentrations on the insulin release from the novel cell lines are displayed in Figures 4.4.1 to 4.4.14. The parental fusion partner CRI-G5 was confirmed as being unresponsive to glucose and this was not improved by the presence of nicotinamide (Fig. 4.4). The results of the cell lines HiCRI-D3c, HiCRI-D3c-p57, HiCRI-D4c-p66, HiCRI-D5c, HiCRI-D6c, HiCRI-D8c, HiCRI-E12c and HiCRI-E15c are restricted to their respective Figures.

HiCRI-D1c: (Fig. 4.4.1)
Although only the neighbouring glucose concentrations of 4 v 8 mmol/l showed significant glucose-stimulated insulin secretion (GSIS), the increase in insulin secretion was consistent between the concentrations of 1 - 8 mmol/l, and gave an overall 1.7-fold increase (P <0.001, by Anova-Tukey).

HiCRI-D2c: (Fig. 4.4.2)
Although only the neighbouring glucose concentrations of 4 v 8 mmol/l glucose concentrations showed significant GSIS, the increase in insulin secretion was consistent between the concentrations of 4 - 16 mmol/l, and gave an overall 1.44-fold increase (P <0.001, by Anova-Tukey).

HiCRI-D4c: (Fig. 4.4.4)
Glucose concentration displayed a consistent negative effect on insulin secretion, with an overall 2-fold decrease between 1 - 32 mmol/l (P <0.001, by Anova-Tukey).

HiCRI-D7c: (Fig. 4.4.7)
The increase in insulin secretion was consistent between the glucose concentrations of 1 - 16 mmol/l, and gave an overall 1.35-fold increase (P <0.001, by Anova-Tukey).

HiCRI-E11c: (Fig. 4.4.9)
GSIS was seen to be significant between 2 v 4 and 4 v 8 mmol/l glucose concentrations, with an overall 1.81-fold increase between 2 - 8 mmol/l (P <0.001, by Anova-Tukey).
HiCRI-E13c: (Fig. 4.4.11)

In the presence of nicotinamide, this cell line displayed a 1.68-fold decrease between 1 - 16 mmol/l glucose concentrations (P <0.001, by Anova-Tukey).

HiCRI-E14c: (Fig. 4.4.12)

This cell line displayed a consistent increase in insulin secretion between 1 - 4 mmol/l glucose concentrations, with an overall 1.43-fold increase (P <0.001, by Anova-Tukey).

HiCRI-E16c: (Fig. 4.4.14)

Although no significant GSIS was seen between neighbouring glucose concentrations there was a steady increase, resulting in a 1.75-fold increase between 1 - 8 mmol/l, when nicotinamide was present (p <0.001, by Anova-Tukey).
Fig. 4.4 Insulin secretion of CRI-G5 parent cell line in response to glucose.

The effect of the indicated glucose concentrations on insulin secretion was measured on CRI-G5 which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-D1c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-D2c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
Fig. 4.4.3 Insulin secretion of HiCRI-D3c cell line in response to glucose.

The effect of the indicated glucose concentrations on insulin secretion was measured on (A) HiCRI-D3c (passage 19) and (B) HiCRI-D3c-p57 (passage 57). Where nicotinamide had been present during culture, it was also present at 10 mmol/l in the static incubation buffer (▲), but was absent for normally cultured cells (■). Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on (A) HiCRI-D4c and (B) HiCRI-D4c-p66. Where nicotinamide had been present during culture, it was also present at 10 mmol/l in the static incubation buffer (▲), but was absent for normally cultured cells (■). Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-D5c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
Fig. 4.4.6 Insulin secretion of HiCRI-D6c cell line in response to glucose.

The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-D6c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-D7c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-D8c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
Fig. 4.4.9 Insulin secretion of HiCRI-E11c cell line in response to glucose.

The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-E11c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-E12c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-E13c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-E14c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-E15c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
The effect of the indicated glucose concentrations on insulin secretion was measured on HiCRI-E16c (passage 19), which had been cultured normally (■) or in the presence of 10 mmol/l nicotinamide (▲). Where nicotinamide had been present during culture, it was also present in the static incubation buffer. Static incubation was for 2 h in KRBB & glucose at 37°C in 5% CO₂ following a pre-incubation of 1 h in KRBB at 37°C in 5% CO₂. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Bonferroni for selected pairs of columns. The mean value from each glucose concentration was selected for testing against the mean value from the immediately lower glucose concentration. Significance is displayed beside the value from the higher glucose concentration.
4.5 Insulin secretion levels of the cell lines.

The data herein is taken directly from the previous ‘insulin release in response to glucose’ assay (Section 4.4). As such the results are for novel cell lines at passage 19, with the exceptions of HiCRI-D3c-p57 (passage 57) and HiCRI-D4c-p66 (passage 66). Displayed in Figure 4.5 are the insulin secretion levels of the novel cell lines from the 2 h static incubation in 5% CO₂ at 37°C in HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.5% BSA (KRBB) and 16 mmol/l glucose. This buffer and the preincubation buffer of KRBB containing no glucose were both pre-equilibrated in 5% CO₂/95% air at 37°C. The pre-incubation step was for 1 h in 5% CO₂ at 37°C. Results were derived, using the Phadeseph insulin RIA kit and protocol, but in all cases the kit primary antibody was replaced with the Biogenesis anti-rat insulin primary antibody and the assay was calibrated against rat insulin standards. Cells were disrupted using 0.1 mol/l NaOH and the cellular protein content of each culture well was determined using a Coomassie protein assay against a standard of BSA prepared in 0.1 mol/l NaOH.

Three of the cell lines showed no significant difference to the parental CRI-G5 when determined by ANOVA with the post-test of Dunnett. When these were compared to CRI-G5 using the Student’s unpaired t-test, all were significantly different (P <0.0001). The range of insulin secretion levels was from half to 53.9-fold that of CRI-G5. Values displayed were derived from the anti-rat insulin RIA, using the values from the Phadeseph based RIA the top value was 72.8-fold that seen with CRI-G5.

The effects of ‘ageing’ were apparent in the HiCRI-D4c-p66 cell line which secreted only 16% of that secreted by the earlier passage HiCRI-D4c cell line. No decrease in insulin secretion was seen between HiCRI-D3c and HiCRI-D3c-p57.
Fig 4.5 Comparison of the insulin secretion of the novel cell lines to the parental CRI-G5, at a glucose concentration of 16 mmol/l.

The insulin secretion values at 16 mmol/l glucose concentration are taken from the previous response to glucose concentrations curves (Figs. 4.4 - 4.4.14). Static incubation was for 2 h in pre-equilibrated KRBB & 16 mmol/l glucose at 37°C in 5% CO₂ following a pre-incubation of 1h in KRBB at 37°C in 5% CO₂. The cell lines are displayed as (A) HiCRI-D1c, (B) HiCRI-D2c, (C) HiCRI-D4c, (D) HiCRI-D4c-p66, (E) HiCRI-D6c, (F) HiCRI-E11c, (G) HiCRI-E12c, (H) HiCRI-E14c, (J) HiCRI-D3c, (K) HiCRI-D3c-p57, (L) HiCRI-D5c, (M) HiCRI-D7c, (N) HiCRI-D8c, (P) HiCRI-E13c, (Q) HiCRI-E15c, (R) HiCRI-E16c. All were assayed at passage 19, with the exceptions of HiCRI-D3c-p57 (passage 57) and HiCRI-D4c-p66 (passage 66). Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are means ± SEM (n = 6). * P < 0.05, ** P < 0.01, *** P < 0.001 determined by ANOVA with the post test of Dunnett, with CRI-G5 as the control.
4.6 Insulin content of the cell lines.

