THE ENCAPSULATION AND TRANSPLANTATION OF ISLETS OF LANGERHANS

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by

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The encapsulation and transplantation of islets of Langerhans.
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Although several clinical human islet transplants have been performed, allograft rejection has been a major problem. Encapsulation in sodium alginate/poly-l-lysine has been proposed as a method to protect the islets from rejection and autoimmune destruction. The aims of this project were to determine optimum capsule composition and to assess encapsulated islet function following transplantation using the spontaneously diabetic BioBreeding (BB/d) rat as a model of autoimmune insulin-dependent diabetes.

The membrane integrity of islets, determined by microfluorometry, was not adversely affected by encapsulation. During perifusion, the stimulation increase of encapsulated islets was decreased relative to controls, but the stimulation index and response time were unaffected. Capsule composition did not affect these results.

The biocompatibility of the capsules was related to their composition. The presence of an outer layer of alginate, and the preparation of alginate used, reduced the severity of pericapsular fibrosis. Capsules implanted in the peritoneal cavity provoked a more severe response than those placed in the renal subcapsular space. BB/d rats displayed a marked response to the capsules.

Transplantation experiments demonstrated that 3000 non-encapsulated or 5000 encapsulated islets were the minimum number required to reverse streptozotocin-induced diabetes when transplanted into the peritoneal cavity. An intense pericapsular fibrosis led to failure of the encapsulated grafts. Further transplants into the BB/d rat were postponed to allow the cause of fibrosis to be investigated.

The alginate was found to be contaminated with protein and this was removed by dialysis. In vitro experiments with the dialysed alginate demonstrated that treatment of the alginate did not affect the viability of encapsulated islets. Biocompatibility studies showed that capsules coated with dialysed alginate remained free from pericapsular fibrosis, even in the BB/d rat, when tested over the time interval which had resulted in the development of severe fibrosis of capsules coated with untreated alginate.

Statement of originality

This is to confirm that the work in this thesis was undertaken in the Department of Surgery, University of Leicester by myself, unless otherwise stated, during the period January 1989 to December 1991, and has not been submitted for another degree at any University.

H.A.Clayton

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CONTENTS

ABBREVIATIONS

ALS	Anitlymphocyte serum
ATS	Antithymocyte serum
BB/d	Spontaneously diabetic BioBreeding rat
BB/ndp	Non diabetes prone BioBreeding rat
BG	Blood glucose
BSA	Bovine serum albumin
CHES	2-[N-Cyclohexylamino]ethane-sulfonic acid
CsA	Cyclosporin A
DTT	Dithiothreitol
FDA	Fluorescein diacetate
HbA_{1c}	Glycosylated haemoglobin
IAA	Insulin autoantibodies
ICA	Islet cell antibodies
IDDM	Insulin-dependent diabetes mellitus
IMS	Industrial methylated spirit
IVGTT	Intravenous glucose tolerance test
LPS	Lipopolysaccharide
MEM	Minimum essential medium
MLR	Mixed lymphocyte reaction
NIDDM	Non insulin-dependent diabetes mellitus
NOD	Non obese diabetic mouse
PBS	Phosphate buffered saline
PI	Propidium iodide
PLL	Poly-l-lysine
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
STZ	Streptozotocin
TBS	Tris buffered saline

CHAPTER 1

ENCAPSULATED ISLET TRANSPLANTATION

Insulin dependent diabetes mellitus results from the selective destruction of beta cells within the islets of Langerhans. This leads to the individual becoming hyperglycaemic, and if insulin injections are not administered the patient will become ketotic and die.

Although the advent of insulin therapy prevented death from the acute metabolic complications of diabetes, many diabetics develop long-term sequelae such as retinopathy, nephropathy and neuropathy. The exact cause of these complications is unknown but there is evidence to suggest that poor control over blood glucose levels leads to glycosylation of essential proteins, ultimately resulting in the conditions listed above.

In an attempt to improve glycaemic control and therefore reduce the possibility of developing complications, some patients have undergone vascularised pancreas transplants to replace the damaged beta cells and provide normal physiological control over blood glucose levels. However, this operation is usually only available to patients who have already received a kidney transplant or who are to undergo a simultaneous pancreas and kidney transplant. This is because the potential side effects of long-term immunosuppression and the risks of the operation are considered to outweigh the benefits of pancreas transplantation early in the course of the disease prior to the development of complications.

Consequently, the possibility of isolating the islets from the pancreas has been considered so that only the tissue required by the patient is transplanted, and by working with a much smaller volume of tissue the potential for immunomodulation of the islets is much

greater than if the whole organ is involved. Experimental studies undertaken to assess the effect of pretreating the islets on graft survival have shown improvements. However, the experience of clinical islet transplantation has been unable to match these experimental findings.

Immunoisolation is an alternative method for extending graft survival. This involves placing the islets inside a semipermeable membrane which allows the passage of insulin, glucose and nutrients but not immunoglobulins or cells of the immune system. Some devices have involved placing numerous islets in a single chamber, but problems have been encountered with poor biocompatibility often leading to occlusion of the membrane. Another approach to the problem has been to encapsulate individual islets into microdroplets formed from a biocompatible material. One of the most commonly used methods of encapsulation is the formation of a three-layered capsule with sodium alginate forming the "biocompatible" inner and outer layers, and poly-l-lysine (PLL) forming the semipermeable middle layer.

Experiments with alginate-PLL encapsulated islets have given mixed results, with some groups reporting long-term graft survival and others reporting failure of the grafts due to intense pericapsular fibrosis. Comparison of the results has been made difficult by differences in capsule composition and variation in the donor-recipient combinations used to assess encapsulated islet graft function. Therefore, the aims of this project were twofold. Firstly, to determine optimum capsule composition in terms of in vitro insulin release characteristics and in vivo biocompatibility. Secondly, to assess the ability of the capsules to protect transplanted islets from allograft rejection and autoimmune destruction. The spontaneously diabetic BioBreeding rat was the model of autoimmune IDDM chosen for these experiments.

In vitro experiments showed that the membrane integrity of the islets, as determined by microfluorometry, was not adversely affected by the process of encapsulation, and that although the absolute amount of insulin released from the encapsulated islets during perifusion was less than from control islets, the proportionate

increase in insulin release in response to increased glucose and the response time of the islets was the same as for controls. Capsule composition did not affect these results.

The biocompatibility of the different capsule compositions was assessed in four strains of rat. The results showed that the presence of alginate reduced the severity of pericapsular fibrosis relative to PLL-coated capsules and that this response could be further reduced depending on the preparation of alginate used. It was also noted that a much stronger response was provoked in the diabetic BB (BB/d) rat than in the WAG/Ola or non-diabetes prone BB (BB/ndp) rat, and that the response to capsules implanted in the peritoneal cavity was greater than those in the renal subcapsular space. Sections of the fibrosed capsules were stained with a panel of monoclonal antibodies to determine the cell types involved. A classical foreign body reaction comprising macrophages and fibroblasts was demonstrated.

Although the results demonstrated that pericapsular fibrosis could be reduced by implantation in the renal subcapsular site, the increase in size of the encapsulated islets relative to non-encapsulated islets meant that there would not be sufficient space to transplant enough encapsulated islets to reverse diabetes, even if both kidneys were used. The peritoneal cavity was considered to be the only site which could provide enough room for the number of encapsulated islets required.

Transplantation studies were undertaken to determine the number of non-encapsulated syngeneic islets required to reverse streptozotocin (STZ)-induced diabetes in the BB/ndp rat when placed in the peritoneal cavity and to compare this with the number of encapsulated islets required. These experiments were preliminary to encapsulated islet transplants into the BB/d rat. The results demonstrated that 3000 non-encapsulated or 5000 encapsulated islets were the minimum number required to reverse diabetes. However, failure of the encapsulated grafts in the BB/ndp rat due to an intense pericapsular fibrosis indicated that the alginate was not biocompatible and could not be used for long-term transplantation studies. Further transplants were postponed until the poor biocompatibility of the alginate could be resolved because any

further transplants would fail due to fibrosis of the capsules and would not provide any additional information on the ability of the capsules to protect the islets.

The final part of the project concentrated therefore on identifying the impurity in the alginate which provoked the fibrotic reaction, and on trying to remove this to improve the biocompatibility. It was found that the alginate was contaminated with a small amount of protein which could have led to fibrosis of the capsules, and a method of dialysis was developed to remove the protein from the alginate. In vitro studies on the dialysed alginate showed that the treatment did not affect the viability of encapsulated islets, and most importantly in vivo experiments indicated that no fibrosis developed round the capsules after three weeks. This time interval was sufficient with untreated alginate to cause intense fibrosis and capsule breakdown.

CHAPTER 2

INSULIN-DEPENDENT DIABETES MELLITUS - A REVIEW

c. Transplantation studies

iii. Human islet transplantation

iv. Foetal islet transplantation

v. Xenotransplantation

vi. Immunomodulation of islets

2.1 INTRODUCTION

This chapter reviews the literature relating to aspects of insulin-dependent diabetes (IDDM), including a brief history of the discovery of IDDM, the various factors thought to be involved in its aetiology, the current methods of treatment, and the complications commonly encountered by IDDM patients. The problems and success rates of intact pancreas transplantation have been reviewed to assess its impact as a potential treatment for IDDM. In addition, the development of islet isolation and transplantation, the success of islet transplantation, and the problems still to be resolved before islet transplantation can be considered as a treatment for large numbers of IDDM patients, are reviewed.

2.2 NON INSULIN-DEPENDENT DIABETES MELLITUS

Although islet transplantation as a treatment for IDDM is the focus of this project, this section explains the condition of non insulin-dependent diabetes mellitus (NIDDM) in order to highlight the differences between the two forms of diabetes.

Also referred to as Type 2 diabetes, NIDDM does not often present with acute metabolic complications and patients are not dependent on insulin therapy to prevent ketonuria. Patients may be asymptomatic for many years with a slow progression of the disease becoming apparent and insulin levels may be normal (National Diabetes Data Group, 1979). Most patients develop NIDDM after the age of 40 and consequently the condition used to be referred to as mature or adult onset diabetes. However, as the condition can occur in young individuals definition of the condition by the age of onset is inappropriate.

Approximately 1.2% of the population in the United Kingdom have diabetes and 75% of these are NIDDM patients (British Diabetic Association, 1988). Genetic susceptibility is known to be a factor leading to the development of NIDDM although environmental factors also play a role in its onset, most notably obesity or weight gain. It is estimated that 60-90% of all Western NIDDM patients are obese

(NDDG, 1979). Thirty percent of NIDDM patients can control their condition by dietary regulation, 50% have to be treated with oral hypoglycaemics, and the remaining 20% require insulin injections to control their diabetes. Complications of the condition are similar to those for IDDM and these will be discussed in greater detail with respect to IDDM.

