INSULIN GENE THERAPY FOR THE TREATMENT OF DIABETES MELLITUS

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Currently available treatments for insulin-dependent diabetes mellitus are often inadequate in terms of both efficacy and patient compliance. Gene therapy offers the possibility of a novel and improved method by which exogenous insulin can be delivered to a patient. This was approached in the present study by constructing a novel insulin-secreting cell line. For the purposes of this work immortalized cell lines were used. Fibroblasts and pituitary cells were transfected with the human preproinsulin gene to create stable lines of proinsulin- and insulin-secreting cells. The effect of known β-cell secretagogues on these cells was investigated, and found mostly to have no stimulatory effect, although IBMX, arginine and $ZnSO_4$ each increased the rate of secretion.

Cyclosporin (CyA) is currently the immunosuppressant of choice for transplant recipients; the effect of this treatment on endogenous β-cell function was assessed both *in vivo* and *in vitro*. Therapeutic doses of CyA were found to reduce plasma insulin concentrations and to impair glucose tolerance. The effect of immunoisolation on insulin release by HIT T15 cells was also investigated. The presence of an alginate membrane was found to severely impair insulin release.

For the first implantation of the insulin-secreting cells, the animal model selected was the athymic nude mouse. This animal is immunoincompetent, and hence the use of an immunosuppressive regimen is circumvented. Graft function was assessed by measurement of plasma human C peptide concentrations, using a highly specific assay. Intraperitoneal implantation of genetically manipulated insulin-secreting pituitary cells into nude mice subsequently treated with a large dose of streptozotocin (STZ) resulted in a significantly delayed onset of hyperglycaemia when compared to control animals. Consumption of a $ZnSO_4$ solution was shown to increase human C peptide release by the implant. Ensuing studies in nude mice examined the efficacy of different implantation sites, and included histochemical examination of the tumours. Aldehyde fuchsin staining and immunocytochemical processing demonstrated the presence of insulin containing cells within the excised tissue.

Following initial investigations in nude mice, implantation studies were performed in CyA-immunosuppressed normal and STZ-diabetic mice. Graft function was found to be less efficacious, possibly due to the subcutaneous implantation site, or to the immunosuppressive regimen. Histochemical and transmission electron microscopic analysis of the tumour-like cell clusters found at autopsy revealed necrosis of cells at the core, but essentially normal cell morphology, with dense secretory granules in peripheral cells. This thesis provides evidence that gene therapy offers a feasible new approach to insulin delivery.

Key phrases: insulin release, human proinsulin C peptide, nude mice, cyclosporin

In memory of my Father

with love

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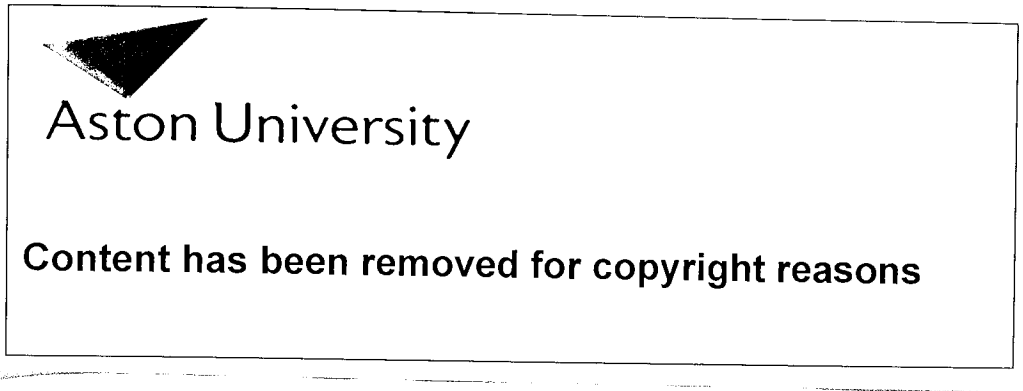
ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic monophosphate
cGMP	Guanosine 3',5'-cyclic monophosphate
CyA	Cyclosporin A
DMEM	Dulbecco's modification of Eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis-(aminoethyl ether) tetraacetic acid
g	Gravity
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPLC	High performance liquid chromatography
IBMX	3-isobutyl-1-methyl-xanthine
ILI	Insulin-like immunoreactivity
ip	Intraperitoneal
iv	Intravenous
PBS	Phosphate buffered saline (without Mg^{2+}, Ca^{2+})
po	Per oral
POMC	Proopiomelanocortin
sc	Subcutaneous
SEM	Standard error of the mean



Roberts 1922

CHAPTER ONE

INTRODUCTION

1.1 Diabetes mellitus - current treatment

Insulin dependent diabetes mellitus (IDDM) is a metabolic disease characterised by severe insulinopenia, hyperglycaemia, glycosuria, weight loss, hyperphagia and polydipsia. The metabolic abnormalities are caused by a deficiency of endogenous insulin due to destruction of the pancreatic β-cells. Prior to the introduction of insulin therapy, individuals developing IDDM died at a young age from severe metabolic decompensation (ketosis). Insulin treatment has provided a means of support for IDDM patients, but this has exposed the long-term vascular and neuropathic complications of diabetes. There is strong evidence that microvascular and neuropathic complications are associated with the hyperglycaemia (Singh and Nattrass 1990, Hanssen 1991), while macrovascular complications are at least partly related to accompanying dysregulation of lipoprotein metabolism (Jay and Betteridge 1991).

IDDM is currently treated by the subcutaneous administration of exogenous insulin, either as a syringe, pen, or pump regimen. IDDM patients display circulating insulin concentrations that are approximately twice normal due to the unphysiological method of administration. In normal individuals, insulin is secreted directly into the portal circulation; the major fraction acts on the liver and is removed by this organ. Hence insulin levels in the systemic circulation are relatively low (Gwinup and Elias 1990). Increased circulating insulin levels are undesirable since excess insulin appears to be atherogenic (Sato *et al* 1989) and may potentiate diabetic angiopathy (Stout 1990). There are several other inherent problems with these regimens. A major concern with any form of insulin therapy is the risk of severe hypoglycaemic reactions

- these cause substantial morbidity and some mortality in IDDM, and may be the limiting factor in attempts to achieve euglycaemia (Cryer *et al* 1989). There are further difficulties with the wide intraindividual variability in absorption (Galloway *et al* 1981), and concern has recently been expressed regarding the use of recombinant human insulin and lack of hypoglycaemic awareness (Pickup 1989). Multiple injection regimens are clinically desirable since they greatly improve diabetic control when compared to conventional treatment (Saurbrey *et al* 1988; Kroc Collaborative Study Group 1988), but these methods demand amounts of skill, motivation and persistence which are often considered to be incompatible with the degree of compliance obtained.

Several novel methods for the administration of exogenous insulin have been investigated. Insulin can be delivered across the nasal mucosa in biologically active doses, but the observed potency is low (Jorgensen and Drejer 1990). Moreover there may be nasal irritation and rhinorrhea, and long-term use may cause detrimental changes to the micro-structure of the nasal epithelium (Pontiroli *et al* 1982, Salzman *et al* 1985). Oral administration of insulin is possible provided that the insulin is protected from proteolytic degradation, for example by polyalkylcyanoacrylate nanocapsules (Damge *et al* 1988), or by polymer coatings with azoaromatic groups (Saffran *et al* 1986). However, oral administration of insulin appears to be unreliable as regards transit time, stability, and biological potency, particularly since disturbances of gastrointestinal tract transit and absorptive function are common in diabetes (Nompleggi *et al* 1989, Hartford, Skyler and Barkin 1990).

All available methods of administration of exogenous insulin have limitations in their usefulness and effectiveness, particularly the lack of an exact reinstatement of a normal rapid and portally-delivered physiological insulin response to changes in glucose supply and demand.

In contrast to administration of exogenous insulin, recent research on the treatment of insulinopenic diabetes has centred on replacement of the defective β-cell with equivalent functional cells, that is by transplantation of pancreatic tissue, isolated islets or β-cell isolates.

Pancreatic transplantation has been found to have a low success rate, and has mostly been limited to uraemic recipients of a renal transplant (patients already obligated to immunosuppression). More than temporary insulin independence has rarely been achieved in patients receiving a pancreatic graft (Sutherland, Chow and Moundry-Munns 1989). This procedure is unlikely to become a routine standard treatment for IDDM (Kemmer, Berger and Grabensee 1992) unless many inherent problems can be solved - the operation is expensive and technically difficult, HLA matching is difficult due to the limitations of graft preservation, there is the problem of exocrine duct management, and there may be recurrence of the original disease process resulting in the destruction of the graft (Sutherland, Goetz, and Sibley 1989).

The ability of implanted isolated islets to reverse diabetic symptoms in experimentally-induced diabetic animals has been well documented (Hegre *et al* 1975, Brown *et al* 1976, McEvoy, Schmidt and Hegre 1978). Many different transplantation

sites have been used, including the liver (via the portal vein), the peritoneal cavity, the subcapsular space of the kidney, and the spleen. Tissue immunogenicity has remained a major barrier to successful islet transplant, although recent pre-transplant treatments to remove antigen-presenting cells carried in the graft have improved graft acceptance; procedures such as ultra violet B irradiation (Hardy *et al* 1984), low temperature culture at 24°C (Lacy, Davie and Finke 1979), or culture in 95% O_2 and 5% CO_2 (Bowen, Andrus and Lafferty 1980).

Islet grafting to spontaneously diabetic animals (eg the BB rat and the NOD mouse) has demonstrated a tissue-specific disease recurrence in the graft (Nomikos *et al* 1986, Prowse, Bellgrau and Lafferty 1986). This may occur in human diabetic recipients of an islet graft even though there are some differences in islet cell antibodies present (Bone 1991).

The total number of islet allo- and xenografts performed in human subjects between 1970 and 1988 was 473, of which only 28% showed any reduction in requirement for exogenous insulin (Hering, Bretzel and Federlin 1988). Only transient insulin independence has been reported in diabetic recipients of islet grafts (Scharp *et al* 1990, Warnock *et al* 1992). More encouragingly, non-diabetic pancreatitis patients who received islet autografts following pancreatectomy have demonstrated insulin independence for over 7 years (Pyzdrowski *et al* 1992).

Implantation of a β-cell isolate is also likely to have drawbacks. Although a β-cell preparation of over 95% pure can be produced using an autofluorescence-activated

cell sorter (Pipeleers *et al* 1991), the response of these cells to glucose is several times lower than that of an equivalent whole islet (Pipeleers *et al* 1982), apparently due to disruptions of tight junctions between cells (Orci 1981).

The use of these transplantation procedures requires the use of an immunosuppressive regimen, which has serious side effects, particularly on endogenous β-cell function (this is discussed in greater detail in Chapter 4). Long-term immunosuppression may impose a greater risk on the patient than the morbidity of existing exogenous insulin therapy (Gray and Morris 1987), and cannot at present be justified, particularly in young and healthy diabetic patients.

The prevention of IDDM has two aspects which must be considered, namely identification of individuals at risk, and their subsequent treatment. Many factors are thought to contribute to the development of IDDM, including genetic factors; up to 15 % of IDDM patients have a 1st degree relative with the disease (Wolf, Spencer and Cudworth 1983). The inherited susceptibility for IDDM involves the HLA region on chromosome 6; these genes code for proteins involved in immune response. Certain HLA DR/DB/DQ alleles are thought to confer (variously) susceptibility and protection from IDDM, although the precise effect varies with racial group (Wassmuth *et al* 1992). The INS gene region on chromosome 11 is also known to be involved in suscepibility to IDDM, although the absolute risk of an individual with a high risk geneotype as described above developing the disease is only 6 % (Todd and Bain 1992), which makes population screening unjustifiable. Furthermore the concordance rate in monozygotic twin studies is only 30-60 % (Pyke 1979, Rotter,

Vadheim and Rimoin 1990) and so other factors beside genetic ones must be important in the aetiology of IDDM. Virus infection has been suggested as an autoimmune trigger, but as yet few specific viral infections have been identified (Turner and Neil 1992). Furthermore, animal models of IDDM display an increased incidence of the disease when maintained in pathogen-free conditions. It may be that autoimmunity is associated with the absence of appropriate stimuli for the induction of tolerance - this hypothesis is supported by the observation that injection with Freunds adjuvant before weaning has a protective effect in these animals (Sadelain *et al* 1990). Hence at present it is impossible to identify individuals at risk of developing IDDM; many with a genetic predisposition will not develop the disease, and in addition sub-clinical diabetes (the presence of islet cell antibodies) may last for many years or undergo remission. Even if an individual likely to develop IDDM could be confidently identified, no truly effective preventative treatment is currently availible. Potential areas for development include the possible blockage of autoimmunity with an artificial peptide, vaccination with T cells specific for the autoantigen, or, more clumsily, generalized immunosuppression (Pozzilli, Signore and Andreani 1992). Trials involving administration of cyclosporin (CyA) have induced remission for up to 3 years (Dupre and Stiller 1989), but the continued use of CyA (especially in children) is undesirable since there is a substantial risk of infections, malignancies and nephropathy (Bach, Feutren and Boitard 1989).

Thus although the life saving effect of existing insulin treatment regimens is undisputed, it can be seen that current therapies for IDDM have their limitations, and approaches involving pancreas, islet and β-cell transplants are proving difficult.

A conceptually new approach to treating IDDM may involve the technique of gene therapy. This type of therapy would offer many advantages over conventional transplantation methods currently undergoing research; for example, since a patients' own cells could be used to express a (prepro)insulin gene, there would be no need for immunosuppression or problem with finding a suitable donor, and the choice of implantation site may enable a more physiological portal delivery of insulin.

1.2 The theory of gene therapy

Current therapies for many genetic and biochemical diseases that occur in man are of limited effectiveness. A conceptually new approach to treating these disorders may involve the technique of gene therapy. This can be defined as the insertion and safe expression of a normal gene into an organism which results in the amelioration of a pathophysiologic condition; the expression of the new genetic information causes correction of the disease phenotype (Anderson 1984). Somatic cell gene therapy involves the introduction of new genetic information into a non-germ-line cell; expression of the new DNA is confined to the individual and not passed on to the offspring. Germ-line gene therapy, in contrast, involves the introduction of exogenous DNA into the nucleus of fertilized eggs. Following *in utero* development of these eggs, the new genetic information is expressed in the offspring and is transmitted to subsequent generations. Germ-line gene therapy presents many technical, legal and ethical problems which preclude use in humans (Juengst 1992) and will not be discussed further in this Introduction. The ethics of somatic cell gene therapy have been debated for many years, but the consensus of opinion is that this type of treatment (ie insertion of genetic material into a human being for the sole purpose

of correcting a severe genetic or biochemical defect) does not differ fundamentally from organ transplantation, and is both ethically acceptable and potentially invaluable in the future treatment of disease (Anderson 1992).

There are two main approaches to somatic cell gene therapy. The first, most popular method, has been referred to as *ex vivo* gene therapy (Grossman and Wilson 1992). This procedure involves implantation of genetically modified (ideally autologous) cells. Cells are removed from the donor, taken into primary culture, and transfected with the gene of choice *in vitro*. The cells are then reimplanted back into the donor. The advantages of this technique are that the characteristics of the modified cells can be elucidated prior to reimplantation, the target cell population can be chosen and well defined, the site of implantation can also be chosen, and immunosuppression should not be required. However, this type of therapy is invasive, requiring at least two surgical procedures (tissue harvest and cell transplantation). Furthermore, the efficacy of this method may be limited by the efficiency of the cell transplantation method used.

An alternative to *ex vivo* gene therapy involves direct delivery of the exogenous DNA to the target cells *in vivo*. Whilst this technique is more attractive for several reasons (it is less invasive and, in theory, simpler to carry out) it is at present less well developed. A concentrated, high titre preparation of either retrovirus or other gene transfer vehicle would be needed. As the mechanisms by which genes are controlled become better elucidated it may be feasible to target selected cells and manipulate

the activity of their regulatory sequences. In this way the required gene could be switched on or off. However, present technology renders this a distant prospect.

The first step when designing a gene therapy protocol is to select the method of transfection to be used to insert the exogenous genetic information into the target cell population. To some extent the method chosen depends upon the cell type to be used. There follows a review of the methods of transfection currently available.

1.3 Methods of transfection

There are several main types of approach for the insertion and expression of exogenous genetic information into a target cell population. These include chemical, physical and viral methods.

1.3.1 Chemical transfection

Mammalian cells will take up DNA in the form of a calcium phosphate co-precipitate (Graham and Van der Eb 1973). Further refinements of this method include additional treatment of the cells with dimethylsulphoxide (DMSO), glycerol, chloroquine or sodium butyrate. The DNA gains entry to the target cells by pinocytosis, and passes through the lysosomes before reaching the nucleus. Exposure to lysosomal enzymes is thought to increase the risk of mutation or deletion of exogenous DNA (Chen and Okayama 1988).

Encapsulating DNA within a liposome carrier enables uptake of the exogenous genetic information by cells following fusion of the liposome with the cell membrane, endocytosis, and integration (Mannino and Gould-Fogerite 1988). Liposomes can be targeted to specific cell types by incorporating, for example, an antibody as an integral membrane protein in the lipid bilayer - this increases delivery of the vesicle contents (Gregoriadis *et al* 1985).

These chemical methods of transfection have several disadvantages. The efficiency of stable transfection is extremely low (maximally 1 cell in 10^5) which necessitates the use of a selectable marker to identify those cells which have incorporated and expressed the exogenous DNA. These methods are also only applicable to certain cell types, in particular to adherent cells, and cells must be actively replicating.

1.3.2 Physical transfection

The exposure of cells to a pulsed electric field (electroporation) enables them to take up exogenous DNA due to the transient formation of micropores in the plasma membrane. Electroporation can have an efficiency of up to 1 %, is suitable for both cell lines and mammalian primary cultures, and the expression of the genetic information may be more stable than with the chemical methods of transfection since the DNA is not exposed to lysosomal enzymes (Andreason and Evans 1988). However, electroporation has deleterious effects on the target cells; conditions of maximal transfection efficiency kill approximately half the cells.

It is possible to microinject DNA directly into the nucleus of a cell. This method is relatively efficient but extremely time consuming. Only 1 cell can be injected at a time, hence this approach is unlikely to be suitable for gene therapy (Capecchi 1980).

It has also been reported that firing DNA-coated tungsten micropellets into tissue has resulted in uptake and expression of exogenous DNA. This method, however, results in a certain amount of damage to the target tissue (Klein *et al* 1987).

Both the chemical and physical methods of transfection just described result in the integration of the exogenous genetic information as multiple tandemly repeated copies, which are relatively stable.

1.3.3 Retrovirally-mediated transfection

The major drawback with the physical and chemical methods for the integration of exogenous genetic information into host cells is their low efficiency of incorporation and expression. However, retroviral mediated gene transfer is much more efficient and stable - most cells in a target population can be infected. Most retroviral vectors are derived from the Moloney murine leukaemia virus (MoMLV) which is an enveloped single strand RNA virus (Cepko, Roberts and Mulligan 1984). The retrovirus virion binds to the cell surface receptors, and its RNA genome enters the cell where it is copied to DNA by the virus encoded reverse transcriptase. This (provirus) DNA then integrates into the genome of the target cell as a single copy (Eglitis and Anderson 1988). The retrovirus has long terminal repeat sequences

(LTR) which contain the viral promoter and enhancers required for the initiation and termination of transcription, and also the sequences needed for viral integration into the target cell genome. There is also a psi (Ψ) region needed for the packaging of the RNA genome into viral particles. The viral genes are called gag, pol and env which encode the following proteins respectively: viral structural proteins, enzymatic proteins (including reverse transcriptase), and the viral envelope protein.

The retroviral vector consists of the exogenous gene of interest (and possibly a selectable marker) with the deletion of the viral genes gag, pol and env (Mann, Mulligan and Baltimore 1983). Once packaged into a virion, the retroviral vector can enter a target cell and integrate its genetic material into the genome of the host, with the consequent expression of the exogenous gene(s). Retroviral vectors cannot package themselves into virions since they lack the viral genes needed for virion formation - they are thus replication-defective. To incorporate retroviral vectors into virions, a packaging cell line (ie with gag, pol and env but without psi) must be used (Danos and Mulligan 1988). Putting a retroviral genome into a packaging cell line creates a producing cell line making virions containing the exogenous RNA.

The main advantage of using retroviruses as vectors is the high efficiency of transformation - up to 100% of cells can be infected, and subsequently express the integrated viral (ie exogenous) gene. There are also however some important disadvantages. Although retroviral vectors are able to infect a broad spectrum of cell types, they are unable to infect non-dividing cells (Miller, Adam and Miller 1990). There is also a tendency towards deletion of sequences during viral replication - the

foreign genetic information may be rearranged or removed from the construct (Dougherty and Temin 1988). A further difficulty with the use of retroviral vectors involves the preparation of a large enough titre which must be at least 10^6 infectious units per ml - titres of 10 -100 fold higher would be needed for most *in vivo* vector delivery.

The safety of retroviral vectors is of the utmost concern. There are 4 main safety issues to consider, namely insertional mutagenesis, recombination with endogenous (latent) retroviral sequences, the transfer of endogenous genetic material, and accidental exposure to replication competent murine retrovirus (Epstein 1991). It should be remembered that the initial target diseases of gene therapy (discussed in a later section) all cause substantial morbidity and mortality, and available treatments are mostly palliative. When considering the use of retroviral vectors for gene therapy procedures, the benefit/risk ratio must be carefully evaluated. It should also be remembered that most therapies currently in use have inherent risks; renal transplants and cancer chemotherapy are both used to treat potentially life-threatening conditions, but both may have deleterious effects on the patient. Kidney transplant recipients have an increased risk of infections and cancer (Blohme 1985); chemotherapy during childhood may induce a cancer in later life (Whitehouse 1985).

Retroviruses and retroviral vectors efficiently integrate into the genome in a random manner, although there appears to be a preference for transcriptionally active regions (Rohdewohld *et al* 1987). Insertional mutagenesis may be defined as the insertion of a retroviral vector which causes disruption or abnormal regulation of a gene.

Disruption of a gene essential for cell survival is a rare event (Cornetta 1992), and in the case of *ex vivo* manipulation of cells prior to reimplantation would simply result in a reduced efficacy of therapy.

A more serious consequence of insertional mutagenesis could involve activation of a proto-oncogene or inactivation of a tumour suppressor gene, with the subsequent development of malignancy. Given the size of the human genome, however, such an event is thought to be unlikely (Weatherall 1991). Many factors would be important in such a scenario, including the number of cells infected, the number of integrations per cell, and the numbers of proto-oncogenes or tumour suppressor genes present. It has been shown that the insertional activation of 1 proto-oncogene by a wild-type retrovirus was not sufficient for the development of malignancy (Temin 1988). Furthermore, unlike retroviruses, retroviral vectors integrate only 1 copy of their genome into each target cell. This distinction between retroviruses and retroviral vectors is important since up to 10 genetic alterations may be involved in the generation of certain human tumours (Callahan and Campbell 1989). This type of serious consequence of the use of retroviral vectors for gene therapy could be controlled by isolation of the cells following implantation for example by containing them in a prosthesis which could be easily removed if required.

Other possible problems include the formation of a replication-competent endogenous human retrovirus, and transfer of murine retroviral sequences contained in the helper cell line.

Generation of replication-competent murine retrovirus may occur, presumably by recombination, with the vector donating its intact psi sequence to the psi-deficient packaging cell genome. However, studies in which primates were exposed to a replication-competent virus during a bone marrow transplant did not demonstrate retroviraemia for 3 years post transplant (Cornetta *et al* 1991, Rosenfeld *et al* 1992). However, one study in which monkeys were infused with bone marrow contaminated with replication-competent retrovirus developed thymic lymphoma after approximately 6 months (Miller 1992). No complications have been observed in 5 patients treated with an adenosine deaminase retroviral vector (Rosenberg *et al* 1990), and there is evidence that murine viral particles are extremely unstable in humans, and therefore unlikely to cause disease (Cornetta *et al* 1990). Nevertheless it is important that retroviral preparations for use in clinical trials are tested extensively prior to implantation.

Several steps can be taken to reduce the risk of generating a replication-competent retrovirus. Firstly, packaging cell lines and retroviral vectors could be redesigned to decrease the possibility of recombination (Miller and Buttimore 1986); for example by placing the gag and pol genes on a separate plasmid to the env gene (Mann, Mulligan and Baltimore 1983). Thus multiple recombinations between the vector and viral genes would have to occur before replicative function could occur. Also careful screening of cell preparations for replication-competent viruses (for example the use of Polymerase Chain Reaction (PCR) which can detect 1 cell in 10^5 carrying a retrovirus) prior to implantation would decrease the risk (Morgan, Cornetta and Anderson 1990).

1.3.4 Adenovirally-mediated transfection

Aadenoviral vectors may offer several potential advantages when compared to retroviral vectors. These vectors have a linear double stranded genome, and can carry large segments of DNA (up to 7.5 kb). Furthermore, they are capable of transferring recombinant genes into non-replicating cells (Berkner 1988). Recombinations are rare, and there are no known associations of human malignancies with adenoviral infections. Moreover, live adenovirus has been used safely as a vaccine (Ballay *et al* 1985). However, disadvantages include possible problems involving immune response since adenoviral genes are present in many vectors, and also instability of gene expression since the vector does not integrate into chromosomal DNA (Crystal 1992).

Several studies in animals have shown encouraging results using an adenoviral vector; the genes for α1-antitrypsin (which is defective in emphysema) and the cystic fibrosis transmembrane conductance regulator CFTR (defective in cystic fibrosis) have both been successfully expressed in the respiratory epithelium following intratracheal introduction (Rosenfeld *et al* 1991 and 1992).

1.3.5 Adeno-associated virally-mediated transfection

Human adeno-associated viruses (AAV) may offer several advantages over the use of retroviral vectors. AAV are ubiquitous in humans, and can be concentrated to titres of over 10^9 infectious units per ml. They are non-pathogenic, and so need to be coinfected with a helper adenovirus for integration to occur (Hermonat *et al*

1984). A potential problem with the use of AAV as vectors include the possibility of contamination with pathogenic helper virus.

1.3.6 Direct *in vivo* delivery

As opposed to the genetic manipulation of cells *in vitro*, direct delivery of the vector *in vivo* would be a far simpler protocol for clinical use if safety and accurate targeting of the DNA could be assured. The use of retroviruses alone may not be suitable for this type of approach as a very high titre would be needed, and also because retroviruses are not tropic for any particular cell type (Friedmann 1989). For this method to be effective, the delivery vehicle would need to be organ specific. It is possible that tissue tropisms could be exploited; for example vectors derived from neurotropic viruses such as rabies or herpes may be suitable for gene transfer into the CNS (Friedmann 1989). Targetable liposomes and erythrocyte ghosts have been used to introduce genes directly into whole animals. *In vivo* liposome-mediated gene delivery has led to the expression of foreign insulin in the liver of recipient rats (Nicolau *et al* 1983, Soriano *et al* 1983). The use of a poly-L-lysine-asialoglycoprotein DNA carrier containing a plasmid encoding the CAT reporter gene when injected iv into rats resulted in liver specific expression of the foreign DNA for up to 72 hours following administration. When injection of the carrier-DNA complex was followed by partial hepatectomy, expression of CAT was detectable for up to 11 weeks post injection (Wu, Wilson and Wu 1989). Tissue specific uptake and expression is possible since hepatocytes have unique cell surface receptors that recognise and internalise asialoglycoproteins. Partial hepatectomy presumably causes persistence of CAT expression by stimulation of hepatocyte regeneration. However, the direct

delivery of a vector *in vivo* has 2 potentially serious drawbacks. The first is that the process is irreversible, and so any deleterious effect observed following administration could not easily be rectified. Secondly there is the possibility of the exogenous DNA entering the germ cells.

1.4 Potential target cells

In order for gene therapy to be successful, it is imperative that suitable target cells are chosen for the expression of the required genetic information. To some extent the ideal target cell depends upon the disease to be treated, but some characteristics are essential for all cells used for *ex vivo* gene therapy protocols. The target cells should be readily accessible, and be amenable to culture *in vitro*. They should also be susceptible to genetic manipulation - certain methods of transfection are more applicable to certain cell types than others, as discussed previously. If the new product requires certain substrates or coenzymes to be maximally effective, the selected target cell should contain them. The method of reimplantation of the cells is also critical for the success of gene therapy ie the site and route by which the cells are replaced into the donor. Some existing clinical procedures can easily be adapted for the replacement of cells. For example bone marrow transplantation and skin grafting would both be feasible methods by which genetically modified cells could be reimplanted back into the original donor. Many different types of cell have been proposed as suitable for *ex vivo* gene therapy. To demonstrate the great potential of gene therapy, there follows a more detailed review of these cells, and the diseases for which they may be utilized.

Haemopoietic stem cells

Stem cells were initially proposed as promising target cells for gene therapy protocols (Anderson 1984) since they are self-renewing. Thus in principle they would pass the new genetic information on to their progeny, and appropriate transplantation procedures are well established in clinical practice. Diseases for which stem cell transfection might be useful include adenosine deaminase (ADA) deficiency and other inborn errors of metabolism. Stem cell transfection may also be applicable to thalassaemias and haemoglobinopathies (Robertson 1986). However there are many problems implicit in the transfection of stem cells. Firstly, it is very difficult to isolate stem cells since they only make up approximately 0.1 % of bone marrow cells, and once identified it is very difficult to infect them with retroviral vectors since most are not actively dividing (Steinberg 1991). When transduced stem cells are returned to the donor, expression of exogenous DNA is transient and unstable. Hence circulating concentrations of the therapeutic product tends to 'wax and wane'. An explanation for this phenomenon is that most totipotent stem cells are out of cycle at any one time (Weatherall 1991), and it may be that those cells transfected (ie in cycle) are almost at the end of their lifespan. Furthermore, stem cells may leave the cell cycle at any time and remain quiesent for long periods. It is therefore likely that any gene therapy protocol using stem cells to express exogenous genes will involve multiple infusions of cells.

Lymphocytes

The use of lymphocytes may circumvent many of the difficulties implicit in using stem cells to express a therapeutic product. T lymphocytes are readily available from

peripheral blood, and are easily manipulated *in vitro*. These cells will proliferate upon exposure to a suitable antigen - this could be used to increase numbers *in vivo* by reimmunisation. T cells have a good longevity, and in monkeys genetically manipulated T cells persisted in the circulation for up to 2 years (Culver *et al* 1991). The genetic modification of tumour infiltrating lymphocytes may be of value in the treatment of cancer, and this is discussed in greater detail later in this Chapter.

Hepatocytes

The liver is the site of expression of many genetic diseases, and hepatocytes can be easily purified and have considerable potential for regeneration. Clinical protocols exist for the transplantation of hepatocytes, and gene marking experiments have been undertaken (Ledley *et al* 1992). Hepatocytes may be suitable target cells for inborn errors of metabolism since they contain many cofactors and substrates essential for correct enzyme function (Ledley 1987, Grompe *et al* 1992). Most hepatocytes are quiescent and differentiated which presents a problem for *ex vivo* gene therapy. Several genes have, however been stably transduced into adult hepatocytes using retroviral vectors, including β-galactosidase (Wilson *et al* 1988) and hypoxanthine phosphoribosyl transferase (HPRT) (Wolff *et al* 1987). The low density lipoprotein (LDL) receptor has also been expressed in hepatocytes, and this promising study is discussed in Section 1.6 of this Chapter. Hepatocytes can be reinfused back to the liver via the portal vein, and will also survive intraperitoneally and intrasplenically (Friedmann 1989). It is also possible to achieve direct *in vivo* delivery of the vector to the liver using liposome mediated gene transfer (Nicolau *et al* 1983).

Pulmonary epithelial cells

The use of retroviral vectors to infect pulmonary cells is at present not possible since lung cells are terminally differentiated and do not replicate. However, adenoviral vectors are particularly suitable for transducing lung tissue since they do not require the cells to be actively dividing for the integration of their genetic information, and also because most adenoviral vectors are tropic for the lung (Crystal 1992). The α1-antitrypsin gene has been expressed in lung epithilial cells from the cotton rat both by *in vitro* and *in vivo* delivery of an adenoviral vector. Expression of α1-antitrypsin was detectable *in vivo* for approximately 1 week (Rosenfeld *et al* 1991).

Endothelial cells

The vascular endothelium has a surface area of approximately 1000 m^2 and direct contact with the bloodstream, and thus the efficient export of a therapeutic protein to the circulation or for delivery to an organ just downstream of the site of implantation should be possible (Nabel, Plautz and Nabel 1991). The use of a retroviral vector has resulted in the expression of human factor IX from rat capillary endothelial cells, and has also shown that these cells are capable of posttranslational processing - γ-carboxylation of factor IX is essential for biological activity (Yao *et al* 1991). Capillary endothelial cells have also been transduced with β-galactosidase - in this study the retroviral vector was infused directly into the artery of a pig, which is site specific gene targeting. β-galactosidase was detectable in all layers of the arterial wall, and expression persisted for up to 21 weeks (Nabel, Plautz and Nabel 1990). Hyperplasia due to catheter-induced injury may increase the efficiency of retroviral transduction in endothelial cells *in situ*. Site specific gene targeting to

endothelial cells may be of value in the treatment of circulatory disorders, for example clotting dysfunction, or potentially for cardiovascular disease where localized expression of an anti-thrombosis agent would be beneficial.

Cells of the central nervous system

Diseases of the central nervous system are not obvious targets for gene therapy since these disorders are often multigenic and multifactorial, and in addition the cells involved are inaccessible, and neurons do not divide and so are resistant to retroviral infection. However it would be possible to transduce an autologous cell and implant that into the brain instead of relying on neurons. It has been shown that fibroblasts transduced to express nerve growth factor protected cholinergic neurons from degradation when implanted into lesioned rat brain (Rosenberg *et al* 1988a). This type of approach may be of use in Alzheimer's disease. A further study reported the transduction of nigral cells to produce tyrosine hydroxylase, which resulted in an increased production of dopamine by these cells. When implanted into the brain of experimentally-induced Parkinsonian rats a 70 % reduction in symptoms was observed (Davies 1992). Protocols such as these may be of great benefit for treatment of these diseases, particularly since current therapy depends (controversially) on the use of foetal tissue (Widner *et al* 1991).

Tumour cells

The inclusion of tumour cells as a possible target for gene therapy may at first glance seem unusual. However, genetic manipulation does not need to provide a missing or deficient product, but can be used to destroy cells or to confer sensitivity to a

specific drug. For example, transducing human leukaemia cells with HPRT confers sensitivity to purine analog antimetabolites (Howell *et al* 1987). This type of approach is discussed in more detail later in this Chapter. Certain cancers (for example retinoblastoma) are caused by inactivation of both alleles of a wild-type tumour suppressor gene. Infection of retinoblastoma cells with a retroviral vector containing the wild-type retinoblastoma suppressor gene leads to diminution of the tumourigenic properties of these cells when implanted into nude mice (Haung *et al* 1988). The inactivation of dominant oncogenes is much more complicated and would require large advances in site-specific targeted mutagenesis.

Myocytes

Muscle may be a suitable target tissue for many gene therapy protocols since it has a rich blood supply, makes up approximately 40 % of the mass of the body, and is readily accessible. Injection of pure RNA or DNA directly into mouse skeletal muscle has been shown to result in expression of reporter genes, including chloramphenicol acetyltransferase, luciferase and β-galactosidase. The expression showed a dose response, and was found to persist for several months. The exogenous genes appear to be present within the myocytes in a circular, extrachromosomal form (Wolff *et al* 1990). Direct injection of DNA into cardiac muscle has also resulted in expression of the exogenous DNA (Lin *et al* 1990). Expression of luciferase has been reported in primate myocytes following plasmid injection, although the level of expression was low (Jiao *et al* 1992). It has been shown that transplant of normal myocytes into a patient with Duchenne muscular dystrophy resulted in localized

production of dystrophin (Law *et al* 1990), and so delivery of a therapeutic product using genetically manipulated myocytes may be a feasible approach.

Keratinocytes and fibroblasts

Genetically modified skin grafts would be a safe way to introduce new genes into a recipient since the graft could be easily removed if neccessary. It has been found that proteins produced by skin grafts can enter the circulation although sometimes in much lower amounts than produced *in vitro*. This appears to be the case for many implantation sites (Palmer, Thompson and Miller 1989). Keratinocytes and fibroblasts can be readily removed from a donor and cultured *in vitro*. Skin grafts could therefore possibly be used to produce therapeutic products both locally (eg growth factors to aid wound healing) or systemically (eg coagulation factors, enzymes or hormones). It has been demonstrated that when transplanted epidermally even large proteins such as apolipoprotein E can diffuse into the circulation (Fenjeves et al 1989). The transduction of human keratinocytes with a retroviral vector containing the human growth hormone (hGH) gene and a mouse metallothionein promoter produced detectable blood concentrations of hGH following implantation into athymic nude mice for over 4 weeks (Teumer *et al* 1990).

1.5 Potential target diseases

As the development of more effective gene transfer techniques progressed, many diseases were deemed suitable for treatment by gene therapy (Cournayer and Caskey 1990). Some of the earliest targets have now been overthrown in favour of new and

previously unthought of disorders (for example CNS dysfunctions and cystic fibrosis). To demonstrate the rapid development of this field of research, I will first briefly consider why certain diseases are now not thought to be suitable for treatment by this approach, and then discuss promising new targets for gene therapy. This section will conclude with a review of clinical gene therapy trials currently underway.

Thalassaemias and haemoglobinopathies were tentatively proposed as suitable targets for gene therapy many years ago (Anderson and Fletcher 1980), mainly because the function of the globin genes was relatively well understood. These monogenic disorders result from an aberrant expression of one of the globin genes; thalassaemia sufferers die in early adulthood due to the effects of iron-loading caused by frequent blood transfusions. It has recently become clear, however, that the organisation and regulation of the globin genes is highly complex, with gene clusters comprising 5 active genes and 2 pseudo genes on chromosome 11 (β-globin chain), and 3 active genes, 1 pseudo gene and 1 gene whose function is as yet unknown on chromosome 16 (α-globin chain) (Steinberg 1991). The position of a gene in relation to its neighbours is also critical for correct expression; regulatory sequences far upstream of the β-globin gene cluster have recently been found to be important in regulating expression (Grosveld *et al* 1987, Sorrentino *et al* 1990). The amount and type of globin chains produced is also very important; 2 α- and 2 β-globin chains are required for correct formation of the haemoglobin tetramer. It is now becoming apparent that these disorders of globin expression are formidable targets for gene therapy. Globin must be produced within the erythrocyte, which necessitates the transduction of haemopoietic stem cells - the difficulties inherent in this approach have been

discussed previously. Futhermore, it is essential that α- and β-globin chains are produced in equal amounts - unbalanced expression of either one is in itself deleterious, and may cause a different disease phenotype.

Much media attention has recently been given to the prospect of gene therapy for the treatment of cystic fibrosis, and to a lesser extent, α1-antitrypsin deficiency. These are two of the most common potentially fatal hereditary diseases (Owen 1992). Cystic fibrosis is inherited as an autosomal recessive, and occurs in approximately 1 in 2500 live births. Clinical manifestations are apparent in many organs, including frequent respiratory infections and pancreatic exocrine insufficiency (both caused by abnormal thick mucus), infertility and early death. 95 % of all morbidity and mortality is due to pulmonary infections. Treatment consists of physiotherapy (to clear pulmonary mucus), antibiotics to fight infection, and pancreatic enzyme replacement. The genetic defect in cystic fibrosis has recently been described (Rommens *et al* 1989) and found to be a chloride channel regulated by a cAMP-dependent protein kinase A. This has been termed the cystic fibrosis transmembrane conductance regulator or CFTR. Although most CFTR appears to be expressed in the sub-mucosal glands of the lung with a lesser amount evident in airway lining cells, correcting the latter (which are accessible via aerosol delivery) may be sufficient. It has been shown that the respiratory manifestations of cystic fibrosis do not recur following heart-lung transplantation (de Leval *et al* 1991), and so gene therapy aimed primarily at the lung may be sufficient to greatly improve the clinical prognosis. *In vitro* studies indicate that transducing as little as 6 % of the airway lining cells may be enough to correct the defect; gap junctions between the cells may allow the

passage of chloride ions (Collins 1992). Several *in vivo* studies involving adenoviral vectors containing the CFTR gene have been carried out. Adenoviral vectors are suitable for this type of system since they can deliver recombinant genes to non-replicating cells (such as those of the pulmonary epithelium), and are also tropic for the lung. Following intratracheal administration of an adenoviral vector containing the CFTR gene to cotton rats, expression of CFTR was observed in the pulmonary epithelium, with the protein detectable for up to 14 days (Rosenfeld *et al* 1992). It is unlikely that CFTR would cause an immune response in the recipients since low levels of it can be found in most cystic fibrosis sufferers; however it is possible that an immune response may be raised to the adenoviral vector. The use of a retroviral vector would ensure efficient integration of the exogenous gene, but the pulmonary epithelial cells would need to be induced to replicate, possibly by oxidant injury. Thus gene therapy for the treatment of cystic fibrosis remains an exciting possibility.

1.6 Human gene therapy trials

Two distinct types of clinical studies involving the implantation of genetically manipulated cells are currently underway on 3 continents. The first type involves so-called gene marking whilst the second is gene therapy itself.

Gene marking

The first transfer of genetically manipulated cells into a patient in 1989 involved gene marking of tumour-infiltrating lymphocytes (TIL). These T cells have been used in the treatment of malignant melanoma - the TIL can be recovered from the tumour,

grown up in large numbers *in vitro*, and given back to the patient along with interleukin 2 (IL-2). This approach has been found to improve the prognosis (Rosenberg *et al* 1988b). A retroviral vector containing the neomycin-resistance gene has been used to mark TIL in order to study their longevity and localization *in vivo* (Rosenberg *et al* 1990).

Further gene marking protocols have been approved for clinical investigation. These include the marking of hepatocytes to assess the efficacy of hepatocyte transplantation (Ledley *et al* 1992), and the marking of HIV antigen-specific killer T cells in order to isolate them for subsequent reinfusion into irradiated AIDS patients (Lupton *et al* 1991, Riddell *et al* 1992).

Gene therapy

The initial target diseases of gene therapy involve the so- called 'housekeeping' genes, that is genes that are expressed constitutively at a relatively low level, and whose tight regulation is not critical (Weatherall 1991). The first clinical trial involving gene therapy was concerned with adenosine deaminase (ADA) deficiency. ADA deficiency causes an increase in 2'deoxyadenosine which is toxic to T and B cells causing a severe combined immunodeficiency (SCID). Treatment involves bone marrow transplantation or weekly injections of bovine ADA, which slightly improves the clinical prognosis. However to totally ameliorate the condition, ADA must be produced within the T cells themselves. The precise regulation of the ADA gene is not critical - ADA levels of 5 - 5000 % of normal are acceptable (Steinberg 1991). The gene therapy protocol utilised the patients' own T lymphocytes infected with a

retrovirus containing the human ADA and neomycin genes (neomycin was used as the selectable marker). The patients (both young children) received infusions of transduced cells at approximately monthly intervals, as well as weekly bovine ADA injections. This study has demonstrated the potential therapeutic value of gene therapy - both patients showed an improvement in clinical situation, with a significant increase in cell circulating ADA levels, and an increased lymphocyte count (Parkman and Gelfand 1991). The modified T cells persisted in the first patient for over 6 months after cell infusions were discontinued.

A further condition caused by a single-gene defect which is a promising target for gene therapy is familial hypercholesterolaemia. This is due to a defective LDL receptor gene in the liver; normally subsequent to receptor binding the lipoproteins are internalized by receptor mediated endocytosis and catabolized in lysosomes. Patients with a defective LDL receptor exhibit hyperlipidaemia and early mortality from coronary heart disease (Grossman and Wilson 1992). Animal studies in which the Watanabe hyperlipidaemic rabbit received an intrahepatic implant of hepatocytes manipulated to express the rabbit LDL receptor demonstrated a reduced cholesterol concentration in the blood for up to 4 months (Chowdhury *et al* 1991). A clinical trial at the University of Michigan has been approved (Wilson *et al* 1992).

The gene therapy protocols just described concern the transfer of exogenous genetic information into a cell with a missing or defective gene; the expression of the new gene ameliorates a pathological condition. A further extension of the gene-marking experiments involves the transfer of a gene to stimulate an immune reaction against,

or otherwise destroy, tumour cells. The gene for tumour necrosis factor (TNF), shown to be a powerful anticancer agent in mice (Asher *et al* 1987), has been transferred into TIL. The administration of TNF to humans causes systemic toxicity before the effective intratumoural concentration is reached. By engineering TNF secretion from TIL, it may be possible to produce a more physiological localized concentration of TNF within the tumour to enhance its immune destruction whilst avoiding systemic side effects; initial studies in mice are promising (Rosenberg *et al* 1992). Ongoing developments in this field involve the addition of a gene to the tumour cells themselves to make them more immunogenic, and hence stimulate the production of more effective TIL. The genes for TNF, interleukins, and γ-interferon have been expressed in murine tumour cells, with initial success (Gansbecher *et al* 1990, Tepper, Pattengale and Leder 1989).

It is not always necessary to remove cells from the patient in order to insert exogenous genes. The first trial to use this technique involved the direct injection of an HLA antigen encoding gene complexed with liposomes into a tumour, with the aim of increasing tumour immunogenicity (Miller 1992).

As an alternative to the use of genetic manipulation to provide a missing therapeutic product or to increase tumour immunogenicity, gene therapy could be used to sensitize tumour cells to a specific prodrug. This type of drug targeting would ideally involve a drug not toxic to the target tissue or to other organs. This form of therapy has been called virally directed enzyme prodrug therapy (VDEPT). Several such VDEPT protocols have been described; cells transduced with the herpes simplex

thymidine kinase gene display sensitivity to the anti-herpes drug ganciclovir, leaving normal cells unaffected, whilst cells manipulated to express cytosine deaminase convert the non toxic compound 5'-fluorocytosine (used as an anti-fungal agent) to the cytotoxic 5-fluorouracil, resulting in the death of the modified cells (Mullen, Kilstrup and Blaese 1992). It has been demonstrated that this type of VDEPT approach can be effective against hepatoma cells *in vitro* (Huber, Richards and Krenitsky 1991).

An exciting use for this technique is due to begin human trials shortly, involving patients with inoperable or inaccessible brain tumours (gliomas). Mouse fibroblast cells were infected with a retrovirus carrying the thymidine kinase gene. Pilot studies in rats have demonstrated the potential of this technique; following administration via an intratumoral injection the infected cells produced virions *in situ* which went on to infect the proliferating glioma cells. Since retroviruses can only replicate in actively dividing cells (a perceived disadvantage of the use of retroviral vectors), healthy non-dividing brain cells were not affected. Subsequent treatment of the rats with ganciclovir (which is metabolised by the thymidine kinase gene to a cellular toxin) caused the complete regression of the tumour in 11 of 14 rats (Culver *et al* 1992). This type of therapy can be expected to be particularly effective in a tissue such as the brain; the brain is relatively immunologically privileged and hence immuno-incompatible vector-producing cells could be expected to display relative longevity.

A further development of this approach is to attempt to confer a survival advantage on certain cell populations. Production of cytotoxic drug-resistant stem cells may be a mechanism by which normal cells could be protected from treatment toxicity. The theory is to render stem cells resistant to specific drugs, thus allowing much larger (and hence more effective) therapeutic doses to be used. An example of this would be the introduction of dihydrofolate reductase into stem cells, which would confer resistance to the chemotherapeutic agent methotrexate (Gutierrez, Lemoine and Sikora 1992). It is also possible to manipulate stem cells to produce certain colony-stimulating factors eg granulocyte colony-stimulating factor (G-CSF). G-CSF is a regulatory glycoprotein essential for the production and activation of neutrophils; administration of G-CSF accelerates the recovery of blood neutrophil count after chemo and radiotherapy. Transfection of G-CSF into fibroblasts subsequently transplanted into nude mice resulted in neutrophilia and an increase in haemopoietic progenitor cells in spleen (Tani *et al* 1989). It may be possible to infect stem cells but there is, however, a problem with this type of approach in that the efficiency of gene transfer into stem cells is very low as discussed previously.