All novel cell lines were assayed at passage 19, with the exceptions of HiCRI-D3c-p57 (passage 57) and HiCRI-D4c-p66 (passage 66). The methods used are described in detail in Sections 2.7.1 and 2.7.4-2.7.5. Cell lines were plated at a constant cell concentration between $4-6 \times 10^4$ cells/well over twelve culture plate wells and cultured for 48 h in 5% CO$_2$/95% air at 37°C in RPMI-1640 & 10% FCS. Cells were then washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.5% BSA (KRBB) and pre-incubated in 0.5 ml of KRBB (pre-equilibrated in 5% CO$_2$/95% air at 37°C) for one hour in 5% CO$_2$ at 37°C. Cells were then washed twice in KRBB, and then washed extensively in PBS pH 7.4 and allowed to air dry. Having given care to the symmetrical placement of each set of 12 culture wells, these were then divided into two sets of 6 culture wells, for each cell line. To one set, 0.25 ml of 0.1 mol/l NaOH was applied to each well and left at 4°C for 4 hours. Protein content determination was then performed on the resultant cell lysates using a Coomassie protein assay against a standard of BSA prepared in 0.1 mol/l NaOH. The other set received in each well 500 µl of acidified ethanol. These wells were kept in air tight conditions overnight at 4°C, followed by one week at -70°C. The acidified ethanol solution was then removed vigourously from each well, centrifuged 1000g for 5 min at 4°C, and 50 µl samples were aliquoted in triplicate and freeze-dried for later insulin measurement by RIA. For the RIA 50 µl RIA assay buffer was added to the freeze-dried aliquots and left for four hours at room temperature and finally vortexed. Insulin measurement was performed using the Phadeseph insulin RIA kit and protocol, but in all cases the kit primary antibody was replaced with the Biogenesis anti-rat insulin primary antibody and the assay was calibrated against rat insulin standards.

Due to the deliberately similar culture and incubation conditions the insulin content of these cells should approximate the content of their respective cells used in the static incubation study, at commencement of the 2 h static incubation. The insulin content of the cell lines are displayed in Table 4.6.
The effects of ‘ageing’ were less apparent on the insulin content as HiCRI-D4c-p66 contained approximately 75% of that of the earlier passage HiCRI-D4c.

Table 4.6 Insulin content of each of the cell lines after a 1h preincubation in KRBB, as a function of cellular protein.

Data is represented as mean ± SEM (n = 6). Also shown is the estimated percentage of the total insulin released during 2h static incubation in KRBB & 16 mmol/l glucose.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Insulin Content (pmol/mg protein)</th>
<th>Percentage of Insulin Content released in 2h incubation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRI-G5</td>
<td>15.04 ± 3.22</td>
<td>28.97 ± 1.34</td>
</tr>
<tr>
<td>HiCRI-D1c</td>
<td>28.29 ± 3.45</td>
<td>7.42 ± 0.13</td>
</tr>
<tr>
<td>HiCRI-D2c</td>
<td>397.6 ± 71.35</td>
<td>7.42 ± 0.13</td>
</tr>
<tr>
<td>HiCRI-D3c</td>
<td>186.35 ± 31.68</td>
<td>37.39 ± 1.50</td>
</tr>
<tr>
<td>HiCRI-D3c-p57</td>
<td>256.03 ± 24.70</td>
<td>28.51 ± 1.17</td>
</tr>
<tr>
<td>HiCRI-D4c</td>
<td>319.39 ± 42.16</td>
<td>28.12 ± 0.51</td>
</tr>
<tr>
<td>HiCRI-D4c-p66</td>
<td>240.06 ± 26.82</td>
<td>5.99 ± 0.23</td>
</tr>
<tr>
<td>HiCRI-D5c</td>
<td>442.78 ± 65.66</td>
<td>29.30 ± 0.91</td>
</tr>
<tr>
<td>HiCRI-D6c</td>
<td>270.23 ± 33.57</td>
<td>20.55 ± 0.64</td>
</tr>
<tr>
<td>HiCRI-D7c</td>
<td>1007.89 ± 24.77</td>
<td>15.29 ± 0.32</td>
</tr>
<tr>
<td>HiCRI-D8c</td>
<td>1008.92 ± 56.35</td>
<td>23.02 ± 0.38</td>
</tr>
<tr>
<td>HiCRI-E11c</td>
<td>95.65 ± 11.24</td>
<td>17.67 ± 0.36</td>
</tr>
<tr>
<td>HiCRI-E12c</td>
<td>150.90 ± 22.47</td>
<td>27.30 ± 1.69</td>
</tr>
<tr>
<td>HiCRI-E13c</td>
<td>504.8 ± 32.56</td>
<td>28.74 ± 0.67</td>
</tr>
<tr>
<td>HiCRI-E14c</td>
<td>274.78 ± 43.57</td>
<td>24.36 ± 0.40</td>
</tr>
<tr>
<td>HiCRI-E15c</td>
<td>497.84 ± 73.25</td>
<td>33.91 ± 2.06</td>
</tr>
<tr>
<td>HiCRI-E16c</td>
<td>1108.75 ± 125.81</td>
<td>21.19 ± 0.68</td>
</tr>
</tbody>
</table>
4.7 Insulin release in response to secretagogues.

This analysis was performed in parallel to the static incubation in various glucose concentrations, the results of which are presented in Section 4.4. As such all novel cell lines were assayed at passage 19, with the exceptions of HiCRI-D3c-p57 (passage 57) and HiCRI-D4c-p66 (passage 66). The methods used are described in detail in Sections 2.7-2.7.5. Cell lines were plated at a constant cell concentration between $4 \times 10^4$ cells /well and cultured for 48 h in 5% CO$_2$ / 95% air at 37°C in RPMI-1640 & 10% FCS. Cells were then washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.5% BSA (KRBB) and pre-incubated in 0.5 ml of KRBB (pre-equilibrated in 5% CO$_2$ / 95% air at 37°C) for one hour in 5% CO$_2$ at 37°C. Cells were then washed twice in KRBB before static incubation was performed for two hours in 5% CO$_2$ at 37°C. The static incubation buffer was KRBB (pre-equilibrated in 5% CO$_2$ / 95% air at 37°C) containing either 2 or 16 mmol/l glucose containing either 20 mmol/l KCl, 1 μg/ml glucagon, and together and separately 0.5 mmol/l 3-isobutyl-1-methylxanthine (IBMX) and 8 mmol/l glutamine. After the static incubation the culture buffer from all cells was assessed for insulin using the Phadeseph insulin RIA kit and protocol, but in all cases the kit primary antibody was replaced with the Biogenesis anti-rat insulin primary antibody and the assay was calibrated against rat insulin standards. Cellular protein content of each culture well was performed by cellular disruption with 0.1 mol/l NaOH followed by analysis using a Coomassie protein assay.

The insulin release in response to the various secretagogues is displayed in Tables 4.7 and 4.7.1. The responses of the different cell lines were seen to vary from being closely analogous, to being very different to the responses seen with CRI-G5. The responses of the cell line HiCRI-E11c were particularly different from both CRI-G5 and the other cell lines.
Table 4.7 The effects of glutamine, IBMX, and glutamine & IBMX on the insulin release of the novel cell lines. Results are from a static incubation in KRBB & glucose & secretagogue of 2 h in 5% CO<sub>2</sub> at 37°C following a pre-incubation of 1h in KRBB in 5% CO<sub>2</sub> at 37°C. Data is presented as the fold increase in insulin release compared to that seen in KRBB & 2 mmol/l glucose, (n = 6) ± SEM.