2.3. INSULIN-DEPENDENT DIABETES MELLITUS

2.3.i Definition of IDDM

Insulin-dependent diabetes mellitus, also referred to as Type 1 diabetes (formerly juvenile onset diabetes), is characterised by acute onset of symptoms including weight loss, polyuria and thirst, with dependency on insulin therapy to maintain life. IDDM patients are prone to ketosis especially if the diabetes is not well controlled (NDDG, 1979; BDA, 1988). Full details of the classification of diabetes and other types of glucose intolerance were standardised by the National Diabetes Data Group in 1979.

2.3.ii Brief history of IDDM

The term diabetes meaning "siphon" was first used by Aretaeus of Cappadocia to describe the condition of "melting down of flesh and limbs into urine". Mellitus meaning "honeyed" was added to describe more fully the disease when in 1674 Willis noted that the urine from diabetic patients tasted sweet "as if imbued with honey and sugar" (Baxter, 1989).

It was in 1898 that Minkowski realised the relationship between the pancreas and diabetes when total pancreatectomy of a dog for the purpose of fatty acid research resulted in the animal developing diabetes, and in 1901 Opie further resolved the problem when he made the connection between damaged islets of Langerhans and diabetes. The discovery of insulin and its ability to alleviate the symptoms of diabetes came in 1921 when Banting and Best finally managed to extract insulin from the pancreas, a task which several others had attempted without success (Luft, 1989).

2.3.iii Incidence rates

Many studies have been undertaken to determine the incidence of diabetes and to assess the relative risks of males and females, different age groups, different social classes and ethnic groups.

A retrospective study in Denmark (Christau et al. 1977) between 1970 and 1974 discovered an annual rate of new cases of 13.2 per 100,000, with 27% more males than females affected. The peak incidence was between 12-14 years for males but slightly earlier for females, with the lowest number of new cases detected between May and July. Areas of lower social status provided higher incidence rates, although such divisions were very loosely defined.

The opposite was discovered by West et al. (1979) in a study in Montreal between 1971 and 1977, with a higher incidence in areas of high socioeconomic level, although the trends of male:female incidence and seasonal peaks were similar. They also noted the incidence in children with diabetic siblings was increased fifteen-fold relative to the general population and that 10% of diabetics had a first degree relative with IDDM.

Fishbein et al. (1982) reported trends in incidence of diabetes in Pittsburgh when 5-9 and 10-14 year olds were considered, but not when all cases presenting under the age of 20 years were studied. The incidence rate in males was noted to have more seasonal patterns than in females. Seasonal patterns were also noted by Fleegler et al. (1979) in a study of three areas in the United States and Melbourne, Australia. Such seasonal variations have been linked with peaks of mumps and rubella suggesting that these infections were the trigger for diabetes onset, but in this study the peaks of rubella and mumps occurred later than those of diabetes onset, and immunisation against these conditions was noted not to have affected diabetes incidence.

Three more recent studies have all noted an increase in incidence rates of IDDM. Joner and Sovik (1991) found the incidence of diabetes in Norway had increased to a mean of 17 per 100,000 per

year in the period 1978-1982 compared with 8.8 per 100,000 per year between 1956 and 1964. Seasonal, geographical and sex variations were also noted. Tuomilehto et al. (1991) also discovered the incidence rate in Finland between 1965 and 1984 in children under 15 years rose by 2.4% annually, with peaks in 1978 and 1983. Similarly, Metcalfe and Baum (1991) found an increase in cases under 15 years in the British Isles from 8 per 100,000 in 1973/74 to 13.5 per 100,000 in 1988, with Scotland having a rate of almost 20 per 100,000 and Eire only 6.8 per 100,000. Peak onset was at 12 years for both sexes and in the months of February and March.

The different rates associated with ethnic groups was highlighted by analysis of data from 24 registries in 15 countries (Diabetes Epidemiology Research International Group, 1988). This demonstrated lower incidence rates amongst black and Hispanic populations than in white populations in the U.S., a three-fold increase in risk amongst New Zealanders of European origin than those of Maori or Polynesian origin, and a two-fold increase in risk amongst Ashkenazic Jewish children living in Montreal compared with those living in Israel, suggesting that an environmental factor was causing an increase in the number of cases. This study also suggested that risk of developing IDDM is related to the average yearly environmental temperature.

2.3.iv Pathogenesis of IDDM

It was in 1948 that von Meyenberg used the term "insulitis" to describe the condition of islets at the onset of diabetes. This refers to the infiltration of lymphocytes in or around the islets. T lymphocytes are the predominant cells, and most of these are $CD8^+$ cytotoxic T cells, although $CD4^+$ helper T cells may be present. Also, IgG-producing B lymphocytes can be detected in the acini surrounding the affected islets (reviewed by Trucco and Dorman 1989). Polymorphonuclear cells may also be present. Not all the islets within the pancreas are necessarily affected, but infiltrating cells have never been detected in islets devoid of beta cells (Cahill and McDevitt 1981).

Gepts has made some of the most notable observations relating to the histology of the pancreas at the onset of IDDM. In 1965, he noted that approximately 10% of the normal number of beta cells were present in recent onset diabetics with these residual beta cells displaying intense hyperactivity. Inflammatory infiltrates were present in the islets in about 70% of cases studied. Individuals with chronic diabetes were noted to have small pancreata with few islets composed of small atrophic cells, few or none of which were beta cells. Further observations by Gepts and DeMey (1978) detailed thin cords of islet cells (not beta cells) actively secreting glucagon and somatostatin in both recent and longer-term cases. In recently diagnosed cases, isletitis was only present if beta cells were still present. These residual cells were demonstrated to have increased RNA synthesis and to be degranulated, confirming the apparent hyperactivity previously described. In this study, it was also noted that with increasing time after onset, there is an increase in the number of atypical islets present in the pancreas. These are almost entirely composed of pancreatic polypeptide cells.

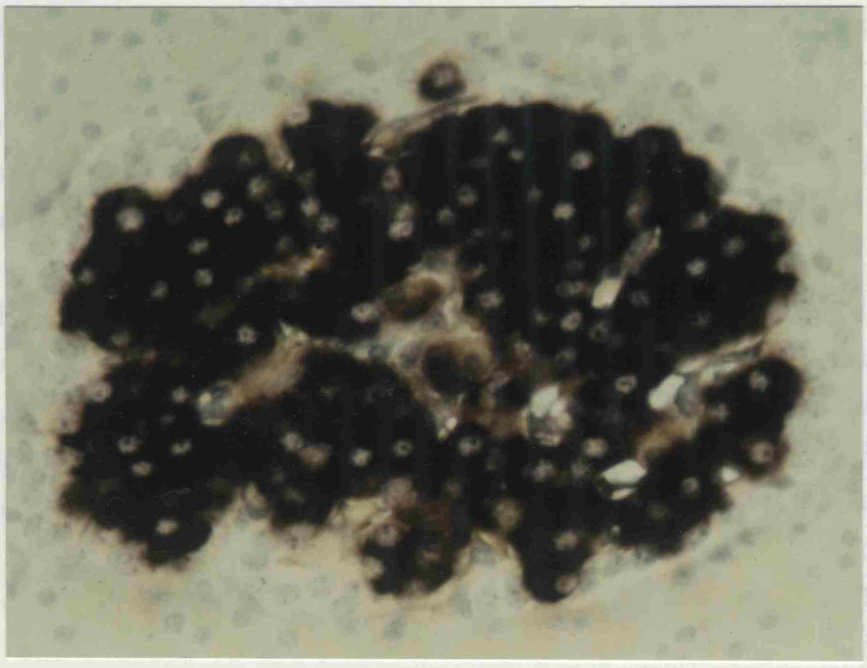
Similar observations were made by Foulis et al. (1986) in a retrospective study of pancreatic tissue taken from young diabetics at post mortem. They found that insulitis was still present in 78% of the individuals with recent onset. Figures 2.1.i and 2.1.ii demonstrate the selective destruction of beta cells within an islet.

2.3.v The aetiology of IDDM

a. Autoimmunity

The connection between IDDM and autoimmunity was made by Irvine et al. (1970) when antibodies to thyroid cell cytoplasm and gastric parietal cell cytoplasm were found to be significantly increased in diabetics without thyroid disease or pernicious anaemia when compared with controls. In 1974, the same group (MacCuish et al.) detected circulating antibodies to islet cells in the sera of five out of twenty patients with IDDM and coexistent autoimmunity, increasing the evidence in support of IDDM being an autoimmune condition.

i.



ii.

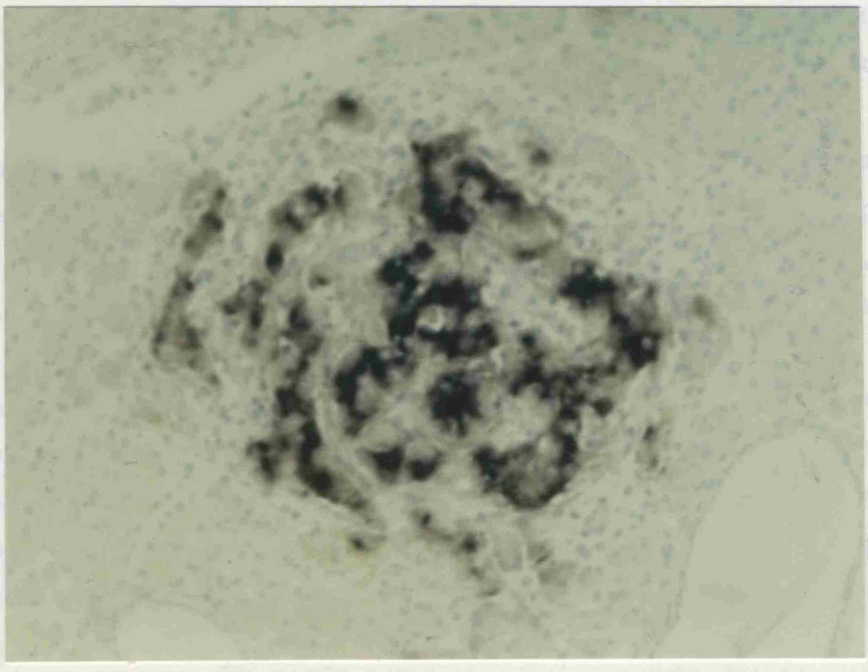


Figure 2.1
Control islet (i) and BB/d islet with isletitis (ii) stained for insulin mRNA using in situ hybridisation.

Also in 1974, Bottazzo et al. discovered that young people with IDDM were three times more likely to have primary myxoedema, thyrotoxicosis and pernicious anaemia than controls. They also noted that 10% of Addison's disease patients had IDDM compared with 1% of the normal population, and that adrenal antibodies occurred thirty times more often in IDDM patients than in controls. Also, 13 patients with multiendocrine deficiencies associated with organ-specific autoimmunity had antibodies to islet cells.