1.7 Gene therapy for IDDM

Research into the field of diabetes has yielded an impressive number of firsts. Insulin was the first protein to be crystallized (1926); its amino acid sequence was determined in 1950 by Sanger; it was the first hormone to be chemically synthesized (1963); the first RIA was developed using insulin as a model by Yalow and Berson in 1959; and insulin was the first protein whose structure was determined by X-ray

crystallography (1972). The insulin gene was one of the first human genes to be cloned (1980), and in 1979 recombinant human insulin became the first therapeutic protein product to be commercially available (Selden *et al* 1987b, Owens, Vora and Dolben 1991, Ashcroft and Ashcroft 1992).

All diploid cells in the human body contain the insulin gene on the short arm of chromosome 11. At present the activation of the endogenous gene in a non-β-cell remains a distant possibility since not enough is yet known about the tissue specificity of gene expression. An alternative approach to gene therapy for diabetes is to insert an exogenous insulin gene (and a suitable promoter) into a suitable target cell population. There are formidable problems to be overcome when considering the design of a genetically modified cell for the treatment of diabetes. The normal function of the endogenous β-cell is considered in the Introduction to Chapter 3; there are several particular elements with the potential for development. It has previously been shown that a surrogate cell can be transfected to produce proinsulin (Selden *et al* 1987b). From a therapeutic viewpoint, a cellular implant that releases proinsulin constitutively offers only a very modest advantage over currently available therapies. However there are several important requirements for the cellular processing and release of insulin which may be amenable to genetic alteration.

Insulin is produced as a prohormone (proinsulin) which is split within the endogenous β-cell by endopeptidases and carboxypeptidase H (Bailyes, Guest and Hutton 1992). Proinsulin has only about 10% of the biological activity of insulin, and exerts most of its effects on the liver. In order for gene therapy for diabetes to be successful, the

first goal must be the correct processing of proinsulin to insulin. There are 2 possible approaches. One involves the use of a target cell that already contains suitable endopeptidases, and the other would involve cotransfection of the insulin gene and an endopeptidase gene simultaneously. Neuroendocrine cells contain secretory apparatus and prohormone processing endopeptidases which could be expected to package and process polypeptide precursors (as discussed in Chapter 3). For example transfection of the murine anterior pituitary cell line AtT20 with the preproinsulin gene results in the constitutive release of correctly processed insulin and C peptide, with a 10 fold increase in the insulin secretory response to cAMP (Moore *et al* 1983). However for a clinical application, the use of fibroblasts or keratinocytes would be more desirable, as discussed previously, and so it may be necessary to transfect a processing enzyme as well as the preproinsulin gene. To date the best characterized prohormone-processing enzyme is Kex2, which is found in yeast. It has been shown that cotransfecting various types of cell which have no endogenous endopeptidase processing ability with the ACTH precursor proopiomelanocortin (POMC) and Kex2 results in the correct processing of the ACTH (Thomas *et al* 1988), and that cotransfection of Kex2 and the protein C precursor into BHK cells (hamster kidney fibroblast cells) results in correct proteolytic cleavage (Foster *et al* 1991). It would be expected that proinsulin would be processed in the same manner since these prohormone precursors each have pairs of dibasic amino acid residues at their cleavage sites (Docherty and Steiner 1982). Recently a mammalian homologue of the Kex2 endopeptidase, PC2, has been isolated from a human insulinoma library (Smeekens and Steiner 1990). This has been found to be a Ca^{2+}-activated protease with a pH optimum of 5.5 (Shennan *et al* 1991), and experiments are currently

underway to cotransfect PC2 with the human preproinsulin gene into fibroblast cells, and to examine the extent of processing that occurs.

A further problem that must be addressed is that of 'closing the loop', or the glucose responsiveness of the cells. From the clinical perspective, an implant of cells that secretes a constant amount of insulin irrespective of the prevailing glucose concentration offers little advantage over existing therapies. The exact mechanism by which the β-cell alters the rate of insulin biosynthesis and release in response to changes in ambient glucose concentrations is very complex, and has not yet been fully elucidated. However certain areas of research are promising a greater understanding of this mechanism. The high capacity glucose transporter GLUT 2 has been reported to be defective in some spontaneous and experimentally induced animal models of diabetes; for example GLUT 2 mRNA expression is reduced in the β-cells of fatty Zucker rats, and the islets of these animals have an impaired insulin secretory response to glucose stimulation (Johnson *et al* 1990). It has been estimated that a 75 % reduction in GLUT 2 causes a decrease in glucose transport to rates which limit glucose metabolism by glucokinase. Hence in these animals increasing plasma glucose concentrations are not matched by an increase in insulin release. It has also been shown that the glycaemic clamping of normal rats at 50 mg/dl glucose (ie hypoglycaemia) leads to very low levels of GLUT 2 mRNA after 4 days, and that hyperglycaemic clamping at 200 mg/dl glucose causes an increase in GLUT 2 mRNA expression of 46 % over 5 days (Chen *et al* 1990). The insulin secreting cell line HIT T15 (which can alter insulin release in response to changes in ambient glucose concentrations) (Santerre *et al* 1981) expresses GLUT 2 whereas the RIN m5F cell

line (which is not responsive to glucose) (Gazdar *et al* 1980) only expresses the low activity GLUT 1 (Inagaki *et al* 1992). Thus transfecting the GLUT 2 gene may be of use in engineering glucose responsiveness by increasing the capacity of the cells to take up glucose; AtT20 cells naturally express the low capacity GLUT 1 glucose transporter. It has been shown that transfecting AtT20 cells with the insulin gene, and subsequently with the GLUT 2 gene results in an enhanced secretory response to glucose (Hughes *et al* 1992) - this is discussed in greater detail in the Discussion to Chapter 3.

However, since the transfection of cells allows not only the therapeutic gene of interest to be chosen, but also the promoter that drives the gene to be selected, it may not be necessary to use glucose as the signal for insulin release. For example the metallothionein promoter contains a heavy metal response element which responds to increasing levels of heavy metal ions by increasing the transcription of the gene (Durnam and Palmiter 1981). Hence in a cell transfected with a plasmid containing the insulin gene driven by a metallothionein promoter, increasing the concentration of zinc or cadmium should result in an accelerated production of insulin. Similarly, the mouse mammary tumour virus promoter is regulatable by corticosteroids (Lee *et al* 1981).

Further components of the β-cell secretory apparatus that may soon be available for transfection into surrogate cells includes the ATP-sensitive K^+ channel which closes due to increases in the ATP/ADP ratio preventing K^+ efflux (Docherty 1991).

1.8 Aims and objectives

The preceding literature review has considered the present state of knowledge of gene therapy. This thesis will examine the potential of this technique for the treatment of insulin-dependent diabetes mellitus. The initial step in any gene therapy protocol is the manufacture of a cell line which expresses the product in a suitable form and amount. Chapter 3 describes such work, and includes preliminary characterization of these cells, particularly concerning the secretory response to known β-cell secretagogues. Chapter 4 comprises an evaluation of the effect of immunosuppression by cyclosporin on endogenous β-cell function, and Chapters 5 and 6 describe the implantation of novel insulin-secreting cells into streptozotocin-diabetic athymic nude mice and immunosuppressed mice respectively.

CHAPTER TWO

MATERIALS AND METHODS

2.0. MATERIALS AND METHODS

2.1 Materials

^{125}I-insulin was purchased from Amersham International, Bucks, rat insulin from Novo Laboratories, Basingstoke, Hants, and insulin binding reagent from Wellcome Research Laboratories, Dartford, Kent. All other reagents were purchased from BDH Chemicals, Poole, Dorset, UK, or from Sigma Chemical Company, Poole, Dorset, UK.

2.2 Animal care

The animals were maintained in an air-conditioned room at 22 ± 2°C, with a light cycle of 12 hours light (0800-2000) and 12 hours dark. A standard rodent pellet diet (Rat and Mouse Breeding Diet No 1, Heygate and Sons, Northampton, UK) and tapwater were provided *ad libitum* throughout the study unless decreed otherwise by experimental procedure.

Animals were caged either singly (nude mice) or in groups of 4 to 6 (MF1 mice and Wistar rats). Body weight and food intake were determined regularly throughout the studies, the latter being taken as the difference in weight between the amount provided 24 hours previously, and the amount remaining in the hopper.

2.2.1 Blood sampling

Blood samples of approximately 60 µl for determination of plasma glucose and insulin were obtained from the cut tail-tip of conscious rats into microfuge tubes pretreated with 500 U/ml heparin. Blood samples were obtained from mice in the

same way and were approximately 30 µl in volume. Blood samples were stored on ice throughout the procedure. The plasma was separated by centrifugation at 15000g for 30 seconds; 20 µl was stored at -20°C with the addition of 20 µl 0.9 % saline for insulin assay, or 40 µl stored at -20°C for human C peptide assay, and 5 to 10 µl used immediately for glucose analysis.

2.2.2 Glucose tolerance tests

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance tests (IPGTT) were carried out on overnight fasted animals. The glucose dose used was 2g/kg body weight, administered as a 40 % w/v solution (ie 5ml/kg). Blood samples were usually obtained immediately before (ie time 0) and 30 and 60 minutes after administration of the glucose load. The plasma was separated for glucose and insulin or human C peptide assay as before.

Oral glucose tolerance test

Oral glucose tolerance tests (OGTT) were performed on overnight fasted animals. The glucose solution was administered by oral gavage; glucose dosage and blood sampling were carried out as in the IPGTT.

2.2.3 Induction of diabetes

Diabetes was induced in overnight fasted mice by intraperitoneal injection of 140 - 200 mg/kg streptozotocin dissolved in citrate buffer (Appendix 1) as a 30 mg/ml solution. The exact dose of streptozotocin used depended upon the severity of hyperglycaemia desired and also to some extent on the particular batch of streptozotocin in use. The streptozotocin solution was kept on ice between

solubization and injection to minimise the deterioration of anomers. Induction of diabetes was confirmed by the development of non-fasting plasma glucose concentrations of approximately 12 mmol/l and above after 4 days.

2.3 Cell culture methods

2.3.1 Cell culture media and supplements

DMEM (Dulbecco's modification of Eagles medium) and RPMI 1640 media, and L-glutamine were purchased from Gibco BRL, Uxbridge, Middlesex; fetal calf serum and and phosphate-buffered saline tablets (PBS) from Flow Laboratories, Scotland; Benzylpenicillin sodium BP and streptomycin sulphate BP were obtained from Glaxo and from Evans respectively; other chemicals from Sigma. Cel-Cult plastic tissue culture ware from Sterilin Ltd., Hounslow, Essex.

Both media used throughout these experiments (DMEM and RPMI 1640) were obtained as 500 ml sterile 1x solutions containing 2.00 g/l or 3.70 g/l bicarbonate respectively, but without glutamine. Media were supplemented by the addition of foetal calf serum to 10 %, L-glutamine to 2 mM, sodium benzylpenicillin to 100 μg/ml, and streptomycin sulphate to 100 units/ml. Supplements were sterilised by filtration and added to 500 ml of basic medium. Supplemented media were stored at 4°C for a maximum of four weeks.

Foetal calf serum was heat inactivated by incubating at 55°C for 30 minutes, and either used immediately or stored at -20°C. L-glutamine was made up as a 200 mM stock and stored in 5 ml aliquots at -20°C. Benzylpenicillin sodium BP and streptomycin sulphate BP were made up as a stock solution containing 10^5 units/ml

benzylpenicillin sodium and 10^5 µg/ml of streptomycin sulphate in distilled water. The stock solution was dispensed into small aliquots and stored at -20°C.

2.3.2 Maintenance and propagation of cell lines

Cells were grown in 90mm culture dishes (Nunclon) with 10 ml of medium at 37°C under 5% CO_2, 95% air. Medium was changed every 2 or 3 days, and cells were passaged when roughly 75 to 90 % confluent. Since cell lines grew at different rates, the "generation time" between passages varied, but was approximately every 7 or 8 days.

Passaging was performed by trypsinization, using a 0.5 % solution of trypsin (Sigma type III, bovine pancreas) diluted in phosphate buffered saline (PBS, see below). Optimal conditions varied for cell lines and to some extent between trypsin preparations. Medium was removed and cells were rinsed with 10 ml of sterile PBS, then 1 ml of diluted trypsin (final concentration 0.250 to 0.125 %) was added. After 2-5 minutes at room temperature trypsinization was stopped by the addition of cell culture medium, cells were dislodged by pipetting, transferred to a 30 ml sterile Universal tube and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in warmed culture medium, and 1 ml of the suspension was added to a 90mm culture dish containing 9 ml of warmed medium, and swirled gently to evenly distribute cells. Cells were normally passaged 1 to 5 (HIT T15) or 1 to 10 (all others).

To prevent constant passaging of cells, liquid nitrogen was used for storage. The cells were trypsinised as described above, and resuspended at a concentration of 10^7

cells/ml of 90% fetal calf serum, 10 % DMSO. 0.5 ml of the resulting suspension was aliquotted into each cryotube, frozen at -70°C overnight, then transferred into the liquid nitrogen for storage. When required, the cryotubes were rapidly thawed at 37°C, the cells were transferred to a sterile 30 ml Universal tube, and 10 ml of complete medium added dropwise. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes, the medium discarded, and the cells resuspended in medium and plated out as desired.

The trypsin stock (0.5 %) was made by dissolving powdered trypsin in distilled water, filtering through Whatman 91 paper and adjusting the pH to 7.5 with NaOH. The solution was sterilized by filtration through a 0.2 μm filter, dispensed into 5 ml aliquots and stored at -20°C.

PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) was prepared by dissolving 1 PBS tablet (Mg^{2+} free, Ca^{2+} free) in 100 ml of distilled water, autoclaved and stored at 4°C.

2.3.3 Cell lines used

The following cell lines were used in this study:

NIH 3T3 — A murine skin fibroblast line

AtT20 — A murine anterior pituitary (corticotrophic) cell line secreting ACTH and β-endorphin (Richardson 1978).

HIT T15	An insulin secreting pancreatic β-cell line derived by transformation of hamster pancreatic islet cells with SV40 (Santerre *et al* 1981)
RINm5F	An insulin secreting β-cell line derived from an implantable rat insulinoma (Gazdar *et al* 1980)

2.4 Analyses

2.4.1 Analysis of plasma glucose

Glucose was assayed by an automated glucose oxidase procedure (Stevens 1971) using a Beckman Glucose Analyser (Beckman Instruments, High Wycombe, Bucks, UK). The analyser uses the oxygen rate method to assay glucose; an oxygen electrode within the reaction well compares oxygen utilization by the sample with oxygen utilization of a standard glucose solution (a standard of 8.3 mmol/l glucose was used). The rate of oxygen utilization is directly proportional to the concentration of glucose in the solution:-

$$\beta\text{-D-glucose} + O_2 \xrightarrow[H_2O]{\text{glucose oxidase}} \text{glucuronic acid} + H_2O_2$$

The H_2O_2 is removed by two further reactions to ensure that it cannot yield back any oxygen to the solution:-

$$H_2O_2 + C_2H_5OH \xrightarrow{\text{catalase}} CH_3CHO + 2H_2O$$

$$H_2O_2 + 2H^+ + 2I \xrightarrow{\text{molybdate}} I_2 + 2H_2O$$

2.4.2 Analysis of plasma insulin

Insulin was measured in 20 µl plasma samples diluted 1:1 with 0.9 % saline. A radioimmunoassay (RIA) procedure was adopted using polyethylene glycol 6000 (PEG) for separation of free from bound antigen. Crystalline human or rat insulins were used as standards.

The general principle of RIA is based on the immunogenic properties of polypeptide hormones such as insulin (Ag); hence a specific antibody (Ab) can be raised when the hormone is administered to a species in which the hormone bears sufficient structural difference from the endogenous hormone to induce an immune response. A standard preparation of purified hormone can be labelled with a radioisotope (Ag* - in this case ^{125}I-insulin), and a known amount added to a limiting quantity of Ab. Unlabelled test hormone is then added; the Ab shows no preference in binding to Ag or Ag*, and since Ab and Ag* are both known constant values, the amount of Ag*-Ab formed is inversely proportional to the concentration of Ag (unknown).

Human or rat insulin standards were prepared by serial dilution in RIA assay buffer (Appendix 1) to give final concentrations of 10 ng/ml to 0.625 ng/ml. Insulin antiserum (IAS) was diluted in assay buffer to give a concentration of 1:30,000. ^{125}I-insulin was diluted in assay buffer to give approximately 10 - 12,000 cpm in 40 µl; typically a 1:20 to 1:10 dilution.

The following tubes were set up:-

total count	- empty
blank	- 80 µl buffer
standards	- 40 µl standard, 40 µl IAS
zero	- 40 µl buffer, 40 µl IAS
samples	- 40 µl sample, 40 µl IAS

All tubes were run in triplicate, except samples which were either duplicate or single. After mixing, tubes were covered and incubated at 4°C for 4 hours. 40 µl labelled insulin was then added to all tubes, which were again mixed, covered and incubated at 4°C overnight. 800 µl PEG buffer (Appendix 1) was added to all tubes except total count; after mixing the tubes were centrifuged at 3000 rpm for 30 minutes. The supernatant was discarded and the tubes allowed to dry for approximately 1 hour. The ^{125}I activity of the pellet was counted on an automatic gamma counter (LKB-Wallac 1282 CompuGamma, London) for 1 minute/tube. This system contains a computer package which produces a spline plot (a graph of counts per minute versus log concentration) of the standards.

2.4.3 Analysis of tissue insulin or C peptide content

The excised samples of pancreata or tumour were weighed and stored deep frozen. They were then extracted using 10 ml or 5 ml acid ethanol/g tissue respectively. (Acid ethanol was composed of 75 ml absolute alcohol, 25 ml dH_2O and 1.5 ml concentrated HCl). The tissue was disrupted by mechanical maceration, incubated at 4°C for 24 hours, then centrifuged at 2500 rpm for 10 minutes. The supernatant

was stored at -20°C until assayed for insulin at a dilution of 1:500, or for human C peptide at a dilution of 1:100.

2.5 Histological processing

The tumours processed for histological staining were treated as follows; briefly, after weighing, the tissue pieces were fixed by incubation in 10 % formal saline (10 % formalin in saline ie 4 % formaldehyde in saline) for 24 hours. The tissue samples were then dehydrated through a series of graded alcohols (70 % ethanol for 2 hours, 90 % for 2 hours, absolute alcohol for 2 hours then finally absolute alcohol overnight). The tissue was cleared in 2 changes of xylene for 2 hours each, then incubated in 2 changes of paraffin wax at 56°C for 3 hours each before setting in a paraffin wax block and incubated at 4°C overnight. Following embedding, sections 3-5 μm thick were cut using a rotary microtome, water-mounted onto slides and air dried at 37°C.

2.5.1. Haematoxylin and eosin staining

Haematoxylin and eosin staining was performed to demonstrate general cell morphology. Prior to staining the sections were cleared of paraffin wax and hydrated by immersion in the following: xylene 2 minutes, 2nd xylene 1 minute, 2 changes of absolute alcohol for 1 minute each, 90 % alcohol 30 seconds, 70 % alcohol 30 seconds, and finally tapwater. The slides were immersed in haematoxylin (Gurr Delafields haematoxylin) for 6 minutes, rinsed in running water for 15 minutes, and then immersed in eosin (water and alcohol soluble yellowish eosin) for 5 minutes. The slides were then washed in tapwater for 3 minutes, and redehydrated and cleared

by a reversal of the procedure described above ie tapwater through to xylene. The stained sections were dried and mounted under Canada Balsam.

2.5.2. Aldehyde fuchsin staining

Staining with aldehyde fuchsin was carried out to demonstrate the presence of insulin. These sections were dewaxed as described above, and then processed as follows. The stain used was Gomori's aldehyde fuchsin (Drury and Wallington 1980), composed of 0.5 g basic fuchsin, 1 ml conc HCl and 1 ml paraldehyde, made to 100 ml with 70 % alcohol. After mixing the stain was left at room temperature to ripen for 24 hours, then stored at 4°C. The counterstain used was Light Green (Drury and Wallington 1980), composed of 0.2 g Light Green, 1 g Orange G, 0.5 g phosphotungstic acid, 1 ml glacial acetic acid, made to 100 ml with dH_2O.

The sections were immersed in aldehyde fuchsin for 15 minutes, washed in 3 changes of 70 % alcohol, then counterstained with Light Green for 3 minutes. The sections were then washed and redehydrated and cleared by a reversal of the procedure described above ie tapwater through to xylene. The stained sections were dried and mounted under Canada Balsam.

2.5.3. Immunocytochemical staining

Sections of tumour were processed for immunocytochemical staining by Dr Irene Green, Department of Biological Sciences, University of Sussex, Brighton.

The method used was the peroxidase - antiperoxidase method as described in Sternberger (1979). Paraffin-embedded tumour sections were dewaxed in xylene for

10 minutes, then rehydrated through graded alcohols (90, 70 and 30 % for 2 minutes each) to tapwater. Sections were rinsed in PBS for 30 minutes, and non-specific attachment of antibody to the tissue was blocked by incubation with 10 % (vol/vol) goat serum for 10 minutes. The primary antibody was diluted in PBS (1:300), applied to the sections and incubated in a humid chamber overnight at 4°C. The antibody used was guinea pig anti-insulin. Controls for specificity were assessed by absorption of the primary antibody with the corresponding antigen, and by the substitution of primary antibody with nonimmune serum for 2 days at 4°C. The sections were exposed to goat anti-guinea pig antibody (diluted 1:300) for 1 hour at 37°C, rinsed in PBS and the anti-guinea pig peroxidase-antiperoxidase conjugate (1:16) was applied for 1 hour at 37°C. After 3 more 5 minute rinses, the peroxidase activity was visualized by a 0.05 % 3,3'-diaminobenzidine tetrahydrochloride dihydrate with 0.02 % hydrogen peroxide reaction for 10 minutes. The sections were finally mounted in glycerine/PBS (1:1).

2.6 Statistical analysis

Results were expressed as mean ± standard error of the mean (x ± SEM). Values within and between groups of animals were compared using Students paired and unpaired t-test respectively. Differences were considered to be statistically significant for $p < 0.05$.

CHAPTER THREE

GENERATION AND INITIAL CHARACTERIZATION OF NOVEL INSULIN-PRODUCING CELL LINES

3.1. INTRODUCTION

The generation of an artificial β-cell does not simply involve the transfection of the insulin gene. There are many other factors involved in the synthesis and secretion of insulin, and although it is as yet not possible to incorporate all of these into a surrogate cell, they may not all be essential. The mechanism by which the β-cell responds to changes in glucose supply and demand by altering insulin biosynthesis and secretion is highly complex. While glucose remains the major stimulus for insulin release, many other substrates, metabolites, hormones and neurotransmitters also exert an effect upon the β-cell. The net result of the many stimuli involved is a precise control of insulin release to adapt to a wide range of physiological conditions.

Insulin biosynthesis

The insulin gene is present in all cell types but is expressed only in pancreatic β-cells (Clark and Docherty 1993) although recently islet-like cell clusters in the duodenal mucosa close to the bile duct have been reported (Bendayan and Park 1991). The tissue specificity of insulin gene expression is not yet fully understood; transcription of the insulin gene is regulated by specific hormones and nutrient secretagogues. Glucose exerts a stimulatory effect on insulin gene transcription (German, Moss and Rutter 1990, Goodison, Kenna and Ashcroft 1991). The short-term control of insulin production involves the translation of preproinsulin mRNA in response to changes in blood glucose levels (Permutt and Kipnis 1972), possibly via increases in cAMP (Charles *et al* 1975). Additionally, the degradation of insulin mRNA in RINm5F cells is specifically inhibited by glucose (Welsh *et al* 1985). Other proteins involved in insulin secretion may also be affected by ambient glucose concentration - fasting has

been reported to cause a reduction in both β-cell glucokinase and GLUT 2 mRNAs, while refeeding induces expression of both genes (Tiedge and Lenzen 1991), although this has recently been disputed (Koranyi *et al* 1992). The concordant regulation of these genes may represent the basis of the physiological regulation of glucose-induced insulin secretion at a transcriptional level.

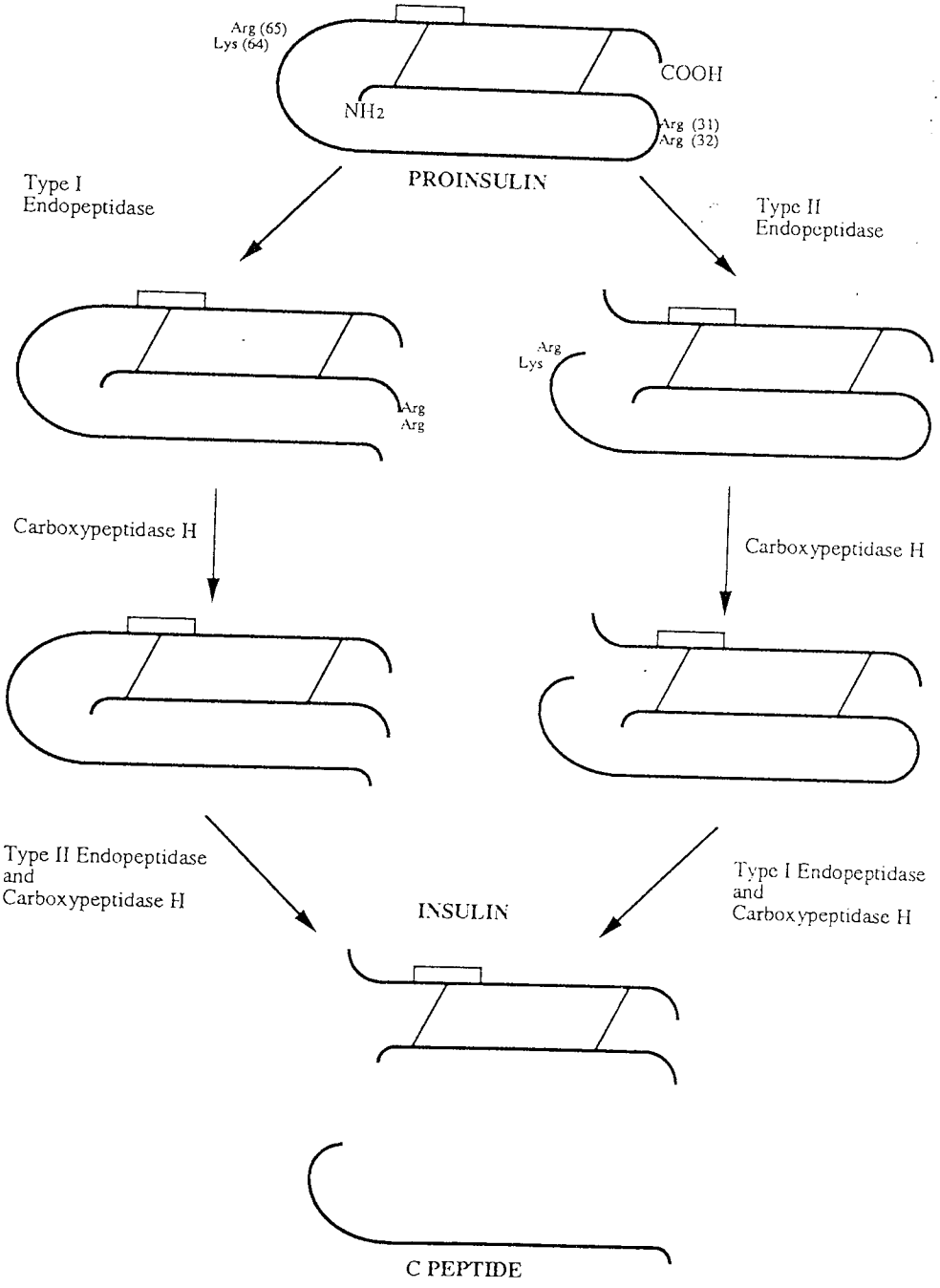
The initial stimulation of insulin biosynthesis which occurs within 20 minutes of exposure to glucose involves translational regulation, probably by increasing the rate of translational initiation (Welsh *et al* 1986). The translation product of insulin mRNA is preproinsulin (proinsulin plus a signal peptide). Translocation across the rough endoplasmic reticulum (RER) membrane results in cleavage of the signal peptide to give proinsulin; this cleavage is mediated by the signal peptidase on the luminal surface of the membrane (Lively 1989). Once inside the RER lumen, the newly-formed proinsulin folds, disulphide bridges are formed, and membrane-bound vesicles transport the protein to the cis elements of the Golgi complex. Within the Golgi, the proinsulin is packaged into immature secretory granules with clathrin-rich coats (Kitabchi, Duckworth and Stentz 1990). These proinsulin-rich immature granules have a neutral internal pH; mature non-coated granules contain mostly insulin plus C peptide and have an acidic pH of 5 - 5.5 (Guest and Hutton 1992). Acidification appears to be an important event in the initiation of prohormone conversion - this may be due to activation of proton translocases (Davidson *et al* 1988). The acidic pH of the granule interior matches the isoelectric point of insulin hence encouraging the formation of insulin crystals, and is also the pH at which the proinsulin-converting endopeptidases are maximally active (Davidson, Rhodes and Hutton 1988, Shennan *et al* 1991). As well as concentrating protons, the granule

membrane concentrates zinc ions, which are essential for crystallising the insulin (Cook and Taborsky 1990).

The proteolytic conversion of proinsulin to insulin occurs due to the presence of 2 endopeptidases: this is shown in Figure 3.1. Type I endopeptidase cleaves exclusively after the Arg^{31} - Arg^{32} site, and type II endopeptidase cleaves after the Lys^{64} - Arg^{65} site. Carboxypeptidase H, present in secretory granules, removes the exposed basic residues (Arg-Arg and Lys-Arg) to produce insulin and C peptide in equimolar amounts plus free arginine and lysine residues (Davidson and Hutton 1987). Small amounts of unconverted proinsulin and proinsulin intermediates are also present.

The mature granules enter a cytoplasmic storage pool; the most recently formed granules are preferentially released, whilst older granules eventually fuse with lysosomes and hence the constituents are recycled (Cook and Taborsky 1990). Insulin release occurs when the granule membrane fuses with the cell membrane, discharging the insulin crystal. Cytoskeletal structures such as microtubules and the cell submembrane microfilamentous web are critical for delivering granules to their membrane release sites (Ashcroft and Ashcroft 1992). As the interior of the cell plasma membrane and the secretory granule both have a negative charge, electrostatic repulsion occurs between the two. Calcium ions, with a positive charge, may facilitate fusion of the granule with the membrane. Contraction of the microfilaments are important to bring the granule close to the plasma membrane - this process is also mediated by calcium (Howell 1984).

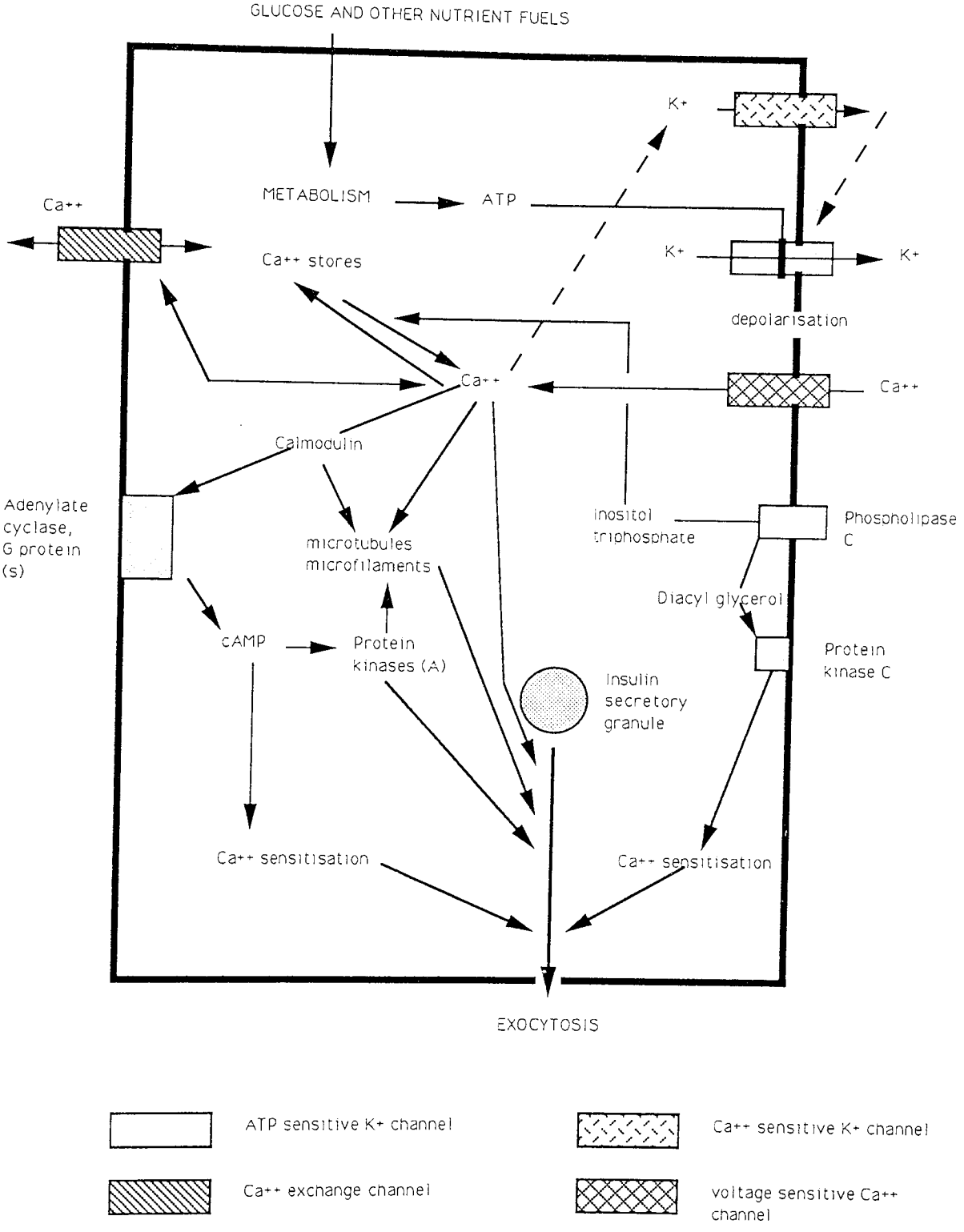
Figure 3.1. Endoproteolytic processing of proinsulin to insulin.



Insulin secretion

The phrase 'stimulus - secretion coupling' is used to describe how extracellular physiological stimuli of insulin secretion (eg glucose and arginine) are transformed into intracellular secondary messengers (eg cAMP and Ca^{2+}) which are known to be vital for insulin release. Figure 3.2 demonstrates the intracellular mechanism leading to insulin release by exocytosis. Briefly, high capacity glucose transporters (GLUT 2) in the β-cell membrane allow free entry of glucose, which is phosphorylated to glucose-6-phosphate by a high affinity hexokinase and a low affinity glucokinase (Malaisse *et al* 1976). However at physiological glucose concentrations, the glucokinase is the main enzyme involved in the phosphorylation of glucose, and may introduce a rate dependence to signal generation for glucose-induced insulin secretion (Lenzen 1992). When extracellular glucose concentrations rise (for example after a meal), increased glycolysis causes an increased flux of intermediary metabolites. The increased concentration of generated ATP (relative to ADP) causes the closure of ATP-sensitive K^+ channels, which in turn reduces the efflux of K^+ ions, and precipitates membrane depolarization (Ashcroft, Harrison and Ashcroft 1984). At a certain transmembranal potential difference, voltage dependent Ca^{2+} channels open, causing an influx of Ca^{2+}, which triggers insulin release (Hughes, Christie and Ashcroft 1987). Glucose also causes intracellular Ca^{2+} mobilization from endoplasmic reticulum stores via phosphoinositide metabolism - levels of diacylglycerol (DAG) and inositol triphosphate (IP_3) increase, possibly by activation of membrane bound phospholipase C. DAG (with Ca^{2+}) activates protein kinase C (PK_C) in the membrane, which increases insulin secretion by sensitizing the secretory mechanism to the existing intracellular Ca^{2+} concentration (Hughes and Ashcroft 1988). IP_3, in the cytoplasm, causes release of Ca^{2+} from intracellular stores.

Figure 3.2. Stimulus-secretion coupling within the β-cell.



The membrane bound enzyme adenylate cyclase is activated by various stimuli (including Ca^{2+} - calmodulin) with G proteins (guanine-nucleotide binding proteins) implicated in signal transduction (Gomperts 1990), and causes conversion of ATP to cAMP. Increasing intracellular levels of cAMP increase insulin release by sensitising the secretory mechanism to Ca^{2+} and also via protein kinase A (PK_A). PK_A may stimulate insulin release by phosphorylation of the voltage dependent Ca^{2+} channel (Hughes and Ashcroft 1992) and also by action on the secretory machinery. PK_A may also be involved in transcription of the insulin gene (Goodison, Kenna and Ashcroft 1991).

Other metabolic signals

Most amino acids can stimulate insulin release to varying extents (Henquin 1987) - the mechanism involved for amino acids such as leucine is likely to be by intracellular metabolism by transamination, resulting in increased ATP. Other amino acids such as arginine may also act via a different pathway, for example through nitric oxide which may affect the G proteins and hence the adenylate cyclase system (Schmidt *et al* 1992). Glucagon (secreted by the α-cells of the islet) stimulates insulin release via a receptor mediated effect on adenylate cyclase, and hence activation of PK_A (Christie and Ashcroft 1985). IBMX stimulates insulin release by inhibition of a phosphodiesterase hence increasing intracellular cAMP concentrations.

The biosynthesis and secretion of insulin are not, however, neccessarily coupled since these processes can be dissociated under certain conditions. The removal of Ca^{2+} from the medium causes inhibition of glucose-stimulated insulin release in isolated islets, whilst insulin biosynthesis is still operational (Pipeleers, Marichal and Malaisse

1973a). The phosphodiesterase inhibitor IBMX potentiates glucose-stimulated insulin release but does not affect insulin biosynthesis (Ashcroft *et al* 1978). Sulphonylurea drugs used for the treatment of Type II diabetes (maturity onset diabetes) stimulate insulin secretion but not insulin biosynthesis (Beck-Nielsen, Hother-Nielsen and Pedersen 1988). Furthermore, the threshold for glucose-induced activation of insulin synthesis (2.5 - 3.9 mM) is lower than that for insulin secretion (4.2 - 5.6 mM) (Pipeleers, Marichal and Malaisse 1973b).

The artificial β-cell

Thus it can be seen that the processes involved in the biosynthesis and release of insulin are extremely complex. Attempting to create a similar system within a surrogate cell is at present not possible, but the subtleties and refinements present within the β-cell may not all be essential to achieve constitutive insulin production and release within a surrogate cell. Many types of cell contain packaging and secretory machinery; some also possess endopeptidase processing activity. If a cell such as this could be grown *in vitro* and the exogenous DNA of interest incorporated into the genome, production, processing and secretion of the novel protein may occur. This Chapter describes such work, and also initial studies on the properties of the transfected cells.

3.2. MATERIALS AND METHODS

3.2.1. Transfection of fibroblasts with the human insulin gene

Chemicals

Geneticin G418 was purchased from Gibco/BRL, Paisley, Scotland.

Cell maintenance

NIH 3T3 fibroblast cells were maintained under standard tissue culture conditions as described in Chapter 2.

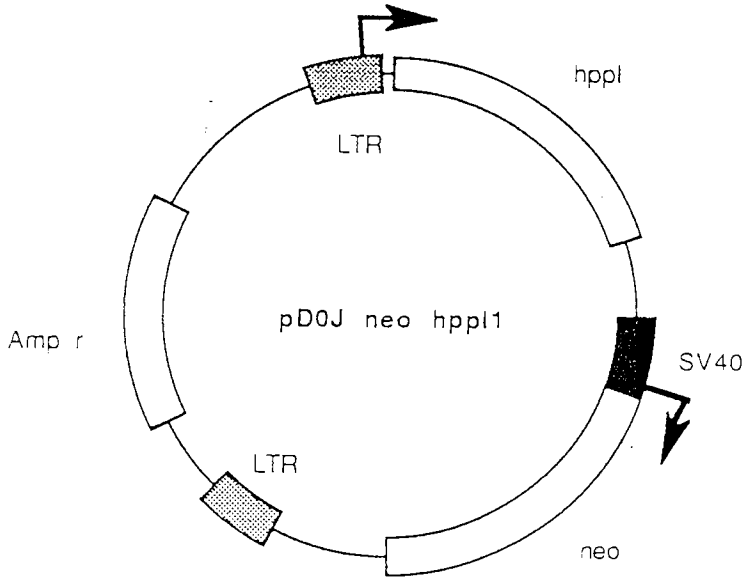
Plasmid construction

The plasmid pDOJhPPI/1 containing the human insulin gene was a kind gift of Dr Neil Taylor, Department of Medicine, University of Birmingham, Birmingham. The structure of this plasmid is shown in Figure 3.3. The human preproinsulin cDNA was cloned into the Eco R1 site and is expressed under the control of the upstream viral long terminal repeat (LTR) which contains a strong viral promoter and enhancer. The neomycin resistance gene is controlled by the SV40 early promoter, and is used as the selectable marker. (The ampicillin resistance gene enables the plasmid to be grown in bacterial cells).

Calcium phosphate cotransfection

A 2x solution of Hepes Buffered saline (HBS) was made up from a 10x stock (8.18 % NaCl, 5.94 % HEPES, 0.2 % Na_2HPO_4, percentages w/v), and the pH adjusted to 7.12 using 1M NaOH. A 2.5 M solution of $CaCl_2$ was made up and sterilised by filtration.

Figure 3.3. Plasmid construct DOJNeohPPI1. (LTR = long terminal repeat, hppI1 = human preproinsulin gene, SV40 = Simian virus early promoter, neo = neomycin resistance gene, amp = ampicillin resistance gene). Diagram not to scale.



Cells were transfected when at 50 to 75 % confluence (ie whilst still rapidly growing). Approximately 4 hours prior to transfection, cells were fed with complete DMEM.

In most cases several replicate transfections were performed. Solutions were made up as follows, where n is the number of replicates.

Solution A: 25 n µl of 2.5 M $CaCl_2$; DNA (usually 5 to 30 µg per plate of cells to be transfected); sterile distilled water to 250 n µl.

Solution B: 250 n µl of 2x HBS.

Solution A was slowly added to solution B, mixed by gentle vortexing for a few seconds and left at room temperature for 15 minutes. A 500 µl aliquot of this solution was added to each cell monolayer, followed by gentle swirling to evenly distribute the precipitate. Cells were incubated overnight at 37°C, then the medium was removed and the cell monolayer was washed once with 10 ml of unsupplemented DMEM and once with 10 ml of PBS. Fresh medium was added and the culture dishes were replaced in the incubator.

Cells were passaged as usual and selection was undertaken by the addition of Geneticin G418 (500 µg/ml) to complete DMEM. After addition of the G418, the cells were left for approximately 10-14 days to allow untransfected cells to die. The surviving colonies were picked using cloning rings and trypsin as described in Chapter 2. Each individual colony was transferred into one well of a 6-well plate with 2 ml of complete DMEM. These G418 resistant cells were allowed to grow for 2-3 days

then an aliquot of the medium was removed for assay of insulin-like immunoreactivity (ILI). Colonies which appeared to release the greatest amount of insulin were chosen for further study.

3.2.2. Effect of secretagogues on proinsulin- and insulin-secreting transfected cells

The transfected AtT20hPPI/1 (insulin-secreting) and AtT20hPPI/D34,35 (proinsulin-secreting) cells used in this study, and in subsequent implantation studies, were a kind gift of Dr Neil Taylor, Department of Medicine, University of Birmingham, Birmingham, UK. The manufacture of these cells has been previously reported (Taylor and Docherty 1992).

Chemicals

Trasylol (Aprotinin) was purchased from Bayer UK Ltd, W Sussex, UK.

Maintenance of cells

The transfected cells (AtT20MtNeohPPI/1 and AtT20MtNeohPPI/D34,35) were maintained under standard tissue culture conditions as described in Chapter 2.

Media containing various known β-cell secretagogues were prepared from complete DMEM, supplemented to give test media with the following concentrations:-

Test media	Glucose (mM)	Additive
Control	10	-
High glucose	16.7	D-glucose
K^+ 15 mM	10	KCl
Ca^{2+} 7.6 mM	10	$CaCl_2.2H_2O$
Arginine 20 mM	10	L-arginine monochloride
IBMX 5 mM	10	IBMX
Zn^{2+} 90 µM	10	$ZnSO_4$
Cd^+ 5 µM	10	CdCl
Glucagon 1 nM	10	

(Additions as in Flatt *et al* 1987).

All media were filter sterilized in the hood. The cells were seeded into 6 well plates at approximately 1.5 x 10^5 cells per well and incubated for 24 hours in complete (ie control) DMEM, 2.0 ml per well. This was then removed and stored at -20°C until insulin assay. 2.0 ml of test media was then added to each well (4 wells per test medium), and a 24 hour incubation at 37°C was carried out. Sampling was performed as before, and a further 24 hour incubation was carried out.

3.3. RESULTS

3.3.1. Results of transfections

Using 3T3 cells, calcium phosphate transfection experiments using the vector pDOJneohPPI/1 were carried out. The initial results of these transfections are shown in Table 3.1. It can be seen that all colonies which demonstrated G418 resistance also produced a material with an insulin-like immunoreactivity.

3.3.2. Effect of secretagogues on proinsulin- and insulin-secreting transfected cells

HPLC analysis of medium in which G418-resistant AtT20 cells had grown demonstrated that these cells released different products - the pMtNeohPPI/D34,35 transfected cells produced mainly proinsulin basally and when stimulated with 1 mM IBMX (Figure 3.4a and 3.4b), and the pMtNeohPPI/1 transfected cells produced mainly correctly processed insulin under basal and stimulated conditions (Figure 3.5a and 3.5b) (Taylor and Docherty 1992).

The secretory response of the transfected AtT20hPPI/D34,35 cells (proinsulin-secreting) and AtT20MtNeohPPI/1 cells (insulin-secreting) to various secretagogues *in vitro* was observed by measurement of insulin-like immunoreactivity in the medium. The first 24 hour incubation was a control period in normal DMEM medium; there were 2 consecutive 24 hour incubations in test medium supplemented as described in the Methods section. Results are presented as change from basal for days 1 and 2 respectively.

Table 3.1 Insulin-like immunoreactivity (ILI) released into the medium by G418-resistant 3T3 fibroblast cells following calcium phosphate transfection with pDOJhPPI/1.

	ILI release by colony (ng/ml)	ILI release/10^6 cells/24hr (ng/ml)
Transfection 1		
colony 1	0.180	-
colony 2	0.220	-
colony 3	0.211	-
colony 4	0.200	-
colony 5	0.167	-
colony 6	0.545	5.4
colony 7	0.187	-
Transfection 2		
colony 1	0.260	1.8
colony 2	0.180	-
Transfection 3		
colony 1	0.185	-
colony 2	0.200	1.5

Figure 3.4a. HPLC elution profile of insulin-like immunoreactivity released by AtT20MtNeohPPI/D34,35 cell line under unstimulated (control) conditions.