<table>
<thead>
<tr>
<th>Modulator: Glucose conc. (mmol/l)</th>
<th>none</th>
<th>Glutamine (8 mmol/l)</th>
<th>IBMX (0.5 mmol/l)</th>
<th>Glutamine + IBMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRI-G5</td>
<td>1.01 ± 0.05</td>
<td>1.84 ± 0.15**</td>
<td>1.09 ± 0.04</td>
<td>1.32 ± 0.07</td>
</tr>
<tr>
<td>HiCRI-D1c</td>
<td>1.18 ± 0.02</td>
<td>1.50 ± 0.12**</td>
<td>1.47 ± 0.10**</td>
<td>1.37 ± 0.01*</td>
</tr>
<tr>
<td>HiCRI-D2c</td>
<td>1.44 ± 0.05</td>
<td>3.26 ± 0.13**</td>
<td>2.63 ± 0.12**</td>
<td>2.77 ± 0.05**</td>
</tr>
<tr>
<td>HiCRI-D3c</td>
<td>0.75 ± 0.03*</td>
<td>1.31 ± 0.05**</td>
<td>1.56 ± 0.03</td>
<td>1.49 ± 0.05**</td>
</tr>
<tr>
<td>HiCRI-D3c-p57</td>
<td>1.04 ± 0.04</td>
<td>1.71 ± 0.08**</td>
<td>1.78 ± 0.08**</td>
<td>2.14 ± 0.06**</td>
</tr>
<tr>
<td>HiCRI-D4c</td>
<td>0.73 ± 0.01</td>
<td>1.68 ± 0.13**</td>
<td>1.66 ± 0.10</td>
<td>1.18 ± 0.07</td>
</tr>
<tr>
<td>HiCRI-D4c-p66</td>
<td>0.60 ± 0.02</td>
<td>2.72 ± 0.11**</td>
<td>2.80 ± 0.06**</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td>HiCRI-D5c</td>
<td>1.09 ± 0.03</td>
<td>3.31 ± 0.12**</td>
<td>2.85 ± 0.03**</td>
<td>1.52 ± 0.03**</td>
</tr>
<tr>
<td>HiCRI-D6c</td>
<td>0.89 ± 0.03</td>
<td>2.36 ± 0.12**</td>
<td>2.11 ± 0.11**</td>
<td>2.05 ± 0.11**</td>
</tr>
<tr>
<td>HiCRI-D7c</td>
<td>1.16 ± 0.02</td>
<td>1.91 ± 0.08**</td>
<td>1.43 ± 0.06</td>
<td>2.23 ± 0.07**</td>
</tr>
<tr>
<td>HiCRI-D8c</td>
<td>0.68 ± 0.01</td>
<td>1.02 ± 0.04</td>
<td>0.95 ± 0.05</td>
<td>1.21 ± 0.06*</td>
</tr>
<tr>
<td>HiCRI-E11c</td>
<td>1.85 ± 0.04</td>
<td>6.25 ± 0.24**</td>
<td>5.84 ± 0.16**</td>
<td>4.00 ± 0.17**</td>
</tr>
<tr>
<td>HiCRI-E12c</td>
<td>1.02 ± 0.06</td>
<td>3.25 ± 0.12**</td>
<td>3.71 ± 0.16**</td>
<td>2.12 ± 0.06</td>
</tr>
<tr>
<td>HiCRI-E13c</td>
<td>1.14 ± 0.03</td>
<td>1.31 ± 0.06</td>
<td>0.89 ± 0.03</td>
<td>1.11 ± 0.07</td>
</tr>
<tr>
<td>HiCRI-E14c</td>
<td>1.27 ± 0.02</td>
<td>2.55 ± 0.12**</td>
<td>2.68 ± 0.05**</td>
<td>2.59 ± 0.04**</td>
</tr>
<tr>
<td>HiCRI-E15c</td>
<td>1.07 ± 0.07</td>
<td>2.02 ± 0.07**</td>
<td>1.72 ± 0.02**</td>
<td>1.66 ± 0.06**</td>
</tr>
<tr>
<td>HiCRI-E16c</td>
<td>1.14 ± 0.04</td>
<td>1.63 ± 0.05</td>
<td>1.31 ± 0.08</td>
<td>1.67 ± 0.09**</td>
</tr>
</tbody>
</table>

Statistical significance was calculated by ANOVA with the post test of Dunnett, where the insulin secretion value seen in KRBB & 2 mmol/l glucose concentration was the control, * P < 0.05, ** P < 0.01.
Table 4.7.1 The effects of KCl and glucagon on the insulin release of the novel cell lines. Results are from a static incubation in KRBB & glucose & secretagogue of 2 h in 5% CO₂ at 37°C following a pre-incubation of 1h in KRBB in 5% CO₂ at 37°C. Data is presented as the fold increase in insulin release compared to that seen in KRBB & 2 mmol/l glucose, (n = 6) ± SEM.

<table>
<thead>
<tr>
<th>Modulator: Glucose conc. (mmol/l):</th>
<th>Cell Line</th>
<th>None</th>
<th>KCl (20 mmol/l)</th>
<th>Glucagon (1 μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>CRI-G5</td>
<td>1.01 ± 0.05</td>
<td>2.12 ± 0.15**</td>
<td>1.64 ± 0.10**</td>
<td>1.10 ± 0.07</td>
</tr>
<tr>
<td>HiCRI-D1c</td>
<td>1.18 ± 0.02</td>
<td>1.59 ± 0.09**</td>
<td>1.10 ± 0.20</td>
<td>0.79 ± 0.05</td>
</tr>
<tr>
<td>HiCRI-D2c</td>
<td>1.44 ± 0.05</td>
<td>2.42 ± 0.15**</td>
<td>2.28 ± 0.19**</td>
<td>1.53 ± 0.04</td>
</tr>
<tr>
<td>HiCRI-D3c</td>
<td>0.75 ± 0.03*</td>
<td>1.34 ± 0.07**</td>
<td>1.62 ± 0.03**</td>
<td>1.03 ± 0.04</td>
</tr>
<tr>
<td>HiCRI-D3c-p57</td>
<td>1.04 ± 0.04</td>
<td>1.65 ± 0.19**</td>
<td>2.00 ± 0.11**</td>
<td>1.43 ± 0.06*</td>
</tr>
<tr>
<td>HiCRI-D4c</td>
<td>0.73 ± 0.01</td>
<td>1.18 ± 0.03</td>
<td>1.12 ± 0.01</td>
<td>0.61 ± 0.06**</td>
</tr>
<tr>
<td>HiCRI-D4c-p66</td>
<td>0.60 ± 0.02</td>
<td>1.81 ± 0.09**</td>
<td>1.47 ± 0.03</td>
<td>1.72 ± 0.19**</td>
</tr>
<tr>
<td>HiCRI-D5c</td>
<td>1.09 ± 0.03</td>
<td>1.21 ± 0.02</td>
<td>1.22 ± 0.05</td>
<td>0.97 ± 0.01</td>
</tr>
<tr>
<td>HiCRI-D6c</td>
<td>0.89 ± 0.03</td>
<td>1.45 ± 0.02</td>
<td>1.33 ± 0.09</td>
<td>1.03 ± 0.03</td>
</tr>
<tr>
<td>HiCRI-D7c</td>
<td>1.16 ± 0.02</td>
<td>1.35 ± 0.04*</td>
<td>1.58 ± 0.13**</td>
<td>1.22 ± 0.12</td>
</tr>
<tr>
<td>HiCRI-D8c</td>
<td>0.68 ± 0.01**</td>
<td>0.88 ± 0.03</td>
<td>1.16 ± 0.05</td>
<td>0.83 ± 0.01</td>
</tr>
<tr>
<td>HiCRI-E11c</td>
<td>1.85 ± 0.04*</td>
<td>5.21 ± 0.24**</td>
<td>5.01 ± 0.16**</td>
<td>1.75 ± 0.04</td>
</tr>
<tr>
<td>HiCRI-E12c</td>
<td>1.02 ± 0.06</td>
<td>3.25 ± 0.07**</td>
<td>2.95 ± 0.04*</td>
<td>1.33 ± 0.02</td>
</tr>
<tr>
<td>HiCRI-E13c</td>
<td>1.14 ± 0.03</td>
<td>1.14 ± 0.08</td>
<td>1.07 ± 0.02</td>
<td>0.87 ± 0.07</td>
</tr>
<tr>
<td>HiCRI-E14c</td>
<td>1.27 ± 0.02</td>
<td>1.99 ± 0.03**</td>
<td>1.59 ± 0.12**</td>
<td>1.28 ± 0.03</td>
</tr>
<tr>
<td>HiCRI-E15c</td>
<td>1.07 ± 0.07</td>
<td>1.93 ± 0.17**</td>
<td>1.72 ± 0.04**</td>
<td>0.97 ± 0.02</td>
</tr>
<tr>
<td>HiCRI-E16c</td>
<td>1.14 ± 0.04</td>
<td>1.41 ± 0.04*</td>
<td>1.26 ± 0.09</td>
<td>1.22 ± 0.09</td>
</tr>
</tbody>
</table>

Statistical significance was calculated by ANOVA with the post test of Dunnett, where the insulin secretion value seen in KRBB & 2 mmol/l glucose concentration was the control, * P < 0.05, ** P < 0.01.
The least effective secretagogue was glucagon, but IBMX displayed large stimulatory effects on the insulin secretion. Although they act through different mechanisms, both glucagon and IBMX raise cellular levels of cAMP, which potentiates glucose induced insulin secretion in islet cells (Henquin and Meissner, 1984; Praz et al. 1983). While the potentiation of glucose action was generally not apparent in the cell lines, the cAMP levels in the cell lines were apparently more susceptible to the inhibition of cyclic nucleotide phosphodiesterases by IBMX than the hormonal action of glucagon.