The actual mechanism of autoimmune islet cell destruction has not been fully determined with several hypotheses based on the detection of antibodies and cells in or close to islets in newly diagnosed diabetics. One suggestion (Trucco and Dorman, 1989) is that an external factor, for example a virus or toxin, causes the release of antigen from the beta cell which is then processed by macrophages and presented in conjunction with a class II molecule to helper T cells. The T cells then stimulate B lymphocytes into producing antibodies against the antigen, which when bound to beta cells in the pancreas will activate the complement cascade leading to cytotoxicity. Cytotoxic T cells then become involved, infiltrating the damaged islet leading to complete destruction of the beta cells.

An alternative hypothesis for beta cell destruction has been proposed by Bottazzo following the detection of class II expression on beta cells which do not normally express these molecules, and the increased expression of class I (Bottazzo et al. 1983; Bottazzo and Dean, 1984). This model does not rely on antigen presenting cells, and suggests that an external agent is not necessary to start the process of islet destruction, but that any environmental factor can trigger the events as long as it has the ability to cause beta cells to express class II antigens on their surfaces. Class II expression has been induced <u>in vitro</u> on normal human donor pancreas by the action of interferon-gamma and tumour necrosis factor alpha (Pujol-Borrell et al. 1987). Destruction of the beta cells would then follow presentation of an antigen in conjunction with class II on the beta cell itself, without the need for antigen presenting cells, and helper T cells would be activated. Increased class I expression could act as a target for cytotoxic T cell activity, and the release of

lymphokines from the T cells would act to enhance HLA expression on the beta cells (Trucco and Dorman, 1989).

Review articles by Trucco and Dorman (1989), Riley (1989) Cahill and McDevitt (1981), Castano and Eisenbarth (1990), Naji et al. (1987), Eisenbarth (1986) and Bottazzo et al. (1989) provide more comprehensive information on autoimmunity with respect to IDDM.

b. Genetic susceptibility

The reviews quoted above relating to autoimmunity, and that by Leslie et al. (1989) deal with the genetic aspects of susceptibility to IDDM in considerable depth, and provide more detailed information on this subject.

In brief, it is the class II HLA genes which predispose to or protect individuals from developing IDDM. It has been determined that amongst people expressing DR3 or DR4 genes the incidence of diabetes is three to five-fold that of the general population, but that if an individual expresses both DR3 and DR4, the risk of developing IDDM is increased by twenty to forty times. Ninety-five percent of Caucasians with IDDM express either or both DR3 and DR4. Also, expression of certain DQ region genes means that the risk of developing IDDM is increased to ninety times (Leslie et al. 1989), but the presence of aspartic acid at position 57 of the DQ beta chain appears to be negatively associated with IDDM. Erlich et al. (1990) have noted that aspartic acid at position 57 does not confer complete resistance to IDDM, and that specific combinations of DQ beta and DR beta sequences may be important in conferring susceptibility or protection. Protection also appears to be gained if DR2 is expressed as very few cases world-wide have been reported of such individuals developing IDDM (Cahill and McDevitt, 1981; Trucco and Dorman, 1989). Studies on families with a child having IDDM have demonstrated that if a sibling shares 2 haplotypes the risk of developing diabetes is 10-20%, if only one haplotype is shared the risk is reduced to 5%, and if neither haplotype is shared the risk is 1% (Eisenbarth, 1986).

Although the genetic aspect is clearly a major factor in determining whether an individual develops IDDM, this is not the only factor involved, as studies with twins have demonstrated that even if one identical twin develops IDDM the other twin will not necessarily go on to develop the condition. In 1981, Barnett et al. found that out of 147 pairs of identical twins, 80 were concordant for IDDM, but that the remaining 67 were not, suggesting that the development of the disease is not solely linked to genetics. A later study by Johnston et al. (1983) typed 106 identical twin pairs, 56 of which were concordant for IDDM. They found that the prevalence of DR3 and DR4 was high in both groups, DR5 and DR7 was low, and DR2 almost absent. However, they also noted that twins expressing both DR3 and DR4 were more prevalent in the concordant group suggesting that possession of both confers greater genetic predisposition to IDDM than only one, and also helping to explain why the concordance rate is perhaps lower than might be expected.

c. Environmental factors

The evidence discussed above clearly demonstrates the influence of genetic predisposition on the development of IDDM. However, it is also clear that this is not the only factor involved, and attempts have been made to define environmental factors likely to be involved in triggering the onset of IDDM.

Viral infections have been implicated as factors which promote the development of IDDM, a theory supported by the seasonal peaks of diabetes onset (Section 2.3.iii) which occur in the winter months when infections are more common. Although this argument may sound plausible, evidence to confirm that viral infections can trigger the onset of IDDM has been difficult to find.

Wilson et al. (1977), Yoon et al. (1979) and Champsaur et al. (1980) have all described cases in which infections with Coxsackie virus have been associated with the onset of IDDM. However, the small number of cases where such an association is apparent suggest that viral infection may not be a common factor in the development of the disease. Helmke et al. (1980) suggested that viral infections are

unlikely to cause IDDM to develop, but an infection in an individual with islets already undergoing autoimmune destruction and with no symptoms of IDDM onset, may be the final insult leading to the onset of overt diabetes.

Bodansky et al. (1986) noted an increase in insulin autoantibodies associated with viral infections, but not islet cell antibodies, and in 1984 Ginsberg-Fellner et al. found that in a study of 241 children with congenital rubella, a condition associated with the development of IDDM, 20% had islet cell cytotoxic antibodies. Thirty of the children had IDDM and 17 had borderline glucose tolerance. Another study by Rabinowe et al. (1986), also on young adults with congenital rubella, demonstrated abnormal T cell profiles and they suggested that this rather than the condition itself could lead to the development of organ-specific autoimmunity.

Other environmental factors implicated in the onset of IDDM include stress and exposure to chemicals and toxins. Castano and Eisenbarth (1990) and Trucco and Dorman (1989) both review the literature relating to the onset of IDDM following exposure to these factors.

2.3.vi Prediction of IDDM

One aspect of current research into diabetes is the possibility of predicting the onset of the disease. This would allow closer monitoring of individuals who were "at risk" so that prompt action could be taken to minimise metabolic changes at onset of the disease, and ultimately the aim would be to administer treatment to prevent IDDM from developing (Riley et al. 1988; Maclaren, 1988).

One of the aspects which has been considered as a predictor of IDDM is the presence of autoantibodies commonly detected in recently diagnosed diabetics, for example islet cell antibodies (ICA) and insulin autoantibodies (IAA). Studies reviewed by Maclaren (1988) showed that in a study of relatives of IDDM patients, individuals who were negative for ICA had a risk of approximately 1% of the disease developing compared with a risk of 40-70% amongst ICA positive

individuals. This risk was further increased if IAA were also present. Tarn et al. (1988) reported similar figures, but added that the relative risk of first degree relatives developing IDDM if the ICA were complement fixing was increased to 188.5 from 75.2 for non-complement fixing ICA.

A more accurate predictor of IDDM appears to be the presence of autoantibodies to a 64kD antigen present on human islet beta cells (Baekkeskov et al. 1987; Atkinson et al. 1988). These are consistently detected in a high percentage of newly diagnosed IDDM patients and in individuals who subsequently develop the disease, they are disease-specific and tissue-specific for beta cells (Atkinson et al. 1988). Maclaren (1988) reported that 100% of children and young adults tested had these antibodies before onset of IDDM, and highlights the need for screening the wider population. Only 15% of IDDM patients have a relative with the disease, but if all young diabetics possess antibodies to the 64kD antigen prior to onset, many potential patients should be picked up by such screening. One possible way to target screening is to include those with HLA markers associated with an increased risk of developing IDDM, but in turn, this depends on typing a large population.

2.4 CURRENT TREATMENT FOR IDDM

2.4.i Subcutaneous insulin therapy

Ever since the discovery and purification of insulin in 1921 by Banting and Best, insulin-dependent diabetics have relied upon daily subcutaneous insulin injections to prevent hyperglycaemia, ketosis and death. In his review on the modern management of IDDM, Nathan (1988) describes the advent of insulin therapy as having "converted IDDM from a disease with an invariably fatal outcome within several years of onset to a chronic disorder accompanied by profound long term complications and reduced lifespan". Put in these rather depressing terms the outlook for diabetics appears to be very poor, but careful control of blood glucose by adherence to an appropriate regime can allow diabetics to lead a full life, and many do not develop serious complications.

The early insulin preparations were extracted from pigs or cattle, and because pig insulin is structurally very similar to human insulin (Nicol and Smith, 1960) it is considered to be less likely to cause antibody formation. Many different insulin preparations are currently available. A study in 1952 by Hallas-Moller et al. looked at the efficiency of new insulin preparations which had different action times according to the interaction of zinc complexed with the insulin. Since then, preparations of single or mixed insulins have become available and are prescribed according to the individual needs of the patient.

The exact type and intensity of insulin therapy wil depend on the duration of diabetes, age of the patient, lifestyle, metabolic stability, the presence of any complications and the preference of the patient (Nathan, 1988).

One of the problems encountered with insulin injections is that because the subcutaneous route is used, the peripheral insulin concentration is higher than the portal level. As the liver is the main target of insulin action, this situation is not ideal (Tattersall, 1988).

One of the major advances in insulin therapy was made with the manufacture of human recombinant insulin by insertion of cloned A and B chain genes for human insulin into the beta-galactosidase gene of E.coli, followed by purification and joining of the two chains (Goeddel et al. 1979). This was the first molecule produced by such technology to become commercially available. Wilson et al. (1985) demonstrated the decreased immunogenicity of highly purified human insulin relative to both highly purified and conventional bovine insulin, and the majority of diabetics in Europe are now using human insulin with relatively few adverse reactions (Gale, 1989).

There has however been one recurring complaint amongst patients relating to the change from animal to human insulin, and that is the loss of warning of hypoglycaemic attacks. Berger et al. (1989) reported that 18 out of 32 patients in a trial with human insulin reported a decreased awareness of hypoglycaemia, compared with 6 out of 32 in the control group receiving porcine insulin. It was also

noted that the hypoglycaemic attacks developed faster with human insulin, presumably due to the slightly slower absorption rate of porcine insulin (Gale, 1989).

The loss of warning of hypoglycaemia with human insulin has become a controversial matter. It has been suggested that better control over blood glucose can lead more easily to hypoglycaemic attacks due to counter regulatory hormones being released at a lower threshold glucose level, so if human insulin does allow better control over blood glucose levels, this could explain the increase in reported attacks (Gale, 1989). Also approximately 20% of diabetic patients lose perception of hypoglycaemic attacks within the first 20 years of onset due to a loss of adrenergic warning symptoms (Grimaldi et al. 1990).

Another matter thought to be related to this loss of warning is the occurrence of sudden death following the change to human insulin. Evidence has shown that this often occurs at night and it is thought to be related to hypoglycaemia, but as this is very difficult to diagnose at post mortem, no definite conclusions can be drawn on the incidence of sudden death with commencement of human insulin therapy (Gale, 1989). This controversy will almost certainly continue until such time that properly controlled studies have been undertaken which conclusively prove the relationship between human insulin and loss of hypoglycaemic warning.