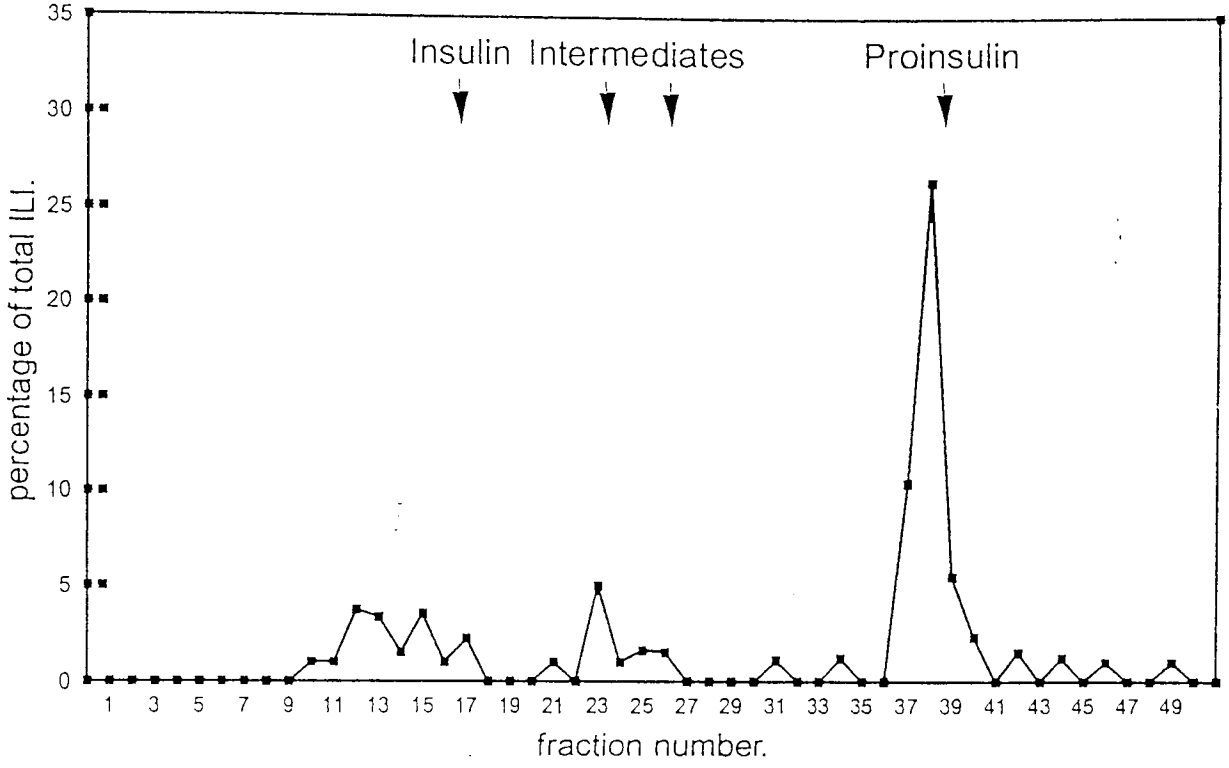


Figure 3.4b. HPLC elution profile of insulin-like immunoreactivity released by AtT20MtNeohPPI/D34,35 cell line under stimulated conditions (1 mM IBMX).

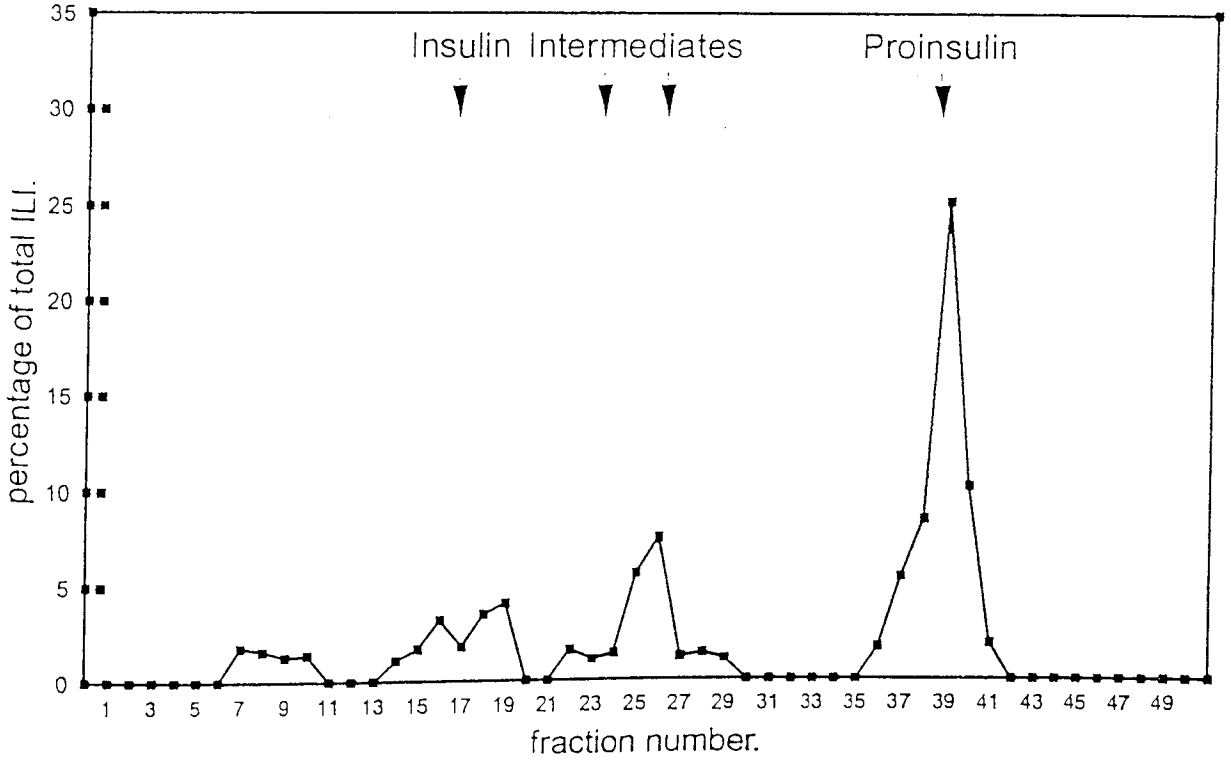


Figure 3.5a. HPLC elution profile of insulin-like immunoreactivity released by AtT20MtNeohPPI/1 cell line under unstimulated (control) conditions.

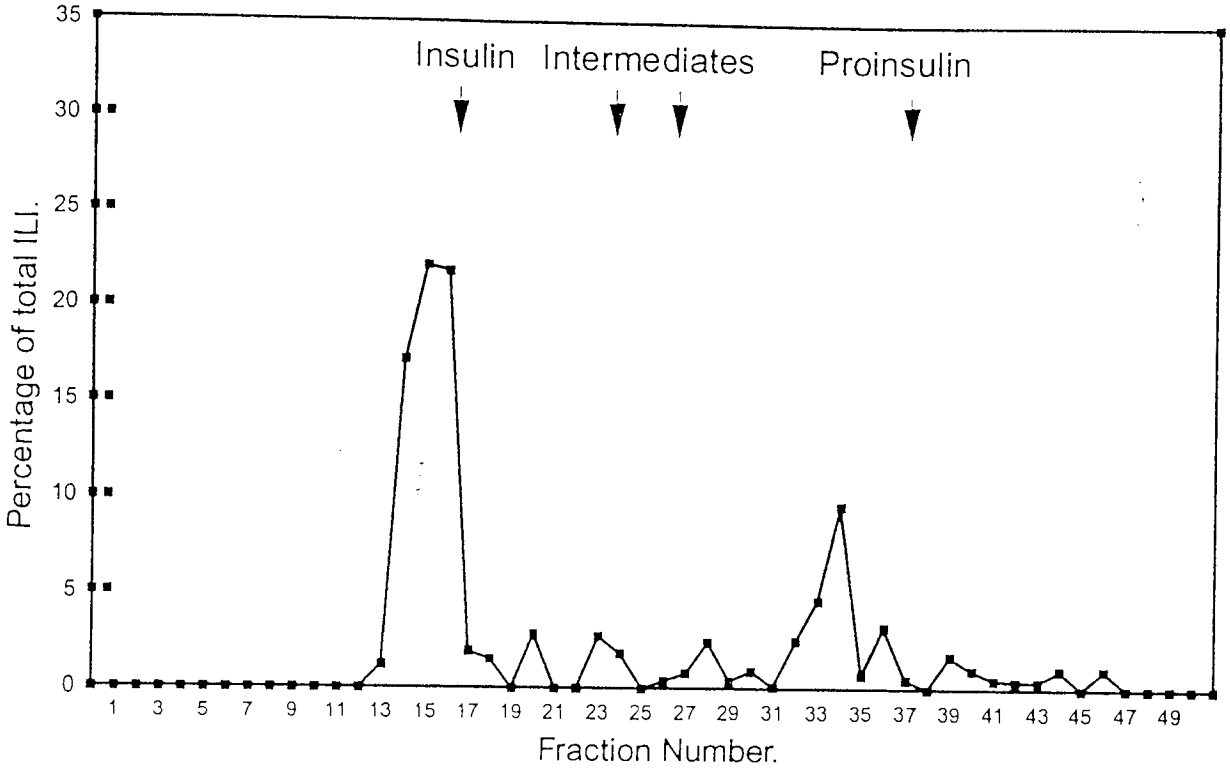
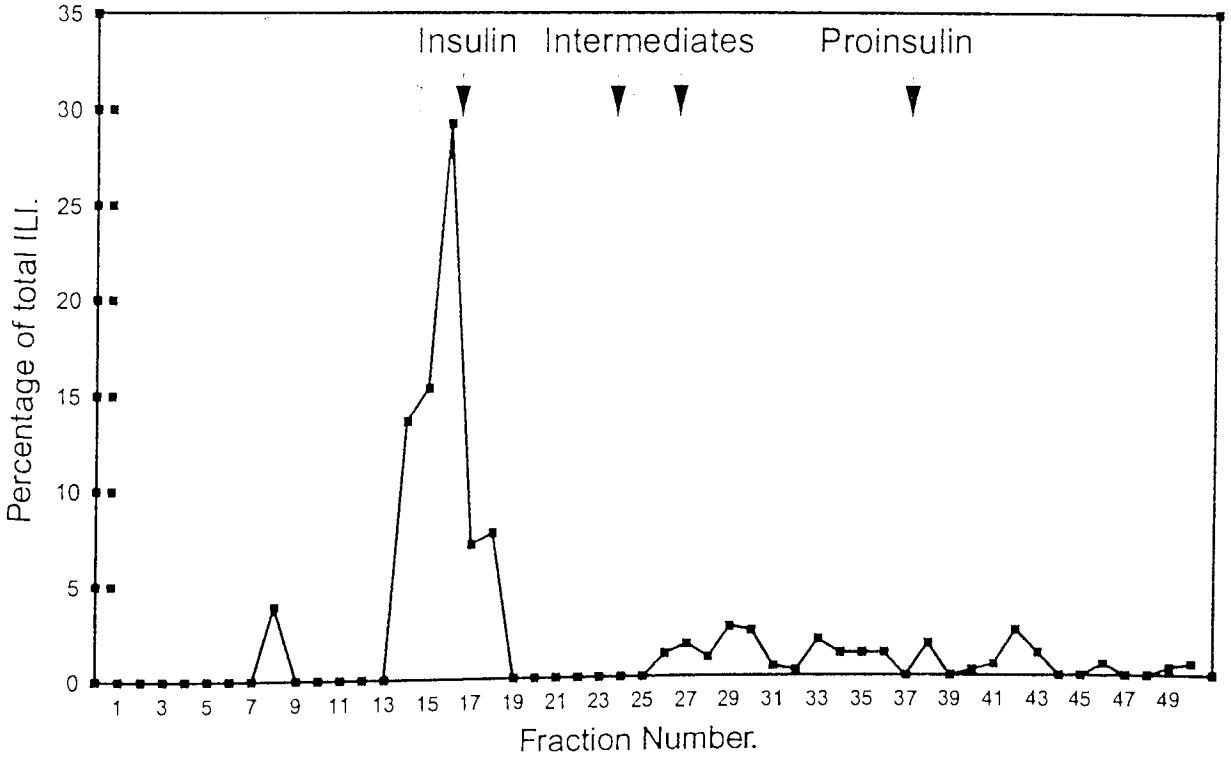


Figure 3.5b. HPLC elution profile of insulin-like immunoreactivity released by AtT20MtNeohPPI/1 cell line under stimulated conditions (1 mM IBMX).



AtT20MtNeohPPI/D34,35 cells

These results are shown in Figures 3.6a and 3.6b.

No significant differences in release of insulin-like immunoreactive material after either the first or second test incubation were found in cells incubated in control medium or in media supplemented with 16.7 mM glucose, 15 mM K^+, 7.6 mM Ca^{2+}, 1 μM glucagon or 5 μM Cd^+.

Cells incubated in medium containing 20 mM arginine showed a significant reduction ($p<0.05$) in proinsulin release between days 0 and 1, but a significant increase between days 0 and 2 ($p<0.001$).

The addition of 5 mM IBMX to the medium caused a significant increase in proinsulin release between days 0 and 1, and days 0 and 2 ($p<0.01$ and $p<0.001$ respectively).

No significant change in proinsulin release was observed in cells incubated in medium supplemented with 90 μM Zn^{2+} between days 0 and 1. However, a significant increase ($p<0.001$) was observed between days 0 and 2.

AtT20MtNeohPPI/1 cells

These results are shown in Figures 3.7a and 3.7b.

No significant differences in release of insulin-like immunoreactive material after either the first or second test incubation were found in cells incubated in control

Figure 3.6a. Insulin-like immunoreactivity (ILI) of medium after culture of 1.5 x 10^5 AtT20MtNeohPPI/D34,35 cells for 24 hours in 2 ml medium containing different agents. Control (column 1) contained 10 mM glucose. Test media contained either 16.7 mM glucose (column 2), or 10 mM glucose. Agents used include 15 mM KCl, 7.6 mM $CaCl_2$, 20 mM arginine, 5 mM IBMX, 90 µM $ZnSO_4$, or 5 µM CdCl (columns 3 to 9 respectively). Results are mean ± SEM (4). [a] $p<0.05$, [c] $p<0.01$ compared to control.

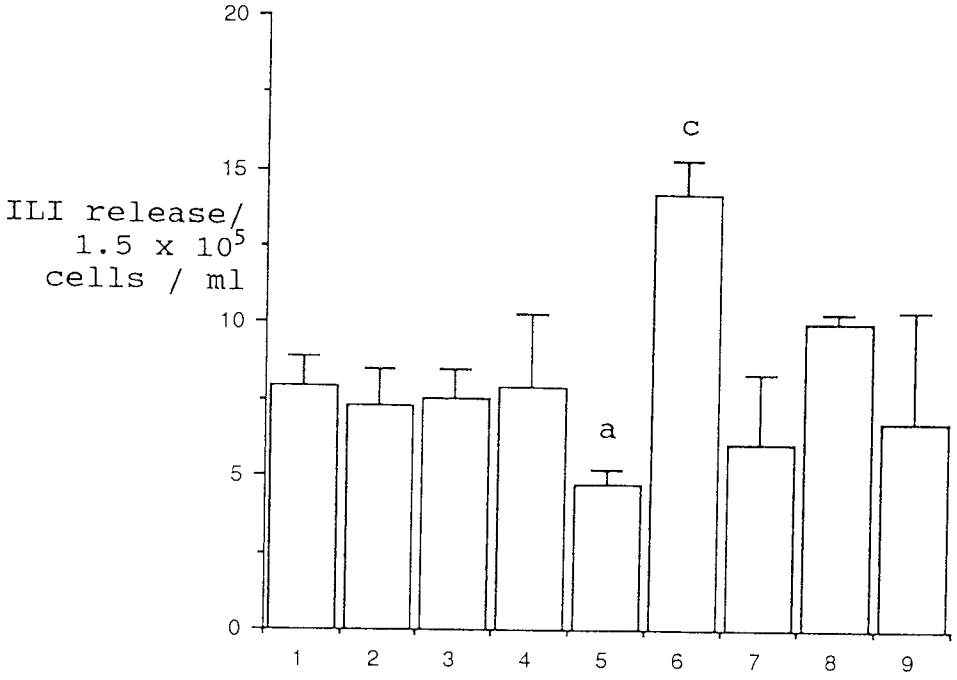


Figure 3.6b. Insulin-like immunoreactivity (ILI) of medium after culture of 1.5 x 10^5 AtT20MtNeohPPI/D34,35 cells for a subsequent 24 hours in 2 ml medium containing different agents. Control (column 1) contained 10 mM glucose. Test media contained either 16.7 mM glucose (column 2), or 10 mM glucose. Agents used include 15 mM KCl, 7.6 mM $CaCl_2$, 20 mM arginine, 5 mM IBMX, 90 µM $ZnSO_4$, or 5 µM CdCl (columns 3 to 9 respectively). Results are mean ± SEM (4). [d] $p<0.001$ compared to control.

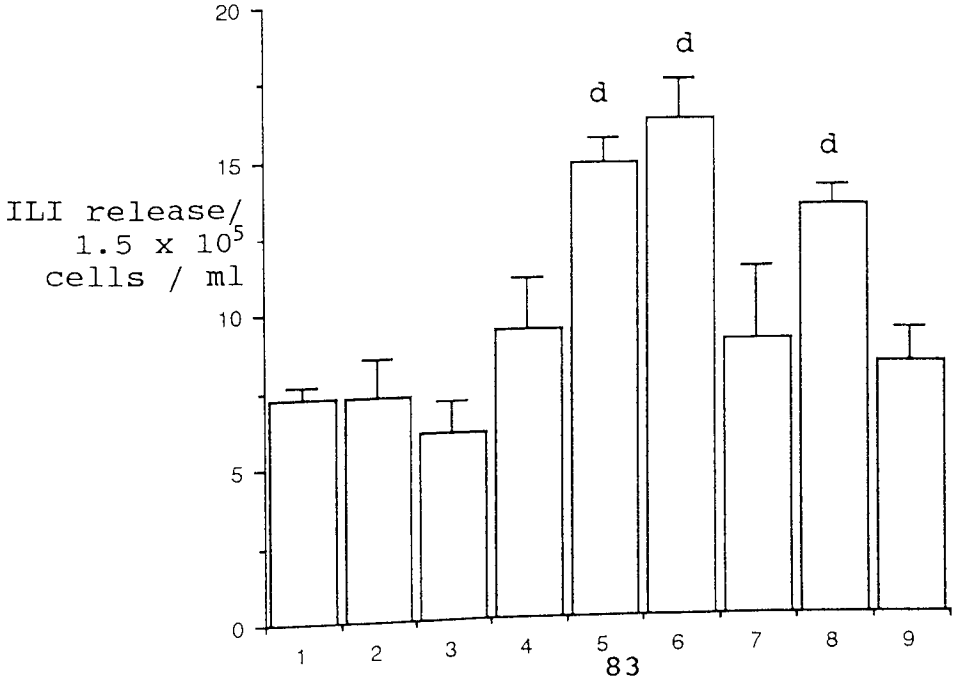


Figure 3.7a. Insulin-like immunoreactivity (ILI) of medium after culture of 1.5 x 10^5 AtT20MtNeohPPI/1 cells for 24 hours in 2 ml medium containing different agents. Control (column 1) contained 10 mM glucose. Test media contained either 16.7 mM glucose (column 2), or 10 mM glucose. Agents used include 15 mM KCl, 7.6 mM $CaCl_2$, 20 mM arginine, 5 mM IBMX, 90 µM $ZnSO_4$, or 5 µM CdCl (columns 3 to 9 respectively). Results are mean ± SEM (4). [a] $p<0.05$, [c] $p<0.01$ compared to control.

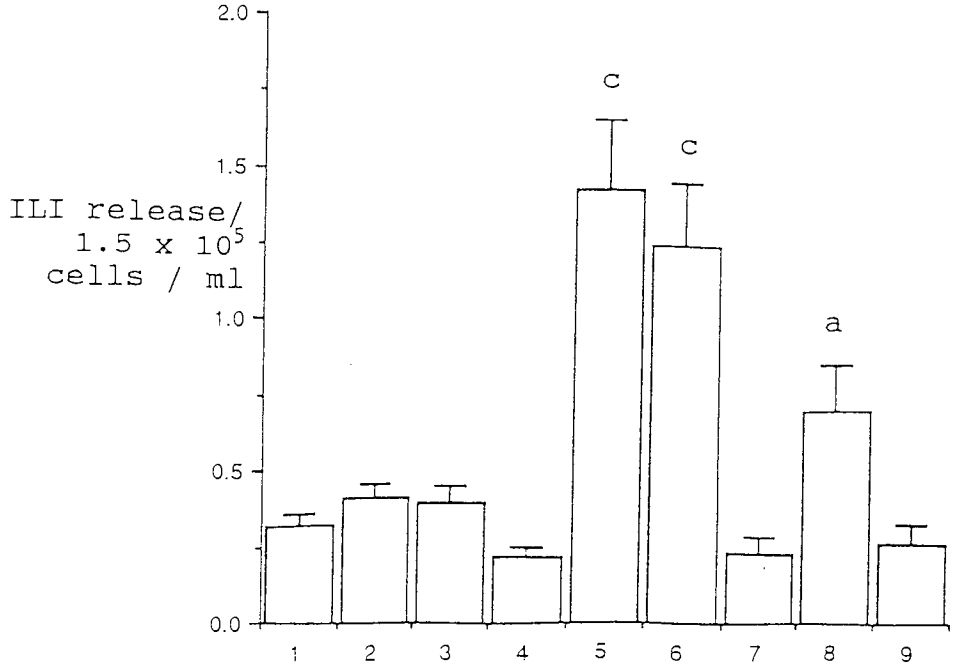
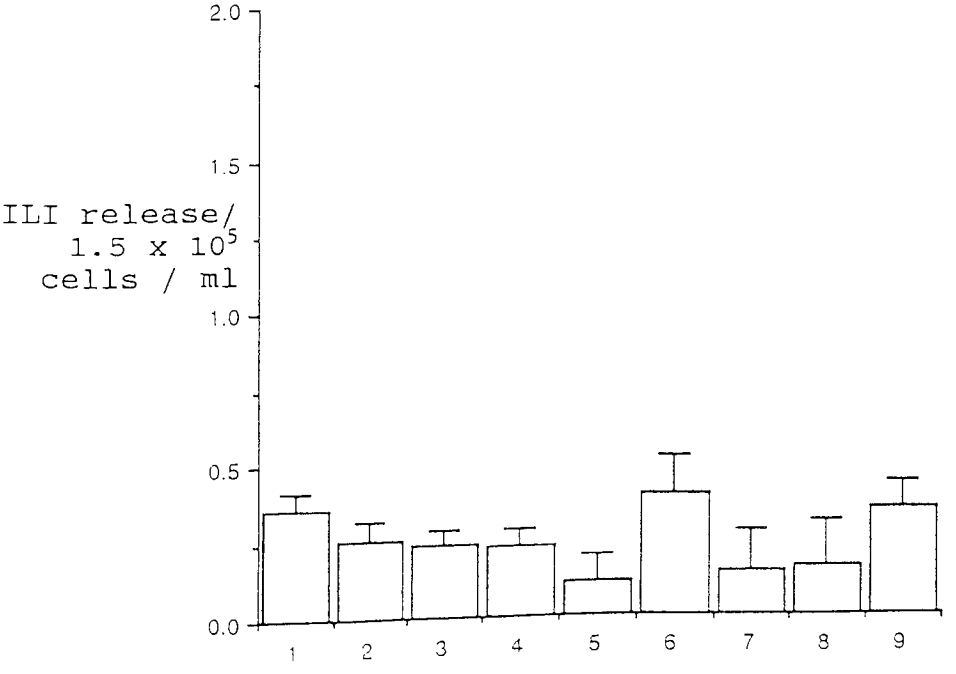


Figure 3.7b. Insulin-like immunoreactivity (ILI) of medium after culture of 1.5 x 10^5 AtT20MtNeohPPI/1 cells for a subsequent 24 hours in 2 ml medium containing different agents. Control (column 1) contained 10 mM glucose. Test media contained either 16.7 mM glucose (column 2), or 10 mM glucose. Agents used include 15 mM KCl, 7.6 mM $CaCl_2$, 20 mM arginine, 5 mM IBMX, 90 µM $ZnSO_4$, or 5 µM CdCl (columns 3 to 9 respectively). Results are mean ± SEM (4).



medium or in media supplemented with 16.7 mM glucose, 15 mM K^+, 7.6 mM Ca^{2+}, 1 μM glucagon or 5 μM Cd^+.

The addition of the test substances 20 mM arginine, 5 mM IBMX or 90 μM $ZnSO_4$ all caused an increase in the release of insulin-like immunoreactive material between days 0 and 1 ($p<0.01$, $p<0.01$ and $p<0.05$ respectively), with no significant differences observed between days 0 and 2.

3.4. DISCUSSION

Transfection studies

The transfection results presented for the 3T3 cells and the AtT20 cells demonstrate that it is possible to manipulate a cell to express an exogenous gene, and produce and release a foreign product. Stable lines were established which released either proinsulin or insulin either constitutively or via a regulated pathway. Fibroblasts are secretory cells (releasing collagen) but do not release large amounts of product, and since they do not possess endopeptidase processing enzymes, cannot process proinsulin to insulin. As may be predicted, the release of proinsulin from these cells was at a relatively low rate when compared to the AtT20 cells. Since proinsulin has only approximately 10 % of the biological activity of mature insulin (Kitabchi, Duckworth and Stentz 1990), the greatest efficacy of an implant upon hyperglycaemia is likely to result from the transplantation of cells releasing processed insulin. The AtT20 cells transfected with the wild-type human insulin gene (the pMtNeohPPI/1 vector) produced correctly processed human insulin (as demonstrated by HPLC analysis), and released it via the regulated pathway (as demonstrated by an increased secretory rate in response to various secretagogues, which is discussed in greater detail below). Hence implantation studies described later in this thesis use this cell line AtT20hPPI/1. The AtT20 cells transfected with a mutated human insulin gene (the pMtNeohPPI/D34,35 vector) where the Arg^{31}-Arg^{32} site was changed to Arg-Gly, and the Lys^{64}-Arg^{65} site was changed to Thr-Arg produced human proinsulin (demonstrated by HPLC analysis) at a high rate, and also released it via the regulated pathway, since changes in secretory rate were also obtained in response to various secretagogues. The observation that certain of these AtT20 cells will

accurately sort novel proteins into the regulated secretory pathway, and furthermore process them correctly is particularly interesting. It is therefore appropriate to consider the mechanisms that are operative in pituitary cells which sort and subsequently process the proopiomelanocortin precursor (POMC).

Sorting into secretory granules

Specialized secretory cells have 2 secretory pathways, namely the constitutive and the regulated pathway. The former comprises the export of proteins to the cell surface directly after synthesis, mainly by a bulk-flow process. The regulated pathway actively sorts peptides into dense core secretory granules for storage prior to release (Kelly 1985). Secretagogues can stimulate the release of only those proteins packaged in secretory vesicles in the regulated pathway; proteins in the constitutive pathway are unaffected (Moore, Gumbiner and Kelly 1983).

The trans-Golgi network appears to be the site where proteins destined for different sites in the cell are sorted from each other, and where the constitutive and regulated pathways diverge (Tooze and Tooze 1986). The endogenous sorting of proinsulin is very efficient - less than 1 % is secreted constitutively (Rhodes and Halban 1987). Cleavage of the peptide hormones occurs after sorting. Immature secretory vesicles have a clathrin-rich coat which is lost upon maturation of the granule. In the AtT20 cell line, POMC is cleaved within the secretory granules, and mature hormone products are secreted by the regulated pathway upon stimulation - over 80 % of ACTH is released by this pathway (Gumbiner and Kelly 1981). In contrast the constitutive pathway mostly releases unprocessed POMC (Gumbiner and Kelly 1982).

Processing is not required for correct sorting - almost all proinsulin secreted by normal islets is released from the regulated pathway. Also, transfection of AtT20 cells with mutant proinsulins (with the C peptide absent or replaced with the C peptide from insulin-like growth factor 1) results in correct sorting into secretory granules, although no cleavage occurs (Powell *et al* 1988). Hence the sorting signal must be within the exposed regions of the A and/or the B chain of the insulin molecule. As yet the signal for sorting proteins into the regulated pathway has not been elucidated; it is thought that constitutive release occurs by default. The signal must be well conserved between cell types and species, since many secretory cells will correctly sort foreign transfected proteins to secretory granules (Bailyes, Guest and Hutton 1992). It is possible that many short segments from different parts of the molecule may comprise the sorting signal, rather than one continuous sequence of amino acids.

It has previously been reported that when AtT20 cells are transfected with an insulin gene, mature (processed) insulin and ACTH are present within the same secretory granules (Orci *et al* 1987).

POMC (and proinsulin) processing

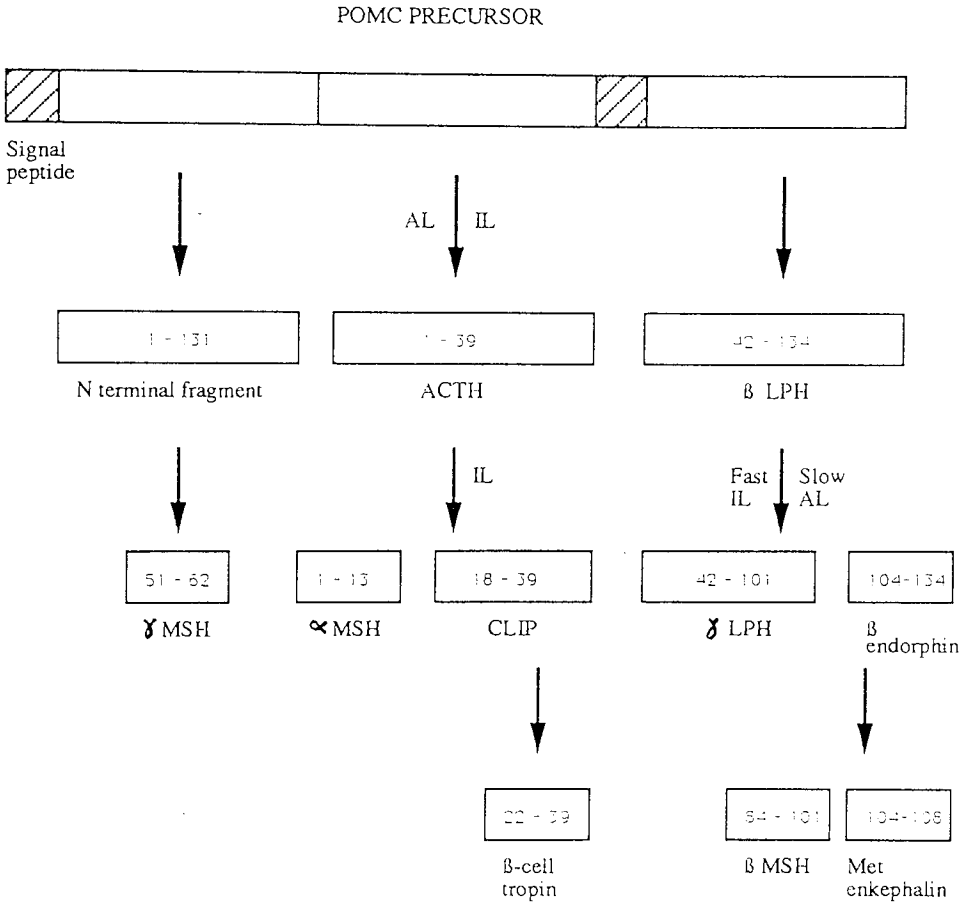
Since proinsulin requires proteolytic cleavage to produce mature insulin (and C peptide), it is possible that transfection of the insulin gene into a cell containing prohormone processing activity will result in the correct processing of the insulin. Neuroendocrine cells synthesize neuropeptides as polypeptide precursors, which lack biological activity, and may give rise to several products. When synthesized in different tissues, post-translational processing can produce different products from the

same precursor. This tissue specific processing often involves endoproteolytic cleavage.

The neuroendocrine cell line AtT20 is derived from the murine anterior pituitary (Richardson 1978). This cell line expresses the POMC precursor. This prohormone can be split endoproteolytically at dibasic amino acid pair sites to form a variety of products - the precise products formed depends upon the tissue in which POMC is expressed (see Figure 3.8) (Hadley 1992). Pituitary cells contain the prohormone-processing enzymes PC1 and PC2, which are homologues of the yeast Kex2 endopeptidase (Bloomquist, Eipper and Mains 1991). In the anterior pituitary, it is mostly PC1 which is expressed, and this expression is inducible by corticosteroids (Day *et al* 1992). The AtT20 cell line processes the POMC precursor to release the N-terminal fragment, ACTH, β-LPH and some β-endorphin (Eipper and Mains 1980). (Cells of the intermediate lobe of the pituitary process β-LPH further to γ-LPH and β-endorphin; ACTH is processed to α-MSH and CLIP). Cotransfection of POMC and PC2 into adrenal cells enabled correct processing of the precursor to γ-LPH and β-endorphin (Thomas *et al* 1991). The AtT20 cell line has been shown to be able to correctly process many precursors following transfection of exogenous genes including proinsulin, proenkephalin, prosomatostatin, and procholecystokinin (Dickerson and Noel 1991). The production of mature peptide from these precursors requires cleavage at Lys-Arg and Arg-Arg dibasic sites, which are also the sites that are cleaved during the processing of endogenous POMC.

Figure 3.8. Endoproteolytic processing of proopiomelanocortin.

(POMC = proopiomelanocortin, ACTH = adrenocorticotropic hormone, LPH = lipotropin, MSH = melanocyte-stimulating hormone, CLIP = corticotropin-like intermediate lobe peptide. IL = intermediate lobe of pituitary, AL = anterior lobe of pituitary). (Hadley 1992).



Effect of secretagogues on insulin- and proinsulin-secreting cells

The results obtained when proinsulin- or insulin-secreting transfected pituitary cells were incubated in media containing various β-cell secretagogues illustrate that these cells do not behave like endogenous β-cells as regards (pro)insulin secretory response.

No change in release of insulin-like immunoreactive material was observed in cells incubated in 16.7 mM glucose, 15 mM K^+, 7.6 mM Ca^{2+}, 1 μM glucagon or 5 μM Cd^+. These substances (with the exception of Cd^+) cause an increase in insulin release from endogenous β-cells. The mechanism by which these secretagogues affect the β-cell have been described in the Introduction to this Chapter; briefly, metabolism of both glucose and leucine causes an increase in the ATP/ADP ratio leading to insulin release, an increase in extracellular K^+ causes increased insulin release by depolarization of the plasma membrane, increased concentrations of Ca^{2+} increases insulin release by a variety of mechanisms, and glucagon mediates its effects via adenylate cyclase.

The inability of these genetically manipulated pituitary cells to respond to certain known β-cell secretagogues suggests that secretion of (pro)insulin from these cells does not occur in a β-cell-like manner, and that parts of the secretory machinery present in β-cells are not active in pituitary cells. The inability of transfected AtT20 cells to respond to an increased ambient glucose concentration with an increased release of insulin is of particular concern when the treatment of IDDM is considered. A constant background infusion of insulin would be of value for the control of excessive hyperglycaemia, but for gene therapy to offer a substantial improvement

over existing therapies, glucose-responsiveness is vital. AtT20 cells express the low capacity glucose transporter GLUT 1. This may be a limiting factor for the uptake and subsequent metabolism of glucose in these cells. It has been shown that transfecting AtT20 cells with the insulin gene, and subsequently with the GLUT 2 gene results in an enhanced glucose responsiveness. These cells demonstrated an increased insulin content (ie increased insulin biosynthesis), and responded to changes in the ambient glucose concentration over a range of 0 to 20 mM glucose with altered amounts of insulin released (and also with altered amounts of ACTH), and potentiated the insulin secretory response to glucose plus forskolin (Hughes *et al* 1992). It is possible that in transfected AtT20 cells with the higher capacity glucose transporter, glucose-stimulated insulin release occurs via a glucose regulated signal for secretory granule membrane fusion and exocytosis; insulin and ACTH are found to be co-localized in the same secretory granules (Orci *et al* 1987). However the maximal insulin release was obtained at approximately 10 μM glucose, with little increase at concentrations up to 20 mM glucose; islets do not respond to concentrations below about 4 - 5 mM glucose. This shift may be due to the limiting factor of insufficient glucose phosphorylation, although AtT20 cells contain an islet-like form of glucokinase which is only present in a few other tissues, namely liver and islets (Hughes *et al* 1991).

No secretory response to 5 μM Cd^{+} was observed in these cells. The exogenous insulin gene within the plasmid is driven by a mouse metallothionein promoter, which is known to contain several heavy metal ion response elements (Thiele 1992). Metallothionein is a metal-binding protein involved in the detoxification of heavy metals, and has a high affinity for cadmium, zinc, mercury and copper. Transcription

of metallothionein can be increased by administration of these metals (Durnam and Palmiter 1981). It would therefore be expected that increasing the extracellular Cd^+ concentration would stimulate the metallothionein promoter resulting in an increased level of transcription of the insulin gene, and therefore an increased release of proinsulin into the medium. However, incubation of these cells in media supplemented with Cd^+ was found to have no effect on insulin release. The transfection of L cells (fibroblasts) with a vector containing a thymidine kinase gene driven by a metallothionein promoter demonstrated that transcription of thymidine kinase was inducible by Cd^+. Incubation of the cells for 14 hours in 20 μM CdCl caused a 2 - 2.5 fold increase in thymidine kinase activity. However, only 5 out of 10 clones tested demonstrated any Cd^+ sensitivity (Mayo, Warren and Palmiter 1982). Other workers have found that fibroblasts transfected with the human insulin gene driven by a metallothionein promoter showed an increase in proinsulin release of between 2 and 3 fold when incubated for 48 hours in medium containing 10 μM $CdSO_4$ (Kawakami *et al* 1992). It seems unlikely that the lack of response to Cd^+ found in this experiment is due to a toxic effect this element on the cells, since other workers have used greater concentrations, albeit for shorter time periods. Since a stimulatory effect of $ZnSO_4$ upon the (pro)insulin release by these cells was observed (discussed below), it would at first glance appear strange that no stimulatory effect of cadmium was observed. However, many binding factors for metal response elements have been described (Andersen *et al* 1990), and recently one factor has been described which appears to be sensitive only to zinc (Labbe *et al* 1991). The binding of this factor, termed zinc activated protein, is maximally activated by 60 - 100 μM $ZnSO_4$ (Thiele 1992), which is in concordance with the concentration used in this experiment.

Increased media concentrations of L-arginine, IBMX and Zn^{2+} all caused a significant increase in release of insulin-like immunoreactive material by both the insulin- and proinsulin-secreting transfected cells at some time points during the study.

IBMX (a phosphodiesterase inhibitor) decreases the rate of degradation of cAMP, which is an important secondary messenger in many types of cell and activates PK_A. Increased concentrations of cAMP are known to increase transcription of the insulin gene (Goodison, Kenna and Ashcroft 1991), and it has recently been reported that adenosine agonists (substances such as 2-chloroadenosine which mediate their effect via adenosine agonists and the cAMP pathway) can induce the production of metallothionein mRNA and protein in liver cells (Xiong *et al* 1992). Increased amounts of metallothionein mRNA were detectable by 2 hours after the start of stimulation. Hence a substance which increases intracellular cAMP concentrations may increase the rate of transcription of the metallothionein gene, presumably via a stimulatory effect on the promoter.

The stimulatory effect of Zn^{2+} is presumably mediated via the heavy metal ion response elements present within the metallothionein promoter in the plasmid, as described above (Durnam and Palmiter 1981). The incubation of fibroblasts transfected with the human growth hormone (hGH) gene driven by a metallothionein promoter in medium containing 100 μM $ZnSO_4$ caused an approximate 10 to 20 fold increase in hGH release after 8 days (Selden *et al* 1986). The transient expression in baby hamster kidney (BHK) cells of the thymidine kinase gene driven by a metallothionein promoter can be induced approximately 20 fold by incubation in 100

μM $ZnSO_4$. (Imbert *et al* 1990) The action of the metal response element(s) within the promoter region appears to be complex, with many combinations being effective for zinc responsiveness (Searle, Stuart and Palmiter 1985). Zinc is also important for the correct crystallization of insulin within secretory granules (Cook and Taborsky 1990).

The insulin-stimulatory effect of L-arginine on these cells is particularly interesting. L-arginine is known to stimulate insulin release from islets (Blachier *et al* 1989). This effect is thought to be mediated by nitric oxide (NO), which is produced (together with L-citrulline) by the action of NO synthase on L-arginine (Schmidt *et al* 1992). NO causes an increase in intracellular cGMP by the activation of soluble guanylyl cylase which converts GTP to cGMP (Feelisch and Noack 1987); cGMP is known to be a mediator of insulin secretion (Laychock 1981), and to be involved in stimulus-secretion coupling, and thus insulin release may partly depend upon nitrinergic signal transduction (Schmidt, Warner and Murad 1992). NO has also been implicated as a mediator of β-cell destruction - cytokines such as interleukin-1β may induce production of NO which attacks intracellular iron-containing proteins resulting in β-cell dysfunction and, ultimately, death (Corbett and McDaniel 1992). In contrast to observations described above, some workers beleive that arginine-induced insulin release and concurrent increases in intracellular cGMP are unrelated (Jones *et al* 1992, Vincent 1992). Increases in cGMP can exert several effects including effects on ion channels, stimulation of cGMP-dependent protein kinases, and both increases or decreases in cAMP. Stimulation of protein kinases causes secretion of insulin (Lowenstein and Snyder 1992). The incubation of isolated islets in media supplemented with L-arginine demonstrated increased cGMP production in a time

and concentration dependent manner, and the addition of a soluble guanylyl cyclase inhibitor (LY83583) abolished the effect of L-arginine on cGMP concentrations and insulin release (Laychock, Modica and Cavanaugh 1991). Exogenous arginine can enhance agonist-induced, NO mediated increases in cGMP in brain slices (Garthwaite 1991), and NO synthase activity has been found to be present in all brain tissues examined to date including cerebellum, cortex, midbrain, medulla and hypothalamus (Forsterman *et al* 1990). It therefore appears possible that the stimulatory effect of L-arginine on these cells is mediated via increases in intracellular protein kinases, which have a generalized stimulatory effect on the secretory machinery of the cell.

Conclusion

This Chapter has demonstrated that non-β-cells can be manipulated to incorporate and express the human (prepro)insulin gene. The manufacture of novel proinsulin- and insulin-secreting cell lines has been described, and the response of these cells to standard β-cell secretagogues, and substances thought to affect the metallothionein promoter has been investigated. Whilst these cells do not behave in a β-cell-like manner (for example no increase in insulin release was observed in response to increasing glucose concentrations) insulin was released in amounts which may be effective in ameliorating experimental diabetes in rodent models.

Since these murine AtT20 cells have been maintained in culture for many years, it is likely that due to antigenic drift, they would provide an immunological challenge when implanted into mice. For this reason investigations into the effects on endogenous β-cell function of immunosuppression with cyclosporin A were undertaken to ascertain any deleterious action. Since gene therapy may also

ultimately involve containment of genetically modified cells prior to implantation, studies were also performed on the efficacy of insulin release by HIT T15 cells following encapsulation within alginate microcapsules. These studies are described in Chapter 4.

CHAPTER FOUR

IMMUNOSUPPRESSION AND IMMUNOISOLATION

4.1 INTRODUCTION

A major focus of current research into the future treatment of IDDM concerns the transplantation of insulin-secreting tissue. This includes whole pancreatic grafts, and implants of isolated islets or β-cell isolates as discussed in Chapter 1. To maintain the function of allogeneic or xenogeneic transplants, immunosuppressive therapy is required to prevent rejection and possibly to prevent destruction of the donor tissue by the original disease process of the host. The eventual aim of gene therapy would be to transfect cells taken from each individual patient and thus circumvent the need for immunosuppression (although it is possible that there may be an immune response by the patient to the 'new' therapeutic product - this has been observed in patients with haemophilia or Christmas disease treated with clotting factors VIII and IX, McMillan *et al* 1988). However, the initial studies described in this thesis have utilized immortalised cultured murine cells, and thus the use of an immunosuppressive regimen is required.

Cyclosporin A (CyA) is now used almost universally for immunosuppression of allogeneic transplant recipients. Recent developments have suggested that the ideal therapy comprises of a drug 'cocktail', ie the combined administration of CyA, prednisone and azathioprine, following a short course of anti-lymphocyte globulins (Pozza and Secchi 1989). Using drugs in combination causes less severe toxicity and fewer side effects, since smaller doses of each can be given.

Mechanism of action of immunosuppressive drugs

Under non-immunosuppressed conditions, rejection of transplanted 'foreign' tissue occurs due to cellular immunity mediated by T lymphocytes and lymphokines. T helper cells ($CD4^+$ T cells) cannot recognise free antigen - to enable recognition, antigen must be presented with MHC class II glycoproteins, on antigen presenting cells (APCs). Graft destruction may occur in 2 ways. Firstly, foreign MHC class II antigens on the graft stimulate T helper cells to help cytotoxic T cells destroy the transplanted tissue. Cytotoxic T cells ($CD8^+$ T cells) recognise the target cells via the foreign MHC class I antigens (transmembranal glycoproteins). Secondly, helper T cells react to the foreign tissue by releasing lymphokines (including interleukin 2), which stimulate macrophages to enter the graft and destroy it. Activated T cells divide rapidly and cause lysis of foreign cells (Roitt, Brostoff and Male 1989).

Azathioprine is a purine antimetabolite and kills T lymphocytes as well as all other rapidly dividing cells. This drug is non-specific and increases the risk of cancers. Prednisone, a corticosteroid, kills T cells by an unknown mechanism, and is also diabetogenic. Anti-lymphocyte globulin causes destruction of mature T cells and their precursors, but has relatively poor specificity and can lead to severe thrombocytopenia (Bonomo and Caccavo 1989).

CyA acts preferentially and reversibly on T lymphocytes, inhibiting lymphokine production and release (Kessler *et al* 1991). The exact mechanism is as yet unknown; membrane events associated with triggering the antigen receptor (eg IP_3 generation or calcium mobilization) are not inhibited. However, the transcriptional activation of lymphokine genes are blocked (Emmel *et al* 1989). CyA, therefore, must interfere

with the function of molecules that transmit signals between membrane events (eg IP_3 generation) and the activation of genes. CyA is a potent immunosuppressive agent whose use has greatly improved the prognosis of transplant surgery. However it has several serious side effects including nephrotoxicity and acute and chronic side effects on renal function (Mason 1990). Thus the use of CyA for pancreas/islet transplant recipients must be carefully evaluated, particularly in view of the liability of diabetic patients to develop nephropathy (Osterby 1992). Several workers have observed a deleterious effect of CyA on the glucose tolerance of transplant recipients (Engfeldt *et al* 1986). These findings are discussed in greater detail in the discussion section of this Chapter. The first section of work described in this Chapter comprises detailed studies of the effect of CyA on glucose tolerance in the rat, and *in vitro* studies using an insulin-secreting cell line, RINm5F.

Immunoisolation

An alternative approach to avoid the problems associated with immunosuppression would be to encapsulate the tissue within a protective biocompatible membrane. If the tissue could be isolated in this way, graft rejection by T lymphocytes, or autoimmune destruction caused by islet cell surface or cytotoxic antibodies present in the plasma of diabetic patients (Dobersen *et al* 1980) could be prevented.

Alginate-polylysine-alginate microcapsules have been used for the immunoisolation and subsequent implantation of several types of tissue. The ability of encapsulated islets to prolong allograft and xenograft survival *in vivo*, and to reverse experimentally-induced diabetes is well documented (O'Shea, Goosen and Sun 1984, O'Shea and Sun 1986, Iwata *et al* 1989, Iwata, Takagi and Amemiya 1992). There

have been conflicting reports concerning the magnitude of the insulin secretory response obtained from islets contained within an alginate microcapsule - some workers report no impairment of insulin secretion compared to free islets (Fan *et al* 1990), whilst others report a substantial decrease in the level of secretion by encapsulated islets (Chicheportiche and Reach 1988, Levesque, Brubaker and Sun 1992).

Alginate-polylysine-alginate microcapsules have also been used to encapsulate cells, including hepatocytes. Implantation of microcapsules containing hepatocytes to recipient rats with hepatic necrosis resulted in a 50 % increase in survival rate compared to controls receiving no implant (Sun and O'Shea 1985).

Microcapsules of this type may therefore prove capable of protecting implanted tissue against both rejection and attack by the original disease process of the (diabetic) host. The second section of work described in this Chapter concerns the encapsulation of HIT T15 cells within an alginate-polylysine-alginate membrane, and the insulin response obtained by incubation in media of different glucose concentrations.

This Chapter therefore investigates two approaches by which transfected cells may be protected from immune attack following implantation across an MHC barrier; by immunosuppression, and immunoisolation.

4.2 MATERIALS AND METHODS

Animals

Adult male Wistar rats of approximately 140 g body weight were used. The rats were maintained under standard laboratory conditions as described previously. A rodent pellet diet (Rat and Mouse Breeding Diet No 1, Heygate and Sons, Northampton, UK) and tapwater were provided *ad libitum* throughout the study unless decreed otherwise by experimental procedure.

Chemicals

The cyclosporin A was a kind gift of Sandoz Pharmaceuticals, Camberley, Surrey, UK. All other chemicals were purchased as previously described.

Experimental procedures

Animals were caged in groups of 6. Body weight and food intake were determined daily throughout the studies.

4.2.1 Cyclosporin administration

The cyclosporin A (CyA) was dissolved in 96 % ethanol then suspended in arachis oil to give a final concentration of CyA 6 mg/ml arachis oil, 10 % ethanol. The test rats were treated with 10 mg CyA/kg body weight/day via the intraperitoneal (ip) route. Control animals were treated daily with a similar volume of vehicle only.