It is well established that islet cells are extremely sensitive to changes in their cationic environment (Wollheim and Sharp, 1981). RINm5F cells have exhibited over a 4-fold increase in insulin secretion in response to depolarization of the plasma membrane by 15 mmol/l K⁺ (Praz et al. 1983). Similar responses were seen with the cell lines HiCRI-E11c and HiCRI-E12c, but were absent from HiCRI-D8c. Hence the cell lines did present a varying degree of responsiveness to K⁺ induced membrane depolarization. It would be perhaps hasty to assume, as discussed in Section 1.16.2, that this is an indication of the presence of functional Kᵦₓ ATP channels in HiCRI-E11c and HiCRI-E12c, but it certainly can not be ruled out (Praz et al. 1983; Macfarlane et al. 1999).

Glutamine was the most potent secretagogue tested, though its effects were potentiated by the co-presence of IBMX, as was also reported for the IgSV195 cell line (Gilligan et al. 1989). The responses to glutamine would indicate that some of the cell lines express the normal islet cell capacity to increase insulin secretion in response to the generation of metabolic flux. It was unexpected that glutamine did not potentiate the insulin secretion in response to increasing glucose concentrations. The relative potency of glutamine, as compared to glucose, may point to a fault in the normally predominant stimulation of insulin secretion due to the presence and metabolism of glucose. This possibility could have been explored further by the use of other initiators of insulin secretion. Indeed it was perhaps negligent not to have also used intermediates of glucose metabolism and substrates of the other metabolic pathways in β cells. These could have included the L-leucine, L-alanine, D-mannose,
fructose, pyruvate, D-glyceraldehyde and 2-ketoisocaproate. As was discussed in Section 1.4 it has been demonstrated in vitro that leucine and arginine are effective insulin secretagogues in the absence of glucose, and therefore qualify as initiators of insulin secretion, and certain intermediates of glucose metabolism, for example D-glyceraldehyde, have been demonstrated to induce insulin secretion (Ashcroft et al. 1978).

Many secretion studies on β cell lines have been performed not in KRBB, but in RPMI-1640 or Dulbecco’s Modified Eagle’s Medium, which are routinely supplemented with glutamine (Hamaguchi et al. 1991; Efrat et al. 1988; Miyazaki et al. 1990). Although these studies were primarily concerned with the relative increase in insulin secretion due to glucose, the presence of glutamine must be considered with respect to insulin secretion levels. In RPMI-1640 containing glutamine and a glucose concentration of 11.1 mmol/l, the HiCRI-E16c cell line secreted insulin in excess of 60 ng/10⁵ cells/h. This high level of insulin secretion is surpassed only by the NIT-1 cell line, with a reported secretion in culture medium of 103 ng/10⁵ cells/h (Hamaguchi et al. 1991).

4.8 Glucagon release of cell lines.

The static incubation of the novel cell lines in varying concentrations of glucose is described in Section 2.7.3 and the insulin secretion measurements in response to the glucose are given in Section 4.4. The static incubation samples of KRBB containing 16 mmol/l glucose were analysed not only for insulin but also for glucagon. 400 μl aliquots from the static incubation culture samples containing 16 mmol/l glucose were pooled for each cell line. The N-terminal glucagon measurement was performed externally at the Dept. of Medicine, Royal Victoria Hospital, Belfast. The assay was a RIA calibrated against porcine glucagon. All novel cell lines were assayed at passage 19, with the exceptions of HiCRI-D3c-p57 (passage 57) and HiCRI-D4c-p66 (passage 66). Cells were then washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.5% BSA (KRBB) and pre-incubated in 0.5 ml of KRBB (pre-equilibrated in 5% CO₂ / 95% air at 37°C) for one hour in 5% CO₂ at 37°C. Cells were then washed twice in KRBB before static incubation
was performed for two hours in 5% CO$_2$ at 37°C. The static incubation buffer was KRBB (pre-equilibrated in 5% CO$_2$ / 95% air at 37°C) containing 16 mmol/l glucose. The levels of glucagon released by the cell lines is displayed in Table 4.8. The cellular protein concentration and secreted insulin values were both estimated by averaging the values of each of these produced by the six culture wells which were pooled to derive the sample for glucagon measurement.
Table 4.8 Glucagon secretion by the novel cell lines.

Glucagon secretion was measured following a static incubation for 2 h in KRBB, preceded by a pre-incubation of 1h. The assay was for N-terminal glucagon. Data are expressed per mg cellular protein, extracted with 0.1 mol/l NaOH. Values are single measurements (n = 1). Also shown is the estimated molar ratio of insulin to glucagon in the same samples (16 mmol/l glucose in KRBB), ± SEM.

<table>
<thead>
<tr>
<th>Glucagon Release (pmol/2h/ mg protein)</th>
<th>Molar ratio of secreted hormones: (insulin/glucagon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRI-G5</td>
<td>0.778 5.6 ± 0.3</td>
</tr>
<tr>
<td>HiCRI-D1c</td>
<td>0.105 20.2 ± 0.3</td>
</tr>
<tr>
<td>HiCRI-D2c</td>
<td>2.947 34.7 ± 1.3</td>
</tr>
<tr>
<td>HiCRI-D3c</td>
<td>0.484 144.0 ± 5.8</td>
</tr>
<tr>
<td>HiCRI-D3c-p57</td>
<td>0.285 256.1 ± 10.5</td>
</tr>
<tr>
<td>HiCRI-D4c</td>
<td>0.708 126.9 ± 2.3</td>
</tr>
<tr>
<td>HiCRI-D4c-p66</td>
<td>0.240 59.9 ± 2.2</td>
</tr>
<tr>
<td>HiCRI-D5c</td>
<td>1.622 79.7 ± 2.5</td>
</tr>
<tr>
<td>HiCRI-D6c</td>
<td>0.511 108.6 ± 3.4</td>
</tr>
<tr>
<td>HiCRI-D7c</td>
<td>0.525 293.3 ± 6.1</td>
</tr>
<tr>
<td>HiCRI-D8c</td>
<td>1.222 190.4 ± 3.1</td>
</tr>
<tr>
<td>HiCRI-E11c</td>
<td>1.509 11.2 ± 0.2</td>
</tr>
<tr>
<td>HiCRI-E12c</td>
<td>1.931 21.3 ± 1.3</td>
</tr>
<tr>
<td>HiCRI-E13c</td>
<td>1.619 89.6 ± 2.1</td>
</tr>
<tr>
<td>HiCRI-E14c</td>
<td>0.598 112.0 ± 1.8</td>
</tr>
<tr>
<td>HiCRI-E15c</td>
<td>1.759 96.1 ± 5.8</td>
</tr>
<tr>
<td>HiCRI-E16c</td>
<td>0.565 416.7 ± 13.3</td>
</tr>
</tbody>
</table>
4.9 Morphologic observations.

Phase-contrast photomicrographs of the cell lines growing in culture are displayed in Figures 4.9.1 to 4.9.7. Also shown is parental CRI-G5 in Figure 4.9.

All the novel cell lines were photographed at passage 20, in 75 cm\(^2\) culture flasks through a phase contrast microscope (Olympus Optical Company, Tokyo, Japan) at x20 lens magnification. For the photography the culture medium was replaced with RPMI-1640.

All the cell lines grew as monolayers, and tightly adhered to culture plastic and neighbouring cells. Most of the novel cell lines appeared morphologically similar to CRI-G5, in that they were fibroblast-like in character. Notable exceptions were:

- HiCRI-D1c (Fig. 4.9.1). These cells were visibly smaller than CRI-G5 cells and in monolayer culture exhibited a 'cobble-stone' pattern more typical of epithelial cell lines.
- HiCRI-D2c (Fig. 4.9.1). These cells appeared frequently as very large and plump, though still maintaining their fibroblast-like character.
- HiCRI-D6c (Fig. 4.9.3). Although, not apparently smaller than CRI-G5, these cells exhibited a 'cobble-stone' pattern in monolayer culture.
- HiCRI-D7c (Fig. 4.9.4). Subculture of this cell line often resulted in foci which, after more than four days in culture tended to form multilayer growth.
- HiCRI-E11c & HiCRI-E12c (Fig. 4.9.5). The cells of both these cell lines appeared to be larger than CRI-G5 cells.
Fig. 4.9 Phase-contrast photomicrograph of CRI-G5 growing in culture. Lens magnification x20.
Fig. 4.9.1 Phase-contrast photomicrograph of HiCRI-D1c (A) and HiCRI-D2c (B) growing in culture. Lens magnification x20.
Fig. 4.9.2 Phase-contrast photomicrograph of HiCRI-D3c (A) and HiCRI-D4c (B) growing in culture. Lens magnification x20.
Fig. 4.9.3 Phase-contrast photomicrograph of HiCRI-D5c (A) and HiCRI-D6c (B) growing in culture. Lens magnification x20.
Fig. 4.9.4 Phase-contrast photomicrograph of HiCRI-D7c (A) and HiCRI-D8c (B) growing in culture. Lens magnification x20.
Fig. 4.9.5 Phase-contrast photomicrograph of HiCRI-E11c (A) and HiCRI-E12c (B) growing in culture. Lens magnification x20.
Fig. 4.9.6 Phase-contrast photomicrograph of HiCRI-E13c (A) and HiCRI-E14c (B) growing in culture. Lens magnification x20.
Fig. 4.9.7 Phase-contrast photomicrograph of HiCRI-E15c (A) and HiCRI-E16c (B) growing in culture. Lens magnification x20.
4.10 Cell line growth rate.