2.4.ii Insulin pumps

Insulin injections, although allowing the majority of diabetics to follow a relatively normal lifestyle, have not prevented the complications of IDDM from developing in many patients. These are thought to be a result of non-physiological blood glucose control, and consequently attempts have been made to provide a more intensive form of therapy which will prevent the complications from occurring. These have included continuous infusions of insulin and the use of implantable pumps.

In 1974, Slama et al. studied the effect of continuous intravenous insulin infusion in seven diabetic patients for between 1 and 5 days and compared the results with the control gained with an intensive regime of 3 insulin injections per day. They concluded that the infusion gave a significant lowering of blood glucose at night, but generally good control was gained with both forms of therapy. They concluded that insulin infusion was feasible in ambulatory patients without the need for blood glucose monitoring for several days. A later study (Pickup et al. 1978) determined the 24 hour metabolic profile of patients treated with subcutaneous insulin infusion and compared this with the profile when the same people were receiving normal injections. They found that the level of control improved in 6 out of 14 patients and was maintained in the same number.

Since these two early studies, several reports have been published relating to various designs of insulin pump. It is difficult to directly compare results as they use different administration routes (intraperitoneal, subcutaneous, intravenous) and some devices are implanted in the patient whilst others are external devices relying on catheters to administer the insulin.

Rizza et al. (1980) reported near-normal blood glucose with continuous subcutaneous insulin infusion in the short term, although the comment was made that the long-term performance and effect on complications was unknown. Librenti et al. (1986) demonstrated the feasibility of constant basal insulin infusion into the peritoneal cavity using an implantable device for 1 year. This did however cause an increase in circulating insulin antibodies and insulin injections were still required at mealtimes to cope with the resulting increase in blood glucose. The group concluded that such a device would need to be fully programmable before this form of therapy would be an advantage.

In 1988, Soltesz et al. studied the effects of continuous infusion on newly-diagnosed diabetic children. Although they were able to achieve restoration of normoglycaemia and correction of some metabolic abnormalities, they also noted a decrease in plasma glucagon and hypoketonaemia. Saudek et al. (1989) reported good

glycaemic control in patients with implantable, programmable insulin delivery devices, and that they were a potential form of therapy for up to 2 years periods. The reservoir had to be refilled every 2 months.

In 1989, Buchwald et al. reviewed the data provided by studies on implanted insulin pumps. Their analysis of the current situation was positive, although there is still a lot of development required, especially in the ability to program the insulin release, before this form of treatment will become widely available.

One potential problem with such devices is the possibility of infection at the infusion site. Tanner and Liljenquist (1988) reported on two patients who had toxic shock syndrome as a result of Staphylococcus aureus infections at their pump infusion sites. They commented that the management of such devices should be fully evaluated to prevent infection.

An aspect of research related to insulin infusion pumps is the development of a glucose sensor which could be implanted in the body so the insulin release system would be controlled by the glucose concentration of the surrounding body fluid. This should mean that better control could be achieved, especially after meals, and there would be no need to have a system which could be programmed. The practical aspects of developing a sensor have not been very successful, however, due to the problems of biocompatibility of the device and failure of the sensor.

One method of controlling insulin release was described by Brownlee and Cerami (1983) which utilised reversibly bound glycosylated insulin complexed to Concanavalin A. The insulin was displaced from the Con A by the presence of glucose and this occurred in proportion to the glucose concentration of the surrounding fluids. A comprehensive review on the automation of blood glucose regulation has been written by Pfeiffer (1987).

2.4.iii Alternative methods of insulin therapy

Many individuals have a fear of needles and injections which may be a reason for some IDDM patients not adhering to their therapy regime. Consequently, efforts are being made to provide insulin in forms which can be taken by alternative routes.

Perhaps the obvious route to make insulin therapy more acceptable is orally, and attempts have been made to produce insulin in a form which can be absorbed through the intestinal wall and exert its action before being degraded. One report (Cho and Flynn, 1989) noted a decrease in blood glucose in short term studies on three patients using an oral form of insulin therapy. Long term studies were also being undertaken.

Attempts are also being made to produce a form of insulin which can be absorbed through the nasal mucosa and could therefore be administered using an inhaler. This involves insulin being combined with a surfactant which is absorbed through the nasal mucosa. A study by Sinay et al. (1990) produced results which demonstrated satisfactory control in some but not all IDDM patients using nasal insulin in a chronic study. Although this type of study provides useful information on the action of different forms of insulin, it is apparent that alternative methods of administering insulin therapy will not be widely available for some time, and properly controlled trials will have to be undertaken to confirm their suitability.

Another method of making insulin injections a more acceptable form of therapy is the development of a "pen" to administer the insulin from a cartridge containing insulin. Several studies have been conducted on the control of diabetes using this method of injection. Spijker et al. (1986) reported equivalent control and hypoglycaemic episodes with conventional therapy and a pen. They also noted that even with a need to increase the number of daily injections from 2 to 4, patients considered the benefits of easier injection, less time taken to inject themselves and easier transport of the equipment outweighed the extra injections. Foss et al. (1990) and Ugart-Libano et al (1990) both reported a decrease in

glycosylated haemoglobin (HbA_{1c}) levels with the pen injections and a higher degree of acceptance by the patients involved in the trial.

2.5 COMPLICATIONS OF IDDM

2.5.i Introduction

Before the discovery of insulin, diabetic patients died within a few years of onset. The introduction and development of insulin therapy, although prolonging the life and increasing the quality of life of most patients, has not been able to prevent the long-term complications associated with the disease, conditions not seen before the advent of insulin therapy because patients did not live long enough for them to develop. The most commonly quoted complications are those of retinopathy, nephropathy and neuropathy, and these will be considered in greater detail in the following sections.

In addition to these conditions, heart attacks and strokes are also common in diabetics. Data published by the British Diabetic Association (BDA, 1988) showed that death from heart attacks was 1.9 times more common in men and 2.7 times in women with IDDM than non-diabetics, but if only the under 45 year age group is considered, the risk increases to 5 and 11.5 times respectively. Similarly, diabetic men have an increased risk of dying from a stroke of 1.5, and in women the risk is two-fold. Diabetic ketosis accounted for 15% of deaths in a group of 448 diabetics who had died. Further analysis demonstrated that this group included 27 unrecognised diabetics, and that there was often a delay in seeking or providing medical attention (Tunbridge, 1981).

One problem which makes it difficult to assess the cause of death amongst diabetics is that death certificates often do not record the diabetic condition of the patient. In 1983, Fuller et al. found that in a group of 2134 BDA members who had died, 33% of the death certificates did not mention diabetes.

Ulceration of the feet is a commonly encountered complication in diabetes, and can be a result of either neuropathy or peripheral vascular disease. It occurs 50 times more often than in non-diabetics, and if peripheral vascular disease is the cause, amputation is often required. Patients over 65 years old with diabetes are 25 times more likely to have had a leg amputated than non-diabetics of the same age group (BDA, 1988).

2.5.ii The cause of diabetic complications

The precise cause or causes leading to the development of diabetic complications have not been conclusively demonstrated, although it is generally thought that poor control of blood glucose will increase the risk of an individual developing these conditions.

In 1976, Gabbay et al. suggested that glycosylated haemoglobin (HbA_{1c}) could be a useful measure of short term blood glucose control as it is a post-translational event which occurs over the 120 day lifespan of the erythrocyte. Since then, HbA_{1c} measurements have generally been quoted as a measure of glycaemic control and many studies have tried to correlate these measurements with the onset and progression of diabetic complications. These studies will be covered in the appropriate sections.

Kennedy and Lyons (1989) reviewed the subject of non-enzymatic glycosylation. Every protein in the body containing lysine or hydroxylysine appears to be affected and monitoring of HbA_{1c} acts as a reflection of the effect on other tissues, as well as providing an indication of recent blood glucose control due to the relatively short half life of haemoglobin. They put forward two hypotheses of how glycosylation of proteins could lead to complications. The first was that the affected site could be essential to the function of the protein or its normal metabolism, and that this could be inhibited by glycosylation. This would be relevant to short-lived proteins with specific functions such as lipoproteins and enzymes. The second suggestion which is more relevant to longer-lived proteins such as collagen is that the structure or physical properties of the protein become altered. Such an effect on collagen could explain why 40% of

diabetic patients experience limited joint movement especially in the smaller joints.

Some studies on specific complications of diabetes have involved the randomisation of individuals into groups receiving either intensive or conventional insulin therapy to determine whether better control of blood glucose levels affects the appearance and progression of secondary diseases. However, there is controversy over whether such an approach is morally acceptable. Some consider that there has been shown to be relatively little difference between groups following the two types of insulin therapy and that rather than further studies being undertaken on a similar line, more effort should be put into educating and motivating patients to achieve the best possible control with the conventional forms of therapy currently available (Mulhauser et al. 1987). The DCCT research group (1988) suggested that studies have demonstrated good control is a benefit to patients, and that long-term studies are required to fully determine the effects of good glycaemic control on the development of complications. The arguments relating to this matter have been reviewed by Ingelfinger (1977).

One possible way to help resolve this problem is with the use of animal models of diabetes, especially as adherence to a particular insulin therapy regime and follow-up of long-term studies would be easier to undertake than with patients. Some studies of this nature have already been undertaken in Edinburgh (Brooks et al. 1980; Tames et al. 1990; Lindsay et al. 1991) and have generally shown that improved control of blood glucose, including the use of continuous insulin infusions, has led to a decrease in HbA_1 levels when compared with animals receiving conventional insulin therapy, but they were still higher than levels in normal control animals.

In addition to the aspect of glycaemic control, it is also thought that genetic susceptibility may affect the risk of an individual developing some complications. This has been suggested for both microangiopathy (Barbosa and Saner, 1984) and for nephropathy (Krolewski et al. 1987).

2.5.iii Microvascular disease

Microvascular complications form the basis of the deterioration apparent in the retina, kidneys and nerves. The changes seen in the blood vessels include thickening of the basement membrane, glycosylation of collagen, impairment of endothelial cell function and loss of pericytes (Hernandez, 1989). These factors then lead to an increase in organ blood flow rate, a decrease in permeability of the vessel walls resulting in the reduced ability of plasma proteins and leukocytes to move across the wall, and the possible impairment of function of endothelial cells. It is also thought that tissue damage may be a result of oxygen acting as a substrate for free radicals producing toxic oxygen (Hernandez 1989).