Blood samples of approximately 60 µl for determination of plasma glucose and insulin were obtained from the tail tip of the rats. Blood sampling was carried out

as described in Chapter 2. The plasma was separated by centrifugation at 15000g for 30 seconds; 20 µl was stored at -20°C with the addition of 20 µl 0.9 % saline for insulin assay, and 10 µl used immediately for glucose analysis.

The CyA treatment was initiated immediately after the first blood sample was obtained. This was taken as day 0 of the study. Blood samples were obtained every subsequent day for 3 days, and analysed as before.

4.2.2 Glucose tolerance tests

Intraperitoneal glucose tolerance test

An ip glucose tolerance test (IPGTT) was carried out on day 2, using overnight fasted rats. The glucose dosage and timing of the blood samples were as described in Chapter 2. The plasma was separated for glucose and insulin assay as before.

Oral glucose tolerance test

An oral glucose tolerance test (OGTT) was carried out on day 3, using overnight fasted rats. The glucose solution was administered by oral gavage; glucose dosage and blood sampling were carried out as described in Chapter 2.

Intravenous glucose tolerance test

An intravenous glucose tolerance test (IVGTT) was carried out on overnight fasted rats after 3 days of CyA treatment.

The rats were anaethetised with sodium pentobarbitone (Sagatal) 60 mg/kg ip, and allowed to rest for 20 minutes to allow plasma glucose levels to stabilize (Bailey and

Flatt 1980). A small vertical incision was then made in the lower right abdomen, and the inferior vena cava (IVC) was located. A time 0 blood sample was obtained from the tail-tip as before, then the glucose load (0.5 g/kg given as a 20 % w/v solution) was injected into the IVC over a period of 30 seconds. Further blood samples were obtained from the tail at 5, 10, 15, 20, 30 and 40 minutes following glucose administration. The body wall was sutured and the skin closed with wound clips. The animals were kept warm throughout under a lamp which has been shown previously to enable a normal core temperature to be maintained.

4.2.3 First phase insulin reponse

The 1st phase insulin response was investigated in non-fasting rats after 4 days of CyA treatment.

The animals were anaethetised as before, and a vertical midline incision was made in the abdomen to expose the hepatic portal vein (HPV). A time 0 blood sample was obtained from the HPV immediately adjacent to the liver via a preheparinised 25G needle. This needle was left in place throughout the experiment for the withdrawal of subsequent blood samples. The glucose load was injected into the IVC (dosage as previously), and further blood samples were taken from the HPV at 3, 5, 7 and 10 minutes. Immediately after the 10 minute sample, a further sample was taken from the hepatic vein. The animals were then killed.

4.2.4 Hepatic extraction of insulin

The hepatic extraction of insulin was investigated in overnight fasted rats after 5 days of CyA treatment.

Anaesthesia, surgery, and iv glucose dosage were carried out as described above. A time 0 blood sample was taken from the HPV. At 20 and 30 minutes following glucose administration, blood samples were rapidly obtained from the HPV, lower abdominal IVC, hepatic vein and lower abdominal aorta via 25G heparinised needles. Plasma was separated and stored for glucose and insulin analysis. A section of the splenic region of the pancreas was removed and stored deep frozen for extraction of insulin.

4.2.5 Effect of CyA on RINm5F cells *in vitro*

Chemicals

RPMI-1640 media and glutamine were purchased from Gibco BRL, Uxbridge, Middlesex; fetal calf serum, penicillin/streptomycin and phosphate-buffered saline tablets (PBS) from Flow Laboratories, Scotland; other chemicals from Sigma. The CyA was a kind gift of Sandoz Pharmaceuticals, Camberley, Surrey. Cel-Cult plastic tissue culture ware from Sterilin Ltd., Hounslow, Essex.

Maintenance of cells

Stock cultures of RINm5F cells were grown in 10cm plates in supplemented RPMI-1640 medium, and maintained under standard tissue culture conditions as described previously in Chapter 2.

Insulin release from monolayer cultures incubated with CyA

The incubation media containing CyA were made up as follows; 1 mg of CyA was dissolved in 100 µl ethanol and 20 µl Tween 80, then made up to 100 ml with complete RPMI to give a final concentration of CyA 10 µg/ml. A further dilution of 1 ml of this medium with complete RPMI gave CyA 0.1 µg/ml.

RINm5F cells were detached from the culture plates as described previously, counted using an improved Neubauer cytometer, and transferred into 6-well tissue culture plates at a concentration of $6x10^4$ cells/ml (with a total volume of 2 ml medium/well). The cells were incubated in RPMI at 37°C for 24 hours, then the medium was removed and stored at -20°C until insulin assay, and replaced with either control medium ie RPMI with vehicle only (ethanol/Tween), RPMI 10 µg/ml CyA, or RPMI 0.1 µg/ml CyA. The cells were incubated as before for 24 hours, and further incubation/sampling was carried out for 4 days.

After the last samples were removed, the cells from each individual well were detached, counted, resuspended in 0.5 ml tris/EDTA (5mM Tris pH 8, 1mM EDTA) and destructed using an ultrasonic probe, then stored at -20°C until insulin assay.

4.2.6 Microencapsulation of HIT T15 cells

Chemicals

Sodium alginate (Kelco Gel LV) was purchased from Kelco, San Diego, California, USA. Cell culture media was from Gibco BRL as before; powdered glucose-free RPMI was purchased from Sigma Chemical Company, Poole, Dorset, UK.

Maintenance of cells

HIT T15 cells were grown in RPMI 1640 medium supplemented as before (supplemented RPMI had a final glucose concentration of 10 mM glucose, or 180 mg/dl). The glucose deficient RPMI was supplemented as previously described, and sterile 50 % (w/v) glucose solution was added to give RPMI with a final concentration of 50, 100 or 200 mg/dl glucose. Prior to the experiment, cells were removed from the plates by trypsinisation, counted, spun down and resuspended in complete RPMI. An aliquot was used to seed 6 well plates at a density of 1 x 10^5 cells/ml. A further aliquot was used for microencapsulation.

Microencapsulation procedure

The microencapsulation procedure used in this study was a modification of the method of Sun and O'Shea (1985), and similar to that described by Fan *et al* (1990).

All solutions described in this section were made from autoclaved materials in a tissue culture hood.

The aliquot of cells was gently centrifuged into a pellet, the supernatant discarded, and the cell pellet was then resuspended in 1.5 % (w/v) sodium alginate in complete RPMI. Spherical droplets were formed by syringe pump extrusion via PP10 tubing mounted on a 25G needle, and collected in 1.1 % $CaCl_2$, resulting in the formation of calcium alginate. Subsequently the gelled droplets were washed in 0.9 % saline before suspension in 0.15% poly-L-lysine (Mr 16,000 to 22,000) for 10 minutes. The droplets were suspended in 0.15 % sodium alginate for 4 minutes, then allowed to react in 55 mM sodium citrate for 6 minutes to reliquify the alginate gel inside the

capsule. The microcapsules were then washed in saline and culture medium before being hand picked into an equal number of wells as occupied by the unencapsulated aliquot of cells. As far as possible the number of capsules in each well was kept constant, so that the number of cells in each well (both encapsulated and unencapsulated) could be assumed to be approximately the same.

Incubations of microencapsulated cells

Both encapsulated and unencapsulated cells were incubated in 2ml complete RPMI 1640 medium at 37°C for 24 hours. The medium was then aspirated using a pasteur pipette and stored at -20°C for subsequent insulin assay. 2 ml of one of the glucose supplemented RPMI media was then added to each well. Further 24 hour incubations and sample collections were carried out for 4 more days.

4.2.7 Analyses

Glucose determination

The analysis of plasma glucose was carried out on 10 µl plasma samples using a Beckman Glucose analyser, as described in Chapter 2.

Insulin determination

Plasma insulin concentration was measured in 20 µl plasma samples using an RIA procedure with PEG separation of free from bound antigen as detailed in Chapter 2.

Analysis of pancreatic insulin content

The excised samples of pancreata were weighed then extracted with acid ethanol as described in Chapter 2. The supernatant was stored at -20°C until assayed for insulin at a dilution of 1:500.

Statistical analysis

Results were expressed as mean ± standard error of the mean (x ± SEM). Values within and between groups of rats were compared using Student's paired and unpaired t-test respectively. Differences were considered to be statistically significant for $p < 0.05$.

4.3 RESULTS

4.3.1 Effect of CyA administration on β-cell function in vivo

Body weight and food intake

The body weights (Figure 4.1) and food intake of control and CyA (10 mg/kg/day) treated rats were not significantly altered during the 5 day duration of the study.

Basal plasma glucose and insulin levels

Basal concentrations of plasma glucose and insulin levels, measured daily over 3 days of treatment with CyA or vehicle only, were not significantly altered, as shown in Figures 4.2a and 4.2b respectively.

Glucose tolerance tests

Intraperitoneal glucose tolerance test

The IPGTT was carried out on overnight fasted rats following 3 days of CyA treatment.

Basal fasting plasma glucose and insulin concentrations were similar in the 2 groups (Figures 4.3a and 4.3b). At 30 minutes post challenge, the plasma glucose concentrations of both groups had increased significantly ($p < 0.001$) from basal and the concentration of the CyA-treated group was significantly ($p < 0.001$) greater than that of the control as assessed by Student's unpaired t test. A similar effect was observed at 60 minutes, when the plasma glucose concentration of the test group were significantly ($p < 0.02$) elevated compared to the control group.

Figure 4.1. Body weights of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square). Results are mean ± SEM (10).

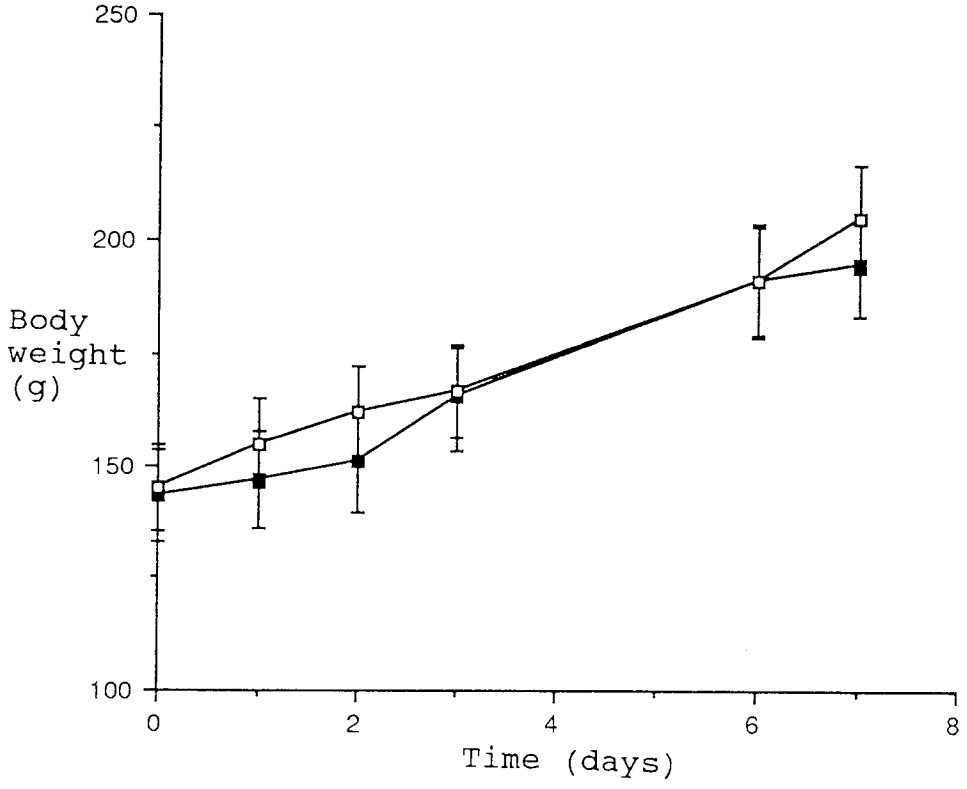


Figure 4.2a. Plasma glucose concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square). Results are mean ± SEM (10).

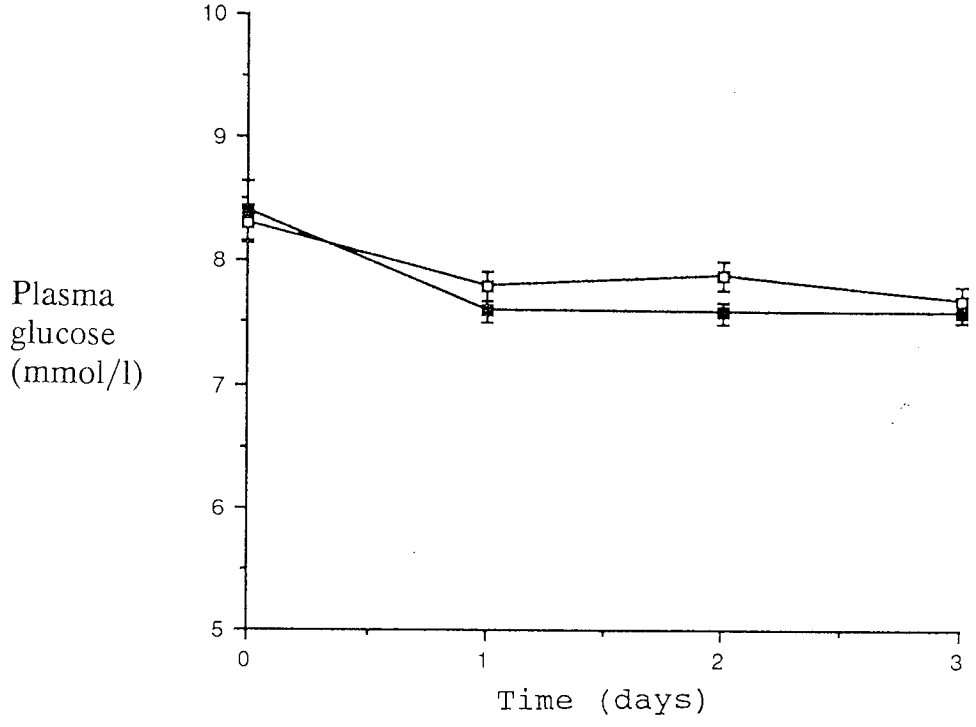


Figure 4.2b. Plasma insulin concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square). Results are mean ± SEM (10).

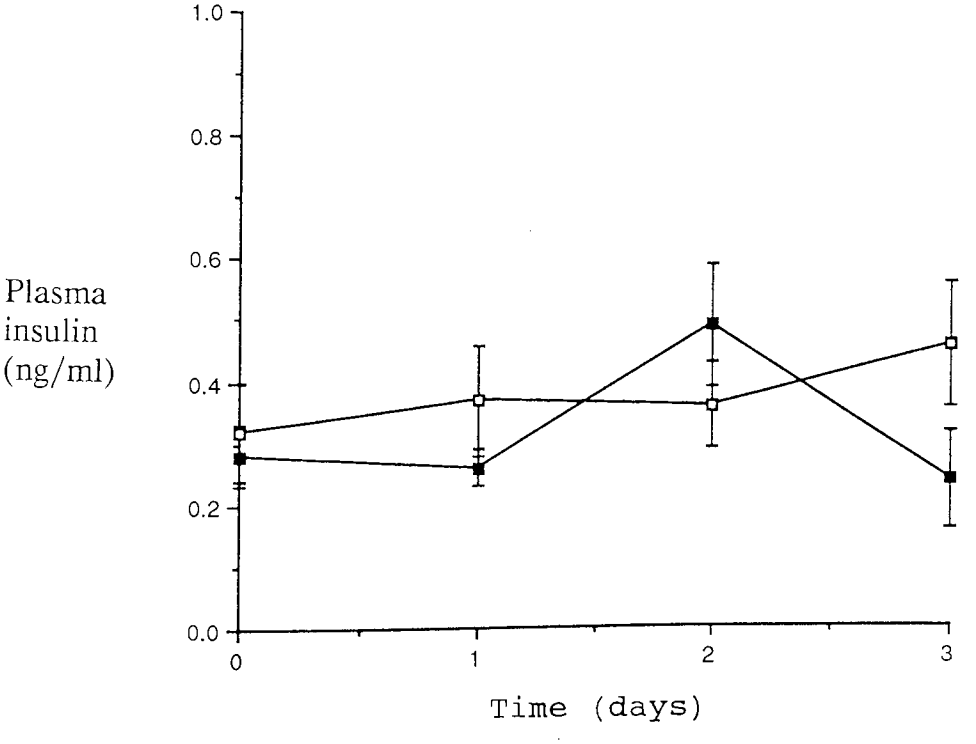


Figure 4.3a. Plasma glucose concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 2 g/kg ip. Results are mean ± SEM (10). [b] $p<0.02$, [d] $P<0.001$.

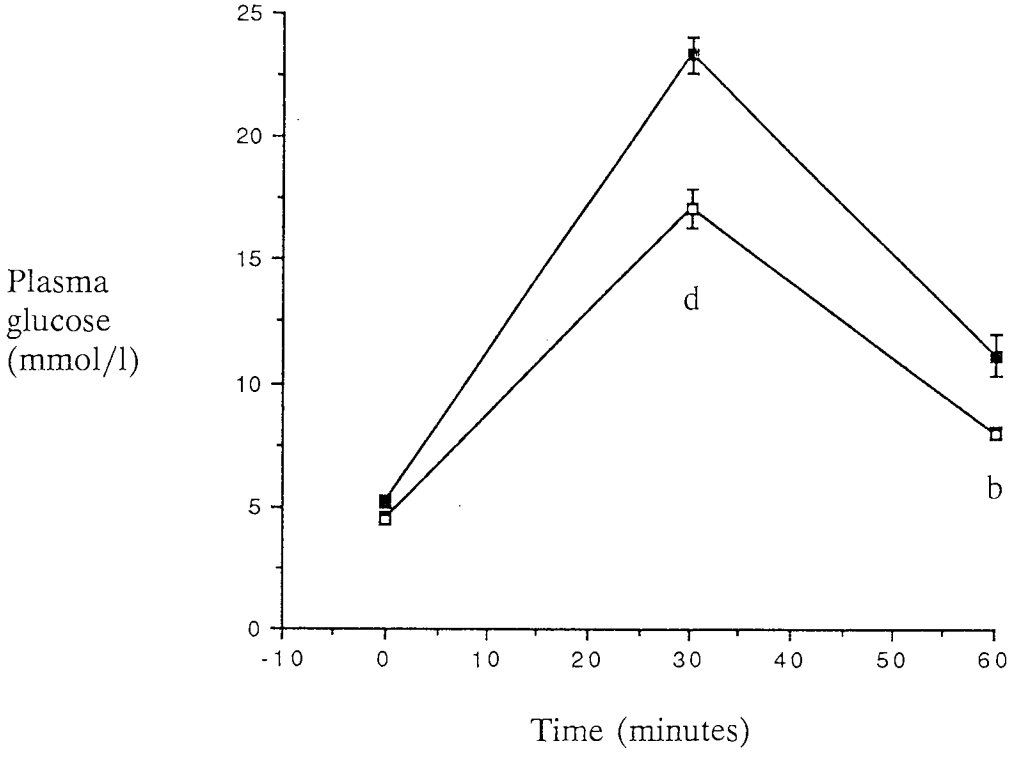
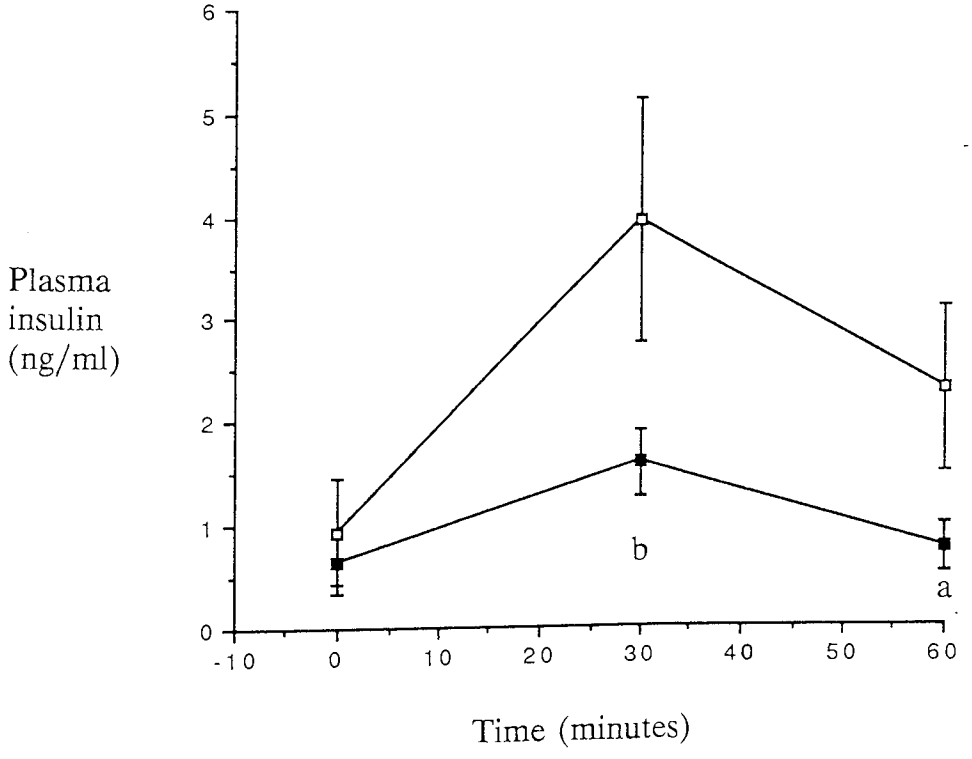


Figure 4.3b. Plasma insulin concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 2 g/kg ip. Results are mean ± SEM (10). [a] $p<0.05$, [b] $p<0.02$.



At time 0, there was no significant difference in plasma insulin concentration between the 2 groups. Both groups demonstrated a significantly elevated ($p<0.05$) plasma insulin concentration by 30 minutes after the glucose challenge. The CyA-treated group showed plasma insulin concentrations significantly below those of the controls at both 30 and 60 minutes ($p<0.02$ and $p<0.05$ respectively).

Oral glucose tolerance test

The OGTT was carried out on overnight fasted rats after 4 days of CyA or vehicle only treatment.

As shown in Figures 4.4a and 4.4b respectively, there were no significant differences in basal plasma glucose and insulin concentrations in the 2 groups at time 0. The plasma glucose concentration of both the CyA-treated and the control group increased significantly after 30 minutes ($p<0.001$) and 60 minutes ($p<0.001$ and $p<0.01$ respectively) compared to basal. The plasma glucose concentration of the CyA-treated group was significantly greater than that of the control group at 30 ($p<0.01$) and 60 minutes ($p<0.05$).

By 30 minutes, CyA and control groups both had increased plasma insulin concentrations compared to basal values ($p<0.05$), but by 60 minutes the plasma insulin concentrations were not significantly different from basal. The plasma insulin concentration of the CyA-treated group was significantly lower than that of the control group at 30 minutes ($p<0.01$); no significant difference was found at 60 minutes.

Figure 4.4a. Plasma glucose concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 2 g/kg po. Results are mean ± SEM (10). [a] $p<0.05$, [c] $p<0.01$

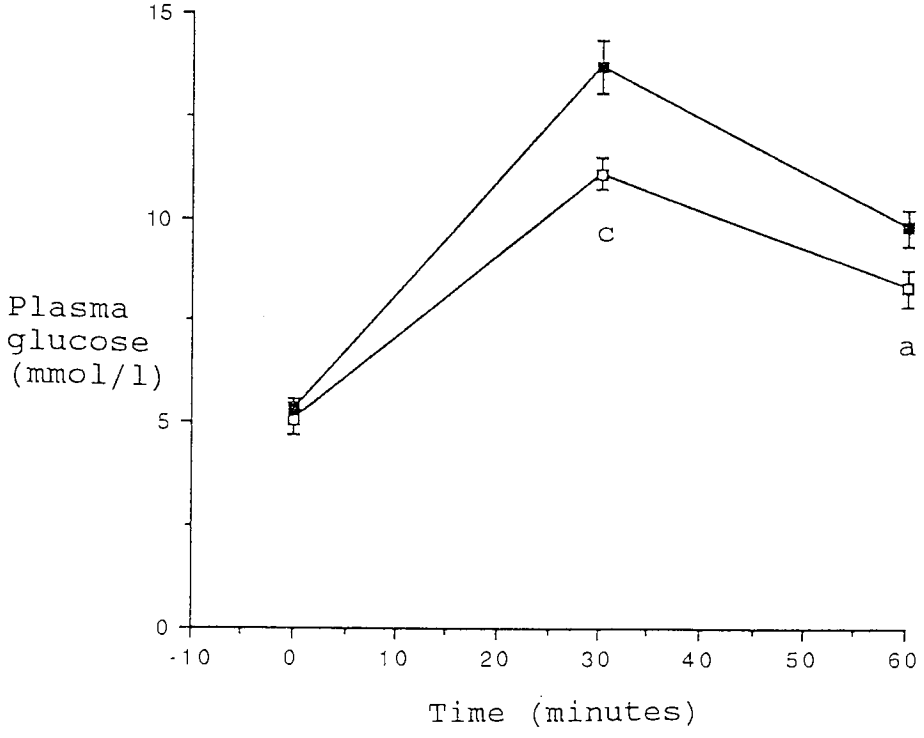
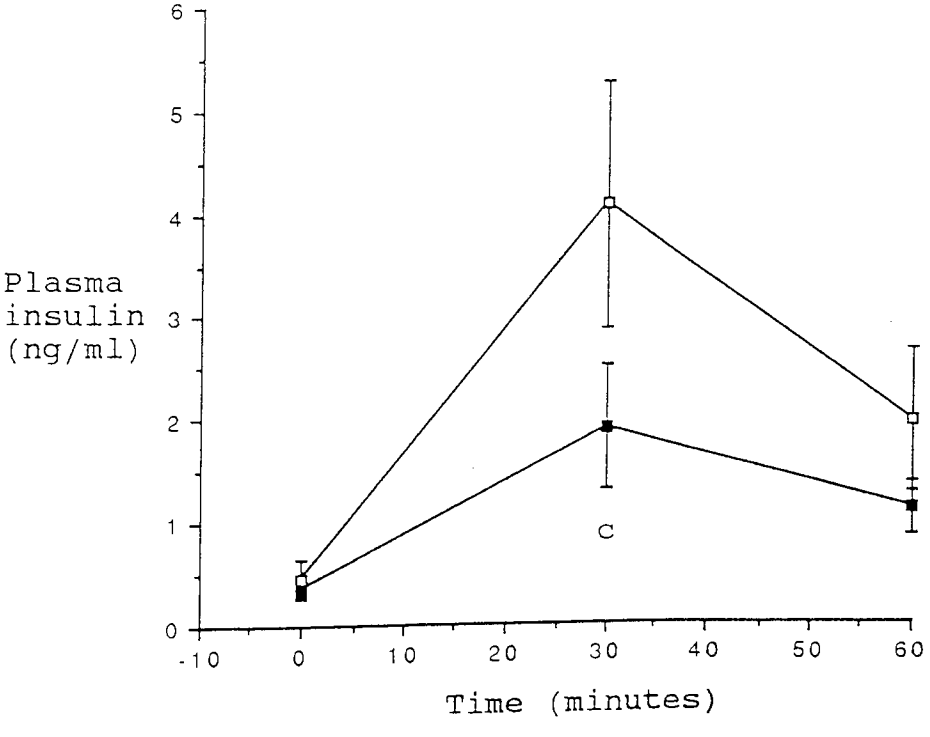


Figure 4.4b. Plasma insulin concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 2 g/kg po. Results are mean ± SEM (10). [c] $p<0.01$.



Intravenous glucose tolerance test

The IVGTT was carried out on day 3 after an overnight fast using pentobarbitone anaesthetised CyA and vehicle only treated rats.

No significant difference in plasma glucose concentrations were observed between the 2 groups at 0, 5, 10 and 15 minutes (Figure 4.5a). At 20, 30 and 40 minutes the plasma glucose levels of the CyA treated group were significantly greater than those of the controls ($p < 0.05$). From the plasma glucose concentrations obtained at 15 and 40 minutes, the rate of glucose disappearance (K value, expressed as %/min) was calculated from the following equation:-

$$K = \frac{\log_e 2 \times 100}{t1/2}$$

(Bailey, Atkins and Matty 1975).

The mean K value of the test group was 0.818 ± 0.07 (4), and that of the control group was 1.182 ± 0.22 (4); these were not significantly different.

No significant difference in the plasma insulin concentrations of the 2 groups were detected at 0, 5, 15, 20, 30 and 40 minutes following the glucose challenge (Figure 4.5b). At 10 minutes, the plasma insulin concentration of the CyA treated group was significantly greater than that of the controls ($p < 0.02$).

Figure 4.5a. Plasma glucose concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 0.5 g/kg iv, with samples taken from the tail tip. Results are mean ± SEM for 3-5 observations. [a] $p < 0.05$.

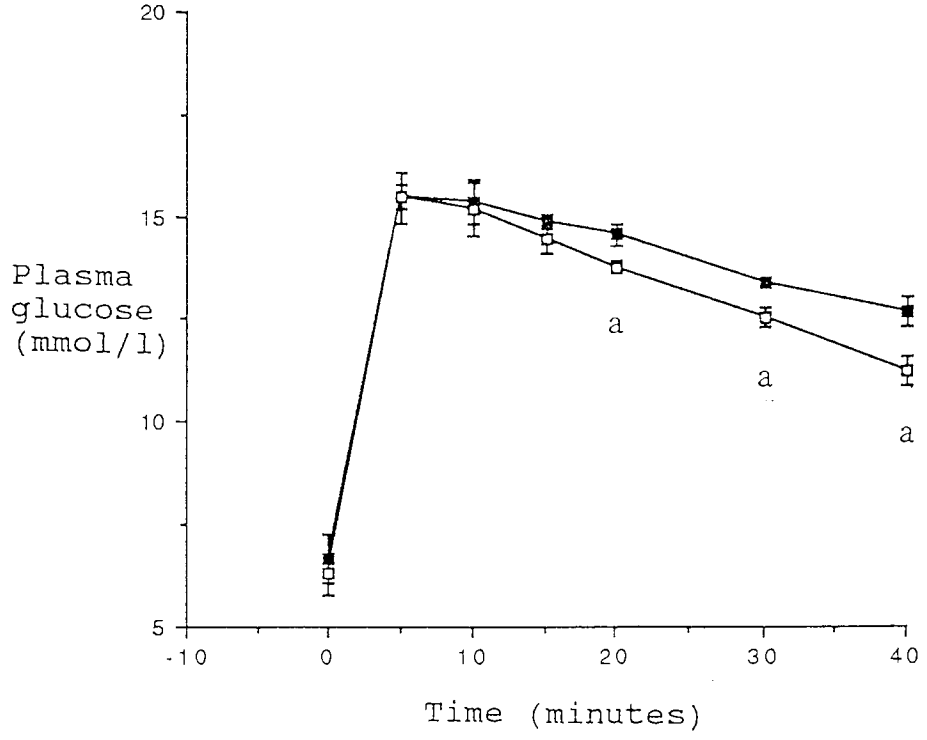
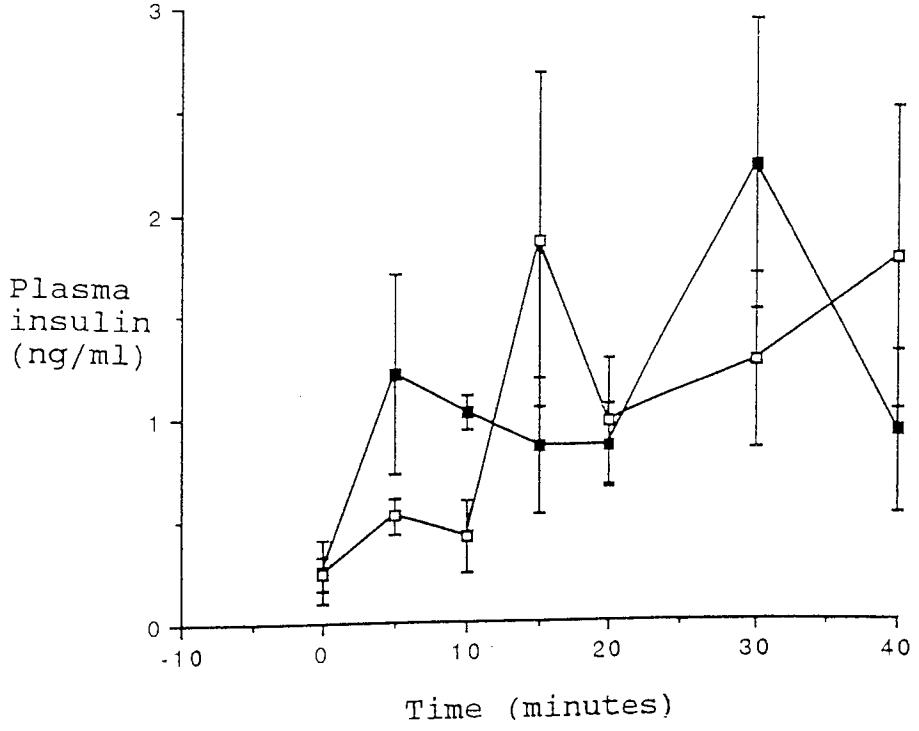


Figure 4.5b. Plasma insulin concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 0.5 g/kg iv, with samples taken from the tail tip. Results are mean ± SEM for 3-5 observations.



First phase insulin response

The 1st phase plasma insulin response to glucose was investigated after 5 days on CyA or vehicle only treatment. The 1st phase of insulin release consists of a sharp peak followed by a decline in the first 10-15 minutes post challenge.

The plasma glucose concentrations in the HPV's of the 2 groups were not significantly different at 0, 3, 5 and 10 minutes following the iv glucose challenge (Figure 4.6a). At 7 minutes, the plasma glucose concentration of the CyA treated group was significantly greater ($p < 0.02$) than that of the control group. Analysis of the 10 minute sample taken from the hepatic vein showed that the CyA treated group had a significantly elevated plasma glucose concentration when compared to the control group ($p < 0.05$), with the test group value being 19.4 ± 0.68 mmol/l (4), and that of the control group 16.9 ± 0.73 mmol/l (4).

No significant difference in plasma insulin concentration was observed between the 2 groups in hepatic portal vein samples taken at 0, 3, 5, 7 and 10 minutes (Figure 4.6b). However the 10 minute sample taken from the hepatic vein showed a significantly reduced plasma insulin concentration ($p < 0.001$) in the CyA treated group when compared to the control group, with the insulin concentration of the test group being 0.32 ± 0.13 ng/ml (5), and that of the control 2.68 ± 0.25 ng/ml (4).

Figure 4.6a. Plasma glucose concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 0.5 g/kg iv, with samples taken from the hepatic portal vein. Results are mean ± SEM for 3-5 observations. [b] $p < 0.02$.

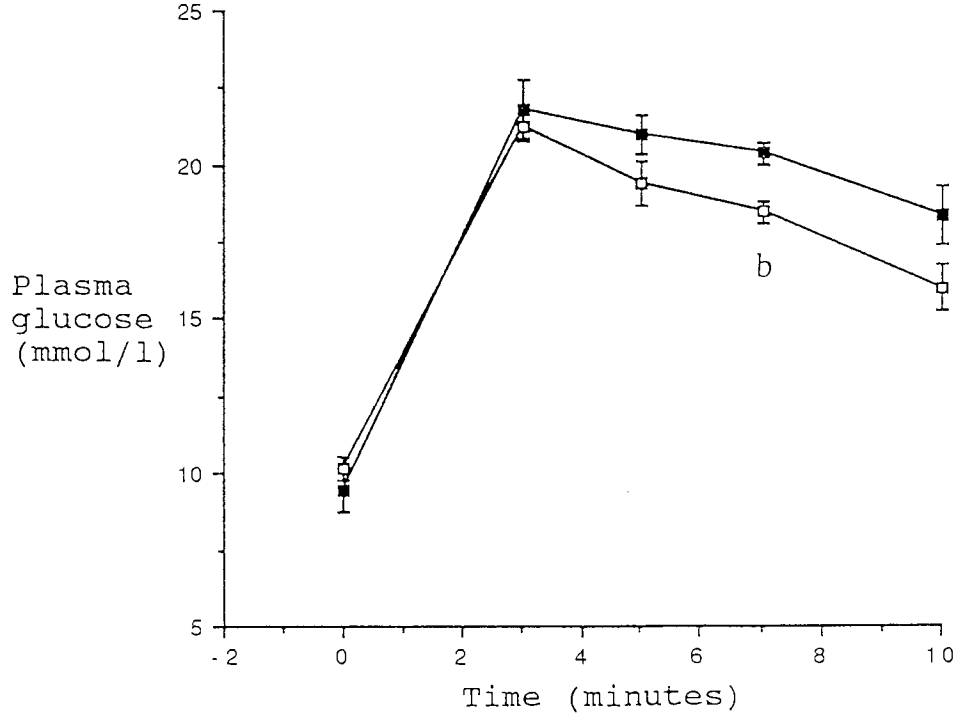
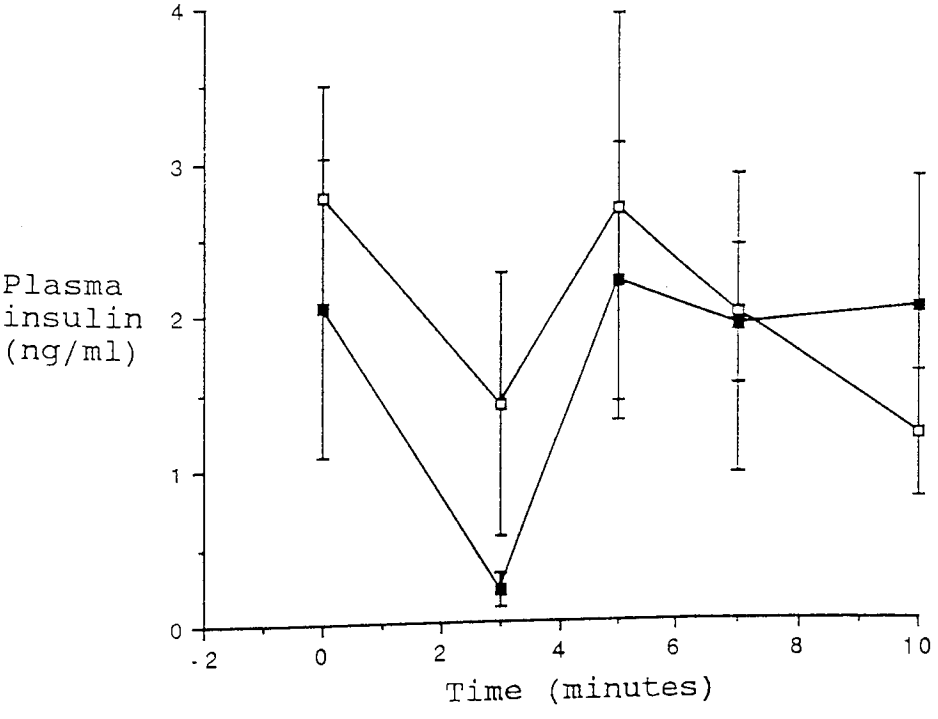


Figure 4.6b. Plasma insulin concentration of rats treated with CyA 10 mg/kg/day (closed square) or vehicle only (open square) following administration of glucose 0.5 g/kg iv, with samples taken from the hepatic portal vein. Results are mean ± SEM for 3-5 observations.



Hepatic extraction of insulin

The results from this experiment are shown in Tables 4.1a and 4.1b. No significant difference in plasma glucose concentration in the hepatic portal vein was found between the 2 groups at time 0. At 20 minutes the plasma glucose level of the CyA treated group was significantly greater than the controls in the HPV, IVC, hepatic vein and aorta ($p<0.05$, $p<0.001$, $p<0.01$ and $p<0.001$ respectively). A similar result was obtained at 30 minutes ($p<0.001$, $p<0.001$, $p<0.01$ and $p<0.001$ respectively).

Analysis of plasma insulin concentrations demonstrated no significant difference between the 2 groups at any time during the experiment.

Assay of pancreatic insulin concentration (expressed as ng/g tissue), shown in Table 4.1c, demonstrated no significant difference between the 2 groups, although the mean value was lower in the CyA group.

4.3.2 Effect of CyA on RINm5F cells *in vitro*

The release of insulin into the medium by Rinm5F cells incubated for 4 days with various concentrations of CyA is shown in Figure 4.7.

No significant differences in insulin release were found at any time between the control cells and those incubated with 0.1μg/ml CyA. However the insulin released by cells exposed to 10μg/ml CyA gradually decreased, becoming significantly lower than that of the controls on day 2 ($p<0.001$). Further decreases of insulin release

Table 4.1a. Plasma glucose concentration (mmol/l) of rats treated with CyA 10 mg/kg/day or vehicle only following administration of glucose 0.5 g/kg iv, with samples taken at 0, 20 and 30 minutes, from the hepatic portal vein (HPV), inferior vena cava (IVC), hepatic vein, and aorta. Results are mean ± SEM (n). [a] $p<0.05$, [c] $p<0.01$, [d] $p<0.001$.

	0′	20′				30′			
	HPV	HPV	IVC	hepatic vein	aorta	HPV	IVC	hepatic vein	aorta
CyA treated	7.5 ± 0.30 (5)	15.5 ± 0.86 (5)	16.1 ± 0.39 (5)	16.4 ± 0.65 (5)	16.7 ± 0.40 (5)	15.8 ± 0.57 (5)	16.1 ± 0.30 (5)	17.6 ± 0.79 (5)	16.9 ± 0.37 (5)
Control	6.0 ± 0.75 (6)	13.3 ± 0.28 (6)	13.3 ± 0.37 (6)	13.5 ± 0.48 (6)	13.2 ± 0.24 (5)	11.4 ± 0.23 (5)	12.4 ± 0.31 (5)	13.7 ± 0.55 (5)	12.2 ± 0.16 (5)
		a	d	c	d	d	d	c	d

Table 4.1b. Plasma insulin concentration (ng/ml) of rats treated with CyA 10 mg/kg/day or vehicle only following administration of glucose 0.5 g/kg iv, with samples taken at 0, 20 and 30 minutes, from hepatic portal vein (HPV), inferior vena cava (IVC), hepatic vein, and aorta. Results are mean ± SEM (n).

	0′	20′				30′			
	HPV	HPV	IVC	hepatic vein	aorta	HPV	IVC	hepatic vein	aorta
CyA treated	0.39 ± 0.19 (4)	2.40 ± 0.88 (5)	0.51 ± 0.19 (5)	0.62 ± 0.23 (4)	0.31 ± 0.09 (5)	1.56 ± 0.42 (5)	0.39 ± 0.07 (5)	0.65 ± 0.21 (5)	0.75 ± 0.18 (4)
Control	0.50 ± 0.21 (5)	1.99 ± 0.26 (6)	0.66 ± 0.14 (6)	0.52 ± 0.12 (6)	0.72 ± 0.18 (7)	2.41 ± 0.83 (6)	0.56 ± 0.33 (5)	1.30 ± 0.38 (5)	0.67 ± 0.23 (5)

Table 4.1c. Pancreatic insulin content of rats treated with CyA 10 mg/kg/day or vehicle only after 5 days of treatment. Results are mean ± SEM (n).

	Pancreatic insulin content (µg/g tissue)
CyA treated	49.3 ± 14.0 (4)
Control	68.5 ± 14.0 (5)

occurred on days 3 and 4 ($p<0.001$ and $p<0.001$). Cells incubated with 10 μg/ml CyA also showed a significantly lower release of insulin compared with cells incubated with 0.1 μg/ml CyA on days 2, 3 and 4 ($p<0.01$, $p<0.001$ and $p<0.001$ respectively).

At the conclusion of the experiment, the cells were harvested, counted, and disrupted to assay for cell insulin content. The results, expressed as ng insulin/10^5 cells, are shown in Figure 4.8.

No significant difference in insulin content was found between the controls and the 0.1μg/ml group. However, the insulin content of the 10μg/ml group was significantly lower than that of controls and the 0.1μg/ml group ($p<0.001$ and $p<0.05$ respectively).

4.3.3 Microencapsulation of HIT T15 cells

HIT T15 cells were seeded into 6 well plates either free or encapsulated within alginate-polylysine-alginate beads, and incubated in 50, 100 or 200 mg/dl glucose RPMI medium over 5 days. Insulin released into the medium is shown in Figure 4.9.

Unencapsulated cells

No significant difference in insulin release was observed between cells in 50 and 100 mg/dl glucose media. Insulin release from cells in 200 mg/dl glucose medium was significantly greater than from 50 mg/dl glucose medium on days 1, 4 and 5 ($p<0.01$,

Figure 4.7. Insulin release into the medium by RINm5F cells incubated in medium containing no CyA (open square), 0.1 μg/ml CyA (closed circle) and 10 μg/ml CyA (open circle). Results are mean ± SEM (4). [d] $p<0.001$ compared to controls.

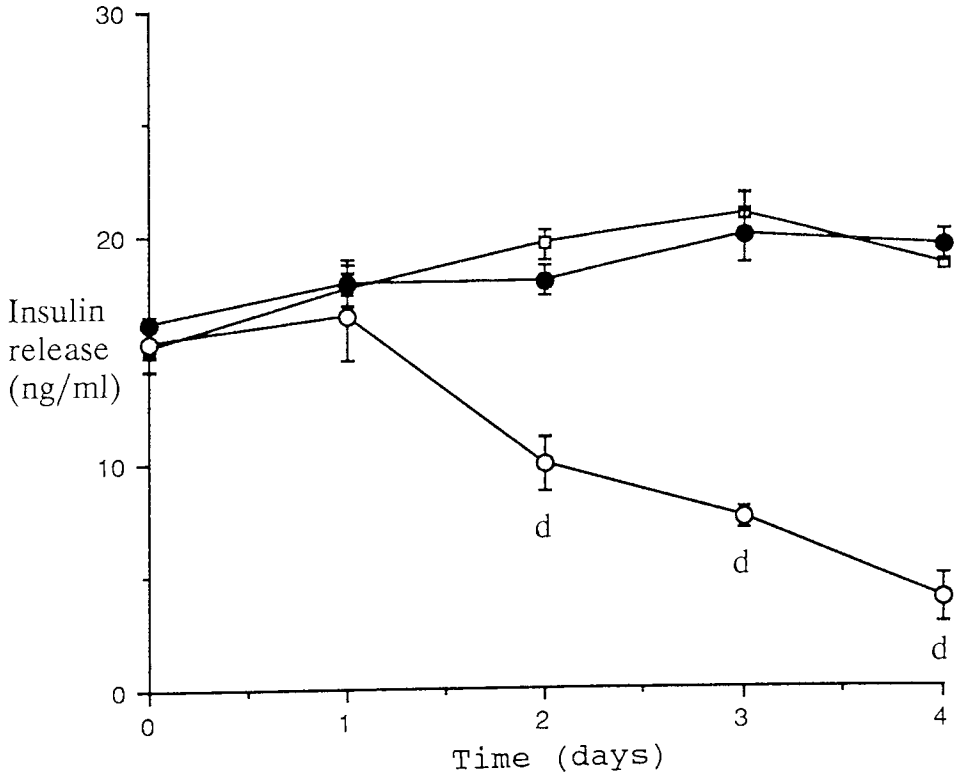


Figure 4.8. Insulin content of RINm5F cells incubated in medium containing either no CyA, 0.1 μg/ml CyA, or 10 μg/ml CyA. Results are mean ± SEM (4). [a] $p<0.05$, [d] $p<0.001$ when compared to controls and CyA 0.1 μg/ml groups respectively.