The daily cell counts and population doubling time of each cell line are shown in Table 4.10.

All novel cell lines were at passage 20, with the exceptions of HiCRI-D3c-p57 (passage 58) and HiCRI-D4c-p66 (passage 67). Precise volumes were used to inoculate five 25 cm² flasks, for each cell line, and the total volume made up to 6 ml RPMI-1640 & 10% FCS. Inoculation was therefore a constant for each cell line and every cell line received between 30 000 and 40 000 cells. Care was given to the symmetrical placement of the flasks in the incubator, where the flasks were cultured as normal, and inspected daily by microscope until cell growth was well established, with approximately 5% confluence. One flask of that each cell line was then harvested and this was regarded as zero hour of day 0. At 24 h intervals the remaining flasks were harvested. Each harvest involved complete separation of the cells from the flasks and complete dispersion of the cells in 3 ml of 0.02% EDTA solution. Flasks which attained greater than 90% confluence, or where there was any sign of the cell monolayer detaching, were regarded as no longer in the exponential growth phase and were not cell counted. Cell counts were analysed by non-linear regression and manual semi-log₂ plots, to ensure that only the exponential growth phase was covered. Hence, data points at the extremities of the analysis time, which flattened the semi-log plot, were removed from the analysis and classed as overgrown.
Table 4.10 Doubling time of each cell line. Determined by non-linear regression analysis of daily total cell counts, measured in 3 ml EDTA solution. Overgrown (OG), counting spoiled by operator error (S).

<table>
<thead>
<tr>
<th></th>
<th>Total cell count (x 1000)</th>
<th>Doubling Time (h.)</th>
<th>95% Limits of confidence (h.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>CRI-G5</td>
<td>141</td>
<td>237</td>
<td>390</td>
</tr>
<tr>
<td>HiCRI-D1c</td>
<td>126</td>
<td>291</td>
<td>693</td>
</tr>
<tr>
<td>HiCRI-D2c</td>
<td>177</td>
<td>297</td>
<td>504</td>
</tr>
<tr>
<td>HiCRI-D3c</td>
<td>123</td>
<td>231</td>
<td>426</td>
</tr>
<tr>
<td>HiCRI-D4c</td>
<td>108</td>
<td>198</td>
<td>360</td>
</tr>
<tr>
<td>HiCRI-D4c-p66</td>
<td>117</td>
<td>234</td>
<td>453</td>
</tr>
<tr>
<td>HiCRI-D5c</td>
<td>141</td>
<td>249</td>
<td>S</td>
</tr>
<tr>
<td>HiCRI-D6c</td>
<td>153</td>
<td>294</td>
<td>570</td>
</tr>
<tr>
<td>HiCRI-D7c</td>
<td>174</td>
<td>309</td>
<td>567</td>
</tr>
<tr>
<td>HiCRI-D8c</td>
<td>135</td>
<td>234</td>
<td>411</td>
</tr>
<tr>
<td>HiCRI-E11c</td>
<td>147</td>
<td>237</td>
<td>384</td>
</tr>
<tr>
<td>HiCRI-E12c</td>
<td>156</td>
<td>255</td>
<td>426</td>
</tr>
<tr>
<td>HiCRI-E13c</td>
<td>126</td>
<td>207</td>
<td>339</td>
</tr>
<tr>
<td>HiCRI-E14c</td>
<td>138</td>
<td>231</td>
<td>372</td>
</tr>
<tr>
<td>HiCRI-E15c</td>
<td>129</td>
<td>273</td>
<td>573</td>
</tr>
<tr>
<td>HiCRI-E16c</td>
<td>159</td>
<td>273</td>
<td>453</td>
</tr>
</tbody>
</table>
4.11 Presence of human antigens.

Triton X-100 solubilized cell suspensions were formed from pools of all the novel cell lines for which CRI-G5 was the fusion partner. The subcloned cell lines discussed in this thesis were pooled with no more than four cell lines combined per pool. These cell lines were harvested at passages 11 and 12, with the exceptions of HiCRI-D3c-p57 (passages 58,59,61) and HiCRI-D4c-p66 (passage 69). The novel cells which were not pursued after human insulin screening were pooled into two pools, according to which pancreas they were established from. These cells were of early passage, with most at passage 2 but a small number were at passage 5. Human islets and CRI-G5 were also analysed, and were like the novel cell lines stored at –70°C.

Solubized cell suspensions were pre-cleared by multiple passes through an affinity column of sheep anti-CRI-G5, and then ran on another affinity column of human antibodies. These were from two pancreas transplant patients with failing glucose toleration tests and high immunoglobulin levels despite immunosuppression and over twenty recently-diagnosed type I diabetics. Elution from this column was performed using 5 ml 20 mmol/l HCl, which was then freeze-dried. Reconstituted samples were run on a SDS-PAGE using a 10% gel, using the discontinuous buffer system of Laemmli. The transfer of proteins from the polyacrylamide gel to solid support was achieved by electro-blotting onto nitrocellulose membrane. Western blotting was then performed using a primary antibody of a pool of human sera consisting of the pancreas transplant failures and newly-diagnosed diabetics. The secondary antibody was sheep anti-human IgG, enzyme labelled with horseradish peroxidase, and development employed Sigma Fast™ 3,3-diaminobenzidine tetrahydrochloride (DAB) tablets.

The developed Western Blot of affinity purified antigens from the novel cell lines is represented in Figure 4.11. No human-specific antigens were detected in the novel cell lines or the pooled samples of the non-characterised novel cell lines.
In repeat analyses the Western blot was deliberately allowed to over-develop to ensure that no antigen bands were missed. Had the presence of an antigen been detected, the cell lines in the relevant pool of samples would have been individually re-subjected to the affinity chromatography, SDS-PAGE and Western blotting. This would have been with the aim of at the very least identifying the cell line containing the antigen and determining the molecular weight of the antigen.

The strategy employed was rather drastic, especially with the thorough pre-clearing with anti-CRI-G5 antibodies. Perhaps it would have been a worthwhile alternative to employ a more sensitive technique, such as screening for human MHC I using the highly specific anti-human MHC I monoclonal antibodies available.
Fig. 4.11 Western Blot of affinity purified antigens from the novel cell lines.

Cell homogenates were subjected to an affinity purification against diabetic human immunoglobulins after pre-clearance with an affinity matrix against CRI-G5 antigens. Purified antigens were then run on 10% SDS-PAGE, followed by Western blotting. The original diabetic human antisera was used as the primary antibody. Development was using 3,3’-diaminobenzidine. Cell homogenates originated from:

(A) CRI-G5
(B) pool of all cell lines from fourth pancreas derived from fusions with CRI-G5.
(C) pool of all cell lines from fifth pancreas derived from fusions with CRI-G5.
(D) pool of HiCRI-D3c-p57, HiCRI-D3c, HiCRI-D4c, HiCRI-D4c-p66
(I) human islet cell-enriched pancreatic cells.
(E) pool of HiCRI-D1c; HiCRI-D2c; and HiCRI-D8c
(F) pool of HiCRI-D5c; HiCRI-D6c; and HiCRI-D7c
(G) pool of HiCRI-E11c; HiCRI-E12c; and HiCRI-E16c
(H) pool of HiCRI-E13c; HiCRI-E14c; and HiCRI-E15c
4.12 Chromosome analysis.

Karyotype analysis of the novel cell lines and the parental CRI-G5 was performed by conventional techniques. The cells used were in the most from the morphologic observations and as such the passage number of each novel cell line was 21. The exceptions were HiCRI-D3c-p57 and HiCRI-D4c-p66 which were analysed at passage 58 and 67 respectively. Whenever extra cells were needed to supplement those already analysed, cryogenic stores were utilised, ensuring that all cells of the same cell line were analysed at the same passage.