Barbosa and Saner (1984) determined that the appearance of small vessel disease is generally related to the duration of IDDM, although a marked individual variation was also apparent. They were unable to correlate the incidence of microangiopathy with any metabolic parameters, implying it is unrelated to glycaemic control, but they did note that men appeared to be more susceptible than women. However, Hanssen et al. (1986) suggested that the progression of microangiopathy in the kidney, retina and nervous system was arrested or retarded after two years of near normoglycaemia, and Camerini-Davalos et al. (1983) when using muscle capillary basement membrane thickness as an indicator of microangiopathy found that over a three year period the basement membrane thickness of a group taking oral hypoglycaemic agents was normal but in those taking a placebo it was increased. This study was undertaken on 41 asymptomatic patients with impaired glucose tolerance or who were judged to be prediabetic, indicating that changes leading to complications may be taking place before the onset of diabetes.

One problem with studying diabetic complications is screening patients to determine whether they have developed the conditions. Deterioration of the retina and increased protein excretion in the urine which indicates the onset of nephropathy are relatively easy to detect, but measuring basement membrane thickness relies on taking muscle biopsies which cannot reasonably be carried out on a regular

basis. Rosenbloom et al. (1981) have however noted that an indicator of increased risk of developing microvascular disease is limited joint mobility in childhood. Analysis of their data demonstrated that the risk of developing microvascular complications after 16 years duration of IDDM if joint mobility was affected was 83% compared with 25% if mobility was normal. This study has therefore defined a population of IDDM patients who may benefit from regular screening for the development of microvascular disease.

2.5.iv Retinopathy

Background retinopathy develops in 1 out of 100 patients during the 5th year of diabetes and after 14 years almost all diabetics are affected, with proliferative retinopathy increasing between 10 and 15 years duration (Krolewski et al. 1987). In 1980, diabetes was the most common cause of blindness in the age group 45-64, and in the same group diabetics are 23 times more likely to be blind than non-diabetics (BDA, 1988).

Disease in the retinal microvascular system is thought to be the cause of retinopathy, with breakdown of the blood-retina barrier being the first change. In addition, glycosylation of the basement membrane collagen, increased membrane protein synthesis and increased synthesis of proteoglycan in the basement membrane are thought to be involved (Hernandez, 1989). It has also been noted that retinal vessels are depleted of pericytes after several years (Krolewsli et al. 1987), and that in poorly controlled diabetes there is an increase in retinal endothelial cell turnover (Cuthbertson and Mandel, 1987).

A study by Leslie and Pyke (1982) demonstrated that in 31 pairs of identical twins concordant for IDDM, 21 had the same of severity of retinopathy, but only 5 of these pairs had the same duration of diabetes. This compared with 35 out of 37 pairs with NIDDM suggesting a strong genetic factor influencing retinopathy in NIDDM but not in IDDM.

Studies to determine whether intensive insulin therapy affects the development of retinopathy have been undertaken. Lauritzen et al. (1983) found that the blood glucose and HbA_{1c} levels fell with continuous insulin infusion relative to conventional therapy, but that retinal morphology deteriorated in both groups. They also noted that the frequency of deterioration was highest in individuals with the best control from continuous infusion although retinal function did increase in this group. Similarly, the KROC collaborative study group (1984) noted more deterioration in a group receiving continuous infusion although they had better control over blood glucose and HbA_{1c} levels. However, in 1989, McCance et al. noted that mean HbA_{1c} levels were strongly correlated with increased severity of retinopathy even when the duration of diabetes was taken into account, and only patients with HbA_{1c} of over 10% had proliferative retinopathy (normal HbA_{1c} range is 4-7%, Krolewski et al. 1987). This last study was carried out over a period of 6 years compared with 8 months and 1 year for the other two studies, and may therefore provide more accurate information about glycaemic control and the development of retinopathy.

A more encouraging aspect of this complication is that if changes in the retina are detected early enough (i.e. usually before the vision has been affected) treatment with lasers can prevent 70% of individuals from becoming blind (BDA, 1988).

2.5.v Nephropathy

Nephropathy is not usually detected until at least 5 years after onset of IDDM, after which the risk of developing the condition rapidly rises, peaks during 10-20 years duration and then falls. It is detected by persistent elevation of albumin in the urine (Krolewski et al. 1987; Hernandez, 1989) and it has been noted that retinopathy almost always preceeds nephropathy and that if this is not the case, an alternative diagnosis to nephropathy may be appropriate (Drury et al. 1989).

It has been found that there is a positive correlation between HbA_{1c} levels and albuminuria and that 2 years of near normoglycaemia

can arrest the progression of the disease (Hanssen et al. 1986). Continuous insulin infusion has been noted to decrease albumin excretion rates (KROC Collaborative Study Group, 1984).

Due to the different risk pattern from that of retinopathy, alternative causes and factors affecting acceleration of the disease have been suggested. These include a genetic predisposition, hypertension, a high protein diet and elevated glomerular filtration rates (Krolewski et al. 1987; Mogensen and Christensen, 1984; Hernandez, 1989).

A study by Tunbridge in 1981 discovered that 19% of deaths in 448 diabetics in 1979 were due to renal disease which was related to the duration of diabetes with evidence of microvascular disease. Once persistent proteinuria is apparent the median survival rate of patients is 10 years and most are dead by 20 years. Amongst individuals who have had IDDM for 20 years approximately 35-40% require dialysis for end-stage renal failure (Hernandez, 1989) and many diabetics are on waiting lists to receive kidney transplants.

In 1976, Mauer et al. reported a study in which 12 diabetic and 28 non-diabetic kidney transplant recipients were followed for at least 2 years. They found that within 5 years of receiving the transplant, 10 of the diabetic patients developed arteriolar hyalinosis lesions, and in 6 of these both the afferent and efferent arterioles were affected. Only 3 of the non-diabetics displayed hyaline vascular changes and these were only in occasional vessels, only involved either the afferent or efferent arterioles and developed after at least 5 years post-transplant.

In another study by Abouna et al. (1983), both kidneys from a diabetic donor with 17 years duration of diabetes, proteinuria and normal serum creatinine were transplanted into two non-diabetic recipients. Histology of the organs showed features associated with established diabetic nephropathy. After 7 months, biopsies of the kidneys showed almost complete resolution of the disease and after a further 7 months both recipients were free from proteinuria. The authors therefore suggested that if IDDM patients with nephropathy were able to receive a pancreas transplant before the onset of end-

stage renal failure, the condition of their kidneys might return to normal. This study adds weight to the argument that poor glycaemic control is a factor which increases the risk of developing nephropathy.

2.5.vi Neuropathy

Impaired nerve function may be detected in up to 60% of diabetic patients, with further problems developing as a result in one third of these. The most common problem is reduced sensation in the feet which can ultimately lead to problems with ulceration as already described. In addition, the thigh muscles may become weak, and the autonomic nervous system may be affected leading to problems with the bladder, the intestines, poor blood pressure control and impotence (BDA, 1988).

An increase in blood glucose is thought to be a factor in causing neuropathy, as with the other complications. One suggested pathway is that accumulation of sorbitol in the peripheral nerves leads to myo-inositol depletion and reduced Na/K ATPase activity, leading in turn to nerve dysfunction and damage. The use of aldose reductase inhibitors can lead to an improvement in conduction velocity suggesting that this enzyme is also involved in some capacity. Glycosylation of nerve proteins and alteration of axonal transport of proteins may also be involved (Ward, 1989).

2.6 PANCREAS TRANSPLANTATION

2.6.i Introduction

The number of IDDM patients with complications and the debilitating or even lethal outcome of these conditions clearly demonstrates that the therapy currently available for diabetics demands improvement. The BDA survey (1988) estimated that in financial terms the health care necessary to screen for retinopathy and end-stage renal failure, and to provide the number of amputations required by diabetic patients cost £30 million per year. In addition, the personal cost to the patient has to be considered, both in terms

of loss of income and the problems of coming to terms with blindness, regular dialysis treatment or loss of a limb, and all the changes in lifestyle which accompany these events.

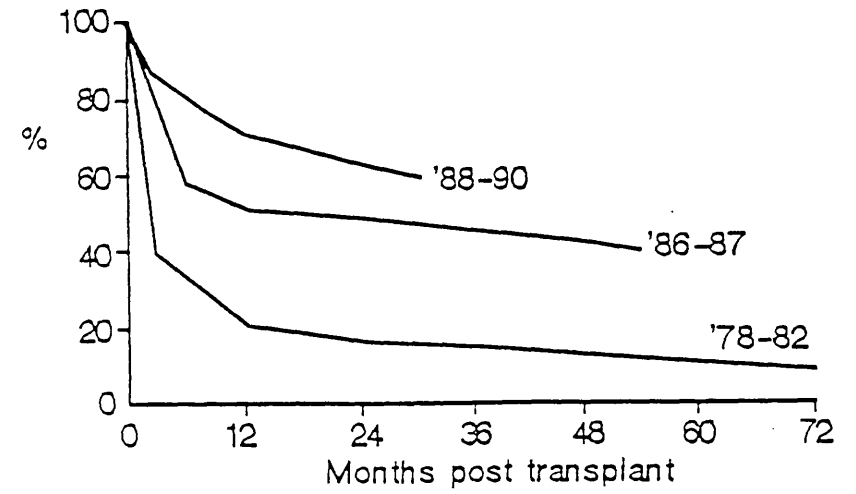
The inadequacy of the currently available treatment has led to the development of techniques to supply diabetic patients with insulin producing cells so that they will no longer need to inject insulin or monitor their blood glucose, and they will regain normal physiological control over their carbohydrate metabolism. The first approach to this problem was the transplantation of the pancreas, either the whole organ or a segment, into diabetics.

2.6.ii Graft survival

The first pancreas transplant was carried out in 1966 by Kelly and Lillehei (Brooks, 1989) and since then until the end of May 1991 a total of 3207 transplants had been reported to the Pancreas Transplant Registry. The number of transplants has gradually increased with over one third of the total number undertaken in 1989 and 1990 (International Pancreas Transplant Registry, 1991). Figures 2.2.i and 2.2.ii detail the graft and patient survival rates respectively since 1966.

Analysis of the transplant data up to 1988 demonstrated that the highest survival rates occurred when simultaneous kidney and pancreas transplants were undertaken, and the lowest rates were in non-uremic patients without a kidney transplant. Increased success was also associated with the use of cyclosporin A (CsA) and azathioprine as immunosuppressive agents, and if there were no DR mismatches between the donor and recipient (Sutherland and Moudry, 1989). The updated data continues to show an improved graft survival rate if a simultaneous kidney and pancreas transplant is carried out, although within this group the patient, kidney graft and pancreas graft survival rates at one year are 89%, 82% and 68% respectively (International Pancreas Transplant Registry, 1991).

i.



ii.