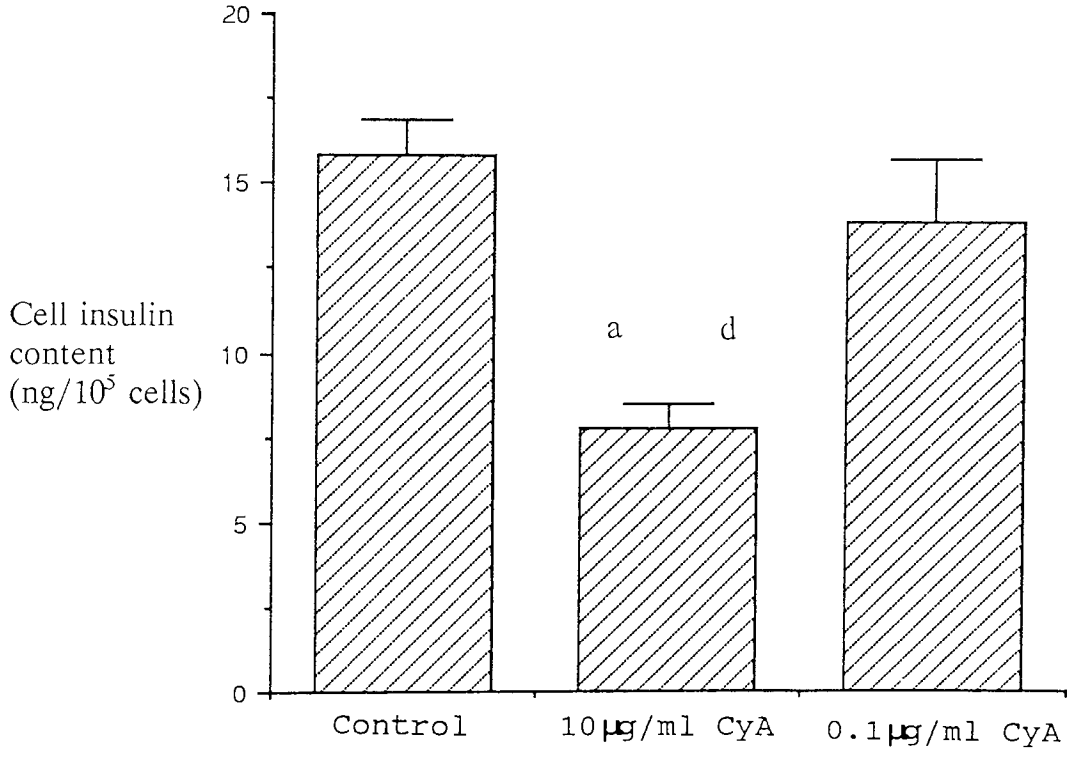
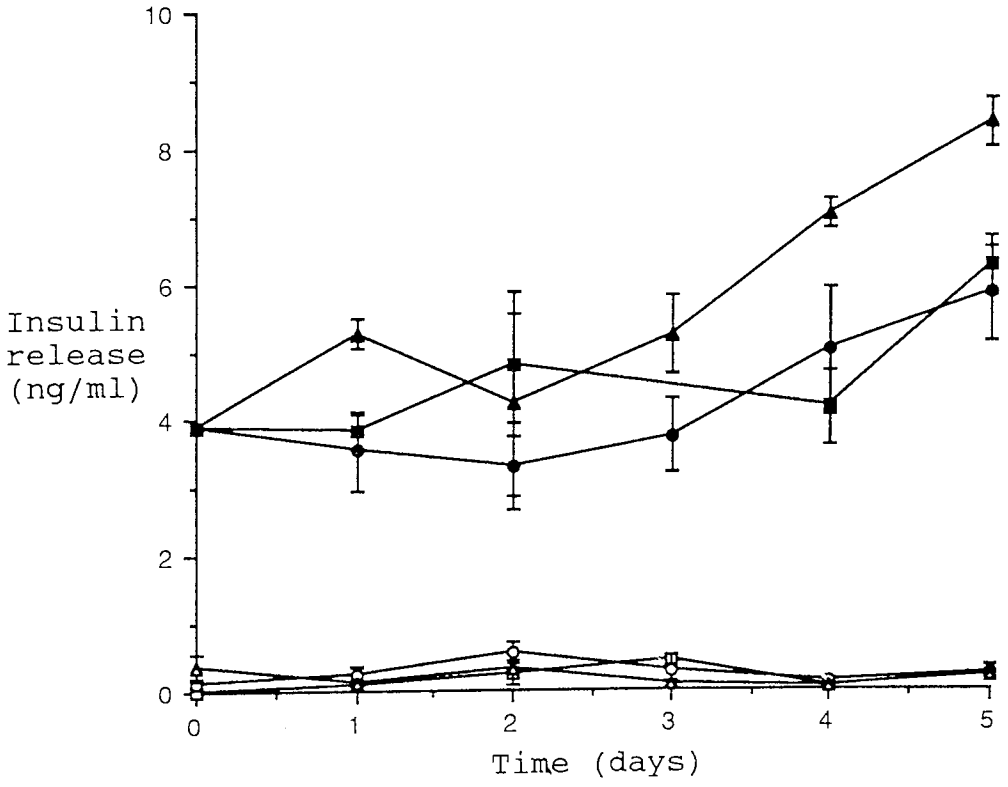


Figure 4.9. Insulin release from HIT T15 cells, either free or encapsulated, in medium containing glucose 50 mg/dl (closed square and open square respectively), 100 mg/dl (closed circle and open circle respectively), or 200 mg/dl (closed triangle and open triangle respectively). Results are mean ± SEM for 3-4 observations. Statistical analysis as described in the text.



$p < 0.01$ and $p < 0.02$ respectively), and from 100 mg/dl glucose medium on days 1 and 5 ($p < 0.05$ and $p < 0.02$ respectively).

Encapsulated cells

Plates 4.1a and 4.1b show photographs of HIT T15 cells encapsulated within an alginate-polylysine-alginate membrane. It can be seen that the exterior of the capsule is smooth and that there is no cell growth outside the bead, and that size is uniform.

Insulin release by cells in 100 mg/dl glucose RPMI was not found to be significantly different to insulin release by cells in either 50 or 200 mg/dl glucose media. Cells incubated in 200 mg/dl glucose RPMI released significantly greater ($p < 0.02$) insulin than cells in 100 mg/dl glucose RPMI on day 1, and significantly ($p < 0.01$) less on day 3.

At all time points, insulin release from cells encapsulated within alginate beads was significantly lower ($p < 0.001$) than that from unencapsulated cells.

Plate 4.1a HIT T15 cells encapsulated within an alginate-polylysine-alginate membrane. Magnification x 50.

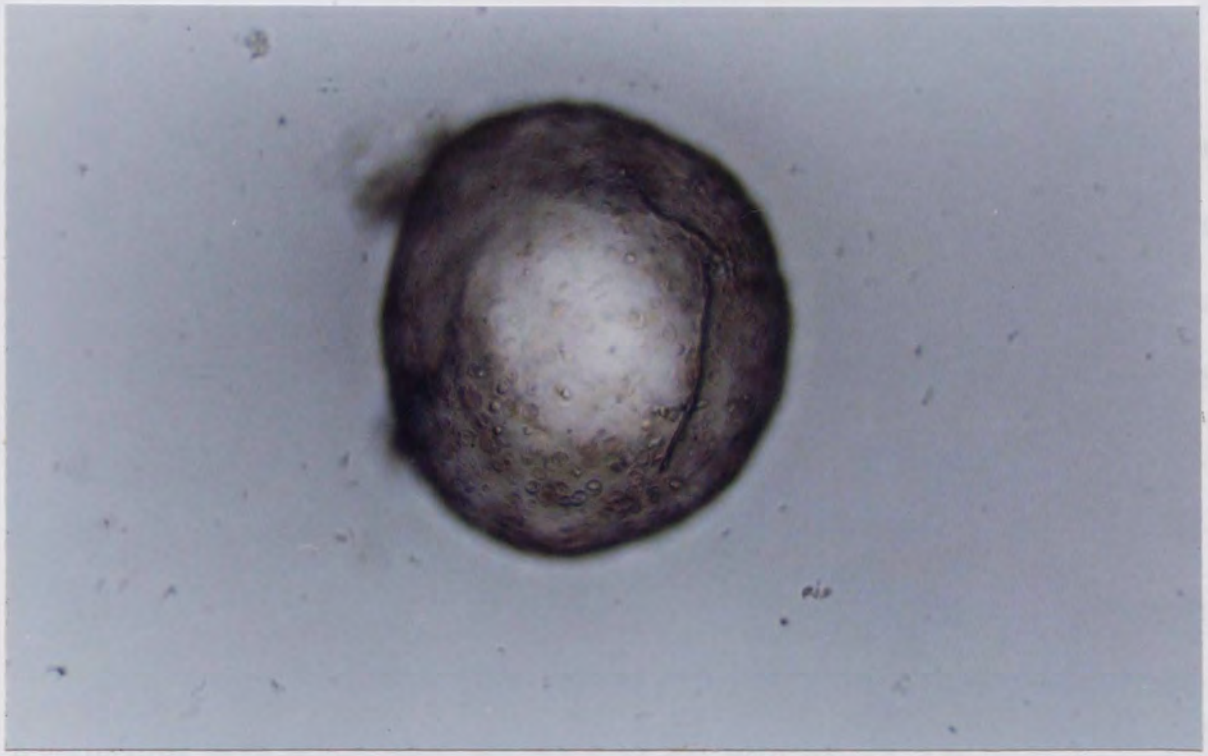


Plate 4.1b Two alginate-polylysine-alginate microcapsules containing HIT T15 cells. Magnification x 50.

4.4 DISCUSSION

Immunosuppression

The first section of work described in this Chapter concerns the administration of a therapeutic dose of CyA (10 mg/kg body weight/day) to rats, and the subsequent examination of various aspects of glucose homeostasis *in vivo*. Further investigations were then carried out *in vitro* using an insulin-secreting cell line, RINm5F, incubated in several different concentrations of CyA.

Little or no effect of CyA was seen on body weight, food intake, or basal plasma glucose and insulin concentrations during the 5 day period of study. Other investigators have found that over a longer (21 day) period, administration of a similar dose of CyA sc prevented weight gain and increased basal glycaemia in the test animals (Bani-Sacchi *et al* 1990).

It was found that on days 3 and 4, the rats exhibited an impaired ip and oral glucose tolerance, with a significantly reduced insulin release. A similar effect on plasma glucose levels was found following an iv glucose tolerance test on day 5, although no difference in plasma insulin concentrations were found. No significant difference in the ivGTT K value (the rate of glucose disappearance from the plasma) was found between the 2 groups, although the glucose concentrations were consistently higher in the test group. Lack of a significant effect on the rate of glucose disappearance (K value) despite an abnormal glucose tolerance curve has been reported previously (Gillison, Bartlett and Curry 1989).

Similar results to those of the IVGTT were found in the 1st phase insulin response experiment, that is a greater mean plasma glucose concentration in the test group (although only significantly raised at 7 minutes) with no significant difference in plasma insulin concentrations. The 1st phase of nutrient-induced insulin secretion is thought to involve the release of insulin already stored. These results suggest that insulin resistance is present, since plasma glucose concentrations in the test group are raised, but no difference in plasma insulin concentrations was found. The 10 minute hepatic vein sample taken from the test group after this experiment showed a significantly reduced plasma insulin concentration. This indicated that a greater proportion of insulin was removed from the blood during passage through the liver in the CyA-treated group, and this effect was investigated more thoroughly in the subsequent hepatic extraction experiment. However, although raised plasma glucose concentrations were found in the test group, no significant differences in plasma insulin concentrations were detected. These data also suggest the presence of insulin resistance, and confirm previous observations; a reduced insulin sensitivity of adipocytes taken from rats pretreated with CyA has been reported (Son *et al* 1988), and insulin resistance in human transplant recipients treated with CyA has been suggested (Mason 1990).

The incubation of RINm5F cells in medium containing CyA 0.1 μg/ml had no effect on insulin release over a 3 day period. However the addition of CyA 10 μg/ml to the medium caused a significant reduction in the amount of insulin released. These data are in accordance with those of other workers:- for example incubation of RINm5F cells in CyA 10 μg/ml has been shown to cause an 80 % decrease in mRNA synthesis, while CyA 0.1 μg/ml had no discernable effect (Eun *et al* 1987).

Taken together, these results suggest that the administration of a therapeutic dose of CyA causes an impaired glucose tolerance, probably via effects upon insulin production and release, and by some degree of insulin insensitivity.

The development of an impaired glucose tolerance in CyA-treated animals has been observed in a wide range of species including rodents, monkeys and humans (Helmchen *et al* 1984, Engfeldt *et al* 1986, Stegall *et al* 1989). Therapeutic doses of CyA cause insulinopenia and a reduced pancreatic insulin content in rodents (Yale *et al* 1985, Bani-Sacchi *et al* 1990, Hahn *et al* 1992). These effects appear to be dose dependent and rapidly reversible upon discontinuation of CyA therapy. It has been suggested that in human transplant recipients the observed deterioration in glucose tolerance following induction of immunosuppression is due to concurrent treatment with corticosteroids (Esmatjes *et al* 1991), but other workers have found a direct deleterious effect of CyA upon these patients (Gunnarsson *et al* 1984, Neilsen, Mandrup-Poulsen and Nerup 1986). There are further conflicting reports on the effect of CyA on glucose homeostasis in humans, but the general concensus of opinion appears to be that CyA does exert a negative influence upon glucose homeostasis (reviewed by Mason 1990).

The mechanism(s) by which CyA exerts this deleterious effect upon endogenous β-cell function is at present unclear. However certain actions of CyA which could have a bearing on insulin release / glucose homeostasis have been described. Inhibition of both insulin biosynthesis and release have been observed in CyA-treated animals (Gillison, Bartlett and Curry 1989). CyA has been found to interfere with Ca^{2+} influx into cells (Draznin *et al* 1988), and to bind to calmodulin (Le Grue *et al* 1986). Thus

there may be a dysfunction of the cell secretory apparatus or other calmodulin-dependent process. However, several cell types (including islets and RINm5F cells) treated *in vitro* with various concentrations of CyA show a general reduction in mRNA production - an 80 % decrease was found in cells incubated in 10 μg/ml CyA. The cells also showed cytoplasmic vacuolization and severe degranulation (Eun *et al* 1987). Prolonged inhibition of mRNA synthesis in β-cells could lead to a decreased proinsulin production. The inhibition of mRNA synthesis is unlikely to be due to a direct effect on transcription; CyA has been shown to have an inhibitory action on nuclear proteins critical for T lymphocyte activation (Emmel *et al* 1989).

An important observation concerning the use of CyA as an immunosuppressant agent for pancreatic or islet graft recipients is that CyA appears to accumulate in β-cells (Lensmeyer *et al* 1991). The use of CyA is further contra-indicated since the use of a therapeutic dose has been shown to inhibit neovascularization of implanted islets; although concurrent treatment with verapamil (a calcium antagonist) abolishes this deleterious effect (Rooth *et al* 1989). The achievement of a graft with an adequate vascular supply would be a critical aspect of a clinical gene therapy programme.

The use of skin grafts as an implantation method for genetically manipulated insulin-secreting cells has been suggested (Docherty 1991). Keratinocytes (and some endothelial cells) require the cytokine basic fibroblast growth factor (b-FGF) in order to proliferate. CyA has been found to inhibit cell proliferation stimulated by b-FGF (Sharpe *et al* 1989). Thus the administration of CyA to the recipient of a graft of genetically modified keratinocytes would be likely to limit the proliferation of these cells, and hence cause a reduced effectiveness of therapy. However, the use of an

autograft of keratinocytes or fibroblasts would remove any requirement for immunosuppression.

Microencapsulation

In vitro studies have shown that islets encapsulated with alginate-polylysine-alginate respond to an increased ambient glucose concentration by increasing insulin release (Fritschy, Wolters and Van Schilfgaarde 1991, Levesque, Brubaker and Sun 1992). It has recently been suggested that islets encapsulated within an alginate membrane are protected from autoimmune destruction when transplanted into an animal model of diabetes - the BB rat (Fan *et al* 1990), and that islets contained within an agarose capsule functioned after implantation into NOD mice (Iwata *et al* 1992). Other workers however have found rapid destruction of encapsulated islets following implantation into BB rats (Cole *et al* 1992), possibly as a result of cytokine action. Cellular overgrowth on capsules implanted *in vivo* has often been reported (Ricker and Stockberger 1986, Fan *et al* 1990).

Alginate-polylysine-alginate capsules have also been used to encapsulate RINm5F cells. The capsules were shown to be permeable to insulin, glucose and other β-cell secretagogues, but impermeable to antibodies and complement factors (Darquy and Reach 1985).

Despite reports that islets encapsulated in alginate microcapsules can secrete insulin at amounts equivalent to those released by free islets (Fan *et al* 1990), the majority of published investigations appear to find some diminution of insulin release (Darquy and Reach 1985, Chicheportiche and Reach 1988). The results presented in this

Chapter support the observation that the magnitude of insulin response from encapsulated insulin-secreting tissue is significantly reduced when compared to equivalent, unencapsulated tissue. It has been suggested that this effect may be due not to the presence of the capsule itself, but to the processing of the tissue during the encapsulation procedure (Levesque, Brubaker and Sun 1992). The use of sodium citrate (which acts as a calcium chelator to reliquefy the capsule interior) has been found to exert a damaging effect upon islets undergoing encapsulation - the substitution of Ca^{2+}-free Krebs buffer plus EGTA for sodium citrate results in a greatly improved insulin release (Fritschy, Wolters and Van Schilfgaarde 1991).

Conclusions

There are many problems to be overcome prior to the development of methods of immunosuppression or immunoisolation suitable for use in diabetes/gene therapy transplantation. Current protocols appear to exert either diabetogenic effects or to reduce the efficiency of insulin release. There are various other methods for the implantation and immunoprotection of cells which may prove to be of value for gene therapy for diabetes. Cell types that have been considered suitable for gene therapy include skin fibroblasts or keratinocytes - as discussed in Chapter 1 these are easy to manipulate *in vitro*, their dermal location may enable efficient export of the product into the bloodstream, and clinical transplantation protocols (by skin grafting) are already in existence. Fibroblasts can contract a hydrated collagen lattice to produce a tissue-like structure containing cells which may be suitable for transplant (Bell, Ivarsson and Merrill 1979). Such a lattice may provide some protection from immune attack, and may facilitate the growth of anchorage-dependent cells *in vivo*. Collagen lattices containing genetically manipulated fibroblasts have been found to

be integrated into the dermal layer of skin following subcutaneous implantation. These lattices however were not found to command a significant vascular supply (Palmer, Thompson and Miller 1989) which is obviously a serious drawback to their use in gene therapy protocols.

CHAPTER FIVE

IMPLANTATION OF INSULIN-SECRETING CELLS INTO NUDE MICE

5.1. INTRODUCTION

The two previous experimental chapters have discussed, respectively, the manufacture of a novel insulin-secreting cell including its response to various secretagogues *in vitro*, and the effectiveness of several immunomodulatory regimens both *in vivo* and *in vitro*. The next step in the investigation of gene therapy as an insulin delivery system is to implant cells *in vivo*. Certain aspects of the experimental design must be carefully considered, namely the animal model to be used, the site of implantation, and the biochemical parameters to be assayed.

Selection of the animal model

As described in Chapter 4, the current immunosuppressive agent of choice for transplant recipients (namely CyA) has considerable deleterious effects upon the endogenous β-cell. For this reason, it was decided that initial studies on the efficacy of insulin gene therapy would utilize congenitally athymic nude mice. Nude (nu/nu) mice have been widely used as recipients for transplanted allogeneic and xenogeneic tissues (Ziedler *et al* 1982). As a consequence of the absence of a thymus, nude mice display lymphopenia and a decline in cell-mediated immune reactions. They are thus immunoincompetent, and a transplant of foreign tissue is not rejected. Nude mice also exhibit certain endocrine abnormalities in thyroid, adrenal, gonadal and pancreatic functions (Shafrir 1990). The effects on β-cell function are considered in greater detail in the discussion to this chapter. It has been reported that hyperglycaemia could not be induced in nu/nu mice by streptozotocin administration (Buschard and Rygaard 1978) although other groups do not support this observation (Paik, Fleischer and Shin 1980).

Site of implantation

In order to minimise the risk of infection to susceptible nude mice, a transplantation site not requiring operative access was deemed most suitable. Since an intraperitoneal (ip) implant of islets has been shown to be effective in ameliorating both experimental (Hegre *et al* 1975) and naturally occuring diabetes (Hegre *et al* 1989), and islets or cells can be easily introduced into the peritoneal cavity via a needle, the ip site was chosen for the first implant. The subcutaneous (sc) site is also easily accessible via an injection, and the second implant described in this Chapter includes an sc implant.

Biochemical parameters

The volume of mouse plasma obtainable from one blood sample is a limiting factor for the number of substances that can be assayed from it. Plasma glucose can be measured accurately using an automatic analyser (see Chapter 2) on a sample of only 5 μl. Thus the remainder of the plasma is available for a further assay. The insulin-secreting AtT20MtNeohPPI/1 cells described in Chapter 3 process human proinsulin by means of an endogenous endopeptidase; the products of this cleavage are human insulin plus human C peptide, released in equi-molar amounts. At the time of protocol design, a highly specific insulin antiserum capable of distinguishing human insulin from mouse insulins was not available. Greater inter-species differences in C peptide amino acid sequence exist between mouse and man, and an antiserum has been raised which is capable of distinguishing between the C peptides from these two species. Thus a highly specific human C peptide assay can be used to recognise human C peptide in a plasma sample containing C peptides from both species. In this way, the release of insulin by the implant can be accurately assessed.

The first section of work described in this Chapter concerns the development and evaluation of a human C peptide RIA, in particular the specificity of the antiserum employed. The final section details 2 separate implantation experiments performed using nude mice, with insulin-releasing cells implanted ip and sc.

5.2. MATERIALS AND METHODS

5.2.1. Human C peptide assay evaluation

Human C peptide was measured in plasma samples of a minimum of 40 µl (maximum 100 µl) made to volume with 6 % NaFAM buffer (Appendix 1) using a modification of a commercial RIA procedure marketed by Novo Nordisk Diagnostics Ltd, Cambridge, UK.

Human C peptide standards were prepared by serial dilution in 6% NaFAM buffer to give final concentrations of 0.5, 0.1, 0.05, 0.025, 0.012 and 0.006 pmol/ml. Human C peptide antiserum (K6; obtained from a rabbit repeatedly immunised with synthetic human C peptide) (Faber *et al* 1976) was diluted in 0.1 % FAM buffer (Appendix 1) to give a final concentration of either 1:5000 or 1:9000. ^{125}I labelled synthetic human Tyr-C peptide was diluted in 0.1 % FAM buffer to give approximately 10,000 cpm in 100 µl; typically a 1:60 dilution.

The following tubes were set up:-

total count	empty
blank	200 µl buffer
standards	100 µl standard, 100 µl antiserum
zero	100 µl buffer, 100 µl antiserum
samples	100 µl sample, 100 µl antiserum

All tubes were run in triplicate except samples which were single. After mixing, tubes were covered and incubated at 4°C for 24 hours. 100 µl ^{125}I-Tyr-C peptide was then added to all tubes, which were again mixed, covered and incubated at 4°C for 24 hours. 1.6 ml of 95 % (v/v) ethanol at room temperature was added to all tubes except total count, and after mixing the tubes were centrifuged at 2000 g for 10 minutes. The supernatant was discarded, and the precipitate washed with 2 ml of a solution composed of 96 ml 95 % (v/v) ethanol, 1.8 ml 0.1 % FAM, and 16.2 ml H_2O. The tubes were again centrifuged at 2000 g for 10 minutes, and the supernatant discarded. 0.5 ml of 0.05 N NaOH was added to all tubes (including total count), and the ^{125}I activity of the pellet counted on a gamma counter for 60 seconds/tube. A standard curve was plotted and the values of the unknown samples were read off.

Evaluation of the assay parameters

The sensitivity of the rabbit anti-human C peptide antiserum obtained from Novo was investigated by assaying standards using 2 different working concentrations of antiserum, namely 1:27,000 and 1:15,000.

The specificity of the human C peptide antiserum was investigated by performing the assay as described above, but with standards of synthetic rat C peptide I used to replace the human C peptide standards (rat C peptide I was a gift from Dr Sheila Hampton, University of Surrey, Guildford, UK).

Further investigations were carried out using rabbit anti-mouse C peptide antisera I (serum 657) and II (serum 660) - these were a gift of N Blume, Novo Nordisk,

Gentofte, Denmark. These were included in the assay with both human and rat standards, and samples of human, rat and mouse plasma.

5.2.2. Results and discussion of human C peptide assay evaluation

The sensitivity of the anti human C peptide antiserum is demonstrated by Figure 5.1. When a working concentration of 1:27,000 was used, the assay was not able to distinguish the lower concentration standards ie the assay was not sufficiently sensitive. However, when the working concentration of antiserum was increased to 1:15,000, an improved standard curve was obtained over the range tested, and hence the sensitivity was increased. A working concentration of human C peptide antiserum of 1:15,000 was used in all subsequent assays.

The specificity of the human C peptide antiserum is demonstrated by Figure 5.2 and 5.3. The standard curve consisting of the closed squares represents the results obtained from the RIA of human C peptide standards with human C peptide antiserum. The standards were spread over a wide range of concentrations (C peptide of 10, 0.5, 0.05 and 0.012 pmol/ml). The open squares represent the results obtained from the same RIA but with rat C peptide I standards and human C peptide antiserum. A standard curve was not obtained.

These results clearly demonstrate that the human C peptide antiserum will bind human C peptide standards and enable a good standard curve to be produced. However, the same antiserum will not bind rat C peptide I (the amino acid sequences of human and rat C peptides I and II are dissimilar as shown in Table 5.1; the sequence homology of rat C peptides I and II to human C peptide are respectively

Figure 5.1. Standard curve for human C peptide RIA using two different concentrations of antibody; 1:15,000 (closed square) and 1:27,000 (open square). Results mean of 3 samples.

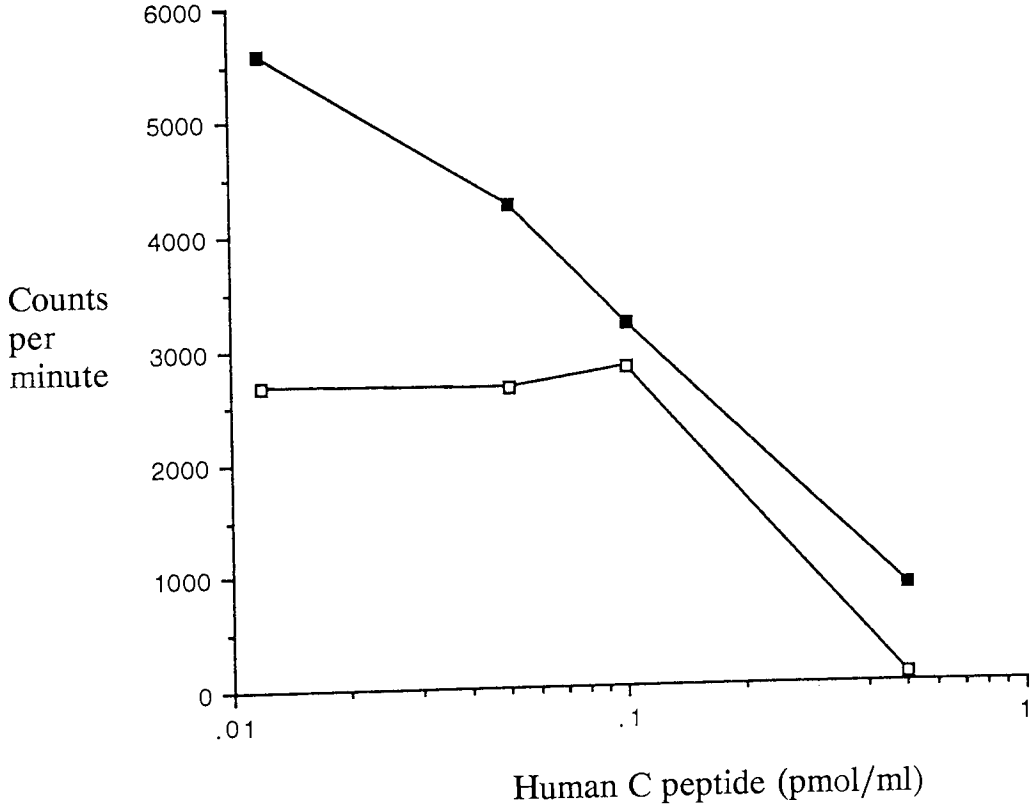


Figure 5.2. Standard curves obtained for human C peptide RIA using human label (diluted 1:60), human antiserum (diluted 1:15,000), and either human standard (closed square) or rat 1 standard (open square) with concentrations as shown. Results mean of 3 samples.

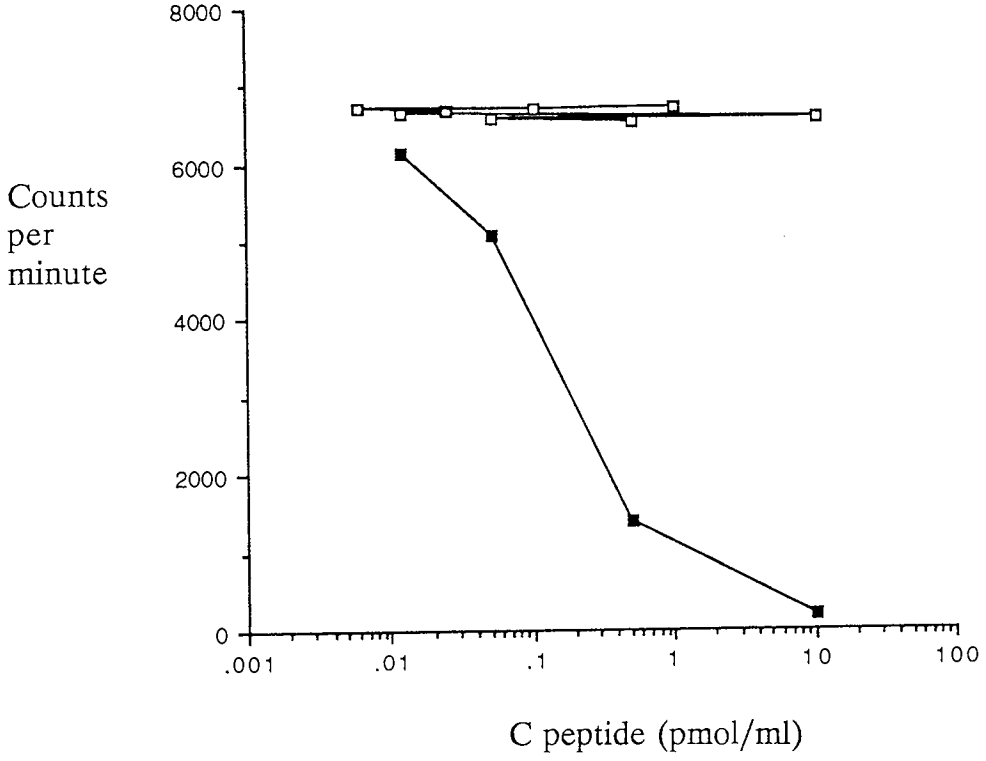
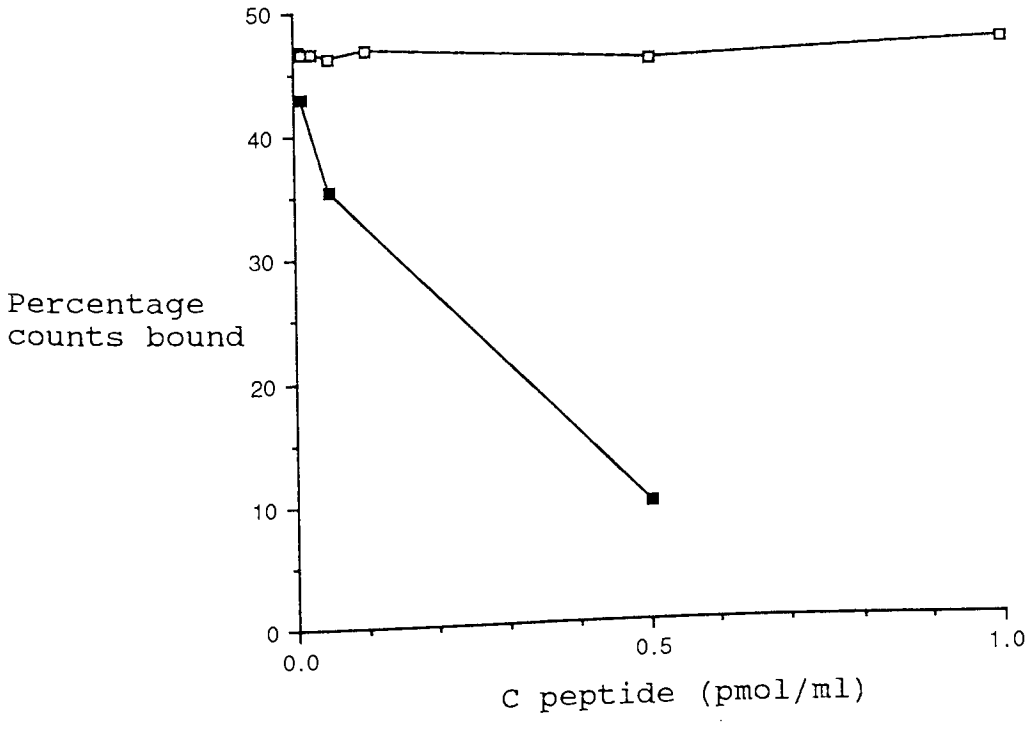


Figure 5.3. Percentage of counts bound from results of human C peptide RIA using human label (diluted 1:60), human antiserum (diluted 1:15,000), and either human standard (closed square) or rat 1 standard (open square) with concentrations as shown. Results mean of 3 samples.



67 % and 70 %). Thus a standard curve was not obtained.

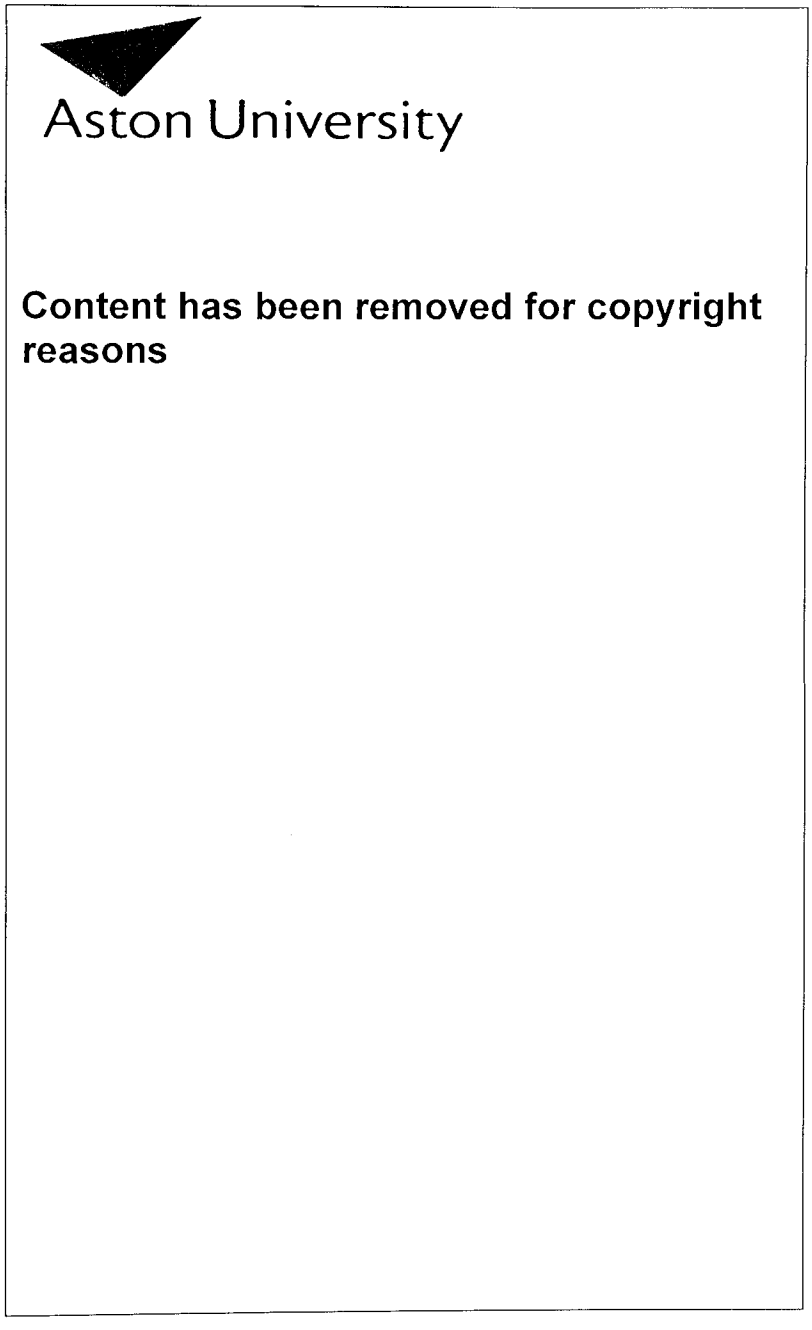
Samples of human, rat and mouse plasma obtained from fed subjects were analysed in this RIA. The human plasma was found to contain C peptide 0.8 pmol/ml (= 2.4 ng/ml), whereas the rat and mouse C peptides were found to be below the lower limit of assay sensitivity.

It can therefore be stated with confidence that the human C peptide antiserum is specific for human C peptide, and in a mixed plasma sample (eg containing rat and human C peptide) the assay will only detect the human C peptide, as no cross reactivity has been found to occur.

A further RIA was performed using antisera raised against mouse C peptides I and II, rat I standard, and human labelled C peptide. The results demonstrated very low counts (<100 cpm) in all tubes. This result suggested that the labelled human C peptide would not compete for binding with the mouse antiserum and rat standard (as would be expected due to the considerable inter-species difference in amino acid sequence), and hence the specificities of the antisera were confirmed.

Using the finalised assay methodology, the coefficient of variation % was found to be 2.7 % for the intra-assay variation, and maximally 11 % for the inter-assay variation of the lower standards.

Table 5.1. Amino acid sequences of C peptides of human and rat I and II proinsulins. Amino acids that are non-homologous with the human equivalent are underlined (Kitabchi, Duckworth and Stentz 1990).



5.2.3. Methods: implantation study one.

Animals

Adult male athymic nude mice of body weight 20-25g were used. The origin and characteristics of these mice have been described elsewhere (Shafrir 1990). The mice were housed isolated in an air-conditioned room which had been previously sterilized. Standard conditions were as previously described. Tapwater and a rodent pellet diet were provided ad libitum unless otherwise required by experimental procedure.

Chemicals

The human C peptide radioimmunoassay antiserum, standards and label were purchased from Novo Nordisk Diagnostics Ltd, Cambridge, UK.

Experimental procedures

The animals were divided into 2 groups for tests and controls, and caged singly. Body weight and food intake were determined regularly throughout the study, the latter being taken as the difference between the amount provided 24 hours previously, and the amount remaining in the hopper.

Blood samples of approximately 80 μl for determination of plasma glucose and human C peptide were obtained from the cut tail-tip of conscious mice into microfuge tubes pretreated with 500 U/ml heparin. Blood samples were stored on ice throughout the procedure. The plasma was separated by centrifugation at 15000 g for 30 seconds; a minimum volume of 40 μl was stored at -20°C for human C peptide assay, and 5 μl used immediately for glucose analysis.

The day on which the cells were implanted was designated as day 0. Plasma samples were obtained for human C peptide analysis on days -7, -2, and -1; and for glucose analysis on day -6.

Oral glucose tolerance test (OGTT)

An OGTT was carried out on day 6. The mice were fasted for 5 hours prior to the test. The glucose dose used was 2g/kg body weight, administered as a 40% w/v solution (ie 5ml/kg) by oral gavage. A blood sample was obtained at 30 minutes following administration of the glucose load, and the plasma used for C peptide and glucose analysis.

Implantation of cells

The cells used for implantation into the test group in this study were AtT20 cells (a murine pituitary cell line) transfected as before with the cDNA pMtNeohPPI/1. These cells were found to secrete human insulin and C peptide, with a negligible proportion of proinsulin (as demonstrated in Chapter 3). Control animals received a similar implant of untransfected rat pituitary cells.

The cells were removed from the plates by trypsinisation and suspended in complete DMEM (as described previously). Immediately prior to implantation, the cells were counted, pelleted by centrifugation and resuspended in sterile PBS to give a final concentration of 10×10^6 cells / ml.

The mice were injected ip with 0.2ml of the cell suspension via a 25G needle (thus each mouse received approximately 2×10^6 cells).

Approximately 5 hours after implantation of the cells, a blood sample was taken from all mice and subsequently assayed for human C peptide and glucose. Further plasma samples for human C peptide and glucose were obtained on days 1 and 5; on day 6 an OGTT was carried out as above, and plasma assayed for human C peptide and glucose. The mice were fasted overnight, and a blood sample was taken for analysis of fasting human C peptide and glucose (day 7).

Induction of diabetes

The mice were treated with streptozotocin 200 mg/kg body weight ip dissolved in citrate buffer (Appendix 1) on day 7 following an overnight fast.

Blood samples were taken for analysis of human C peptide and glucose on days 9, 14, 16, 19, 23, 27 and 29. Plasma glucose only was measured on day 12, and human C peptide only on day 26. On day 20, plasma glucose and human C peptide were measured following an overnight fast.

Animals that developed excessive weight loss following streptozotocin administration were killed by cervical dislocation (1 test mouse on day 19, and 2 more on day 20; 3 control mice on day 13, and 1 more on day 14). The study was terminated on day 29, and all remaining animals were then killed.

Analyses

Glucose and human C peptide were analysed as described previously.

Statistical analysis

Results were expressed as mean ± standard error of mean (x ± SEM) followed by the number in parentheses (n). Values between the 2 groups were compared using Student's unpaired t-test, and differences were considered to be statistically significant for $p < 0.05$.

5.2.4 Methods: implantation study two

Animals and chemicals

Animals and chemicals were as previously described.

Experimental procedures

Body weights and the food and fluid intake of the mice were determined regularly as described previously. Blood samples were obtained and stored as before. The animals were injected with streptozotocin 100 mg/kg body weight ip dissolved in citrate buffer (Appendix 1) on day -10 following an overnight fast. A further dose of 40 mg/kg ip was given on day -3.

Implantation of cells

The day on which the cells were implanted was designated as day 0. The animals were divided into 4 equi-glycaemic groups to serve as ip tests, sc tests, controls which received an ip implant of rat pituitary cells, and a group of mice that received an ip implant of G418-resistant cells (the 'latent' group). The cells used for the ip and sc test groups were AtT20MtNeohPPI/1 cells as described previously. A further group received an implant of AtT20 cells transfected with the same vector and which *in*

vitro had been found to be G418 resistant but not to release any insulin or proinsulin. These cells were termed latent. The cells were prepared for implantation as before. The mice were injected ip, or sc between the shoulder blades with 0.2 ml of a cell suspension in PBS via a 25G needle - each mouse received approximately $5x10^6$ cells. Control mice received cells ip.

Blood samples for analysis of human C peptide and glucose were taken on days 2, 4, 7, 11 (following an overnight fast - ip and control groups only), 14, 16, 21, 23, 25 and 30. Animals developing excessive hyperglycaemia were treated with human protophane insulin sc between the shoulder blades; a dose of 100 - 150 U/kg was found to be necessary for the control of hyperglycaemia.

Between days 11 and 25 the drinking fluid of all mice was replaced with a solution of $ZnSO_4$ 500mg/l. On day 25, the mice were returned to tapwater as the only source of drinking fluid.

Oral glucose tolerance test (OGTT)

An OGTT was performed on day 8 in the ip and control groups only, as described previously. Plasma samples were taken for glucose and human C peptide analysis.

Autopsy

On day 30 the mice were killed by cervical dislocation. During the course of the study, a visible tumour-like cell cluster developed at the site of implantation, and on autopsy these were found to be discrete clusters of cells either subcutaneous or inside the abdominal wall, with a well developed vascular supply and external capsule.

Each tumour was excised and either processed for histological staining or extracted with acid ethanol.

Acid ethanol extraction of tissue

After weighing, the excised tumours of all mice were extracted with acid ethanol, as described in Chapter 2. The supernatant was then assayed for human C peptide at a dilution of 1 / 100.

Histological processing

Tumours processed for histological staining were cleared, dehydrated, embedded in paraffin wax, and cut into sections 5 μm thick using a rotary microtome as described in Chapter 2. The sections were subsequently stained with either haematoxylin and eosin, aldehyde fuchsin or processed immunocytologically, also as described in Chapter 2.

The tumours from this study were processed, sectioned and stained with haematoxylin and eosin by Mr Barry Simms, the Department of Histology, General Hospital, Birmingham, and the immunocytological processing was carried out by Dr Irene Green, Department of Biological Sciences, University of Sussex, Brighton.

Analyses

Plasma glucose and human C peptide were assayed as described previously. Statistical analysis was also performed as before.

5.3. RESULTS

5.3.1. Results of implantation study one

Following injection of streptozotocin on day 7, the control animals rapidly became hyperglycaemic, and exhibited substantial hyperglycaemia-related mortality at an early stage of the study. For this reason, data pertaining to the control group is only shown up to and including day 12.

Body weights

The body weights of the mice remained fairly constant throughout the course of the study - this is shown in Figure 5.4. It was noticable that there was no appreciable loss of weight following administration of the streptozotocin on day 7. No statistically significant differences were found between the two groups.

Food intake

The mean food intake of the mice fluctuated during the study between 3.0 and 6.8 g/mouse/day, with the lowest value found on the day following administration of the cells. Food intake is shown in Figure 5.5. Towards the end of the experimental period the general trend was increasing consumption in accordance with the manifestation of hyperglycaemia. By day 9 the food intake of the control group was significantly ($p<0.001$) greater than that of the test group.

Plasma glucose

Plasma glucose concentrations of the mice throughout the course of the study are shown in Figure 5.7, and the values obtained from an OGTT and following an

Figure 5.4. Body weight of nude mice implanted ip with AtT20MtNeohPPI/1 cells (closed squares) or isolated pituitary cells (open squares). Results mean ± SEM for 10 - 11 observations.

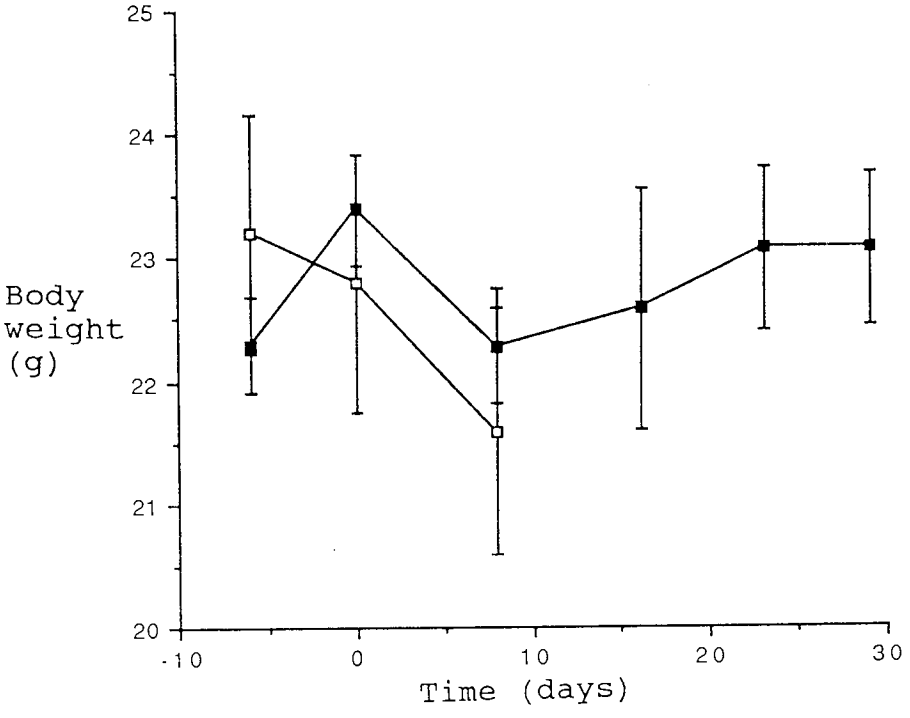


Figure 5.5. Food intake of nude mice implanted ip with AtT20MtNeohPPI/1 cells (closed squares) or isolated pituitary cells (open squares). Results mean ± SEM for 10 - 11 observations. [d] $p<0.001$

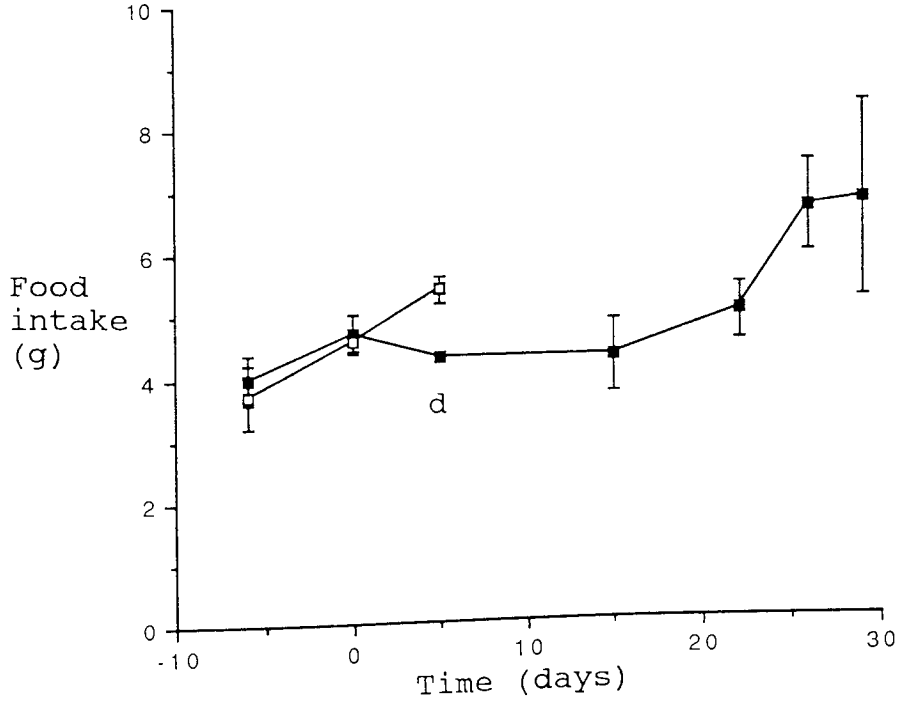


Figure 5.6. Plasma human C peptide concentration of nude mice implanted ip with AtT20MtNeohPPI/1 cells (closed squares)
Results are mean ± SEM for 10 - 11 observations. Line i indicates normal plasma levels of C peptide in humans, line ii indicates lower limit of assay sensitivity.