All 75 cm² culture flasks were allowed to grow to greater than 75% confluence, before mitosis was arrested in metaphase using Colcemid®. The cells were then swelled in 75 mmol/l KCl, before being ‘fixed’ by repeated washes in fixative [75% methanol, 25% glacial acetic acid]. Stored for at least one week at -20°C in this fixative, chromosome spreads were obtained by dispersion in fresh fixative which was then allowed to fall from a height of 5 cm onto one end of a glass microscope slide, with the drop encouraged to spread over the remainder of the slide. Giemsa stained chromosome spreads were mounted in DPX and protected by cover slip, and were viewed on a Leica Leitz Diaplan microscope under a 100x oil immersion lens. Counting was performed in the most part visually, though some chromosome spreads were also photomicrographed through the same microscope and then counted.

The distributions of the chromosomal numbers of the various cell lines are displayed in Table 4.12. The modal chromosomal number of each distribution is shown in bold, except for HiCRI-D4c-p66 for which two modal chromosomal numbers were recorded. Also shown are photomicrographs of metaphases containing the modal chromosomal number for: CRI-G5 (Fig. 4.12.1); HiCRI-D3c (Fig. 4.12.2); HiCRI-D8c (Fig. 4.12.3); HiCRI-E15c (Fig. 4.12.4); and the HiCRI-E14c metaphase containing only 31 chromosomes (Fig. 4.12.5).
Table 4.12 Distribution of the chromosomal numbers of the various cell lines, seen in twenty metaphases. Modal chromosomal number is given in bold.

<table>
<thead>
<tr>
<th>Number of metaphases with the shown chromosomal number.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRI-G5</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HiCRI-D1c</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HiCRI-D2c</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HiCRI-D3c</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HiCRI-D3c-p57</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HiCRI-D4c</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HiCRI-D4c-p66</td>
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<tr>
<td></td>
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<tr>
<td>HiCRI-D5c</td>
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<td></td>
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<tr>
<td>HiCRI-D6c</td>
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<td></td>
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<tr>
<td>HiCRI-D7c</td>
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<td></td>
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<tr>
<td>HiCRI-D8c</td>
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<td></td>
</tr>
<tr>
<td>HiCRI-E15c</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>HiCRI-E16c</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Fig. 4.12.1 Photomicrograph of a CRI-G5 metaphase.
Fig. 4.12.2 Photomicrograph of a HiCRI-D3c metaphase.
Fig. 4.12.3 Photomicrograph of a HiCRI-D8c metaphase.
Fig. 4.12.4 Photomicrograph of a HiCRI-E15c metaphase.
Fig. 4.12.5 Photomicrograph of a HiCRI-E14c metaphase.

Chromosomal number = 31
4.12.1 Chromosome *in situ* hybridisation.

Chromosome spreads prepared in parallel with those in Section 4.12. But were not stained in Giemsa stain and left uncovered for two months. The assay was supplied in kit form from Oncor, with the probe specific for Total Human DNA. The probe was labelled with biotin, and the detection system was fluorescein labelled avidin counter-stained with 20 μl of propidium iodide at a final concentration of 0.3 μg/ml in Antifade biotin labelled. Coverslips were applied to slides before viewing on a Leica Leitz Diaplan microscope, with an appropriate dual band pass filter, and viewed under 100x oil immersion lens. Excitation was provided by a fitted 50 watt mercury fluorescent bulb.

Controls of human lymphocytes showed complete hybridisation along the full length of all the chromosomes and an unbroken FITC signal was observed over interphase nuclei. No FITC positive fluorescence was seen on either metaphase spreads or interphase nuclei from any of the cell lines. The cell lines: HiCRI-D1c; HiCRI-D2c; HiCRI-D4c; HiCRI-D5c; HiCRI-D6c; and HiCRI-E16c were re-assessed externally. The same results were obtained using the same kit probe but with the digoxygenin detection system.
Chapter 5

Discussion.
5.1 Islet cell isolation.

The presence of glycerol in the isolation buffer did noticeably improve yields of islets, but this was counteracted by the difficulty in dissociating the islets when glycerol had been used. To the extent that glycerol offered no noticeable improvement in single islet cell yields.

5.2 Fusion parameters

The use of glycerol in the isolation buffer and the presence of 16 μmol/l thymidine in the selective medium were seen to be of vital importance. The utilisation of either of these resulted in complete failure to establish cell lines. Glycerol is used in the field of cryopreservation, where it stabilises plasma membranes and protects against cell damage from water crystals. The effects of glycerol were possibly due to this stabilising action of plasma membranes counteracting the efforts of PEG to fuse these membranes.

The successful selective medium was undoubtedly azaserine & hypoxanthine, from which all the cell lines were established. The addition of thymidine was sufficient to completely reverse this, resulting in no cell lines at all. It is not clear why thymidine had such drastic effects on the fusions, nor if the failure of HAT was due in part to aminopterin, as thymidine was also present in this medium. HAT containing 16 μmol/l thymidine was used as the selective medium in three of the previous fusions to establish islet cell lines (Boyd et al. 1982; Takaki et al. 1984; Aponte et al. 1989). Of these, only the fusions performed by Boyd et al. involved human islet cells, although this was insulinoma tissue. It is conceivable that human β cells are more susceptible to thymidine than rodent β cells, and that the malignant and hence nucleotide hungry nature of the human insulinoma made these cells less susceptible to high levels of thymidine. This susceptibility to thymidine appeared to be limited to the immediate post-fusion period as, since establishment, the novel cell lines have been cultured in HAT. The fragility of hybridoma cells post-fusion is well established, hence the raising of FCS levels to 20% and the use of the small chambers of the 96-well culture plates.
It was hoped that the use of PDH-1 myeloma cell line would be successful as a fusion partner. Not only because of the possible genetic content of PDH-1, but also its robustness, ease of handling, and lack of insulin-secretion. The lack of success was probably due to the phenotypic difference between lymphocyte and β cell, although this is not definite as RINm5f was not that much more successful as a fusion partner. The sheer number of failed fusions using PDH-1 would however cast doubt on whether it was at all suitable for this sort of trans-species, trans-phenotype fusion.

CRI-G5 was undoubtedly the most successful fusion partner, both in terms of numbers of cell lines established and the insulin-secretion levels of these cell lines.

The parameters of the batch of FCS and the type of PEG showed no noticeable effect on the establishment of cell lines. This was somewhat unexpected as these factors, particularly the FCS, are usually very telling in hybridoma fusions. It would thus appear that the differing content of cytokines in various batches of FCS played no overall negative or positive role in the establishment of the β cell lines. The presence of IBMX and glucagon likewise showed no noticeable effect on the establishment of cell lines but the use of culture medium with 11.1 mmol/l glucose concentration did appear more successful than a 5.55 mmol/l concentration. This success was based on the number of cell lines attained, rather than the characteristics of the cell lines.

Classic hybridoma PEG fusion would employ centrifugations of 500g for 10 min. Observance of islet cell supernatants during the wash stages revealed this was not sufficient centrifugation to pellet out all the islet cells. Therefore the more extreme centrifugation of 1200g for 30 min was employed for the fusions. Post-fusion viable-cell counts and the establishment of the cell lines confirmed that this was an applicable centrifugal force. Likewise the use of a 1:3 ratio of islet cell to fusion partner cells was confirmed as successful in establishment of the β cell lines, but it was not known how this compared to when ratios of 1:1 and 3:1 were used.
Further work would be required to clarify parameters such as the glucose concentration of the fusion culture medium, the ratio of cells to be fused, and the centrifugal force used to fuse the cells. Although they have proven successful, this success was based on quantity and not the characteristics of the cell lines established. The latter two parameters play a major role in the number of cells fused to form a single hybrid cell, which would therefore have significant effects on the characteristics of hybrid cell lines. The much weaker forces previously employed (Boyd et al. 1982; Takaki et al. 1984; Aponte et al. 1989) would favour the fusing of smaller numbers of cells into the one hybrid cell, rather than the more extreme force used in this project.

5.3 Species of secreted insulin species.

Fourteen original cell lines secreted insulin that neither matched the rat nor human insulin control curves when analysed on the parallel RIA human insulin screen. The presence of two species of insulin present in the same sample was already known to cause this failure to match ‘the ratio of derived insulin values’ of either control, as seen in Fig. 3.3. Hence it was considered a possibility that the original novel cell lines consisted of a mixed population of cells, some of which secreted human insulin while others secreted rat insulin.