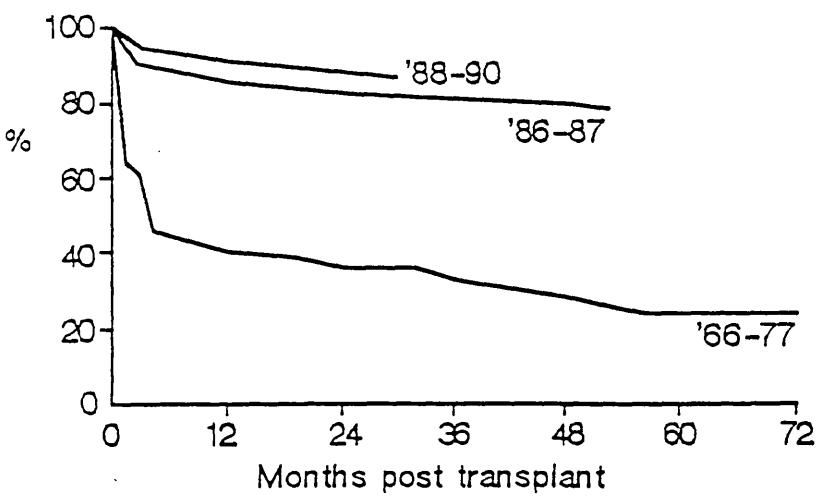


Figure 2.2
World pancreas (i) and patient (ii) survival rates by era from December 1966 to October 1990

Studies reported by Sutherland et al. (1984) and Sibley et al (1985) were designed to assess the success of transplants where the donors were living-related and donated a segment of their pancreas to prevent the need to immunosuppress the recipient. In the first study, pancreas segments from four non-diabetic donors were transplanted into their monozygotic twins. Two of these failed apparently due to autoimmune destruction of the islets, 1 partially failed at which time immunosuppression was started, and the fourth was successful due to the use of immunosuppression from the time of transplantation. In the second study which included 3 pairs of HLA-indentical twins and one HLA-identical sibling, impaired graft function was noted after 6-12 weeks with complete loss of function developing in 3 recipients and the fourth requiring low dose insulin therapy. Examination of the organs demonstrated mononuclear cell infiltration only in islets which contained beta cells.

These two reports indicated the problems of using organ segments from related donors showing that they did not avoid the use of immunosuppression of the recipient. However, data presented by Sutherland et al. (1984) demonstrated that the patient survival rate in recipients of pancreas segments from a relative was 93% compared with 78% with cadaver donors, and the graft survival rates were 43% and 20% respectively. They also noted that the success rate was enhanced if the recipient had already received a kidney from the same donor and if azathioprine was used as the immunosuppressive agent.

Several studies have reported on the effect of receiving a simultaneous kidney and pancreas transplant relative to a pancreas transplant after a kidney transplant, a pancreas transplant alone or a kidney transplant alone. Brooks (1989) noted that a simultaneous transplant could lead to more complications as a result of the operation. Both he and Groth (1989) highlighted the fact that if both organs are transplanted together, kidney function tests provide a good monitor of rejection. However, if only the pancreas is transplanted, a rise in blood glucose signalling rejection usually becomes apparent when it is too late to save the organ. Groth (1989) quotes patient survival rates of 95-100% and graft success rates of 70-80% at 1 year when both organs are transplanted together. He also noted that a successful kidney and pancreas transplant gives the

patient freedom from dietary restrictions which are still necessary if only a kidney is transplanted in cases of end-stage renal failure. Brons et al. (1989) commented that recipients of both organs were rehabilitated in a short time, but those with only a kidney found difficulties in adjusting their diabetic control and that their quality of life was only marginally improved.

A more detailed study by Sutherland et al. (1988) on the relative success of combined pancreas and kidney transplants showed that for the period from November 1984 onwards graft survival was 48% for pancreas alone, 37% for a pancreas received some time after a kidney transplant and 73% for simultaneous transplants, indicating a considerable improvement if both organs were received together. The patient survival rates were similar in all cases.

Nakache et al. (1989) undertook a study to determine in more detail the quality of life of recipients following combined pancreas and kidney transplants or kidney transplants alone. The criteria they used to assess the patients included lost workdays, days spent in hospital, payment of sickness pensions and modification of activities normally carried out. Although both groups felt that their physical activity was good, the group receiving the combined transplant had a better quality of life as judged by the above factors, and in addition they were able to eat a normal diet.

One problem with pancreas transplantation is that because the organ consists of both exocrine and endocrine tissues, and it is only the latter which are required, measures have to be taken to ensure safe removal from the body of the unwanted exocrine secretions. In 1973, Gliedman anastomosed the pancreatic duct into the ureter which appeared to overcome the problems of pancreatitis, necrosis and abscess formation observed when the exocrine drainage was into the urinary tract.

Currently, the common procedure is to anastomose a segment of donor duodenum to the bladder. Sutherland et al. (1988) determined that from 1984 to 1988 the graft success rate was 58% for drainage into the bladder from a cadaver donor, 51% for enteric drainage of a related donor organ and 29% for enteric-drained cadaver organs. A

comprehensive explanation of the technical aspects of pancreas transplantation is included in the review by Sutherland (1981).

2.6.iii The effect of pancreas transplantation on diabetic complications

Inevitably, pancreas transplant recipients have been studied to monitor the effect of the graft on their complications. Ramsay et al. (1988) in a study of 22 patients with successful pancreas transplants and 16 with failed transplants showed no difference in the progression of retinopathy, and that transplantation had failed to halt or reverse the condition. However, Sutherland et al. (1988) reported from the Registry data that retinopathy was stable in 59% of recipients 1 year after transplant.

Studies by Bohman et al. (1985) and Bilous et al. (1989) have both indicated that transplanted kidneys are more likely to show signs of diabetic nephropathy if a pancreas is not received either simultaneously with or soon after a kidney transplant.

The effect of pancreas transplantation on neuropathy is perhaps less clear as in 1987 Solders et al. reported no reversal of diabetic neuropathy after 2 years of normoglycaemia following a transplant, while van der Vliet et al. (1988) reported a slight improvement in some parameters and stabilisation in others after 1 year (34 patients) and 2 years (11 patients) post-transplant. A problem with studies of this type however is the severity of the complications prior to transplantation may be different and it is possible that in some patients the conditions have become so severe that the normoglycaemia achieved by a successful transplant occurs too late to affect the state of the disease (Groth, 1989).

In 1988, Pyke commented that before a decision could be made on whether pancreas transplantation had any affect on the development of complications a properly controlled trial needed to be undertaken. However, in 1990 he suggested that the size of the trial required would be unrealistic and that pancreas transplants should only be carried out in patients already with a kidney transplant or receiving

a kidney at the same time as the pancreas, to prevent the need for immunosuppressive therapy for the pancreas alone. He also commented that ultimately the decision should be taken by the patient following advice as there is a risk involved in the operation, and this would not allow a randomised trial to be undertaken.

2.6.iv Immunosuppression

In order to prevent rejection of a grafted organ, it is essential to give the recipient immunosuppressive treatment. The studies already described which used pancreas segments from HLA-identical donors did not remove this requirement due to autoimmune attack of the beta cells, and a report by Sutherland et al. (1989) demonstrated that immunosuppression was necessary if HLA-identical pancreas transplants were to be successful.

The most commonly used protocol is that of cyclosporin A (CsA) together with azathioprine and prednisone. Some groups also use a short course of antilymphocyte globulin (Brooks, 1989). However, serious side effects can develop from immunosuppressive treatment. When the benefit of a transplant is life-saving or has a profound effect on the quality of life of the recipient, for example with kidney, heart or liver transplants, the potential problems associated with immunosuppression are relatively unimportant. However, such risks are considered too great relative to the potential benefits of a pancreas transplant in a diabetic who ultimately may not develop any severe complications. Consequently, the operation is usually restricted to patients receiving or already with a kidney transplant who would, therefore, require immunosuppression, for patients with very severe complications, or with severe problems managing their diabetic condition (Groth, 1989).

It has been suggested that prednisone may antagonise peripheral insulin action leading to the development of insulin resistance, and chronic steroid therapy of the type used in immunosuppression is the major determinant of residual reduced insulin action (Luzi et al. 1990). Viviami et al. (1989) found that azathioprine decreased

insulin release and islet insulin content *in vitro* using concentrations normally detected in human blood.

One of the major side effects of CsA is that it is nephrotoxic (Assan et al. 1985). Groth (1989) reported data from a study in which a pancreas transplant was given to patients with diabetic nephropathy about 1-2 years before they would normally be expected to receive a kidney transplant, in order to prevent this requirement. However, it was discovered that the kidneys were unable to tolerate the CsA treatment due to their damaged state and a decline in renal function was noted. Some groups undertaking simultaneous kidney and pancreas transplants do not use CsA for immunosuppression until there are signs that the kidney is functioning (Brooks, 1989).

Another factor in the use of CsA in pancreas and islet transplantation is that many studies have been undertaken which demonstrate it has a detrimental effect on islet function. *In vitro* experiments on isolated islets have generally shown that CsA inhibits insulin secretion, even at therapeutic doses (Andersson et al. 1984; Martin and Bedoya, 1990; Nielsen et al. 1986; Robertson, 1986).

Some experiments have also been undertaken to determine the *in vivo* effects of CsA on islet function. Alejandro et al. (1989) noted a decreased response to glucose in dogs treated with CsA and that this persisted for 4 months after the treatment was stopped. Kneteman et al. (1990) found a decrease in insulin synthesis, islet insulin content, glucose-stimulated insulin release and vascularisation of the islet grafts. They suggested that the effect is dose-related and is reversible. Helmchen et al. (1984) and Bani-Sacchi et al. (1990) both noted ultrastructural changes specific to beta cells and the induction of hyperglycaemia. The latter study stated that the effect of the drug was to depress protein synthesis. It was also thought to cause a defect in granulogenesis, although no signs of beta cell degeneration or death were noted.

Gunnarsson et al. (1984) noted a deterioration in glucose metabolism in 6 consecutive patients following a change in immunosuppressive therapy from azathioprine to CsA. However, this was accompanied by an increase in C-peptide release, so the suggestion

was that the CsA was inducing insulin resistance rather than a toxic effect directly on the islets. The problem was reversed when the dose was reduced.

In recent years, considerable efforts have been made to find alternative forms of immunosuppressive therapy. One agent which has been tested for its effect on islets is FK506. This is a macrolide produced by Streptomyces tsukubaensis and was discovered in Japan in 1984. Starzl et al. (1989) showed that FK506 had salvaged 7 out of 10 livers undergoing rejection episodes and that in two patients with a kidney transplant and one with a pancreas and kidney transplant, no rejection episodes or serious side effects were noted. There was no evidence of hypertension or nephrotoxicity, and the only complaints were of transient nausea and vomitting.

Studies by Carroll et al. (1991) and Tze et al. (1990) both found a decrease in insulin release in vitro with islets exposed to FK506 but only at much higher doses than would be used clinically. Yasunami et al. (1989) reported that FK506 treatment on days 0, 1 and 2 after transplantation of islets into rats prolonged graft survival, but the drug became toxic at a high dose. This was confirmed by Ricordi et al. (1991) who also highlighted the need to determine whether chronic FK506 treatment would lead to accumulation in the body tissues and subsequent toxicity. In vitro mixed lymphocyte reaction (MLR) studies by Eiras et al. (1990) have helped to determine a theoretically suitable dose for humans. The mechanism of action of the drug is thought to be by inhibition of T cell stimulated IL-2 production and T cell proliferation (Bierer et al. 1990), important aspects in the sequence of events leading to rejection of tissue (Krensky et al. 1990).