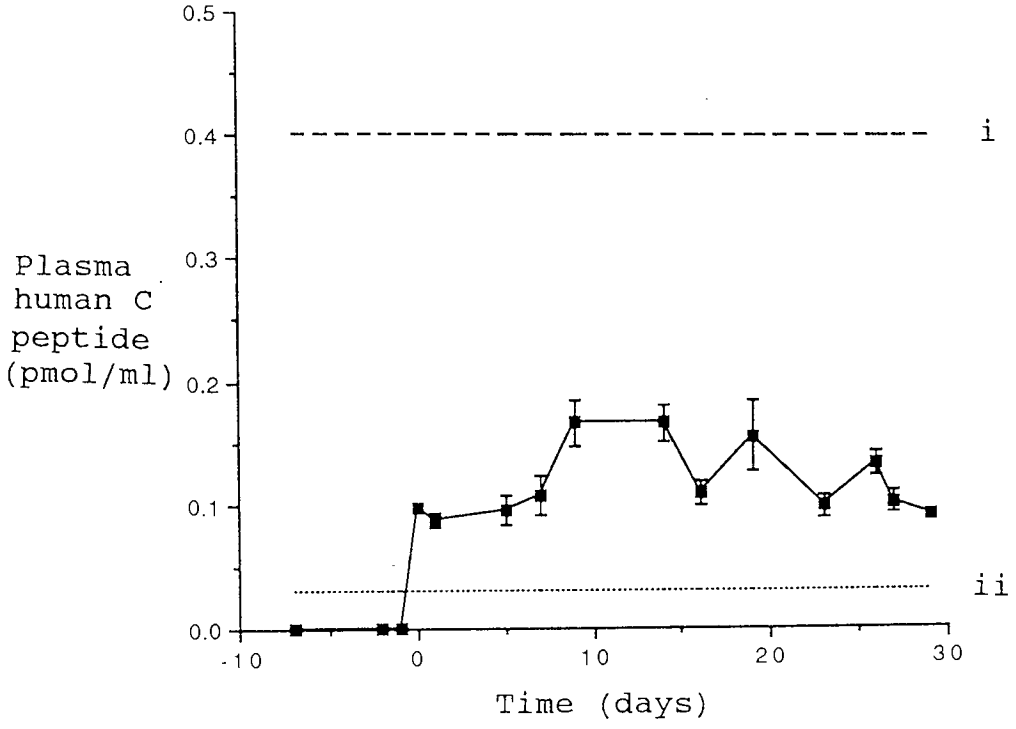


Figure 5.7. Plasma glucose concentration of nude mice implanted ip with AtT20MtNeohPPI/1 cells (closed squares) or isolated pituitary cells (open squares). Results are mean ± SEM for 10 - 11 observations. [c] $p<0.01$, [d] $p<0.001$

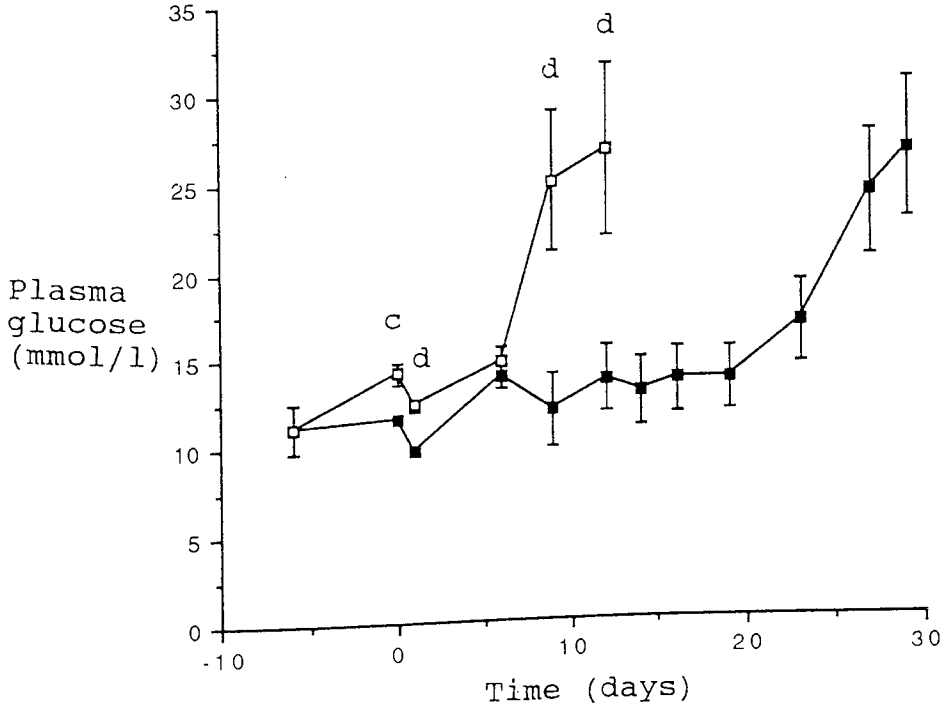


Figure 5.8. Plasma glucose concentration of nude mice implanted ip with AtT20MtNeohPPI/1 cells (closed bar) or isolated pituitary cells (open bar), following an overnight fast, and 30 minutes after an oral glucose challenge of 2 g/kg. Results are mean ± SEM for 10 - 11 observations. [d] $p<0.001$

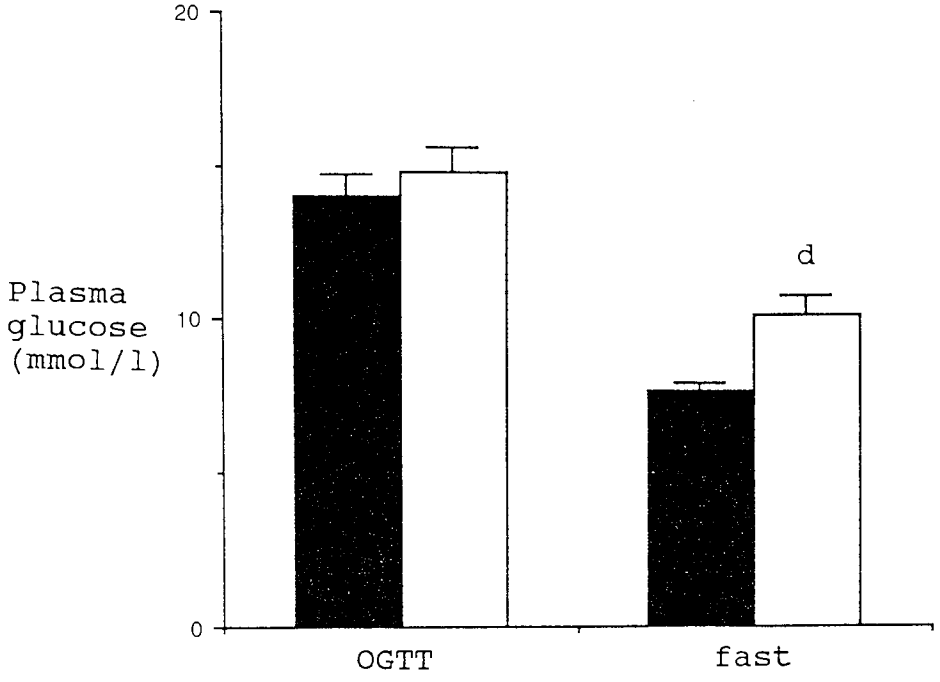
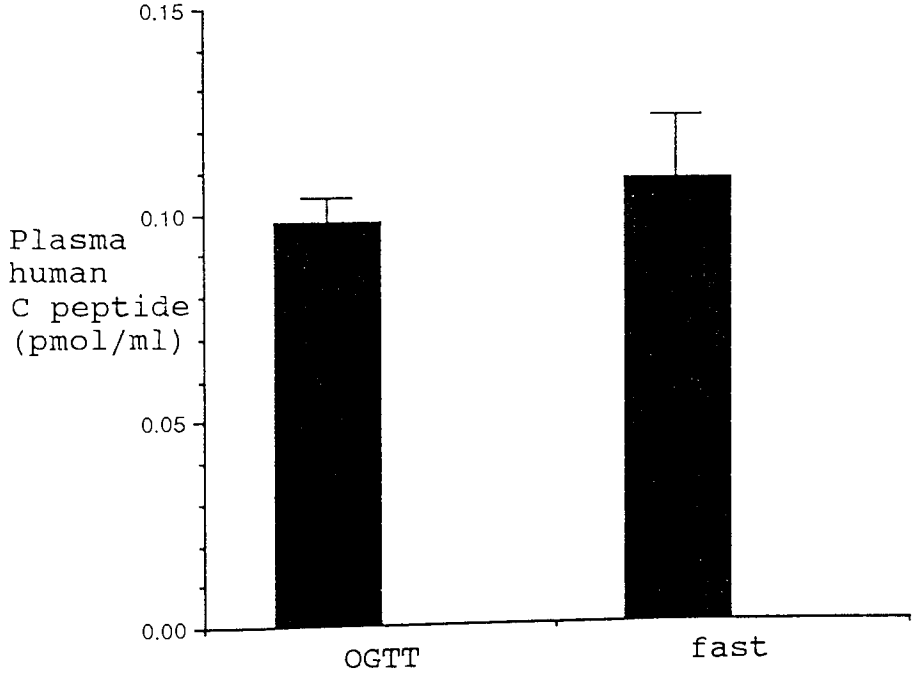


Figure 5.9. Plasma human C peptide concentration of nude mice implanted ip with AtT20MtNeohPPI/1 cells (closed bar) following an overnight fast, and 30 minutes after an oral glucose challenge of 2 g/kg. Results are mean ± SEM for 10 - 11 observations.



overnight fast are shown in Figure 5.8.

No significant difference in plasma glucose concentration was found between the 2 groups of mice on days -6 or 6. However on days 0, 1, 9 and 12 the plasma glucose concentration of the control group was significantly greater ($p<0.01$, $p<0.001$, $p<0.001$ and $p<0.001$ respectively) than that of the tests.

An OGTT carried out on day 6 showed no significant difference in plasma glucose concentration 30 minutes post challenge between the groups.

An overnight fast on day 7 revealed that the control group had a significantly elevated plasma glucose concentration ($p<0.001$) when compared to the test group.

Human C peptide

It has been noted earlier in this Chapter that the C peptide RIA used in this study is capable of differentiating human C peptide from rat/mouse C peptides, such that only the human C peptide is detected in a plasma sample containing human and rat/mouse C peptides.

The lower limit of assay sensitivity was determined by calculating 2 x the standard deviation of the zero standard value, and adding this to the mean: all points under this value were excluded since it was not possible to confidently distinguish them from zero. Hence all samples that were found to be below 0.03 pmol/ml were arbitrarily called zero.

The results obtained from this specific assay demonstrate no detectable human C peptide in the test mice before the implantation of cells, and no detectable human C peptide in the control mice at any time during the study. In all samples taken from day 0 (5 hours after cell implantation), to the end of the study on day 29, human C peptide was detectable in the plasma of the test mice. This is shown in Figure 5.6. The maximum mean concentration found was 0.167 pmol/ml on days 9 and 14; a sample of fed human plasma analysed in the same assay was found to contain 0.4 pmol/ml human C peptide. Thus the maximum mean concentration found in the test group was approximately 42% of that found in a normal fed human.

The C peptide concentration of the sample obtained from the OGTT carried out on day 6 is shown in Figure 5.9. No human C peptide was detected in the control group. Following an overnight fast on day 7, human C peptide was detected in the plasma of the test group only; this is also shown in Figure 5.9.

5.3.2. Results of implantation study two

Body weights and food intake

No significant differences in body weights or food intake were observed between the four groups of mice during the study (data not shown). Body weight gradually decreased, and food intake gradually increased over the 30 days, consistent with the development of hyperglycaemia.

Plasma glucose

The plasma glucose concentrations of the four groups of mice are shown in Figure 5.10.

The plasma glucose concentration of the control group remained fairly consistent throughout the study. No obvious decrease was observed during the period of $ZnSO_4$ ingestion.

Prior to the provision of $ZnSO_4$ in the drinking water, the plasma glucose concentration in the ip group (the mice that received an ip implant of insulin-secreting AtT20 cells) increased, but towards the end of the period of zinc ingestion began to decline. After the removal of zinc, the mean plasma glucose level increased again.

Little change in plasma glucose concentration was observed in the group receiving a sc implant of insulin-secreting cells during the 30 day period of study, although a small decline was seen towards the end of the period of $ZnSO_4$ administration.

The group that received the implant of latent cells did not demonstrate much fluctuation in plasma glucose concentration, although during the period of $ZnSO_4$ ingestion, the mean level decreased slightly.

30 minutes after an OGTT carried out on day 8, the plasma glucose concentration of the ip group was found to be 13.0 ± 1.43 (7) mmol/l, with that of the control group being 12.4 ± 0.66 (6) mmol/l. These results are not significantly different.

Figure 5.10. Plasma glucose concentration of streptozotocin-treated nude mice implanted with AtT20MtNeohPPI/1 cells ip (closed square) or sc (open square), latent AtT20MtNeohPPI/1 cells ip (closed circle), or isolated pituitary cells ip (open circle). Results are mean ± SEM for 7-4 observations. Vertical axis is plasma glucose (mmol/l); horizontal axis is time (days).

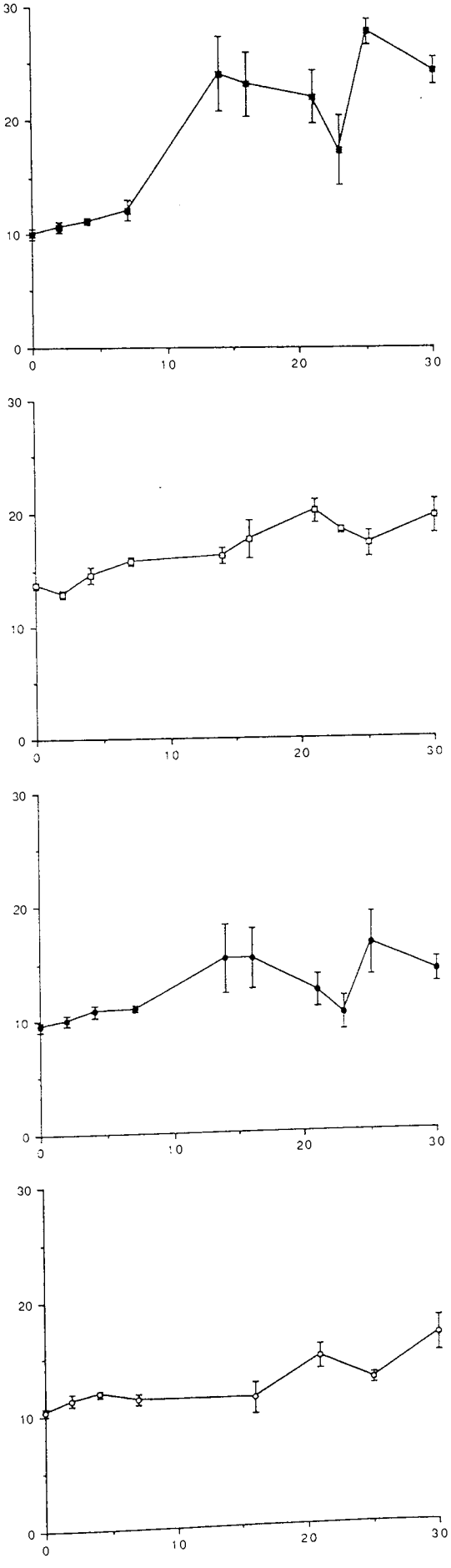
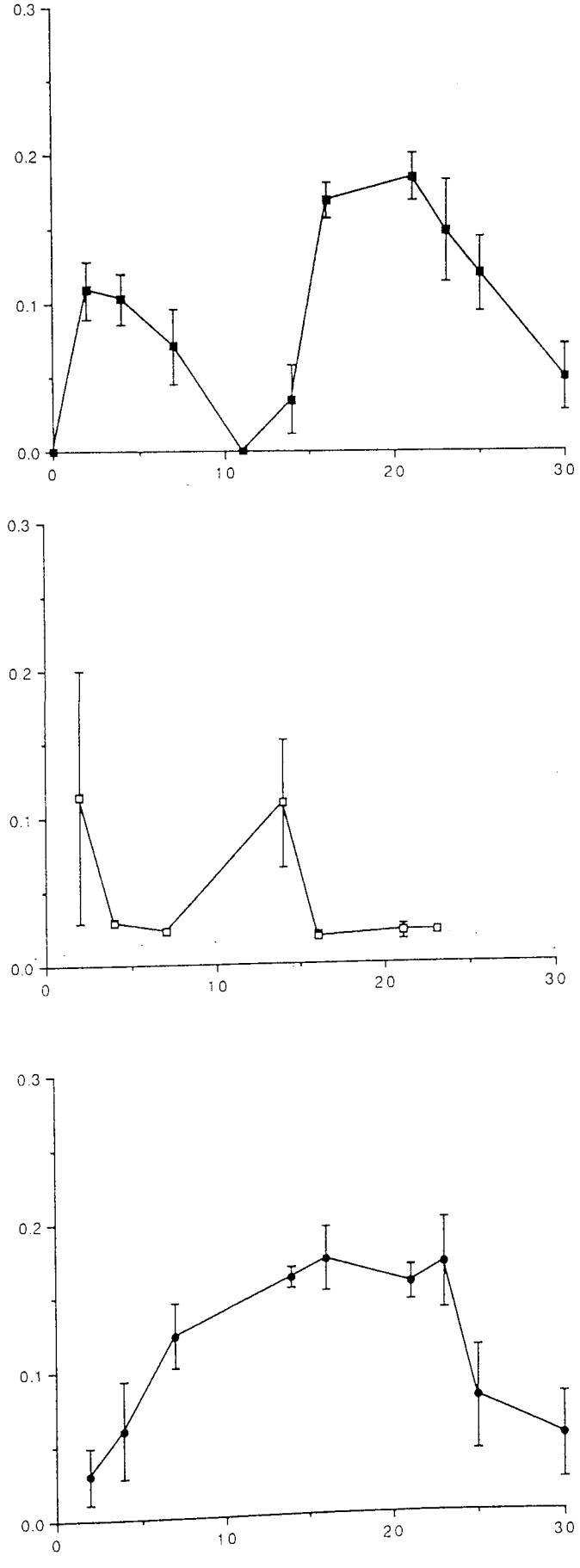


Figure 5.11. Plasma human C peptide concentration of streptozotocin-treated nude mice implanted with AtT20MtNeohPPI/1 cells ip (closed square) or sc (open square), latent AtT20MtNeohPPI/1 cells ip (closed circle). Results are mean ± SEM for 7-1 observations. Vertical axis is plasma human C peptide (pmol/ml); horizontal axis is time (days).



Following an overnight fast on day 11, the plasma glucose concentration of the ip group was found to be 9.1 ± 0.91 (7) mmol/l, whilst that of the control group was 9.7 ± 0.88 (6) mmol/l. These results are not significantly different.

Plasma human C peptide

The plasma human C peptide concentrations found in the 4 groups of mice throughout the study are shown in Figure 5.11.

No human C peptide was found in the plasma of the control group (the group implanted with isolated rat pituitary cells) at any time during the study.

It is revealing to examine the data qualitatively. The response of the group receiving an implant of insulin-secreting cells sc was poor, with only 2 animals from a total of 5 showing any detectable human C peptide in their plasma at any time during the study. From an initially high level on day 2 the mean concentration quickly declined, followed by a slight increase concurrent with the provision of $ZnSO_4$ in the drinking water, and then declined rapidly to levels that became undetectable by day 25.

The mice that received an implant of identical cells to the ip site demonstrated similar results, but with greater amounts of human C peptide. From a moderate initial concentration (human C peptide was detectable in 7/7 mice), the levels declined steadily until day 14, after which (again concurrent with the provision of $ZnSO_4$ in the drinking water), the mean concentration increased markedly. This increased plasma level was sustained for over 7 days before beginning to decrease.

The pattern observed in the group receiving an ip implant of so-called latent cells was completely dissimilar to the groups described above. *In vitro* these cells were found to release no substance with insulin-like immunoreactivity. However *in vivo* human C peptide was detected in the plasma of these mice, although initially in very low concentrations. This steadily increased until day 16, after which a rapid decline was observed. There did not appear to be any obvious response to $ZnSO_4$ in the drinking water.

30 minutes after an OGTT on day 8, no human C peptide was detectable in the control group, whilst the concentration found in the test group was 0.095 ± 0.027 (7) pmol/ml.

Following an overnight fast on day 11, human C peptide was not detectable in either the control or the ip test group.

Autopsy

At the end of the study, the implantation sites of the ip test group were investigated. Tumour like cell aggregations were found adhering to the peritoneal wall. These were excised and extracted with acid ethanol for subsequent assay of human C peptide content, or subjected to histological processing, all as described in Chapter 2.

The mean weight of the tumours removed from the ip group was 56.17 ± 13.3 (4) mg. Plate 5.1 shows the tumour removed from one mouse, and Plate 5.2 shows further tumours excised at autopsy. The mean human C peptide content of these

Plate 5.1. Nude mouse with excised tumour after 30 days growth *in vivo*.

Plate 5.2. Tumours removed from nude mice after 30 days growth *in vivo*.

tumours was found to be 1.72 ± 0.46 (4) pmol human C peptide / g tissue.

Histochemistry

Haematoxylin and eosin stained sections of a tumour-like cell aggregation removed from the ip site, shown in Plates 5.3 and 5.4, demonstrates apparently normal cell morphology with some central necrotic areas.

Aldehyde fuchsin staining of the same tumour sections (Plates 5.5, 5.6, 5.7 and 5.8) demonstrates the presence of insulin-containing cells, illustrated by the darkly stained areas. These appear to be unevenly distributed throughout the tumour, with darkly stained areas close to blood vessels and the external surface of the tumour.

Immunofluorescence staining of the tumour sections (Plates 5.9, 5.10 and 5.11) also demonstrates the uneven distribution of insulin-containing cells within the tumour. The fluroescent areas appear to be localized either under the capsule or around blood vessels.

Plate 5.3. Haematoxylin and eosin stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 250.

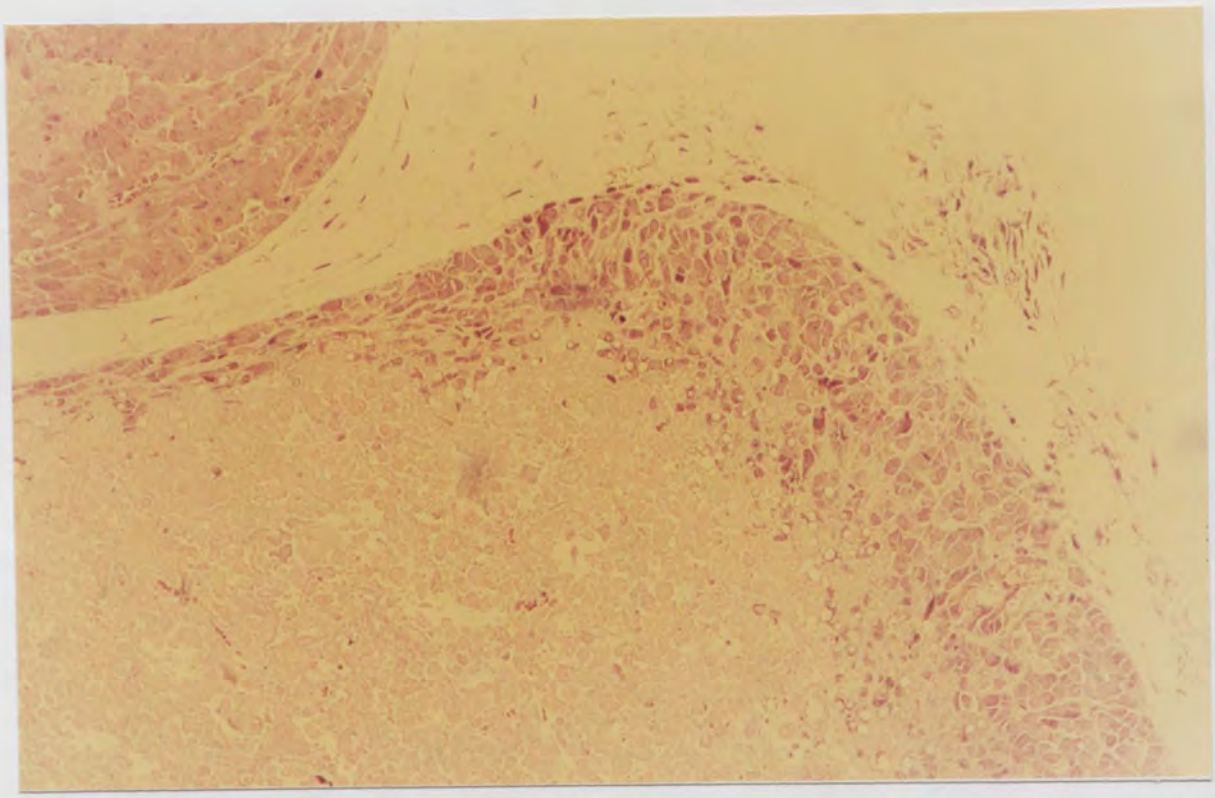


Plate 5.4. Haematoxylin and eosin stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 250.

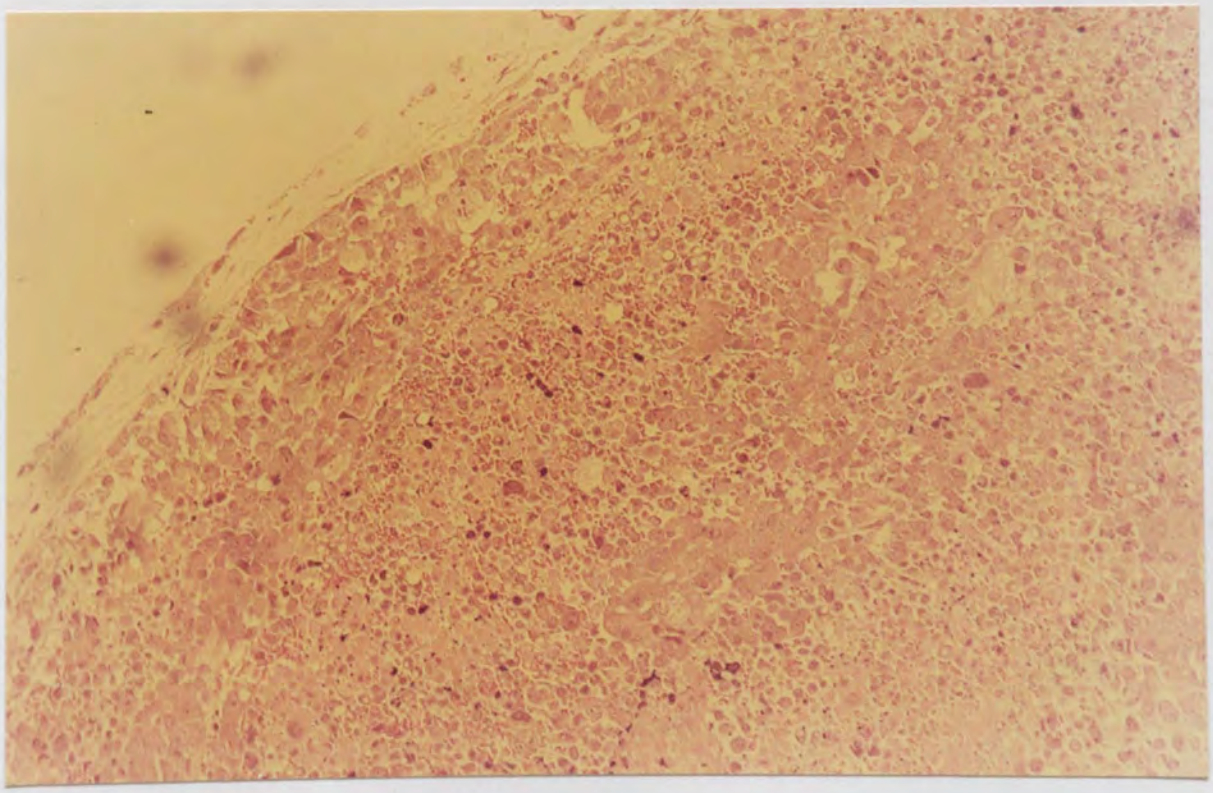


Plate 5.5. Aldehyde fuchsin stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 250.

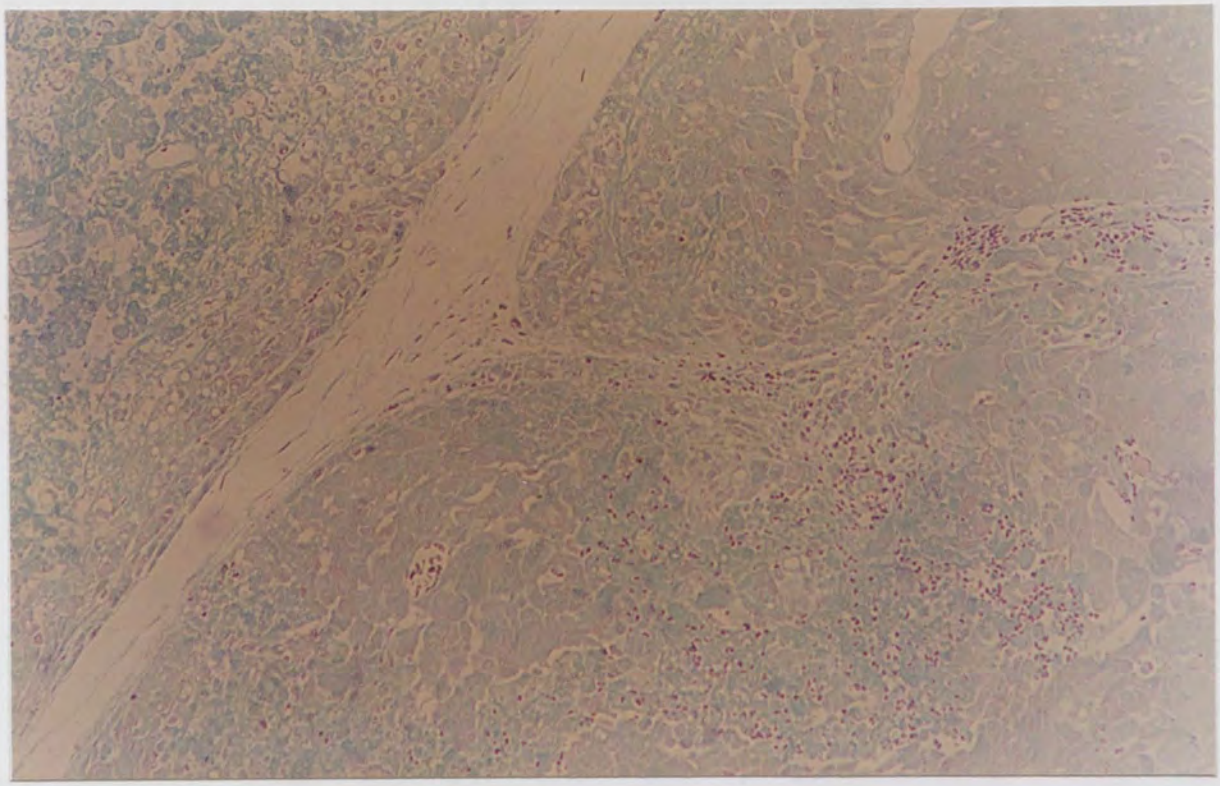


Plate 5.6. Aldehyde fuchsin stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 250.

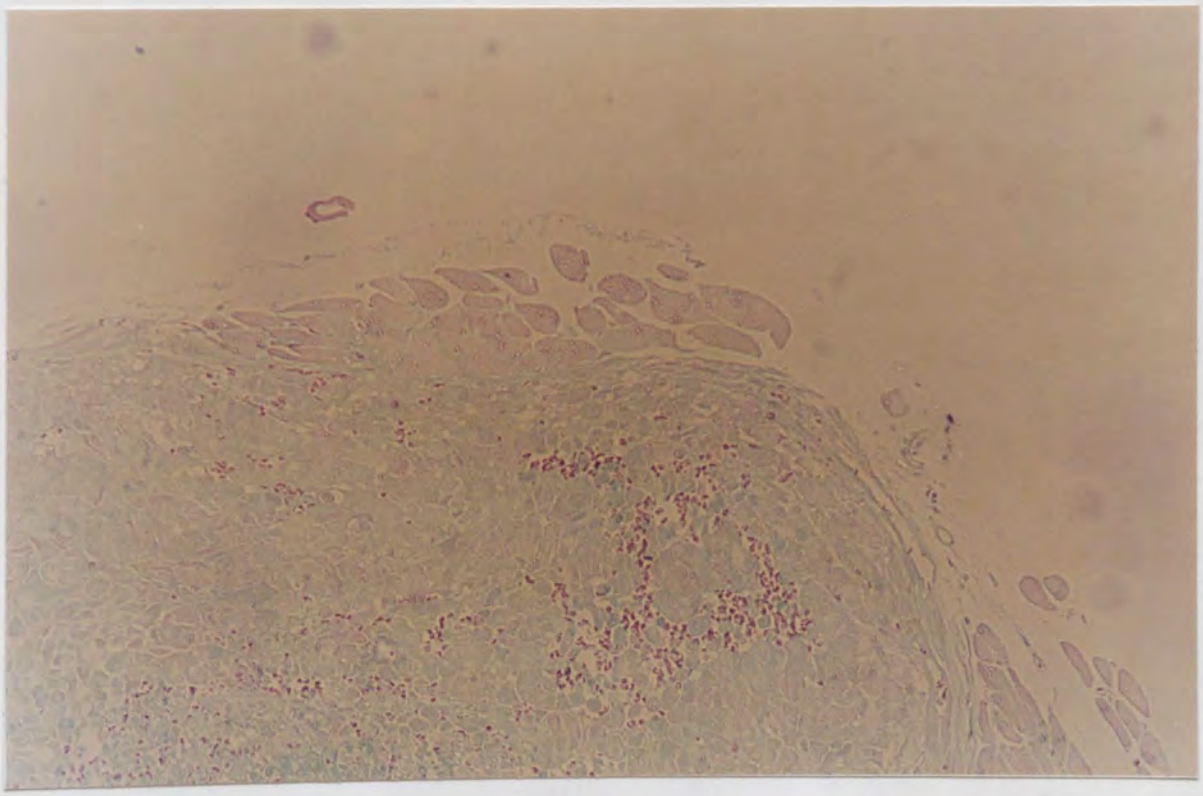


Plate 5.7. Aldehyde fuchsin stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 250.

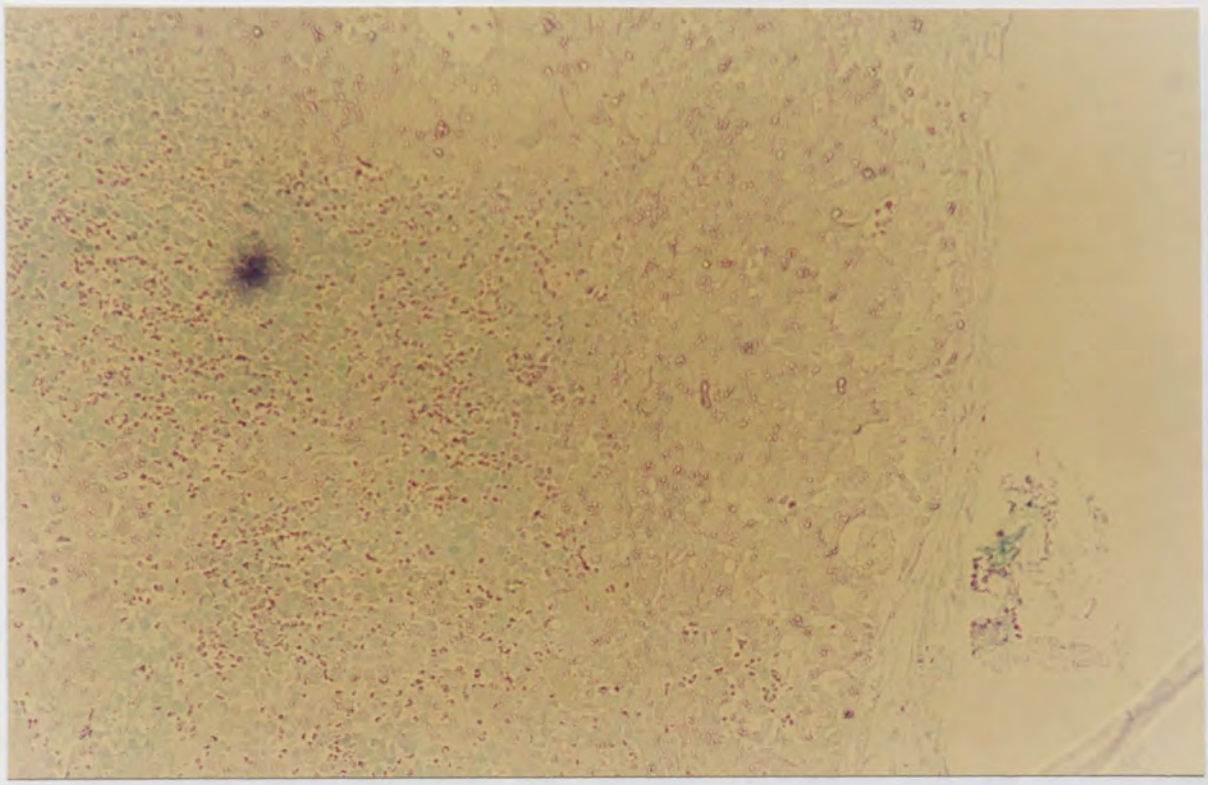


Plate 5.8. Aldehyde fuchsin stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 250.

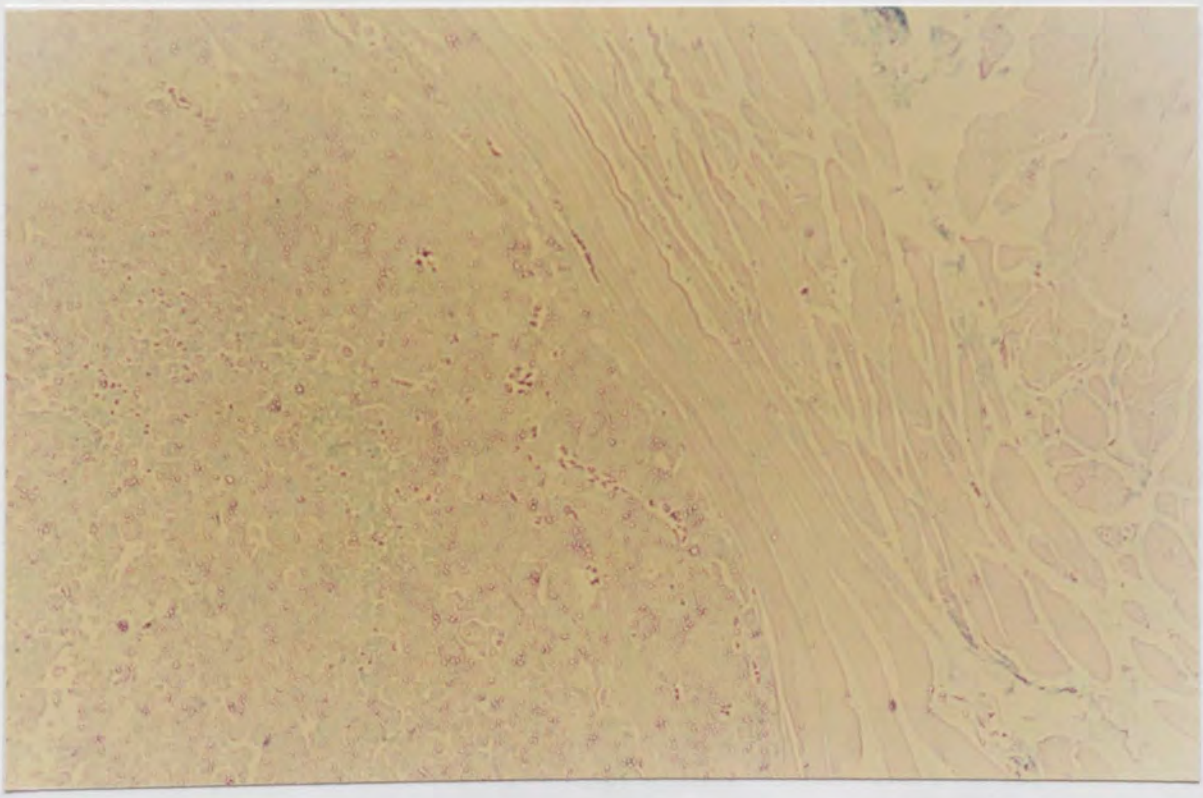


Plate 5.9. Immunocytochemically stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 500.

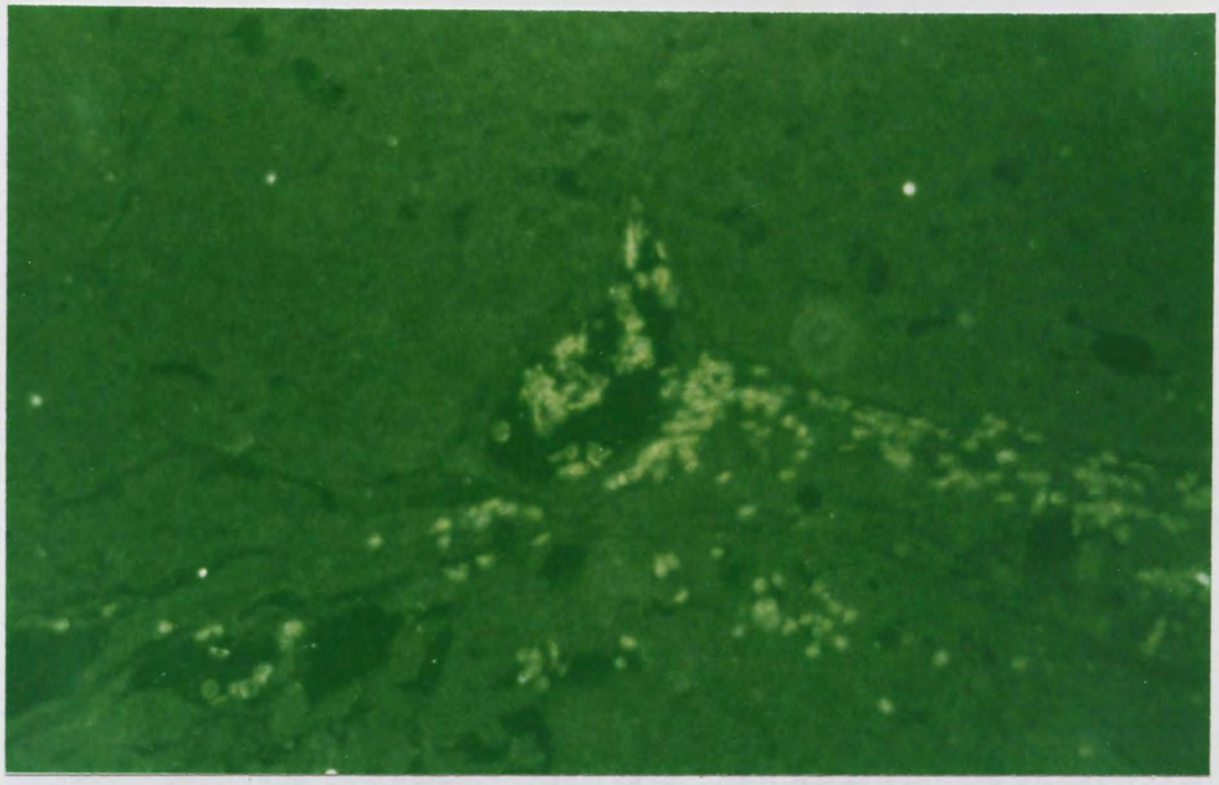


Plate 5.10. Immunocytochemically stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 500.