Subcloning was expected to have resulted in cell lines which secreted either rat or human insulin, or no insulin. Hence the continued detection after subcloning of secreted insulin which matched neither the rat nor the human insulin controls was unexpected. The novel cell lines, like the parental CRI-G5, tightly adhere to culture plastic and each other and despite the use of EDTA, dissociation is rarely total. It was possible that subcloning was unsuccessful due to this failure to dissociate, but it was unlikely that all the subclones should be thus affected. It was, however, still plausible that the same monoclonal cells were secreting more than one species of insulin (Lund et al. 1993; Besnard et al. 1989; Clark et al. 1997).

External assessment of the insulin secreted by five of the subclones revealed that the failure to match the rat or the human insulin controls in terms of ‘the ratio of derived
insulin values', was not due to the presence of more than one species of insulin, as human insulin was not detected. Although the other subclones were not similarly tested for human insulin, the unexpected failure of subcloning to isolate human insulin secretion from any of the subclones implied that human insulin was most probably not secreted by any of the subcloned cell lines.

As to the cause of the failure to match the rat insulin controls in terms of 'the ratio of derived insulin values', the possibilities were therefore limited. Point mutations in insulin are rare but still encountered (Steiner et al. 1990). Such a mutation could have appeared in rat or human insulin and either would have failed to be recognised in the external human insulin specific assay. However, the plausibility of all the subclones suffering similar insulin mutations is beyond belief and extremely unlikely. Human proinsulin could be present. Although detectable in the parallel RIA used for screening, human pro-insulin was not detectable in the external human insulin specific assay. Indeed the only other PEG fusion involving human β cells resulted in the secretion of human proinsulin (Boyd et al. 1982). However, the relative cross-reactivities to human proinsulin of the anti-insulin antibodies utilized in the parallel RIA would not support this possibility. Most plausibly the cause was due to a change in the insulin-like immunoreactive profile secreted from novel cell lines. Where the ratio of rat I to rat II insulin, or the levels of rat proinsulin or proinsulin precursors, were different from the rat standards and the CRI-G5 control.

5.3.1 The insulin-like immunoreactive profile secreted by the cell lines.

The HPLC performed and reported by Efrat et al. (1988) utilized $^3$H-leucine and $^{35}$S-methionine labelling, with monitoring based on radioactivity counts. They also monitored optical absorbance at 210 nm, and although they did not show their results, they reported that mature insulin I and II could be detected at this wavelength. This detection could not be repeated in this study, most probably due to the small amounts of insulin present compared to that of Efrat et al. Although they did not state the amounts, their source was sonicated cells and hence the entire cellular content of insulin.
As the HPLC analysis of the secreted insulin-like immunoreactive profiles was unsuccessful, the possibility that changes in the profiles were the cause of the unusual ‘ratio of derived insulin values’ remained unanswered. However, the mere fact that these values were different from that seen with the parental CRI-G5 was added confirmation of the novelty of the cell lines.

5.4 Insulin release in response to glucose.

In a ‘normal’ glucose-stimulated insulin secretion (GSIS) curve, as seen with perifused islets, increasing glucose concentrations will increase insulin secretion from a basal level until a maximum plateau is reached. Only one cell line HiCRI-E14c exhibited the ‘normal’ increase from basal to a plateau of insulin secretion, though HiCRI-E11c was not far from exhibiting this ‘normal’ secretion curve as well.

The response of the cell lines to glucose was seen to be varied. Some cell lines mirrored the fusion partner CRI-G5, in that they exhibited no GSIS. Other cell lines did present some GSIS over small ranges of glucose concentrations, but this was never greater than a 1.81-fold increase. This level of increase is comparable to the BRIN cell line established by electrofusion concentrations (McClenaghan NH et al. 1996). Increased glucose concentrations often resulted in decreased insulin release, particularly at the 16 and 32 mmol/l glucose concentrations, and as a result the GSIS plots were often erratic. The causes of the erratic nature of the plots and the negative effect of increased glucose concentrations on insulin secretion are unclear, but have been previously reported with the INS-1, INS-2, HIT-5B5 and monolayer cultures of islet cells (Asfari et al. 1992; Santerre et al. 1981).

The culture and stimulation of the cell lines in the co-presence of nicotinamide had varying effects. It was seen to increase or decrease the erratic nature of some insulin secretion plots and was also responsible for decreased insulin secretion with increased glucose concentrations. It did, however, have a positive effect on the GSIS of HiCRI-E16. The main effect of nicotinamide was to alter of insulin secretion levels. The effects of nicotinamide were thought to be due to its presence during the
culture of the cell lines, rather than during the static incubations. Cell growth in culture, as witnessed by the measurement of cellular protein, was improved when nicotinamide was present.

The insulin secretion plots of the later passage cell lines displayed differences from the earlier passage cell lines, indicating some influence of ‘ageing’. Though these differences were reduced by the use of nicotinamide.

It was possible that GSIS was missed by not analysing below 1 mmol/l glucose concentration, as seen in other β cell lines (Newgard, 1996; Hohmeier et al. 1997; Gilligan et al. 1989; Hamaguchi et al. 1991). GSIS was, however, exhibited over a glucose concentration range of 1 -16 mmol/l. Hence GSIS was displayed in some of the cell lines above the physiological threshold glucose concentration of 5 mmol/l (Lindström, 1984; Eizirik et al. 1988). This would indicate that these cell lines contained the necessary predominance of the glucose transporter GLUT-2 and/or the level of glucokinase required for GSIS above a glucose concentration of 5 mmol/l (Tal et al. 1992; McClenaghan et al. 1998).

5.5 Insulin secretion levels of the cell lines.

The insulin secretion levels of the cell lines were the most noticeable confirmations of novelty, with up to 72-fold increase over the level seen with CRI-G5. What caused such extreme differences was unclear, but it was plausible that the novel cell lines contained and/or expressed more insulin genes than the CRI-G5 cell line. This cell line was derived from the RIN tumour, which has been reported to express only the rat I gene (Fiedorek et al. 1990), thus expression of the unexpressed rat II gene could be responsible for the increased insulin secretion levels. Multiple copies of insulin genes can be expressed at the same time (Clark et al. 1997). Although it is discussed more fully in a Section 5.12, chromosome analysis did indicate that some of the cell lines had most probably resulted from cell fusions involving a human islet cell and at least two CRI-G5 fusion partner cells. Hence the expression of multiple copies of the rat insulin genes could also explain the large increase in the insulin secretion levels of the novel cell lines.
5.6 Insulin content of the cell lines.
The range and scale of the cellular insulin content was analogous to that seen with the insulin secretion levels and, with a few exceptions, the percentage of the content released during the static incubation was similar to that of the parental CRI-G5. The low percentage of content released by HiCRI-D4c-p66 would suggest that the effect of ‘ageing’ was on the secretion rather than the synthesis of insulin. HiCRI-E16c contained the most insulin, estimated to be approximately 7% of the content of native β cells (Lernmark, 1974; Ishihara et al. 1993). The content of the cell lines was determined after a pre-incubation of 1 hour, and so the true content of the cell lines would be greater than the stated values.

5.7 Glucagon release of cell lines.
The co-secretion of glucagon and insulin by CRI-G5 was confirmed and all of the novel cell lines exhibited this undesirable co-secretion to varying degrees. The levels of glucagon secretion were seen to range from HiCRI-D4c which secreted less than 14% of that secreted by CRI-G5, to HiCRI-E12c which secreted approximately 2.5-fold the glucagon levels secreted by CRI-G5. However, when the molar ratio of secreted hormones was considered, all the novel cell lines displayed an improvement on the parental CRI-G5. The maximum ratio of 416 was seen for cell line HiCRI-E16c, which was approximately 74-fold the ratio seen for CRI-G5. The molar ratios of secreted hormones compared well to the previously reported values of other β cell lines. These values were: approximately 500 in βTC cells (Poitout et al. 1995); and approximately 50 in HIT-T15 cells (Diem et al. 1990), although these molar ratios were determined in the absence of glucose.

The effects of ‘ageing’ were noticeable in HiCRI-D3c-p57 and HiCRI-D4c-p66 as both secreted less glucagon than the earlier passage cell lines. However, this decrease in glucagon secretion did not always result in an improvement in the molar ratio of secreted hormones.
5.8 Morphologic observations.