Other forms of immunosuppression currently being studied include the perfusion of the organ to be transplanted with antibody to leukocyte common antigen (Brewar et al. 1989). This has been associated with decreased rejection and increased allograft function in kidney grafts. Cosimi et al. (1990) found that a monoclonal antibody to ICAM-1, a molecule thought to be important in the pathogenesis of allograft rejection, reversed some rejection episodes in kidney allografts in cynomolgus monkeys.

Very encouraging results have been described by Goldman et al. (1991) with the use of OKT3 in cadaver kidney transplants. They compared graft success when azathioprine and prednisone were used in conjunction with CsA or with OKT3. When OKT3 was used the number of patients without rejection episodes was 41% relative to 23% with CsA, the total number of rejection episodes was lower in the group taking OKT3, but this was most marked in the first month post-transplant with rates of 21 out of 55 compared with 41 out of 52 with CsA. The cumulative effect was a graft survival at 18 months of 92% with OKT3 and 79% with CsA. Both groups had patient survival rates of 94%. The only problem noted with the use of OKT3 was an increase in the number of infectious episodes.

It will be some time before new forms of immunosuppression become widely available as more clinical studies will be required to confirm their action and safety. Some of the results quoted above are encouraging, however, both due to the effect on graft survival and the apparent reduction in serious side effects.

2.7 ISLET TRANSPLANTATION

2.7.i Introduction

Although the success of pancreas transplantation is gradually increasing, there are still problems with this technique, including the fact that it requires a major operation, immunosuppression is necessary thereby reducing the number of patients considered to be suitable candidates for the operation, and the whole organ is transplanted when the exocrine tissue which forms the largest part of the organ is not needed and in fact causes problems due to the need to drain the exocrine secretions.

These factors have led to the development of methods to try to isolate the islets of Langerhans from the pancreas so that only the required tissue has to be transplanted, allowing a much more simple, less invasive technique for transplantation. It is also hoped that using isolated islets will allow attempts at immunomodulation, thus preventing the need for immunosuppression of the recipient, and making the technique available to diabetic patients before the

development of complications, as the dangers of long-term immunosuppression would not apply.

2.7.ii Animal studies

A lot of developmental work into the isolation, purification and transplantation of islets of Langerhans has been undertaken using a variety of animal models. Some of this work will now be reviewed.

a. Animal models of diabetes

Rodents have been commonly used for islet studies and both chemically-induced and spontaneously-occurring models of diabetes are available.

The chemical streptozotocin (STZ) has been one of the most commonly used chemicals to induce diabetes in rodents. It is an N-nitrosomethylamide isolated from Streptomyces achromogenes and has a direct toxic action on beta cells (Horton et al. 1977). Junod et al. (1967) described the sequence of events following injection of STZ. After 7 hours, massive beta cell necrosis and cellular disintegration was described, with other cells in the islets and the exocrine tissue remaining intact. Macrophages were also noted to be actively phagocytosing the beta cells. One major problem with STZ, however, is that beta cells can regenerate after treatment, so it is essential in islet transplantation experiments that the pancreas is assessed for signs of beta cell regeneration. Alternatively, the graft can be removed to determine whether the blood glucose of the recipient remains low indicating beta cell regeneration (Lake et al. 1989), otherwise false positive results could be generated. Recovery of beta cells has been demonstrated in studies by Brekke et al. (1983), Chicheportiche et al. (1990a), Kruszynska et al. (1986) and Richardt et al. (1984). Other methods for the induction of diabetes include the injection of alloxan or certain viruses, and these have been reviewed by Bailey and Flatt (1986) and Gottlieb et al. (1988).

A more recent development in the use of animal models in diabetes research is the discovery of spontaneous mutations causing the development of diabetes in rats and mice. Although the list of diabetic animals demonstrating beta cell degeneration with possible ketosis and beta cell hyperplasia, with obesity and without ketosis is quite extensive (Bailey and Flatt, 1986) it is the BioBreeding (BB) rat and non-obese diabetic (NOD) mouse which are the most commonly used models of diabetes as these both show beta cell degeneration with ketosis, and are thought to provide the closest models to the human condition.

The BB rat was first discovered at the BioBreeding Laboratories, Ottawa, Canada following an increase in deaths amongst a colony of Wistar rats. Once diabetes mellitus was discovered to be the cause of the deaths, selective breeding was established to provide a line of diabetic animals which could be maintained by daily insulin injections (Parfrey et al. 1989). Since that time, many studies have been undertaken to determine the nature of the condition in these animals. Diabetes onset usually occurs between 50 and 120 days (mean approximately 70 days) and has roughly equal incidence between the two sexes (Nakhooda et al. 1976). The time of onset can vary between colonies and between different lines in the same colony (Parfrey et al. 1989).

Studies by Seemayer et al. (1982) and Nakhooda et al. (1976) helped to determine the events occurring in the islets resulting in the metabolic effects associated with diabetes onset. Infiltration of the beta cells occurs by activated lymphocytes and macrophages (Lee et al. 1988a; Nagy et al. 1989; Appels et al. 1989; Hahn et al. 1988; Hananberg et al. 1989; Hardy et al. 1986; Kiesel et al. 1986; Kolb et al. 1988), and the beta cells display marked degranulation and necrosis. The alpha and delta cells are not normally affected. In many respects, the BB rat and the human insulin-dependent diabetic are very similar. For example, the condition is not associated with obesity, autoantibodies are present (Brogren et al. 1986; Pipeleers et al. 1987; Tanguay et al. 1988), it is associated with MHC genes (Colle et al. 1981; Colle et al. 1986; Gunther et al. 1989), immunosuppressive agents can prevent onset of the disease (Baquerizo et al. 1989; Brayman et al. 1986; Laupacis et al. 1983; Mahon et al.

1988; Oschilewski et al. 1985; Satoh et al. 1988), it is associated with other autoimmune diseases (Lee et al. 1988b), and class II expression on the endocrine cells of the BB rat may occur (Dean et al. 1985), although this is in dispute (Timsit et al. 1989).

One major difference between the conditions in the rat and humans is that the diabetic BB rat is lymphopaenic (Elder et al. 1983; Greiner et al. 1987; Guttmann et al. 1983; Poussier et al. 1982), although a study by Like et al. (1986) has suggested that this does not occur in all breeding lines.

In addition to the diabetic BB rat, a line of non-diabetes prone BB rats has been developed in which the incidence of diabetes is less than 1% (Gottlieb et al. 1988), and which are not lymphopaenic. The availability of these animals means that the same strain of rat can be used to conduct control experiments, rather than having to use ordinary Wistar rats as controls.

The NOD mouse was derived between 1974 and 1980 from a line of JcI-ICR mice which developed cataracts. Diabetes develops from the 12th to the 30th week and similarly to the BB rat and human, diabetes onset is associated with polydipsia, glycosuria, weight loss, hyperglycaemia and ketoacidosis. Also the NOD mouse displays insulitis and autoantibodies to islet cells and insulin (Lampeter et al. 1989). As with the BB rat, a non-diabetes prone line has been developed (Gottlieb et al. 1988). NOD mice are more similar to the human diabetic than the BB rat as they are not lymphopaenic. However, a major difference not seen with the rat model is that female NOD mice are more prone to developing diabetes, with approximately 80% of females and only 10-20% of males becoming diabetic by 30 weeks of age (Gottlieb et al. 1988). Insulitis has been demonstrated in many males, although they still do not go on to develop diabetes (Lampeter et al. 1989). In addition, a study by McInemey et al. (1991) has shown that class II antigen expression in islets of NOD mice was only detected on lymphoid cells infiltrating the islets and not on the endocrine cells.

Although neither the BB rat nor the NOD mouse is identical to the condition seen in the human disease, the similarities are

considerable and this means that both models are considered to give a closer indication of human IDDM than the chemically-induced models of diabetes.

b. Isolation and purification of islets

The islets of Langerhans are scattered throughout the exocrine tissue and have been estimated to comprise approximately 1.5% of the total mass of the organ (Gepts and I'nt Veld, 1987). Consequently, techniques have been sought to maximise both the number of islets which can be isolated from the pancreas and the purity of the islets following isolation.

Early techniques involved the dissection of islets from the exocrine tissue (Hellerstrom, 1964) and ligation of the pancreas causing atrophy of the exocrine tissue allowing the islets to be dissected out (Keen et al. 1965). Also in 1965, Moskalewski described the digestion of the guinea pig pancreas with collagenase such that intact islets could be retrieved from the digested exocrine tissue. This method was adapted for use in the rat by Lacy and Kostianovsky (1967). Their method involved the distension of the pancreas in situ with Hanks solution, followed by resection and mincing when placed in a solution of collagenase. The minced pancreas was left for 20 minutes at 37^{o}C then washed with Hanks solution. The islets could then be retrieved from the digest either by hand-picking under a microscope using a glass loop giving a yield of 75-100 islets in 20-25 minutes, or by centrifugation on a discontinuous sucrose gradient giving 200-300 islets per pancreas. Studies on islets prepared by the sucrose gradient method gave variable results, however, and it was suggested that hand-picking was preferable.

Many changes to this method have been made, but perhaps the two most notable ones are the distension of the pancreas with collagenase solution (Sutton et al. 1986) rather than placing the minced organ into collagenase, and the development of density gradients for the purification of the islets from the digest, with both Ficoll (Ballinger and Lacy, 1972; Gray and Watkins, 1976; Nash et al. 1976;

Kemp et al. 1973) and bovine serum albumin (Lake et al. 1986; Lake et al. 1987; Zucker et al. 1989) used for this process.

A review by Gray (1989) highlights the importance of maximising the purity of islets as exocrine tissue is thought to be thrombogenic, and it is suggested that apparent necrosis of the exocrine tissue may lead to the release of enzymes which could damage the transplanted islets. In addition, exocrine tissue expresses class II molecules and this might be expected to initiate an immune reponse against both the exocrine and islet tissue. Studies by Gotoh et al. (1986) demonstrated that some hand-picked pure islet transplants lasted for over 100 days, whilst those deliberately contaminated with lymph nodes and ductal tissue were rejected soon after transplant indicating that purity of the islets may affect the success of the transplants.

c. Transplantation studies

Many islet transplantation experiments have been undertaken using various animal models, and these have included studies to determine the optimum site for transplantation, the number of islets required to reverse diabetes, and the effect of pre-treating the islets to reduce their immunogenicity in order to prolong allograft survival.