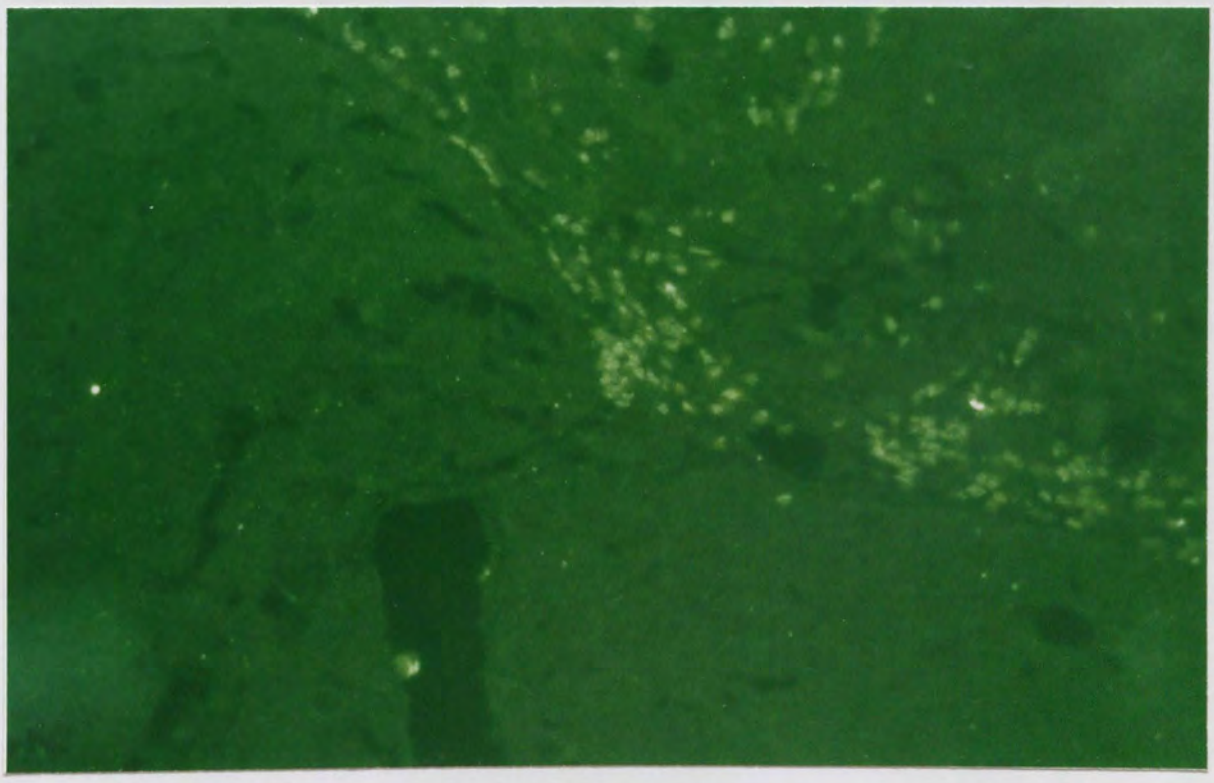
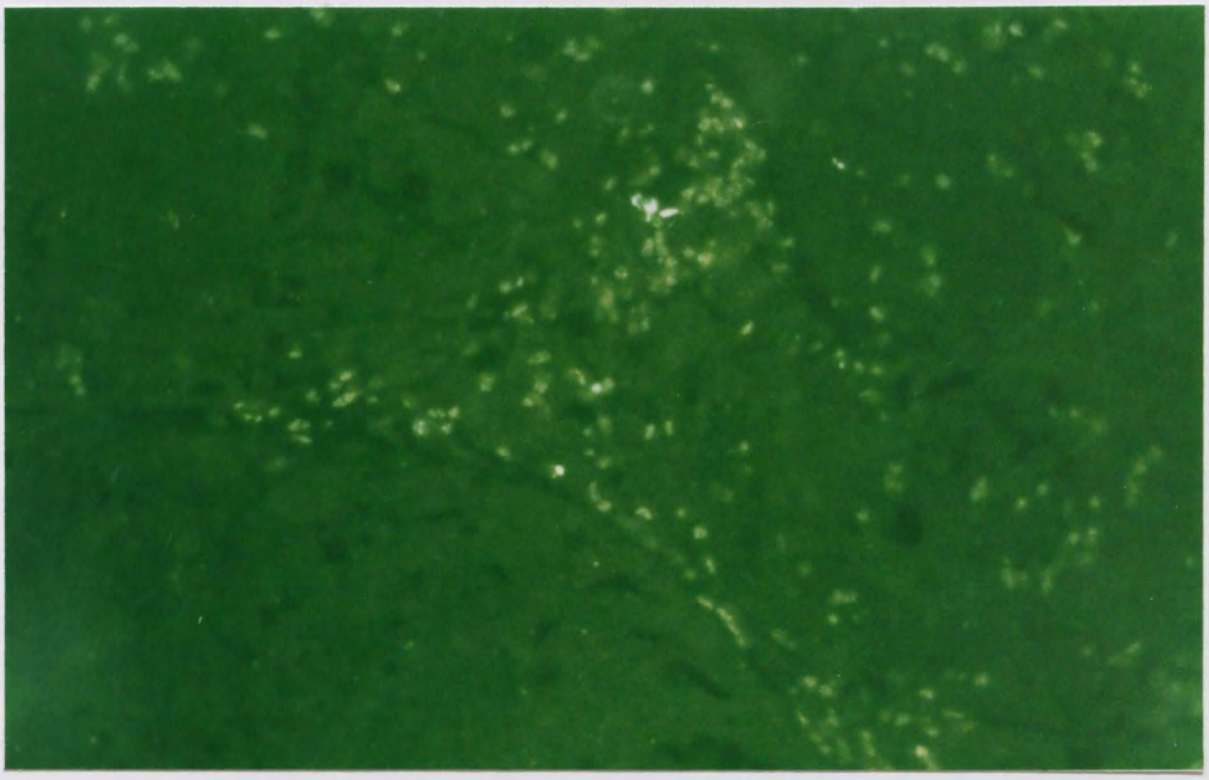


Plate 5.11. Immunocytochemically stained section of an ip tumour removed from a nu/nu mouse after 30 days growth *in vivo*. Magnification x 500.



5.4. DISCUSSION

Implantation study one

The work described in this section relates to the first feasibility study involving the ip transplantation of insulin-secreting cells into nu/nu mice. Following implantation of the cells, diabetes was induced in the mice with a large dose of streptozotocin in order to assess the effect of the implant on the development of hyperglycaemia and other manifestations of experimental diabetes. Early reports that nu/nu mice were impervious to streptozotocin (explained by a dependence of streptozotocin action on thymus function) (Buschard and Rygaard 1978) proved inaccurate. Subsequent workers found that ip administration of a large dose of streptozotocin (200 mg/kg body weight) resulted in the development of a rapid and severe hyperglycaemia within 2 days of injection - mean plasma glucose values of 500 mg/dl (28 mmol/l) were reported (Paik, Fleischer and Shin 1980). Male mice were found to be more susceptible to the streptozotocin than females. On the basis of this work, a streptozotocin dose of 200 mg/kg body weight was selected for use in this study since it could be expected to efficiently induce experimental diabetes.

As mentioned in the Introduction to this Chapter, nu/nu mice display certain endocrine abnormalities, including effects on endogenous β-cell function. These effects include moderate hyperglycaemia, an impaired glucose tolerance, low basal and low glucose-stimulated plasma insulin levels, and an elevated glycohaemoglobin when compared to heterozygous littermates. These effects are only present in male nu/nu mice between the ages of 6 to 12 weeks (Ziedler *et al* 1982, Ziedler *et al* 1984). Thymus implantation from heterozygous littermates causes only moderate

improvement in glucose homeostasis (which can be correlated to T cell function), and so these abnormalities are not due solely to immunoincompetence. It has been reported that although pancreatic insulin and glucagon levels are normal in hyperglycaemic nu/nu mice, somatostatin levels are increased, and more D cells are present. *In vitro* studies indicate that insulin release from an isolated nu/nu pancreas is essentially normal, thus the reason for reduced plasma insulin concentrations and a reduced insulin response to glucose *in vivo* remains to be clarified (Shafrir 1990).

Throughout the 12 days that the control animals were included in the study, no difference in body weights was observed between the 2 groups, but by day 12 the control group had demonstrated a greater level of hyperphagia than the tests, which is consistent with the development of experimental diabetes.

Prior to implantation of the cells, (the day -6 sample), no difference in plasma glucose concentration was found between the 2 groups. The day 0 sample was taken 5 hours after the implantation; in this sample and subsequent samples taken on days 1, 9 and 12, the plasma glucose concentration of the test group was significantly lower than the control group. Severe hyperglycaemia developed in the control group by day 9 (2 days after streptozotocin administration), and after day 12 this group demonstrated substantial hyperglycaemia-related mortality. No difference in oral glucose tolerance was found on day 6, but following an overnight fast on day 7 the test group had a significantly lower plasma glucose concentration than the controls. A similar pattern has been reported previously; proinsulin-releasing fibroblasts implanted into diabetic mice caused an immediate effect on fasting plasma glucose concentrations, but there was no discernable effect on fed glucose concentrations

until at least 14 days following implantation (Kawakami *et al* 1992).

Using a highly specific human C peptide RIA, no human C peptide was detectable in the control group at any time during the study, or in the test group prior to cell implantation. From the day 0 sample onwards, human C peptide was detected in the plasma of the test mice over the 30 days of the study. The mean level fluctuated, but did not seem to be markedly affected by an OGTT on day 6, an overnight fast on day 7, or the eventual development of hyperglycaemia by day 30. The persistence of human C peptide in the plasma after an overnight fast suggests that the implant continued to release insulin despite non-feeding by the mice; this resulted in a significantly depressed plasma glucose concentration in the test group on the morning following the fast. The presence of human C peptide in the plasma of the test mice indicates that the ip implant of genetically modified insulin-secreting cells survived *in vivo* and continued to release human insulin plus C peptide. The products of these cells were also able to enter the circulation from the ip site, possibly achieving a physiological route of delivery by passing into the portal circulation. The results clearly demonstrate that development of hyperglycaemia following streptozotocin administration was delayed in the test mice. Since human C peptide was detected in the plasma of these mice throughout the study, it is reasonable to assume that equi-molar amounts of human insulin were also released, and that this insulin helped to control the hyperglycaemia following streptozotocin administration. However, hyperglycaemia did eventually occur in the test group by day 30 (approximately 15 days after the control group), and this was accompanied by severe insulin resistance (determined previously by means of an insulin sensitivity test). Streptozotocin-treated animals are known to display insulin-resistance (Nishimura *et al* 1989). It can

therefore be postulated that the insulin released constitutively by the implant considerably slowed the development of high plasma glucose levels, although eventually the amount of insulin released was not sufficient to completely prevent the occurrence of frank hyperglycaemia, particularly since insulin resistance is known to have been present, and hence the efficacy of any insulin present would be reduced.

The test mice received an implant of insulin-secreting AtT20 pituitary cells. As mentioned in the Discussion to Chapter 3, these cells produce, process and release various peptides derived from the POMC precursor, including ACTH and opiates. In a clinical situation, a non-pituitary cell type would ideally be used, but for the purposes of this prototype study AtT20 cells were utilized because they contain endopeptidase activity, and have the capability to process proinsulin to insulin. Hence an effect of pituitary peptides upon glucose homeostasis must also be considered. It has previously been reported that AtT20 cells transfected with the human insulin gene release processed human insulin in amounts equivalent to about 1 % of that of ACTH (Moore *et al* 1983).

In vivo, ACTH stimulates the synthesis and release of glucocorticoids from adrenal tissue; this is mainly cortisol in humans but corticosterone in rodents. The binding of ACTH to high affinity receptors on the plasma membrane of adrenocortical cells causes activation of adenylate cyclase which increases intracellular cAMP, and then activation of phosphoprotein kinases which in turn activate the conversion of cholesteryl esters to cortisol or corticosterone (Hadley 1992). Rising concentrations of glucocorticoid in the plasma causes inhibition of ACTH release. Glucocorticoids are bound in the plasma to corticosteroid-binding globulin (CBG); bound steroids are

physiologically inactive (Ganong 1985).

Acute administration of ACTH causes a decrease in plasma glucose and an increase in plasma insulin - these effects are not however necessarily related since the glucose level remains depressed after the insulin levels have returned to normal (Bailey and Flatt 1987a). However the main effect of increased circulating ACTH concentrations is stimulation of glucocorticoid release. Glucocorticoids produce metabolic effects antagonistic to those of insulin; a prolonged excess can cause diabetes (so-called steroid-induced diabetes). Administration of glucocorticoids causes prolonged hyperglycaemia (mediated by increased gluconeogenesis, potentiation of glucagon and catecholamine action, impaired peripheral glucose utilization, and insulin resistance), an impaired glucose tolerance, a mild insulinogenic effect, and a β-cell cytotropic effect (Lenzen and Bailey 1984).

In addition to ACTH, AtT20 cells also release β-endorphin. This peptide binds to opiate receptors, and causes increased plasma glucose levels (possibly via increased glucagon levels) and increased insulin concentrations (Bailey and Flatt 1987b). Increased circulating concentrations of opiates appear to be important contributory factors in the development of hyperglycaemia and hyperinsulinaemia in some diabetic states such as the obesity/diabetes syndrome of the ob/ob mouse (Khawaja *et al* 1990).

Hence implantation of ACTH / opiate secreting cells will cause increased endogenous concentrations of ACTH, β-endorphin and glucocorticoids (corticosterone), with resultant effects on glucose homeostasis. Animals treated with

streptozotocin may be able to maintain a near-normal basal plasma insulin level but are unable to respond to a glucose challenge with an increased insulin output since they have no insulin reserve (Bailey *et al* 1986). It is therefore unlikely that corticosterone could cause an increase in plasma insulin in streptozotocin treated animals. Thus the hyperglycaemic / insulin resistant effects are likely to occur independently of any effect on plasma insulin levels.

In conclusion, this first study using nude mice has demonstrated the original premise that a non-β-cell which has been transfected with the human preproinsulin gene can be used as a source of exogenous insulin to at least partially replace normal endogenous pancreatic function when implanted into an animal model of diabetes.

Implantation study two

The second nude mouse implantation experiment described here differs from the first in numbers of cells implanted, and also in the sites of implantation used. 5×10^6 insulin-secreting AtT20 cells were implanted ip and sc; control rat pituitary cells were implanted ip. Also 5×10^6 G418-resistant non-insulin-secreting AtT20 cells were implanted ip. The results obtained from this study, whilst not as clear-cut as those from the previous study, are nevertheless interesting.

The human C peptide concentrations found in the plasma of the mice provided an indicator of graft function. No human C peptide was detected in the control group at any time, demonstrating that the source of human C peptide was the implant of genetically modified cells. The sustained (albeit low) concentrations of human C

peptide detectable in the plasma of the mice over the 30 days of the study illustrated prolonged graft survival *in vivo*. The detection of human C peptide in a tumour removed from an ip group mouse on day 30 indicated that at least some of the cells were still viable, and that the exogenous gene was still functional. This is supported by both aldehyde fuchsin and immunocytochemical staining of a section of a similar tumour, in which insulin-positive cells were identified. These cells were not however distributed evenly throughout the tumour, but appeared localized to certain areas, particularly around blood vessels and close to the exterior of the tumour. This would suggest that the supply of oxygen and / or nutrients may have been limiting to some areas of the tumour, leading to cellular dysfunction and death. Furthermore, haematoxylin and eosin staining of a section of tumour demonstrated the presence of necrotic areas.

The initially high human C peptide concentration found in the ip group declined steadily until the provision of $ZnSO_4$ in the drinking fluid. The mean level then rose; this was sustained for approximately 5 days, concurrent with a small decrease in plasma glucose between days 14 and 23. A further decline in human C peptide was then observed.

Although the sc group received an implant of the same number of cells as the ip group, human C peptide concentrations were initially much lower, and not sustained beyond day 23. An increased amount of human C peptide in response to $ZnSO_4$ was, however, noticable. Only slight fluctuations were observed in the plasma glucose concentration, although a small decrease in the mean value was seen towards the end of the period of $ZnSO_4$ administration.

AtT20 cells which were found to be G418-resistant following transfection, but which *in vitro* released no insulin were also used for ip transplantation. The mice that received an implant of these latent cells were subsequently found to have a substance with human C peptide-like immunoreactivity in their plasma. The anti-human C peptide antiserum K6 utilized in this RIA has a crossreactivity with human proinsulin of approximately 75 % (Priddle, personal communication), and so this substance could be human C peptide, human proinsulin, or a mixture of the two. The starting concentrations were initially low, but increased steadily until day 23, after which a rapid decrease was evident. No apparent effect of $ZnSO_4$ was discernable, but a small decrease in plasma glucose concentration was seen between days 14 and 23. These latent cells did not behave like the insulin-secreting AtT20 cells implanted either ip or sc.

No significant difference in plasma glucose concentration was seen between ip and control groups 30 minutes following an oral glucose challenge. The concentration of human C peptide found in the ip group was similar to that found 24 hours previously, indicating that the insulin secretion by the implant is unaffected by increases in ambient glucose concentration.

The increased concentrations of human C peptide found during the inclusion of $ZnSO_4$ in the drinking fluid is striking. As discussed in Chapter 3, the human insulin gene in the plasmid is driven by a metallothionein promoter, which contains a zinc-reponsive element (Durnam and Palmiter 1981). It appears that increased ingestion of zinc causes increased plasma zinc levels, which stimulate transcription of the insulin gene via an effect upon the promoter, and results in increased release of

insulin and C peptide. This observation gives rise to the interesting possibility that the metabolic signal for insulin release may not necessarily have to depend on rising glucose concentrations.

The reduced efficacy of the sc site compared to the ip site has been reported previously; sc implantation of human growth hormone (hGH) secreting fibroblasts resulted in much lower and more transient plasma hGH when transplanted into normal mice. hGH was detectable in the plasma of the sc mice for only 7 days; hGH was detectable in the ip group for over 12 (Selden *et al* 1987a).

There are several possibilities as to the poor effect of the implants upon glycaemia. The mice were pretreated with a moderate dose of streptozotocin prior to cell implantation, with the aim of inducing a mild diabetes. This may have resulted in increasing β-cell stress and deteriorating pancreatic sufficiency. Once the development of frank hyperglycaemia had begun, the insulin released by the implanted cells was not sufficient to control it. Insulin resistance was also present.

Since greater numbers of cells were used in this study (compared to the previous nude mouse study), greater amounts of ACTH and β-endorphin would also be released into the plasma of the mice. The deleterious effect of these substances on glycaemic balance has been discussed previously. Furthermore, greater amounts of glucocorticoid would be released. This is inactive when bound to corticosteroid-binding globulin (CBG) in the plasma; it is known that levels of CBG can be limiting (Ganong 1985), and perhaps in this case the amount of CBG present was insufficient to bind all glucocorticoid, resulting in increased amounts of unbound (physiologically

active) glucocorticoid in the plasma, with resultant steroid-induced effects upon the development of diabetes.

Conclusion

The work described in this Chapter has given an indication of the feasibility of insulin provision from transplanted genetically manipulated cells. Following implantation of these cells into immunoincompetent mice, the survival of the implanted cells and the correct functioning of the exogenous gene were ascertained by a specific assay of human C peptide in the plasma. This was detectable for up to 30 days. Some beneficial effects on glycaemia were observed, and an increased *in vivo* secretory response to elevated concentrations of zinc were noted.

Whilst nude mice are valuable as transplant recipients since the use of immunosuppressive regimens can be avoided, in order to gain a fuller understanding of the potential of gene therapy, it is necessary to repeat these experiments in normal animals. The next Chapter is concerned with the implantation of insulin-secreting cells into MF1 mice.

CHAPTER SIX

IMPLANTATION OF INSULIN-SECRETING CELLS INTO IMMUNOSUPPRESSED MF1 MICE

6.1. INTRODUCTION

The previous Chapter has detailed preliminary investigations into the feasibility of gene therapy for the treatment of diabetes mellitus using athymic nude mice as the animal model. Whilst this work has produced promising and valuable results, the 'acid test' of this approach would involve the implantation of cells into immunocompetent animals (that is animals requiring immunosuppression) which is a more clinically realistic situation, at least until a primary culture procedure is employed. Whilst the ultimate aim of insulin gene therapy would be the implantation of genetically modified autologous cells, these pilot studies utilize transfected cell lines. Although these are derived from a rodent source, they would present antigens which stimulate an immune response when implanted *in vivo*. Thus the use of an immunosuppressive regimen is mandated.

Chapter 4 has described how the use of Cyclosporin A (CyA) can affect the efficacy of β-cell function both *in vivo* and *in vitro*. CyA, however, is currently the immunosuppressive agent of choice for transplant recipients, and is at present the only widely available immunosuppressant drug (although FK 506 is now becoming more popular). This Chapter describes the implantation of insulin-secreting cells into streptozotocin-diabetic MF1 mice, with CyA used as the immunosuppressive agent. Since the CyA is administered ip, an sc implantation site (between the shoulder blades) was chosen. This Chapter also investigates the zinc-responsiveness of the cells once *in situ*, and in addition, histological and electron microscopic examinations of the tumours have also been performed.

6.2. MATERIALS AND METHODS

6.2.1. Methods: study three

Animals

Adult male MF1 mice of approximately 20 g body weight were used. All other conditions were as previously described.

Chemicals

The Cyclosporin (CyA) was a gift of Sandoz Pharmaceuticals, Camberley, Surrey, UK, and the human C peptide RIA antiserum, standards and label were purchased from Novo Nordisk Diagnostics Ltd, Cambridge, UK.

Experimental procedures

Body weights and food and fluid intake were determined twice during the 9 day study. Blood samples were obtained and stored as before.

Diabetes was induced in overnight fasted animals by ip injection of 140 mg/kg streptozotocin as described in Chapter 2, on day -12. The mice were treated daily with CyA for immunosuppression from day -2 at a dose of 10 mg/kg/day ip. The CyA was administered as a 4 mg/ml solution in arachis oil / 10% ethanol as described in Chapter 4.

Implantation of cells

The day on which the cells were implanted was designated as day 0. The mice were divided into 2 equi-glycaemic groups to serve as tests and controls. The cells used

for the test group were AtT20MtNeohPPI/1 cells. Control animals received AtT20 cells transfected with the same plasmid but that had been found to release no insulin in vitro (although antibiotic resistance was present - these cells are the 'latent' AtT20 cells as described in Chapter 5). The cells were prepared for implantation as described previously. The mice were injected sc between the shoulder blades with 0.2 ml of the cell suspension in PBS via a 25G needle - the test mice received approximately 16 x 10^6 cells each, and the controls received approximately 10 x 10^6 cells each.

Blood samples for analysis of glucose were taken on days -9 and 0, and for glucose and human C peptide on days 1, 2, 5, 7 and 9.

Between days 2 and 5, the drinking water of all mice was replaced with a solution of $ZnSO_4$ 500 mg/l as the only source of drinking fluid. On day 5 the mice were changed back to tapwater only, and CyA therapy was also discontinued on this day.

On day 9 all animals were killed.

Analyses

Analyses of glucose and human C peptide were carried out as described previously. Statistical analysis was also performed as before.

6.2.2. Methods: study four

Animals and chemicals

Animals and chemicals were the same as described for study 1 above, plus histochemical stains purchased from BDH, Poole, Dorset, UK.

Experimental procedures

Body weights and food and fluid intake were determined regularly as previously described. Blood samples were taken and stored as before. The mice were injected with streptozotocin 140 mg/kg ip dissolved in citrate buffer (Appendix 1) on day -15 following an overnight fast. From day -2 onwards all animals were treated daily with CyA 10 mg/kg ip in arachis oil and 10% ethanol (as Chapter 4).

Implantation of cells

The day on which the cells were implanted was designated as day 0. The mice were divided into 2 equi-glycaemic groups to serve as tests and controls. The cells used for the test group were insulin-secreting AtT20MtNeohPPI/1 cells as described previously. The cells were prepared for implantation as before and injected subcutaneously between the shoulder blades via a 25G needle. Each test mouse received approximately 10 x 10^6 cells. Control mice received a similar injection of rat pituitary cells minced with 0.25% trypsin, centrifuged and resuspended in PBS.

Blood samples for analysis of plasma glucose and human C peptide were taken on days 0, 1, 3, 6, 10 and 14. On day 3 the drinking water of all mice was replaced with $ZnSO_4$ 500 mg/l as the only source of fluid. This was discontinued on day 11 when the mice were returned to tapwater. On day 26 the control animals received an

implant of AtT20MtNeohPPI/1 cells to the same site - each mouse received approximately 30 x 10^6 cells.

Histological investigations

On day 13, 2 of the test animals were killed due to excessive hyperglycaemia. A tumour-like cluster of cells was found at the implantation site. Portions of each of these 2 tumours were selected for further microscopic examination. The tissue was processed for section cutting and subsequent histological staining by clearing, dehydration and embedding in paraffin wax as described in Chapter 2. Sections were stained with haematoxylin and eosin to demonstrate general cell morphology, and further sections were processed for immunohistological staining for insulin, also as described in Chapter 2. Additional portions of these tumours were processed for transmission electron microscopy to examine intracellular structure, as described below.

Processing for transmission electron microscopy

Tumours were processed for transmission electron microscopy by Mr Paul Stanley, Department of Physiology, University of Birmingham, Birmingham.

The sections of tumour were minced manually into pieces 1-2 mm in diameter, and fixed overnight in 2.5 % gluteraldehyde in phosphate buffer. The samples were then washed in phosphate buffer for 15 minutes prior to incubation in 1 % osmium tetraoxide solution in phosphate buffer for 1 hour. Dehydration was achieved by incubation through a series of graded alcohols for 2 x 15 minutes each as follows; 70 % ethanol, 90 % ethanol, 100 % ethanol, 100 % dried ethanol, and propylene oxide.

The samples were then incubated in propylene oxide/epoxy resin (50:50 v/v) for 30 minutes in a rotator. (The resin used was Agar 100 Epon substitute). The resin was removed and replaced with fresh resin and left for a further 30 minutes in a rotator, before placing the sample in a vacuum oven at room temperature for 20 minutes. Finally the samples were placed with fresh resin into the embedding moulds, and polymerised overnight at 60°C. Transmission electron microscopy was then performed using a Joel electron microscope.

6.3 RESULTS

6.3.1. Results: study three

Body weights and food intake

No significant differences in body weights or food and fluid intake were observed between the 2 groups at either time point (data not shown).

Plasma glucose

No significant differences in plasma glucose concentration were found between the 2 groups at any time during the 9 days of observation as shown in Figure 6.1.

Using a paired t test, no significant differences were found in the plasma glucose values of the control group on adjacent days. The plasma glucose concentrations of the test group was found to increase significantly between days 1 and 2 ($p<0.02$). The test mice were given $ZnSO_4$ 500 mg/l as the only source of drinking fluid between days 2 and 5. Between these days, the plasma glucose concentration of the test group decreased significantly ($p<0.05$), by approximately 10 mM, whilst there was no discernable effect on the plasma glucose concentration of the control group. All other time points were not significantly different.

Plasma human C peptide

No significant difference in plasma human C peptide concentration was found between the 2 groups at any time during the study as illustrated in Figure 6.2.

Figure 6.1. Plasma glucose concentration of CyA-treated MF1 mice implanted sc with AtT20MtNeohPPI/1 cells (closed squares) or 'latent' G418-resistant AtT20 cells transfected with the same vector (open squares). Results are mean ± SEM (n=6 for tests, n=5 for controls).

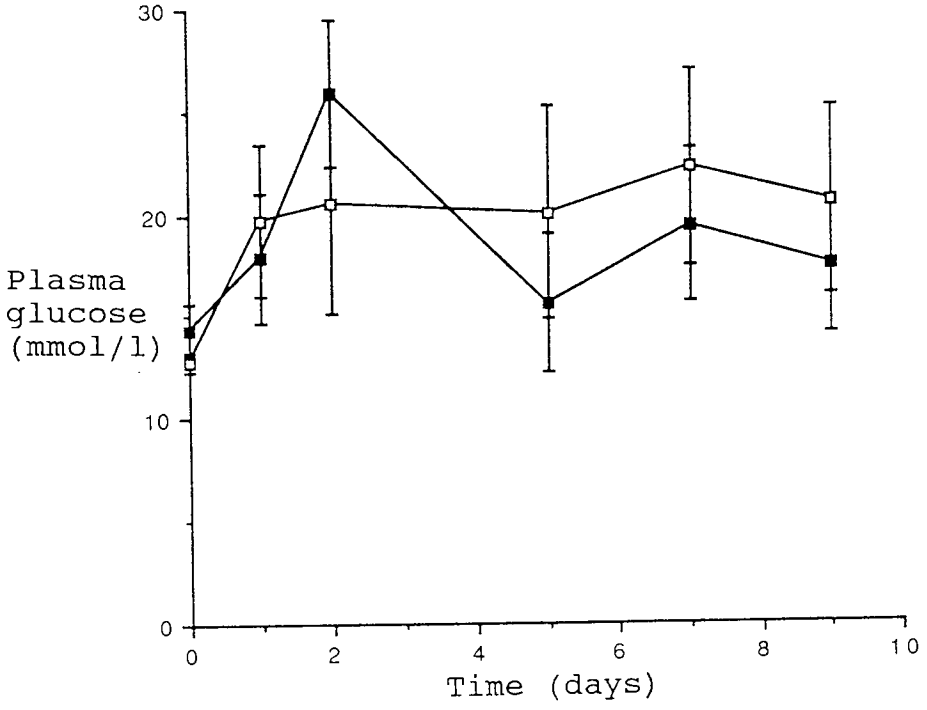
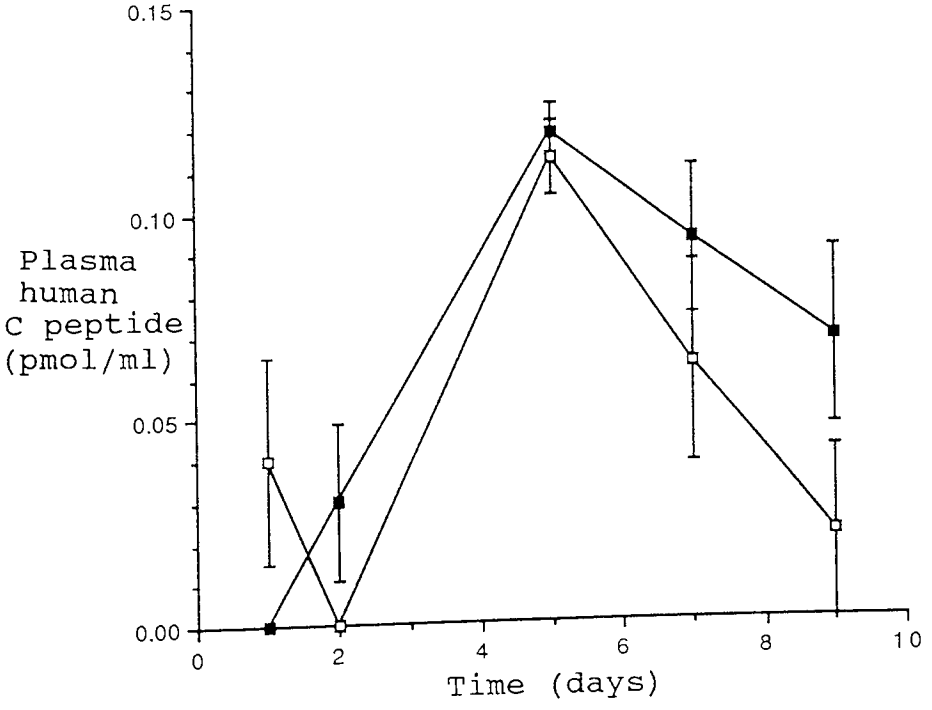


Figure 6.2. Plasma human C peptide concentration of CyA-treated MF1 mice implanted sc with AtT20MtNeohPPI/1 cells (closed squares) or 'latent' G418-resistant AtT20 cells transfected with the same vector (open squares). Results are mean ± SEM (n=6 for tests, n=5 for controls).



Comparison of plasma human C peptide concentrations within each group on consecutive days using Student's paired t test demonstrated no significant differences except between days 2 and 5 when a significant increase was observed in the tests ($p<0.02$) and the controls ($p<0.001$). This increase corresponds to the introduction of $ZnSO_4$ as the only source of drinking fluid. A gradual (non-significant) decrease in plasma human C peptide concentrations was observed on subsequent days, coinciding with the reintroduction of tapwater, and also with the cessation of CyA therapy.

6.3.2. Results: study four

Body weights and food intake

No significant difference in body weights or food intake was found between the 2 groups during the course of the study (data not shown), although between days 0 and 13 the mean body weight of the test group decreased by 0.9 g, whilst that of the control group increased by 2.1 g.

Plasma glucose

No significant difference in plasma glucose concentrations were found between the groups up to and including day 14 after which all test mice were killed, as shown in Figure 6.3.

It is interesting to examine the data subjectively. Following implantation of the cells (approximately 10×10^6 cells per mouse ip), the mean value for the plasma glucose concentrations of the test animals increased (although not significantly), while there

Figure 6.3. Plasma glucose concentration of CyA-treated MF1 mice implanted sc with AtT20MtNeohPPI/1 cells (closed squares) or isolated pituitary cells (open squares) on day 0. Control mice were implanted sc with AtT20MtNeohPPI/1 cells on day 26. Results are mean ± SEM for 7-2 observations.

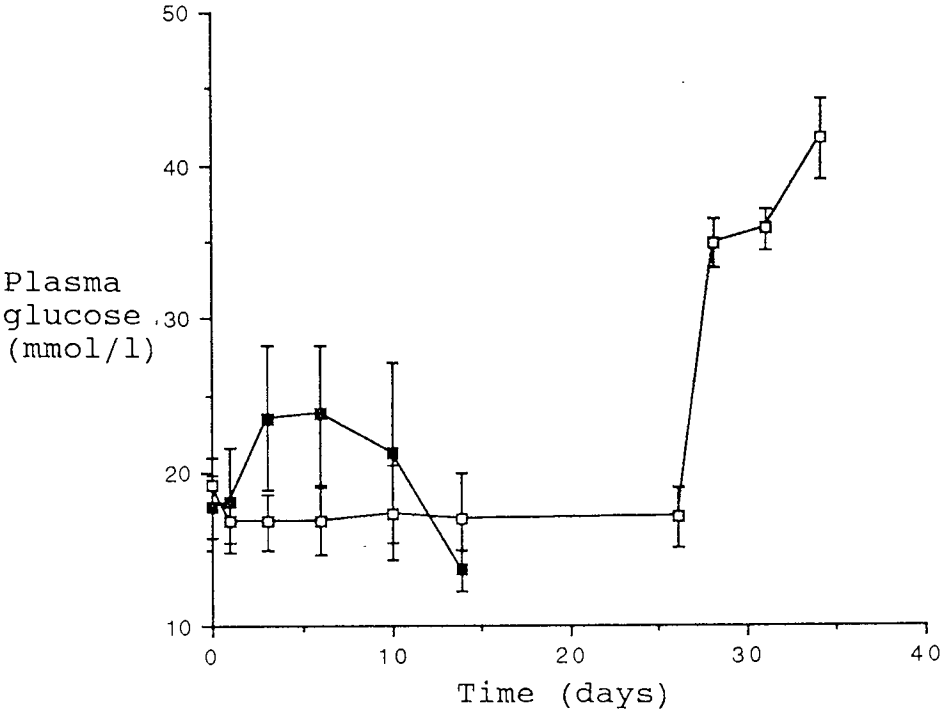
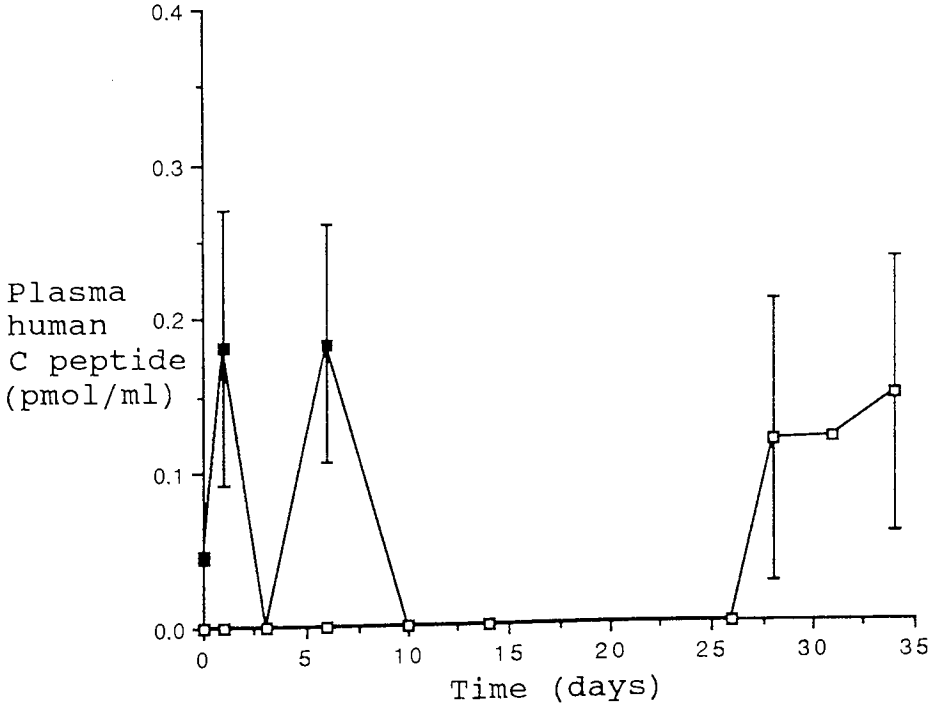


Figure 6.4. Plasma human C peptide concentration of CyA-treated MF1 mice implanted sc with AtT20MtNeohPPI/1 cells (closed squares) or isolated pituitary cells (open squares). Control mice were implanted sc with AtT20MtNeohPPI/1 cells on day 26. Results are mean ± SEM for 4-1 observations.



was no similar pattern discernable in the control group. However following implantation of a large number (30 x 10^6 cells per mouse ip) of test cells into the control group on day 26, the plasma glucose concentration of the control group rapidly became very hyperglycaemic.

Plasma human C peptide

Human C peptide was immediately detectable in the plasma of the test mice following sc implantation of the insulin-secreting AtT20MtNeohPPI/1 cells. The levels detected were transient, and none was detectable from day 10 onwards. No human C peptide was found in the plasma of the control mice until day 28; these concentrations were also transient and not found in all mice; plasma human C peptide concentrations are shown in Figure 6.4.

Weights of tumours

Two test animals were killed on day 13 due to excessive hyperglycaemia. The implantation site on both mice was examined, and a large tumour-like cell cluster was found at each. This is shown in Plate 6.1. The tumours were large, had apparently commanded a good vascular supply from the brown fat pad, and had a well defined capsule. The mean weight of these tumours was 365.2 ± 0.80 mg (2). Upon examination, the centre of each tumour was found to be necrotic. These tumours were processed for histological examination, as described below.

Three further tumours were subsequently removed and extracted with acid ethanol. The mean weight of these tumours was 190 ± 39.2 (3) mg. The human C peptide content was found to be 0.96 ± 0.486 (3) pmol human C peptide/g tissue.

Plate 6.1. MF1 mouse with subcutaneous tumour *in situ* after 13 days growth *in vivo*.

Histochemistry

Hamatoxylin and eosin stained sections of the tumours are shown in Plates 6.2 and 6.3. The tumours can be seen to have large necrotic areas (stained pink) with some healthy areas (stained mostly purple). A well developed vascular supply can be seen, and also the presence of a capsule surrounding the aggregation of cells. Areas around the capsule and blood vessels appear to be the most healthy.

Immunocytological staining

Sections of the tumours processed for immunocytological staining are shown in Plates 6.4 and 6.5. Plate 6.4 was stained with control serum; there is no obvious fluorescence. Plate 6.5 was stained with anti-insulin serum, and there are fluorescent areas visible. These results demonstrate that insulin-secreting cells are present within these cell clusters removed from the sc site.

Transmission electron microscopy

Transmission electron microscopy of the peripheral healthy mantle of a tumour is shown in Plates 6.6 to 6.12. Plates 6.6 to 6.8 demonstrate apparently healthy tissue. Nuclei with clear nuclear membranes are visible, and well defined mitochondria and secretory granules (with and without crystalline contents) are also apparent, as described in the appropriate legends. Plates 6.9 and 6.10 show the onset of tissue degeneration, with pycnotic nuclei, and the start of cytoplasmic breakdown. Plates 6.11 and 6.12 show a section of the tumour which is necrotic, with amorphous cytoplasmic structures and remnants of membranal structures. This variation in tissue structure is in accordance with the macroscopic examination of the tumours, when large central areas of necrosis were identified.

Plate 6.2. Haematoxylin and eosin stained section of a tumour removed after 13 days growth *in vivo* from a CyA-treated MF1 mouse implanted sc with AtT20MtNeohPPI/1 cells. Magnification x 250.

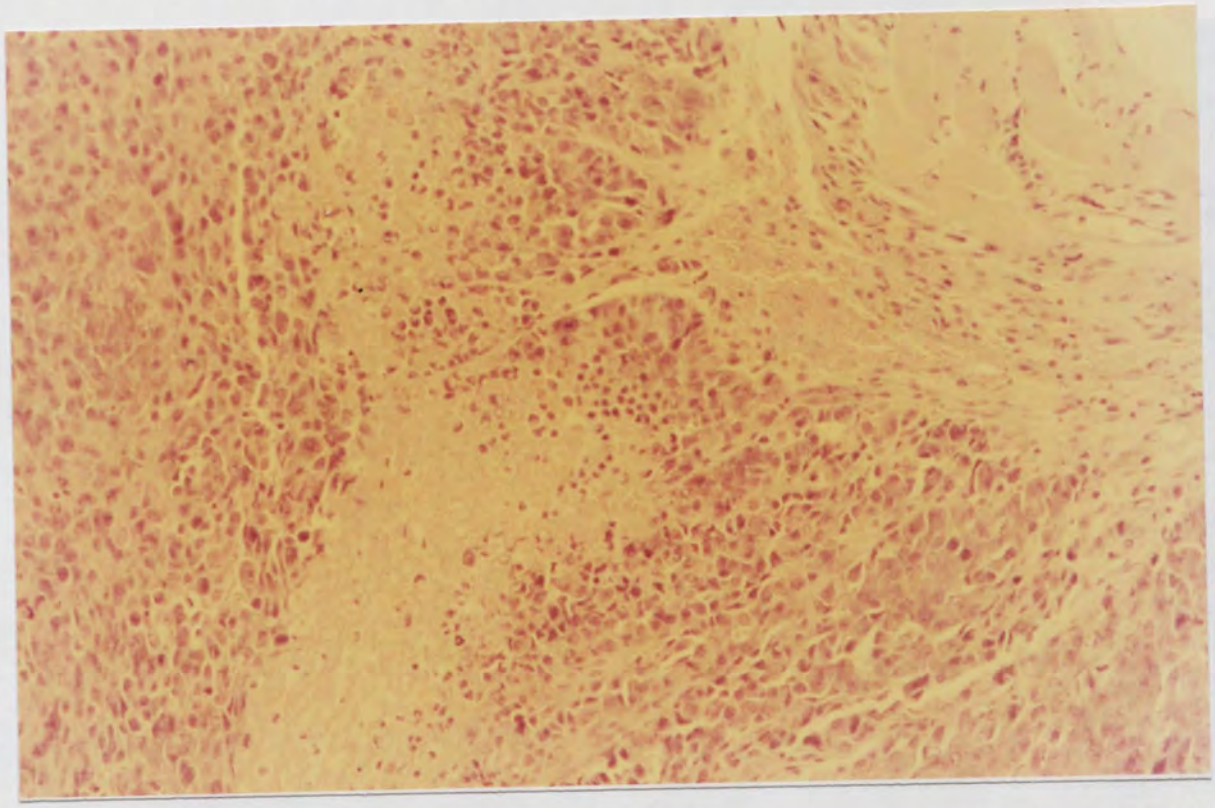


Plate 6.3. Haematoxylin and eosin stained section of a tumour removed after 13 days growth *in vivo* from a CyA-treated MF1 mouse implanted sc with AtT20MtNeohPPI/1 cells. Magnification x 250.

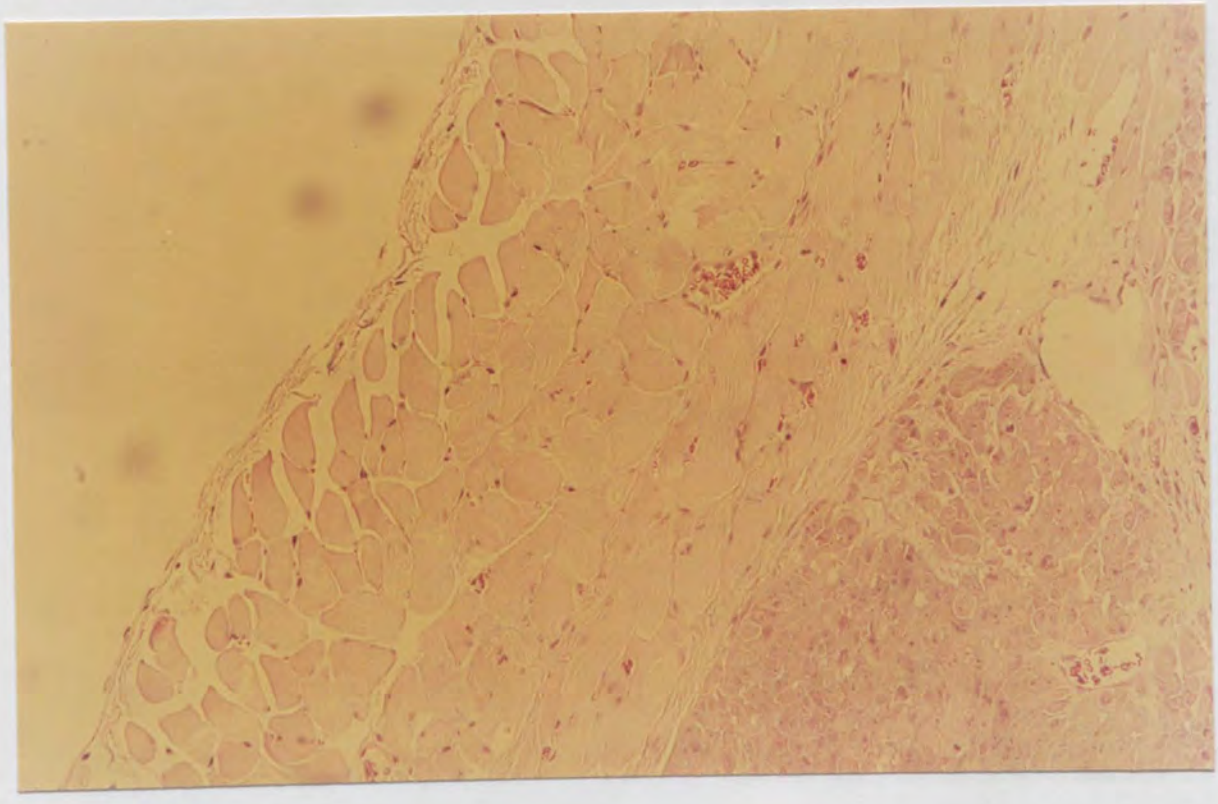


Plate 6.4. Immunocytochemically processed section using control serum of a tumour removed after 13 days growth *in vivo* from a CyA-treated MF1 mouse implanted sc with AtT20MtNeohPPI/1 cells. Magnification x 500.

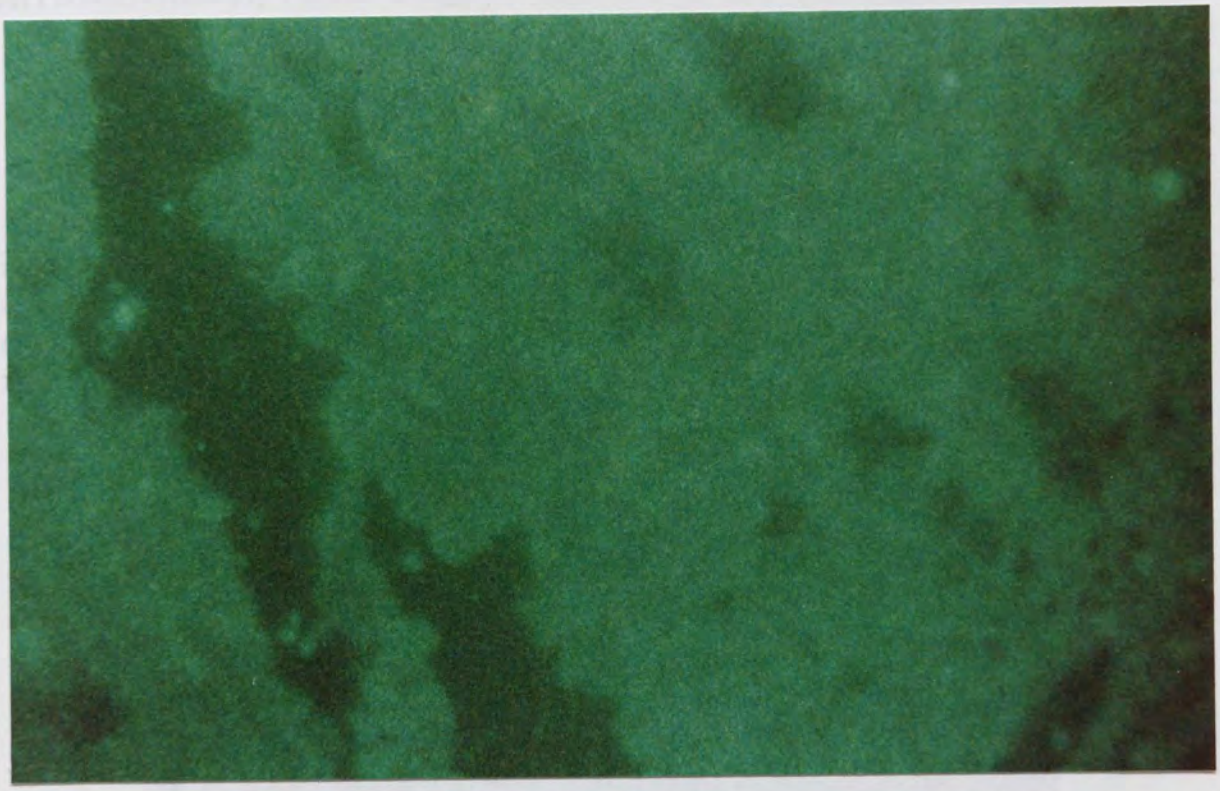
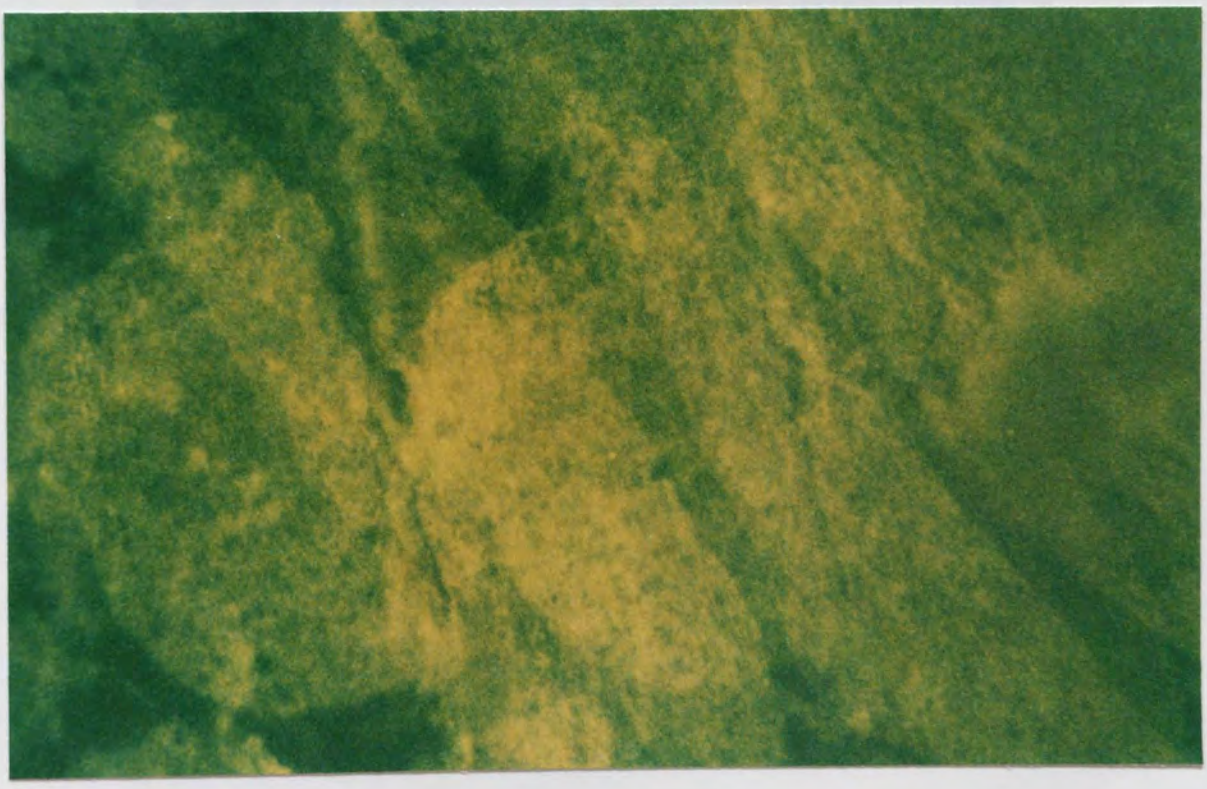


Plate 6.5. Immunocytochemically processed section using anti-insulin serum of a tumour removed after 13 days growth *in vivo* from a CyA-treated MF1 mouse implanted sc with AtT20MtNeohPPI/1 cells. Magnification x 500.