Morphologic observations alone were enough to confirm the novelty of some of the cell lines. In particular HiCRI-D1c and HiCRI-D6c presented not the usual fibroblast-like growth pattern but a 'cobble-stone' pattern more typical of epithelial cell lines.

5.9 Cell line growth rate.

The cell lines presented a range of doubling times between 19.38 h and 34.71 h, with a doubling time of 31.85 h for CRI-G5. The most rapidly replicating cell line HiCRI-D1c was shown previously to secrete the lowest levels of insulin, but HiCRI-E15c also had a short doubling time whilst secreting high levels of insulin. The HiCRI-D3c cell line passages both exhibited similar doubling times and similar insulin secretion levels. HiCRI-D4c-p66 did have a shorter population doubling time than the earlier passage HiCRI-D4c, and the later passage cell line was shown previously to have reduced insulin secretion. Hence, although no inverse relationship can be drawn between growth and insulin secretion when analysed between different cell lines, the late passage cell lines may point to this inverse relationship when analysed in the same cell line.

5.10 Absence of detected human antigens.

Although no human-specific antigens were detected in the novel cell lines or the pooled samples of the non-characterised novel cell lines, this does not preclude the possibility that human antigens or other human molecules were present. As seen with the anti-insulin antibodies used previously in the RIA's, there is a large degree of cross-reactivity between different species. The anti-CRI-G5 antibodies were used to pre-clear the cell line samples of antigens found in this rat β cell line. These antibodies would have cross-reacted with any antigenically similar structures found in human β cells, and these would have also been removed from the samples. Because of this antigenic similarity these antigens are not human-specific. Other human-specific molecules which do not illicit an immune response, such as normally exposed peripheral membrane proteins, would not have been detected by the human
antisera. Although the control sample of human islet cells was seen to work well, exocrine pancreas was undoubtedly present in this sample, and the antisera from the failed pancreas transplant patients would have detected exocrine antigens. As such it was the best control that could be reasonably attained, but it was not an ideal control. So, despite the failure to detect any non-CRI-G5 antigens, the absence of human molecules was not necessarily proven.

5.11 Chromosome analysis.

Despite subcloning, the chromosome distribution range of the cell lines was quite wide and was also observed, albeit to a lesser extent, in CRI-G5. Clearly chromosomes were discarded after subcloning. The chromosome loss observed in CRI-G5 implies that this phenomenon was in part due to ‘ageing’ of the cell line in culture. The added factor of instability due to excessive chromosome content after cell fusion would explain the greater distribution observed in the hybrid cell lines (Takaki et al. 1984; Scott et al. 1992).

The differences in the chromosome distribution of the cell lines, compared to CRI-G5, confirmed the novelty of the cell lines. All the cell lines exhibited a modal chromosome number greater than CRI-G5, except HiCRI-E11c which had the same modal number as CRI-G5. Although HiCRI-E11c displayed relatively low levels of insulin secretion, no correlation was found between this expression of cellular function and modal chromosomal number. Throughout many of the previous studies the effects of ‘ageing’ have been more visible in HiCRI-D4c-p66 than HiCRI-D3c-p57. Whereas the chromosome distributions of both the HiCRI-D3c cell lines are similar, the greater difference seen between the HiCRI-D4c cell lines may explain the root cause of the ageing effects.

Several metaphases were observed with chromosomal numbers greater than 100. In conjunction with results from the chromosome in situ hybridisation study, this number would imply that the original hybrid cell was probably formed by the fusion of at least three cells, at least two of which were CRI-G5 fusion partner cells. The high levels of insulin secretion seen in the cell lines may therefore have been due to
the insulin genes from two or more CRI-G5 fusion partner cells being present and expressed in the hybrid cell lines (Clark et al. 1997).

It was unclear why several of the metaphases contained very low numbers of chromosomes, such as the 31 chromosome metaphase observed for HiCRI-E14c (Fig. 4.12.5). The occurrence of chromosomal numbers below that seen with CRI-G5 may point to a fault in the mechanisms of DNA and/or cell replication. If a fault in cell replication was present, with one cell receiving more DNA than the other, this may offer another explanation for the metaphases displaying the very large (>100) chromosomal numbers.

5.11.1 Chromosome in situ hybridisation

Although no human chromosomes or chromosomal fragments were detected, the absence of human DNA was not proven. The core problem with this analysis was the lack of a suitable positive control of rodent DNA containing human chromosomal fragments. The untilized control of human lymphocytes was therefore inadequate, particularly since any human DNA was expected to represent only a small fraction of the total cellular DNA (Gravekampf et al. 1987).

The continued ability of the cell lines to grow in selective media was a sure indication that the H(G)PRT enzyme was present. It was possible that the H(G)PRT enzyme was obtained from the expression of previously unexpressed CRI-G5 DNA, or that reversion mutations occurred. But the statistical chances of this occurring in all the cell lines and never occurring in the CRI-G5 cell line, which was continually susceptible to selective media, is very slim (Campbell, 1984). Hence, it was most probable that the human DNA coding for the H(G)PRT enzyme was present within at least some of the cell lines, and yet was undetected. The failure to detect human chromosomes, which were definitely present in hybrid cell lines, has been previously reported (Boyd et al. 1982).
5.12 Concluding remarks.

The major aim of this project was to see if PEG fusion could be utilised to establish a novel and stable human-insulin-secreting hybrid β cell line. It was hoped that human β cell characteristics would be expressed in this hybrid cell line. While stable insulin-secreting β cell lines were successfully established, human β cell characteristics were not detected.

The novelty of the cell lines was confirmed during the characterisation of the cell lines, as witnessed by differences from characteristics of the CRI-G5 parental fusion partner cell line. All the cell lines were seen to differ from CRI-G5 in at least some if not all of the following characteristics: glucose-stimulated insulin secretion; insulin secretion levels; response to common insulin secretagogues; insulin content; insulin-like immunoreactive profiles; glucagon secretion; general morphology; growth rates; and chromosome analysis. The cell lines themselves displayed a wide and varied range of characteristics, with no two cell lines exactly similar.

The effects of prolonged culture was assessed in two of the cell lines. HiCRI-D4c-p66 did display a large decline in hormone secretion and loss of chromosomes. However, HiCRI-D3c-p57 should no obvious signs of ‘ageing’ after over one year in culture and was therefore a very stable cell line.

All but one of the cell lines were originally selected solely on the basis of suspected human insulin secretion. These cell lines represented only a fraction of the total number of cell lines generated, amongst which specific screening for desired functions such as glucose-responsive insulin secretion may have proven even more fruitful.

At the onset of this project all attempts to generate a human β cell line had failed and looked destined to continue to fail. The rapid advance in the technology of molecular engineering of cells led to the birth of iterative engineering, which specifically achieved what PEG fusions could only achieve by chance, (and slim chance at that). The one shortcoming of this new technique was the lack of a human β cell to
engineer. The generation of NES2Y was in its own right a major breakthrough, but the subsequent creation of NISK9 by iterative engineering of NES2Y (Macfarlane et al. 1999) will surely prove to be an even greater milestone. It has certainly rendered the strategy employed in this project obsolete.

Perhaps the greatest characteristic of NES2Y was that it was not perfect, but was deficient in a just few factors; Kir6.2; SUR1; and PDX1 (Macfarlane et al. 1999), which provided focus for research into PDX1.

Surely many of the iteratively engineered animal β cell lines will also prove to have deficiencies present and may allow for yet further breakthroughs in our understanding of insulin secretion. Just such work has already highlighted the importance of several factors necessary to obtain good glucose-responsive insulin secretion (as discussed previously in Sections 1.5.1, 1.11.7 and 1.16.1). These include a predominance of the glucose transporter GLUT-2 as opposed to GLUT-1; a high glucokinase (the glucose sensor) to hexokinase enzyme ratio; adequate Kir 6.2 and SUR1 expression to form functional $K_{ATP}$ channels; and adequate PDX1 expression to provide the mechanisms by which glucose modulates both insulin promoter activity and endogenous insulin mRNA levels and thereby provide the capacity to replenish insulin stores following secretory stimuli (Tiedge et al. 2000; Macfarlane et al. 2000; Iizuka et al. 2000).

It will remain to be seen over time if NES2Y and NISK9 become widely available to researchers, and how useful these cell lines will be in terms of diabetic antigen studies.

As for the novel β cell lines reported herein, they represent a panel of β cell lines with different characteristics, which hopefully may prove useful as models for further studies into β cell function and dysfunction.
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