Plates 6.6. to 6.12. Transmission electron microscopy performed on sections of a tumour removed after 13 days growth *in vivo* from a CyA-treated streptozotocin-diabetic MF1 mouse implanted sc between the scapulas with 10 x 10^6 AtT20MtNeohPPI/1 cells. Magnification as described in individual legends.

Plate 6.6. Normal cell from the periphery of the tumour showing undamaged nucleus and nuclear membrane, with intact mitochondria, and some dense staining and hollow vesicles from which the content has presumably been lost in processing. Magnification x 4800.

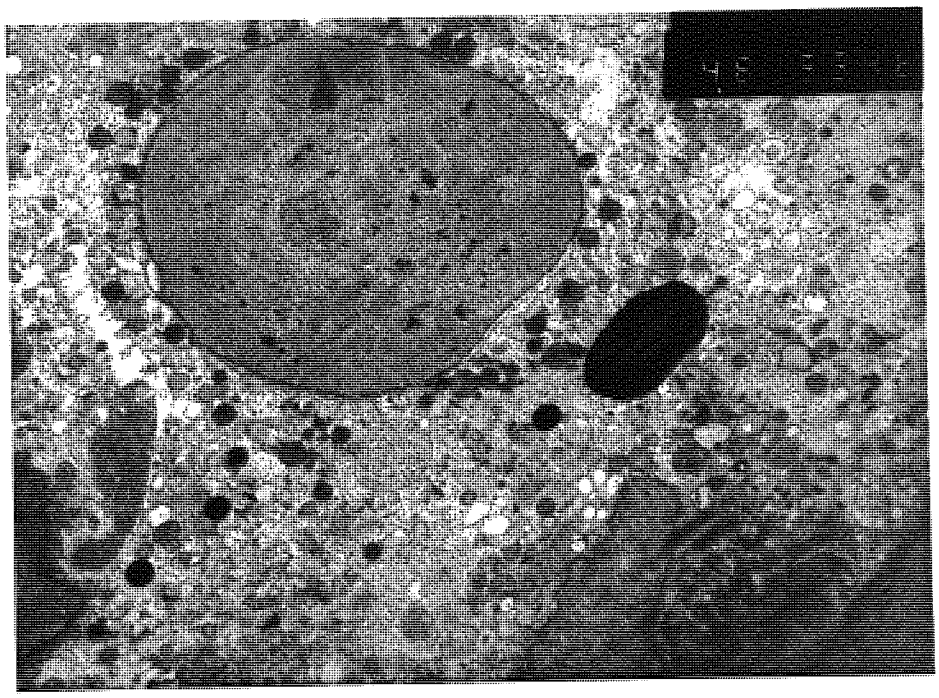


Plate 6.7. Similar morphology to Plate 6.6. with plentiful mitochondria and dense core secretory granules, and showing exclusion of material at the plasma membrane. Magnification x 4800.

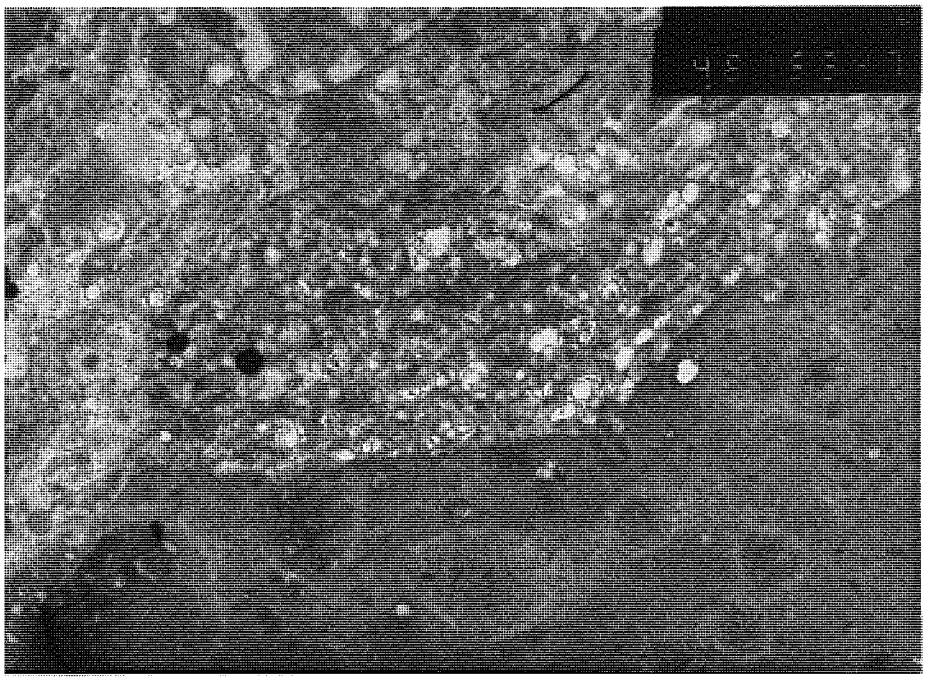


Plate 6.8. Also similar to Plate 6.6. with a healthy appearance. Magnification x 9800.

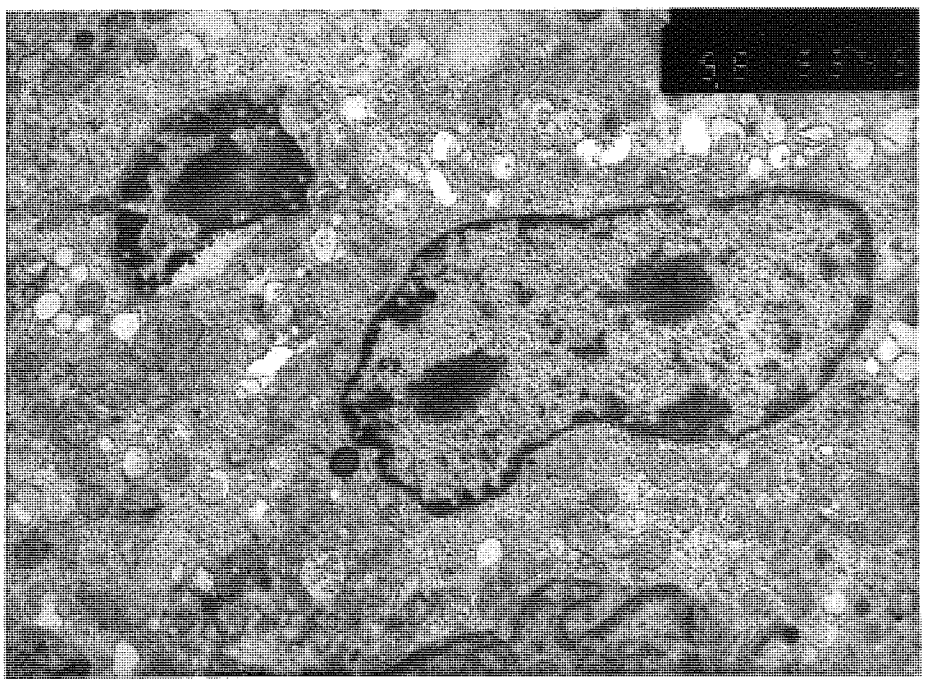


Plate 6.9. This cell from near the periphery of the tumour shows evidence of disruption of nuclear conformation and disorganisation of cytoplasmic content. Magnification x 9800.

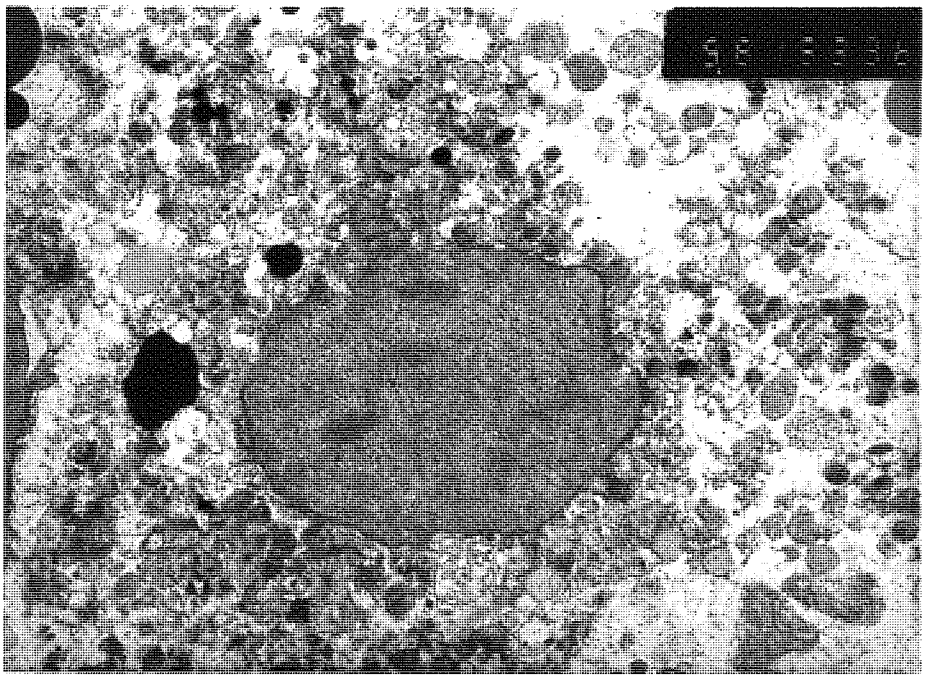


Plate 6.10. Similar morphology to Plate 6.9. with nuclei of a distinctly abnormal appearance. Magnification x 3600.

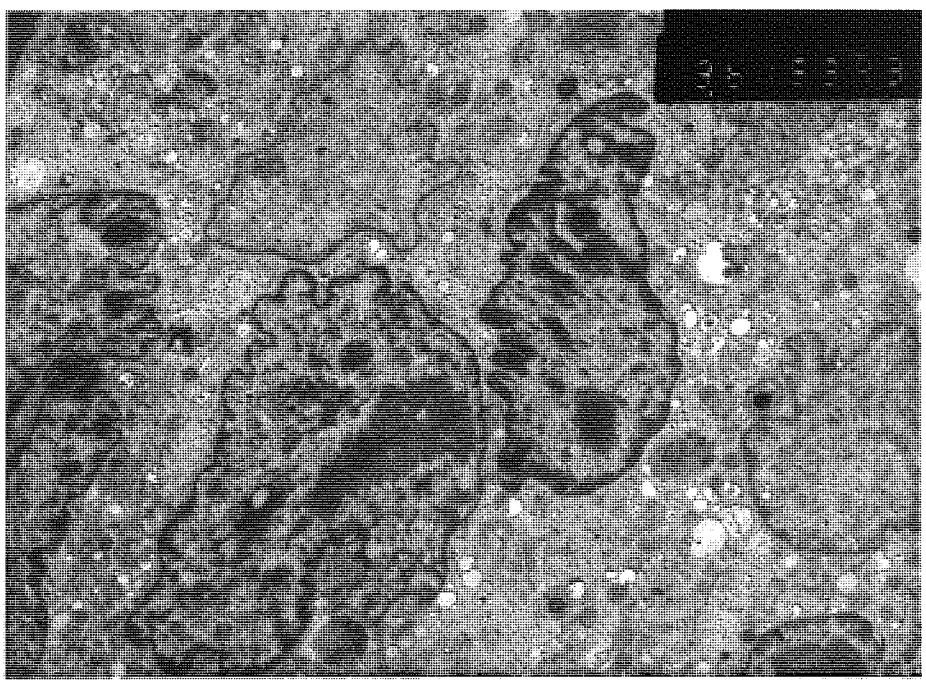


Plate 6.11. This tissue from the interior of the tumour shows cellular necrosis in which nuclei and some associated residual cytoplasm are interspersed with inclusion bodies and the remnants of cytoplasmic and membranal structures. Magnification x 1900.

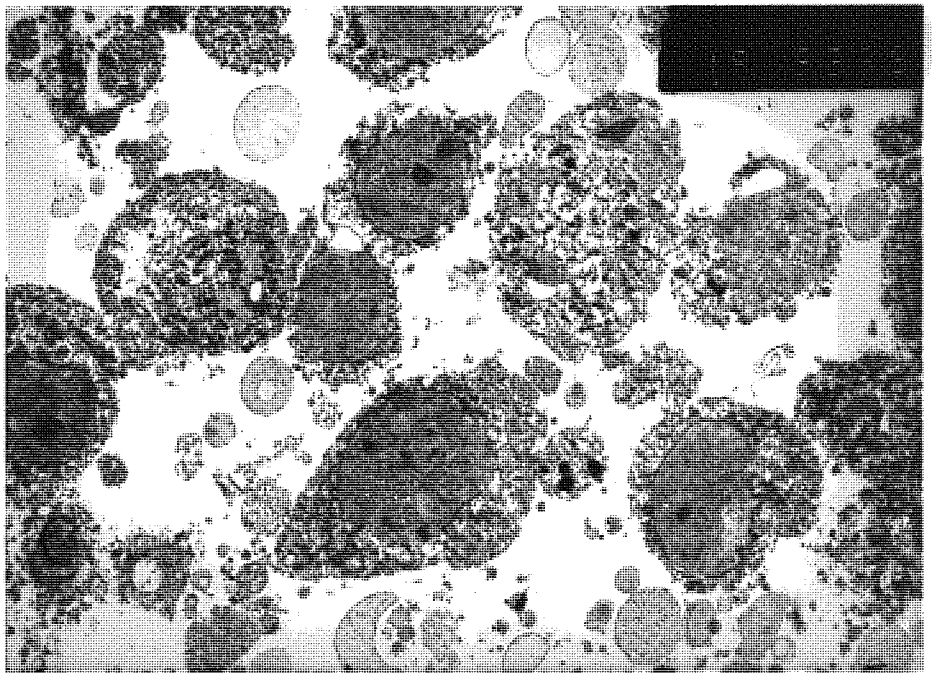
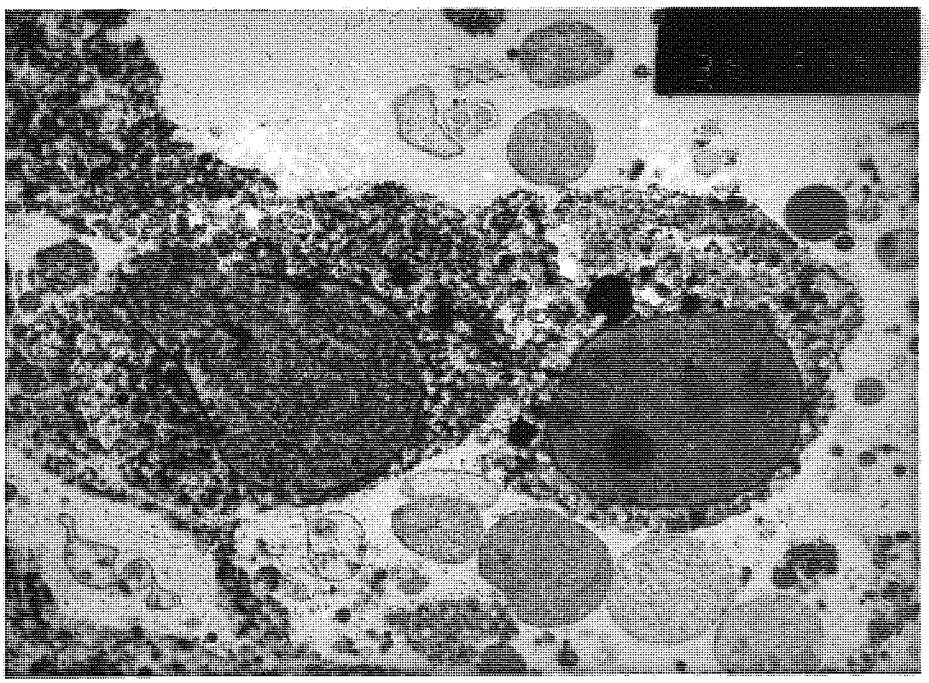


Plate 6.12. Necrotic tissue similar in appearance to Plate 6.11. Magnification x 3600.



6.4. DISCUSSION

Study three

Injection of streptozotocin 140 mg/kg ip on day -10 invoked a mild hyperglycaemia (a plasma glucose concentration of approximately 13 mmol/l) by day 0. During the study, no differences were found in body weight, food intake or plasma glucose concentrations between the test group (implanted with insulin-secreting transfected AtT20 cells) and the control group (implanted with G-418 resistant transfected AtT20 cells which did not secrete insulin when cultured *in vitro*). Following the administration of $ZnSO_4$ to the mice, the mean plasma glucose level of the group implanted with the insulin-secreting cells decreased significantly, with a concurrent increase in plasma human C peptide. Since the plasmid containing the human insulin gene includes a metallothionein promoter (which is known to contain a heavy metal ion response element), it appears that the increased concentration of Zn^{2+} in the plasma of the mice had increased the activity of the promoter, leading to increased release of human insulin and therefore increased release of human C peptide. This larger release of insulin may contribute to the reduction of hyperglycaemia in this group. Zinc is also important for the correct crystallization of insulin within secretory granules (Cook and Taborsky 1990). The acute administration of a large dose of $ZnCl_2$ to hyperglycaemic rats caused a significant and rapid decrease in plasma glucose concentrations; plasma insulin levels were not measured (Shisheva, Gefel and Shechter 1992).

The control group however (recipients of an implant of G-418 resistant cells), also exhibited detectable human C peptide in the plasma. These animals received an

implant of cells initially intended as controls which were subsequently found to release human C peptide *in vivo* as described in Chapter 5. These cells also demonstrated an increased secretory response to Zn^{2+}, but with no attendant effect on glycaemia. It is possible that these cells are releasing human proinsulin - this would be detected by the human C peptide antiserum, but *in vivo* would have only a small effect (maximally 10 % of that of mature processed insulin) on glycaemia.

Study four

Following the implantation of a large number (10×10^6) of the insulin-secreting AtT20 cells into the test mice, these animals developed an increased plasma glucose. No such effect on glycaemia was observed in the control group that received an implant of isolated rat pituitary cells. This effect on glycaemia is unlikely, therefore, to be due simply to stress or to an effect of the sc injection, but may be due to the release of ACTH and endorphins by the AtT20 cells (as described in Chapters 3 and 5). Since these mice were streptozotocin diabetic, they would have no insulin reserve with which to respond to any insulin secretagogues (Bailey *et al* 1986), and hence the insulin-increasing action of these pituitary peptides (Bailey and Flatt 1987a) would be ineffective, whilst the plasma glucose increasing effects would still be operational. This theory is supported by the observation that following implantation of an even larger number of these insulin-releasing AtT20 cells into the control mice on day 26, these mice also became rapidly very hyperglycaemic.

Relatively low concentrations of human C peptide were found in the plasma of the test mice, and not all of these mice had a detectable plasma concentration of human C peptide. None was detected in the control mice prior to day 26. Since greater

numbers of cells were implanted into these mice (compared to the nude mice used in Chapter 5) it would be expected that greater amounts of human C peptide and insulin would be exported to the circulation. It has previously been reported that whilst 10/10 nude mice showed a response to an ip implant of proinsulin-releasing fibroblasts, only 5/10 streptozotocin-diabetic, immunosuppressed mice reacted in a similar manner (Selden *et al* 1987b). The mice were immunosuppressed with anti-thymocyte serum and dexamethasone; the authors attribute the variable results as a failure of immunosuppression due to a wide inter-individual response to the drugs. In this study, similar results were observed. Potentially there may be an inadequacy of the immunosuppressive regimen used, although the dose used was as recommended by the manufacturers (Mason 1990), and, as shown in Chapter 4, was sufficient to cause deleterious effects upon endogenous β-cell function.

It appears that the sc implantation site may not be as efficient as the ip site as regards the concentration of human C peptide detected in the plasma (compare with Chapter 5). The relative inefficiency of the sc site has been observed previously (Selden *et al* 1987a). Several possibilities have been suggested as contributing to this reduced efficiency, including lack of adequate perfusion (thus causing a deficiency of nutrients and oxygen, and hindering the export of product to the circulation), or inadequate cell adhesion (particularly important if the cells are anchorage-dependent). It is possible, but unlikely, that the export of substances released by the tumour is inefficient, since both the macroscopic examination of, and haematoxylin and eosin staining of the tumour (Plates 6.2 and 6.3) revealed a well developed vascular supply. Upon examination following excision, the tumours were found to be large (the weights of tumours removed from MF1 mice were approximately 10 fold

heavier than those removed from nude mice) but very necrotic, with an apparently healthy 'mantle' of cells under the capsule surrounding a large central mass of dead tissue. The human C peptide content of the tumours removed from MF1 mice was approximately half that of the tumours removed from nude mice when expressed as pmol human C peptide/g tissue. Immunocytochemical processing of the tissue demonstrated the presence of fluorescent staining insulin-positive cells (Plate 6.5) compared to no fluorescence following processing with control serum (Plate 6.4).

Therefore it appears that the cells grew faster than they could properly be supported, as typified by many types of tumour (Spector 1989). The greater degree of vascularization of these sc tumours (when compared to the ip tumours described in Chapter 5) may be explained by their proximity to the brown fat pad. Indeed cells implanted at sc locations not immediately above the interscapular brown fat depot did not produce identifiable tumours, and the cells were not recovered at 14 days after implantation despite the presence of human C peptide in the circulation. Fat is known to release a lipid-soluble angiogenic factor; it has been shown that intracorneal injection of a solution prepared from cat omental fat resulted in considerable neovascularization in the corneas of recipient rabbits (Goldsmith *et al* 1984). Fat taken from a subcutaneous site has been found to possess a comparable angiogenic effect, and furthermore this angiogenesis has been found to be dependent upon prostaglandins since the administration of indomethacin abolishes the formation of new blood vessels (Silverman *et al* 1988). A potent angiogenic factor, monobutyrin, has been recently been isolated from differentiated adipocytes (Dobson *et al* 1990) and is thought to be important for correct development of adipose tissue (Ailhaud, Grimaldi and Negrel 1992). It has been demonstrated that monobutyrin

is released in large amounts during periods of lipolysis (Wilkison, Choy and Spiegelman 1991). Concurrent with the development of hyperglycaemia in these mice, some breakdown of fat depots would occur, and hence it is possible that the degree of tumour growth and considerable amount of vascularization observed in tumours removed from the sc site is due at least in part to this angiogenic factor. Interestingly, CyA has been implicated in the inhibition of neovascularization following islet grafting (Rooth *et al* 1989).

Transmission electron microscopy of an outer portion of a tumour removed from a test mouse demonstrated that some of the tissue was healthy in appearance, with some secretory granules present which contained crystalline material and otherwise essentially normal cell morphology (Plates 6.6, 6.7 and 6.8). Further sections of the tumour were found to be undergoing cellular breakdown and death with a progressively deteriorated cell morphology leading to an amorphous inner region.

In this study, no stimulatory effect of $ZnSO_4$ was found on plasma concentrations of human C peptide in the test mice. This is in contrast with the previous MF1 study, and may be due to an insufficient number of viable cells to make an impact on circulating human C peptide concentrations.

Conclusion

This Chapter has described the subcutaneous implantation of insulin-secreting pituitary cells into normal immunosuppressed MF1 mice. As in the previous Chapter, the *in vivo* function of the implant was assessed by measurement of plasma human C peptide concentrations. The circulating levels of human C peptide obtained were

lower and more transient than those obtained in nude mice, although a stimulatory effect of zinc was observed. ACTH and other pituitary peptides released by AtT20 cells may exert a deleterious effect on glucose homeostasis, which makes interpretation of the results more complex. Histological examination of the tumours revealed healthy tissue around the periphery with a central region of necrosis. Immunocytochemical processing of a section from the periphery showed that insulin-containing cells were present. Transmission electron microscopy demonstrated that the intracellular structure of cells from the periphery of the tumour were essentially normal, whilst cells from other more central areas were undergoing cellular breakdown.

CHAPTER SEVEN

DISCUSSION

7. DISCUSSION

7.0. Introduction

This thesis has considered various preliminary aspects of the feasibility of insulin gene therapy for the treatment of diabetes mellitus. The current treatment of IDDM by subcutaneous insulin injection regimens (although life-saving) can, at present, be considered to be inadequate, both in terms of efficacy and patient compliance. Investigations concerning novel routes for the delivery of insulin (for example administration either orally or nasally) appear to be beset with considerable problems, and have not progressed further than limited clinical trials. Approaches involving transplantation of pancreatic or islet tissue have so far achieved little lasting success, although recently exciting results (for 1 patient only) have been reported (Scharp *et al* 1990). Thus the search for conceptually new methods for the long-term administration of insulin remains justified, particularly in view of the morbidity and mortality caused by IDDM, and also the considerable costs of the existing treatment. It has been estimated that the cost of treatment of diabetes mellitus comprises over 4 % of the total health care budget of the UK (Williams 1991), plus additional costs incurred from absenteeism, and related health problems such as coronary heart disease.

The initial step in any gene therapy protocol involves the selection of, firstly, the therapeutic gene of interest, secondly, the promoter to drive transcription of the gene, and thirdly, the cell into which the vector can be introduced. Chapter 3 described the manufacture of novel proinsulin- and insulin-secreting cell lines, both a fibroblast line and pituitary lines, and the response of these cells to various secretagogues *in vitro*.

These results demonstrate that it is possible to introduce and express the human preproinsulin gene in a non β-cell, and that the use of a neuroendocrine target cell (the pituitary AtT20 cell line) enabled the correct packaging of the proinsulin, and subsequent processing to mature insulin. Thus a careful choice of target cell type is likely to be critical if gene therapy (for any disease) is to be effective in the clinical situation. The incubation of proinsulin- and insulin-secreting AtT20 cells in medium containing various secretagogues demonstrated that these genetically manipulated cells do not behave in a β-cell-like manner. No increase in proinsulin or insulin release was observed when cells were incubated in raised concentrations of glucose, Ca^{2+}, K^{+}, or glucagon. These substances are known to increase insulin secretion by normal islets. However, these engineered cells did increase insulin secretion in response to raised concentrations of IBMX, arginine and Zn^{2+}. The precise mechanisms by which this acceleration of secretory rate may be mediated is examined in detail in the discussion to Chapter 3; briefly, the effect of IBMX is mediated via increases in intracellular cAMP, arginine may exert a stimulatory effect via increases in nitric oxide, and the action of zinc is due to a stimulatory effect upon the metallothionein promoter driving the insulin gene. These results give rise to the interesting possibility that the *in vivo* regulation of a novel insulin-secreting cell may not necessarily depend on glucose, but instead perhaps on another activator, depending upon the selection and responsiveness of the promoter used to drive the exogenous (insulin) gene.

Chapter 4 was concerned with evaluating the effect of immunosuppression and immunoisolation on insulin release *in vivo* and *in vitro*. The use of a therapeutic dose of cyclosporin (CyA) was found to severely affect glucose tolerance in treated

animals; the effect appeared to be mediated mainly through an inhibition of insulin production and release, although some experiments indicated the presence of insulin resistance. These data were supported by experiments *in vitro* using the insulin-secreting cell line RINm5F. Whether CyA would affect insulin release by the transfected cells *in vivo* has not been assessed in the present study. However it appears to be unlikely since these transfected cells are not β-cells - CyA is known to accumulate within β-cells (Lensmeyer *et al* 1991) - and insulin release from these genetically modified cells does not involve precisely the same mechanisms as the endogenous β-cell.

The microencapsulation of cells within an alginate-polylysine-alginate membrane has been suggested as a means by which implanted tissue could be protected from immune attack *in vivo*. The encapsulation of HIT T15 cells within such a membrane was found to greatly reduce the amount of insulin detectable in the incubation medium when compared to unencapsulated cells. This method of immunoprotection therefore appears unsuitable for use in the treatment of diabetes, however the ultimate aim of clinical gene therapy would involve transfection of the patients' own cells, thus obviating any requirement for immunosuppression or immunoisolation.

Once the manufacture of an insulin-secreting cell line had been accomplished, and the effect of the immunosuppressive regimen on endogenous β-cell function had been elucidated, the function of the cells *in vivo* was investigated. For the initial *in vivo* studies, the animal model selected was the athymic nude mouse. This animal is immunoincompetent, and has been used as a recipient for many types of implanted tissue. The male nude mouse has been reported to display a mild hyperglycaemia

between certain ages, which is discussed in Chapter 5. Animals were rendered diabetic by the administration of streptozotocin, which induced a permanent hyperglycaemia. Some workers had reported that the action of streptozotocin was thymus-dependent, and that hyperglycaemia could not be induced in nude mice by the use of this drug (Buschard and Rygaarde 1978). However, the results obtained in Chapter 5 support other reports that streptozotocin can be used to induce hyperglycaemia in nude mice (Paik, Fleischer and Shin 1980).

The first implant into nude mice described in this thesis compared ip implantation of insulin-releasing AtT20 cells with implantation of isolated pituitary cells alone. This prototype study demonstrated that the genetically modified cells could survive *in vivo,* and that they continued to process and release insulin (as demonstrated by detection of human C peptide in the plasma of the mice) for up to 29 days. The implant appeared to significantly delay the onset of streptozotocin-induced hyperglycaemia, despite the presence of insulin resistance.

The second study using nude mice supported the observation that genetically modified cells could survive *in vivo* and continue to export products, and provided additional information about the organisation and activity of the implanted cells. The subcutaneous implantation site was found to be less efficacious than the intraperitoneal site. G418-resistant cells that did not secrete insulin *in vitro* were found to release a substance with human C peptide-like immunoreactivity following implantation *in vivo*. This substance may be proinsulin since an increased plasma concentration was seen following zinc administration, but with no effect on glycaemia. Histological examination of a tumour-like cell cluster removed from the ip site of a

nude mouse revealed that the peripheral cells were healthy with a necrotic core, and aldehyde fuchsin staining and immunocytochemical processing demonstrated the presence of insulin-containing cells distributed unevenly throughout the tumour.

The data obtained from the 2 studies utilizing CyA-immunosuppressed mice (mice with an intact thymus) demonstrate that an implant of insulin-secreting cells will continue to function *in vivo* as proven by sustained detection of human C peptide in the plasma, and by immunocytochemical processing of a section of tumour removed at autopsy. Transmission electron microscopy demonstrated that whilst some portions ot the tumour had a normal and healthy intracellular structure, some parts were undergoing cellular breakdown. Secretory granules with crystalline contents were observed. Although the MF1 mice were treated with streptozotocin prior to cell implantation, the reduced efficacy of the cells is unlikely to be due to the action of streptozotocin, which is a specific β-cell toxin. However, the diabetic environment and deteriorating glycaemic control probably render the cells less able to sustain the functional activity of cells in less stressful conditions.

7.1. Overview of results

As summarized in Table 7.1, this thesis describes the results of studies in which insulin-secreting pituitary (AtT20) cells transfected with the human insulin gene have been implanted into animal models of diabetes. The production of insulin by these novel cells following implantation *in vivo* (assessed by the use of a specific human C peptide assay) has been observed. The plasmid used to transfect these cells contained a metallothionein promoter to drive the insulin gene. The presence of metal response elements within this promoter enabled the *in vivo* stimulation of the

Table 7.1. Summary of the effects of cells used for implantation into either athymic nude mice or CyA-immunosuppressed MF1 mice. Cells used were either control cells (isolated rat pituitary cells), insulin-secreting AtT20MtNeohPPI/1 cells, or 'latent' AtT20 cells (G418-resistant AtT20MtNeohPPI/1 cells).

Cells used	Growth in G418	Insulin release *in vitro*	Response *in vitro* (IBMX)	C peptide found *in vivo*	Implantation Site	Implantation Animal	Immunological status	Response to zinc *in vivo*	Cell growth *in vivo*
Control cells	-	-	-	no	ip	nu/nu	athymic	no	no
AtT20 hPPI/1	yes	yes	yes	yes	ip	nu/nu	athymic	yes	tumour
AtT20 hPPI/1	yes	yes	yes	yes (low)	sc	nu/nu	athymic	slight	large tumour
Latent AtT20	yes	no	no	yes	ip	nu/nu	athymic	no	tumour
AtT20 hPPI/1	yes	yes	yes	yes (low)	sc	MF1	CyA treated	yes	large tumour
Latent AtT20	yes	no	no	yes (low)	sc	MF1	CyA treated	no	large tumour

rate of transcription by the administration of $ZnSO_4$ in the drinking fluid of the mice. Following the introduction of ZnSO4, elevated circulating concentrations of human C peptide were detected both in nude mice and CyA-treated MF1 mice.

Several patterns within the data are common to all the implantations described in this thesis. The first is the observation that all plasma C peptide concentrations described are substantially lower than would be predicted from the known insulin release of the same number of cells *in vitro*, even taking into account the shorter biological half-life *in vivo*. It has previously been shown that cells implanted *in vivo* secrete only 2 % of the quantity of factor IX released by the same number of cells *in vitro* (Palmer, Thompson and Miller 1989). This may be due to extensive cell death or dysfunction either prior to implantation or *in vivo*, perhaps due to anoxia, trauma sustained during the implantation procedure, lack of adequate nutrients or growth factors, or to inactivation of the exogenous gene(s) (Palmer *et al* 1991). It has also been suggested that the net charge of the molecule may influence its ability to cross the basement membrane and enter the circulation (Kazama *et al* 1988). Many other workers have also observed an extremely low, or even undetectable, concentration of product in the serum of the animal following transplantation, despite the detection of a high level of secretion *in vitro* (Selden 1987a, Jensen and Bolund 1991, Teumer, Lindahl and Green 1990, Morgan *et al* 1987).

The second pattern seen throughout the results reported in this thesis is the great inter-individual variation seen in response to cell implantation, particularly in terms of plasma glucose and human C peptide concentrations, and the extent of tumour growth. This wide variation in results is especially noticeable in the two studies

utilizing CyA-treated MF1 mice - this may be partly attributable to the sc implantation site chosen, or to the immunosuppressive regimen affecting individual mice differently (Selden *et al* 1987b). Definitive interpretation of the results obtained is therefore difficult.

7.2. Gene therapy for IDDM

IDDM is an extremely challenging target disease for treatment by gene therapy, for a number of reasons. Firstly, as β-cells are subject to autoimmune attack during the development of diabetes, expression of the exogenous (therapeutic) gene cannot be achieved in the native cell, necessitating the selection of an alternative target cell population. Secondly, insulin is produced as a prohormone which must be correctly cleaved by endopeptidases for optimal biological activity. Thus the target cells chosen should contain endopeptidase activity, or co-transfection with suitable processing enzymes is likely to be essential. Thirdly, the concentration of insulin in the plasma is critical - levels that are too high or too low have serious damaging metabolic effects. This narrow therapeutic range for the actions of insulin is in contrast to other target diseases of gene therapy - for example plasma levels of adenosine deaminase of 5 - 5000 % of normal can be both effective in ameliorating the condition, and also safe (Steinberg 1991). Export of insulin from the site of implantation to the circulation is essential, and a portal delivery (which is physiologically normal) would be preferable (Zinman 1989). Furthermore, whilst a constant concentration of insulin in the plasma may be useful in certain situations (for example control of nocturnal hyperglycaemia), for truly effective treatment of diabetes it would be necessary to 'close the loop' by engineering a stimulus-secretion

response, thus altering the level of gene expression and insulin secretion in response to changes in the ambient concentration of glucose. However, the metabolic signal for insulin release by genetically manipulated cells may not be restricted to glucose - it may be possible to sensitize the transfected cells to another metabolite or ion.

A key factor in the success of gene therapy for IDDM is obviously the selection of the optimal target cell. Many types of cell have been suggested to date - these are described in Chapter 1. It has been shown that neuroendocrine cells which contain endopeptidase activity are capable of sorting and processing many propeptide precursors, including insulin, correctly (Dickerson and Noel 1991). However, the AtT20 cells utilized here also release quantities of ACTH and β-endorphin which themselves may be deleterious to the glucose homeostasis of the recipient animal. Hence for a clinical application of gene therapy another target cell is likely to be more suitable. Fibroblasts and keratinocytes have been suggested as ideal target cells since they are manipulable *in vitro*, amenable to genetic modification, and implantation to the subcutaneous site would be relatively simple and non-invasive. A disadvantage for the use of fibroblasts is that they may have a limited secretory capacity, do not release products from secretory vesicles (via the regulated pathway), nor do they contain endopeptidases, and hence cannot process proinsulin to insulin (Moore *et al* 1983). Fibroblasts do however contain furin, which is a Kex2 homologue (Hatsuzawa *et al* 1990), and has a very wide tissue distribution (Bloomquist, Eipper and Mains 1991). Furin is a protease involved in processing constitutively released and membrane proteins, and cleaves precursors at dibasic sites. In addition, furin displays substrate specificity since it requires an arginine residue at

position -4 upstream of the cleavage site (Hatsuzawa *et al* 1992). Site-specific mutagenesis of the dibasic residues Arg^{31}-Arg^{32} or Lys^{64}-Arg^{65} may enable furin to cleave both sites and hence produce mature insulin and C peptide, or reduce the need for two endopeptidases to one. Alternately, the co-transfection of a suitable endopeptidase such as PC2 along with insulin may enable correct cleavage to occur; this has been demonstrated following the cotransfection of the ACTH precursor (POMC) and the endopeptidase Kex2 into cells with no endogenous endopeptidase activity (Thomas *et al* 1988).

There may be a problem with the use of fibroblasts (and potentially with other cell types) concerning the *in vivo* inactivation of transfected genes. It has been shown that although retrovirally-transfected fibroblasts implanted sc into rats survived for many (>8) months, expression of the introduced genes declined rapidly to undetectable levels after 1 month (Palmer *et al* 1991). A similar loss of expression was not observed in transfected cells maintained in culture over the same time period.

It has previously been shown that fibroblasts transfected with the human insulin gene can secrete proinsulin, and that when large numbers of these cells are implanted ip into nude mice, there is a decrease in plasma glucose concentrations over a 24 day study (Selden *et al* 1987b). Interestingly, following ip implantation of the same number of cells into normal but immunosuppressed mice, an effect on glycaemia was only observed in 50 % of the recipients. The authors attribute this to the intra-individual variation of response to immunosuppression. Similar results were reported in Chapter 6 of this thesis, where it was concluded that the sc site may not be as

efficacious as the ip site as regards the export of product into the circulation. In general, the investigations reported herein demonstrate that more promising results were obtained from studies utilizing nude mice than from those involving CyA-treated MF1 mice.

A further study involving the implantation of proinsulin-secreting fibroblasts into diabetic mice included a subsequent transfection step with a mouse T cell antigen CD8.2. Following ip implantation, recipient mice became normoglycaemic after approximately 14 days; treatment with anti-CD8.2 monoclonal antibody caused a rapid increase in plasma glucose levels to pre-implantation values (Kawakami *et al* 1992). This approach may represent an important safety feature by which genetically manipulated cells could be destroyed *in situ* even after implantation to a relatively inaccessible site.

7.3. Regulation of gene therapy protocols

Since several clinical gene therapy trials have already been performed, mostly in the USA (Anderson 1992), detailed guidelines on the regulation of trials and safety considerations have been published by the FDA. Since gene therapy involves the administration of biological materials to a patient, it is difficult to list specific regulations, and each trial must therefore be reviewed entirely separately (Epstein 1991, FDA 1991). The major concern is safety: the guidelines suggest extensive preclinical testing including characterization of the cells for purity and phenotypic markers, and quantification of the products released (both the therapeutic product and any other substance made by the cell). The use of a suitable animal model is required to examine the *in vivo* stability, longevity, and regulation (if any) of

exogenous gene expression. In addition, information on any adverse effects, localization of cells to a particular organ, inappropriate release of the product, or tumourigenicity is required. Theoretically, if the implant produces non-self epitopes of the product (caused for example by mutation, polymorphism or aggregation) these may be immunogenic. Even recombinant human materials could theoretically induce a response in patients previously deficient (and hence not exposed); the administration of recombinant factor VIII (McMillan *et al* 1988) and human insulin (Grammer *et al* 1987) has been shown to stimulate an immune reaction in some recipients.

The guidelines suggest further approaches to safety so that in the case of development of any adverse effects of the implant, exogenous gene expression could be down-regulated, or the cells could be inactivated or destroyed. Such approaches may involve the use of cells that are dependent upon a specific growth factor which could subsequently be withdrawn, the conferral of drug sensitivity on the cells prior to transplantation (for example by co-transfection with the herpes simplex thymidine kinase gene which confers sensitivity to ganciclovir), or possibly the induction of transcription of an anti-sense insert. Furthermore, the cells could be contained in a prosthesis to make them easier to remove (although the use of alginate-polylysine-alginate membranes can seriously affect the level of secretion of the cells, as discussed previously), so more biologically applicable forms of containment are required.

7.4. Concluding remarks

In addition to being a potential target disease for gene therapy, diabetes may also be considered a very useful model system, the study of which will reveal much valuable information that is both relevant and applicable to the effective treatment of other diseases. The insulin gene and the systems by which it is regulated are relatively well understood, and the products (proinsulin, insulin and C peptide) can be readily quantified *in vivo*, and inter-species differences can also be identified. A problem that has hindered many gene therapy programmes to date is the lack of a suitable animal model (Kolberg 1992); both naturally occurring (the BB rat and NOD mouse) and experimentally-induced (streptozotocin) models of diabetes are available for study. Furthermore the action of the hormone *in vivo* can be rapidly ascertained by measurement of plasma glucose concentrations, and various effects upon other metabolic parameters.

Insulin gene therapy for the treatment of diabetes mellitus remains an attractive, if distant, alternative to current treatments and areas of research. A major problem with approaches involving transplantation of pancreatic tissue - that of destruction of the graft by the original disease mechanisms in the recipient (Sutherland, Goetz and Sibley 1989) - would be circumvented by the use of surrogate cells in gene therapy protocols. Furthermore, shortages of donor tissue, and the morbidity of currently available immunosuppression regimens could also be avoided. However, in order for insulin gene therapy to become a clinically realistic prospect, several key areas require further development. The transfection and subsequent reimplantation of autologous cells will be essential if immunosuppression is to be avoided; it has been suggested that in young and healthy diabetic patients the morbidity caused by

immunosuppressive regimens outweighs the risk of exogenous insulin therapy (Gray and Morris 1987). However, the genetic manipulation of primary cultures is notoriously difficult (Selden *et al* 1987b); how realistic is it to aim to treat every IDDM patient with an implant of his own genetically modified cells? How long would such an implant release insulin at an appropriate rate? The selection of different target cells, such as fibroblasts, will alter the protocol; cell harvesting and primary culture may be more straightforward (skin grafting is a treatment routinely in use for burns patients), but the necessary genetic manipulations may become more complex - the co-transfection of insulin plus an endopeptidase such as PC2 (to enable processing of proinsulin to insulin) and / or a high capacity glucose transporter such as GLUT 2 (to improve the insulin secretory response to glucose) may be desirable. More effective methods of transfection may be needed and it may be essential to use an automated transfection procedure.

Success in experimental animals is a prerequisite for clinical trials. However the scale-up from rodent to human is often not simply a matter of magnitude; the first successful islet graft in rodents was carried out almost twenty years ago (Hegre *et al* 1975), but lasting success has yet to be achieved in human patients. The avoidance of autoimmune attack by use of non-β-cell material may contribute to the success of transplantation since islet autografts to non-diabetic pancreatitis patients have been efficacious (Pyzdrowski *et al* 1992).

The sequence of the human insulin gene was determined only twelve years ago (Bell *et al* 1980), yet in that time it has become possible to manipulate the gene, to demonstrate expression following transfection into foreign (surrogate) cells, and even

to achieve insulin release following implantation of these cells into animals. Preliminary steps in the creation of a secretory response to glucose (Hughes *et al* 1992), and in conferring prohormone processing activity to cells without endogenous endopeptidases (Thomas *et al* 1988) have been accomplished. The rapid pace at which developments in molecular biology are occurring may make the prospect of successful gene therapy for diabetes seem less remote.

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APPENDIX ONE

BUFFERS

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Krebs-Bicarbonate buffer

NaCl	120.00	mmol/l
$NaHCO_3$	5.00	mmol/l
$Na_2HPO_4.2H_2O$	1.80	mmol/l
KCl	4.50	mmol/l
$NaH_2PO_4.2H_2O$	0.20	mmol/l
$MgSO_4$	1.00	mmol/l
$CaCl.2H_20$	2.50	mmol/l
HEPES	15.9	mmol/l
BSA	0.20	g/l
D-glucose	5.56	mmol/l

Citrate buffer

$C_6H_8O_7.H_2O$	0.1	M	2 parts	pH 4.8
$C_6H_5O_7Na_3.2H_2O$	0.1	M	3 parts	

Insulin RIA phosphate buffer

$NaH_2PO_4.2H_2O$	6.2	g/l	
BSA	5.0	g/l	pH 7.4
NaN_3	1.0	g/l	

Insulin RIA separation (PEG) buffer

Polyethylene glycol 6000	200	g/l
γ-globulin	1.0	g/l
Tween 20	0.5	ml/l

0.1 % FAM buffer (0.04 M phosphate buffer)

$Na_2HPO_4.2H_2O$	5.77	g/l		
$NaH_2PO_4.H_2O$	1.05	g/l	to volume	
BSA	1.00	g/l	with	pH 7.4
Sodium thiomersalate	0.24	g/l	dH_2O	

6 % NaFAM buffer

NaCl	6.00	g/l	to volume	
BSA	59.0	g/l	with	pH 7.4
			0.1% FAM	