The earliest report of islet transplantation in rats was by Ballinger and Lacy (1972). They used both the intraperitoneal and intramuscular sites to implant the islets and were able to demonstrate vascularisation and function of the islets in the latter. Other studies using the peritoneal cavity as the implantation site have been undertaken by Kemp et al. (1973), Reckard et al. (1973), and Gray and Watkins (1976). The results from these studies were mixed with most showing either poor or no function of the islets.

Other sites used for implantation of islets have included subcutaneously (Kemp et al. 1973; Gray and Watkins, 1976), the spleen (Feldman et al. 1977; Finch et al. 1977; Franklin et al. 1979; Hayek et al. 1988; Hayek et al. 1990), the liver via the portal vein (Kemp

et al. 1973; Pipeleers et al. 1978; Gray and Watkins, 1976; Feldman et al. 1977; Franklin et al. 1979; Haug et al. 1988; Hiller and Klempnauer, 1989; Kamei and Yasunami, 1989) and under the kidney capsule (Haug et al. 1988; Hiller and Klempnauer, 1989; Ar'Rajab et al. 1989; Gray et al. 1986; Reece-Smith et al. 1981; Gray et al. 1989; Hayek et al 1988; Kamei and Yasunami, 1989; Lake et al. 1989; Loftus et al. 1991).

It is very difficult to assess the relative success of these studies as different strains of rat and mice have been used, and the weights of the recipients and the number of islets transplanted are often different or not quoted. In general, however, the renal subcapsular space and the intraportal route are the preferred sites for islet transplantation.

Several studies have been undertaken to determine the fate of islets transplanted into the BB rat. The results from these studies have been mixed. Chabot et al. (1987) reported good long-term graft survival following UVB irradiation of the islets and treatment of the recipient with CsA provided that the donor islets were MHC-mismatched, and that the recipients were long-term and not recent onset diabetics. Hegre et al. (1989) and Woehrle et al. (1986) also found that MHC-mismatched grafts were successful but that MHC-matched islets were destroyed by autoimune disease recurrence. However, studies by Prowse et al. (1986) and Weringer and Like (1985) both reported destruction of the transplanted islets regardless of their MHC status.

Pace et al. (1989) were able to demonstrate long-term morphological and functional integrity of allografts and Selawry et al. (1987 and 1988) reported experiments in which MHC-compatible islets functioned for over 80 days. Markmann et al. (1989) demonstrated that the use of monoclonal antibodies specific for T cytotoxic lymphocytes protected transplanted islets from autoimmune destruction, and Naji et al. (1979) also acheived extended graft survival by using antilymphocyte serum. Implantation site has also been demonstrated to be important in determining graft survival, with islets transplanted into the renal subcapsular space functioning for

over 100 days, but islets injected intraportally failing between 4 and 45 days post-transplant (Woehrle et al. 1987).

These studies demonstrate that although the BB rat provides a good model for the study of human diabetes, there are still conflicting results following islet transplantation which need to be clarified if the results are to be of use in human islet transplantation. One factor which may affect the outcome of experiments on different BB rat colonies is the genetic differences which have arisen as animals have been sent to different centres to start new colonies (Prins et al. 1990).

Larger animals have also been used to study islet transplantation, most notably the dog. Alejandro et al. (1986) followed-up 15 dogs for 24 months post-transplant, and 3 of these had normal blood glucose readings for over 15 months. Failure of the grafts was thought to be a result of transplantation of insufficient islet mass. This was confirmed by Kaufman et al. (1990) who found that both islet mass and transplantation site were important with fewer islets being required if transplanted into the liver or spleen rather than the renal subcapsular space. In contrast, Toledo-Pereya et al. (1985 and 1987) found that the renal subcapsular site was good for islet transplantation in the dog. Warnock and Rajotte (1988) and Warnock et al. (1989) used both the liver and spleen for islet implantation in dogs. They found that they could get enough islets from one donor to reverse diabetes, although with long-term follow-up delayed graft failure became apparent, especially in the hepatic site. This did not appear to be related to the original number of islets transplanted.

The cynomolgus monkey has also been used by Professor Morris's group in Oxford as a close model of the human to determine the fate of islet transplants. They have found some success with intraportal transplants with two grafts functioning for up to 9 months, although in three animals graft deterioration monitored by fasting blood glucose levels was apparent 3-4 months post-transplant (Gray et al. 1987). In addition, renal subcapsular transplants only appeared to be successful if the islets were handpicked to ensure purity, but success was more probable if the spleen was used as the implantation

site when the islet preparation was not very pure, or if the number of islets transplanted was marginal. Good graft function was achieved when the intraportal route was used, and it was noted that early graft function tended to indicate the likely success of the transplant (Gray, 1990).

There has been vast numbers of studies reporting on experimental islet transplantation in various animal models. The above information is only intended to give a general overview of some of this work and is not a comprehensive review of all the work involved. More detailed information can be obtained in the reviews by Sutherland (1981), Gray and Morris (1987a), Yderstroede (1987) and Jung and Merrell (1990).

2.7.iii Human islet transplantation

Experimental islet transplantation has had some success, especially in smaller animals, however the same degree of success has proven to be difficult to repeat in the human situation.

The review of clinical islet transplantation by Hering et al. (1988) includes information on all the reported human islet transplants. Out of a total of 473 transplants only 75 involved the transplantation of adult allogeneic tissue, with the remainder using fetal or xenogeneic tissue. Complete information was not available for all of the cases included, but it is clear from the report that success has been very limited. Some of the recipients were able to stop exogenous insulin therapy for a while, and some have been able to reduce the amount of insulin they need to inject, but no cases reported were of patients who had become long-term insulin independent.

Since this review was written there have been several reports of series of human islet transplants. In 1991, Scharp et al. reported details of their first 9 intraportal islet allografts. Three of these did not have renal failure and all rejected the islets within two weeks of transplantation, even with the use of prednisone, azathioprine and CsA for immunosuppression. The next 3 had established kidney grafts so were already receiving immunosuppressive

drugs and this was supplemented with antilymphocyte globulin for 7 days. One of these patients rejected at 2 weeks and the other two, although not becoming insulin-independent did show signs of islet function for up to 10 months. A delayed C-peptide response in these patients implied that an insufficient islet mass had been transplanted. The remaining three patients were also kidney recipients, one of which rejected the islets at 25 days. The other two received a second transplant of islets, one with seven day cultured islets and the other with cultured and cryopreserved islets. The first of these was functioning at 184 days but had not become independent of insulin therapy, but the second had stopped insulin injections and had a good C-peptide response at 154 days.

Tzakis et al. (1990) reported a series of islet transplants in 9 patients who had also received a liver following the removal of their liver, pancreas, spleen, stomach, duodenum, proximal jejunum and terminal ileum. FK506 was used to immunosuppress the patients and intravenous insulin infusion was also administered. Six of the patients survived with normal liver function, one of these was insulin independent and four required occassional insulin at night. C-peptide was detectable in all patients although none demonstrated normal insulin secretory kinetics indicating transplantation of a suboptimal islet mass. The nature of the condition in these patients, however, could not be directly related to the situation encountered in IDDM patients receiving an islet transplant.

Warnock et al. (1989) reported on two patients receiving islets and a kidney simultaneously. C-peptide was detected although they still required insulin. The onset of CMV infection led to the immunosuppressive dose having to be decreased and this caused a decrease in C-peptide. The same group (Warnock et al. 1991) reported the case of a patient receiving a simultaneous kidney and islet transplant with fresh islets from the kidney donor and cryopreserved islets from four other donors being used. Anitlymphocyte globulin, prednisone, CsA and azathioprine were used to immunosuppress the patient. Insulin injections were gradually tapered off to nothing and at 2 years post-transplant the graft was still functioning with good C-peptide levels. Figure 2.3 shows the number of islet grafts reported to the Transplant Registry.

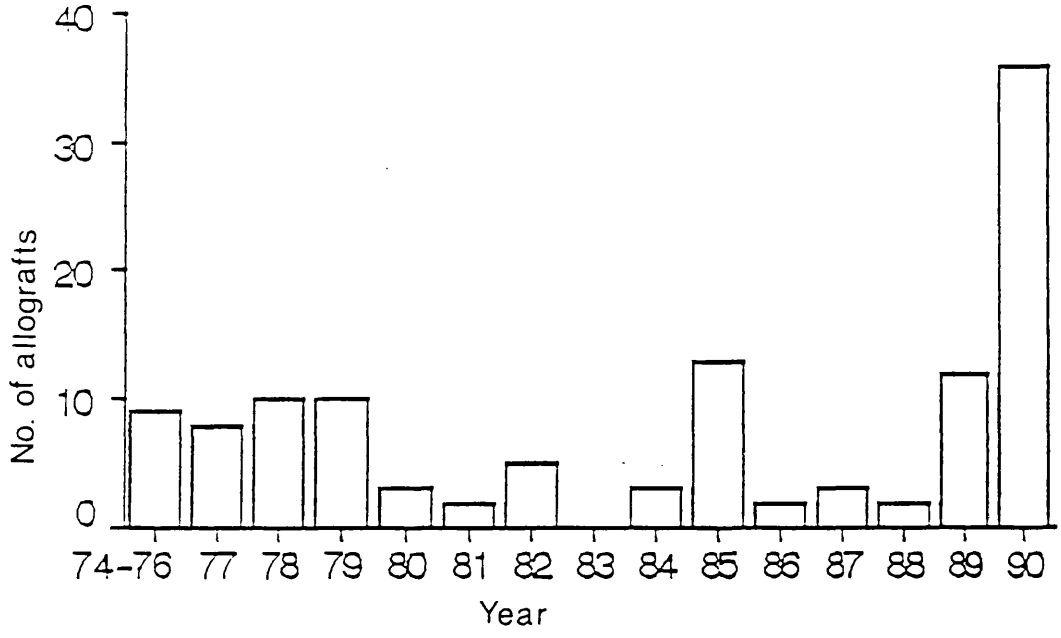


Figure 2.3
Number of adult islet allografts by year from 1974 to 1990

The results from the human islet transplants are generally disappointing and several reasons have been put forward to explain the poor success rate of the transplants. Cuthbertson and Mandel (1990) have suggested that poor vascularisation due to chronic diabetes could be the reason for graft failure. Most transplants in animals are performed after a short period of diabetes, but those transplants undertaken after the animal has been diabetic for some time tend not to function as well, although this can be improved by peritransplant insulin infusion. The authors suggest that failure could be avoided by performing transplants earlier in the course of the disease and in conjunction with a period of accurate diabetic control. It has also been noted that more islets may be required per kg of body mass when immunosuppression is used to ensure a greater chance of success (Scharp et al. 1991).

The problem of comparing results from different trials has also been noted by Scharp et al. (1986 and 1989). They concluded that a concensus is needed on islet transplantation as there are so many variables involved in the purity of the islets, the state of the patient, the methods of immunosuppression used, and the criteria used to determine function of the islets.

Although improvements in the function of human islet grafts have been made with some patients being able to reduce their insulin requirements or becoming insulin independent for a period of time, it is clear however that further improvements are required before human islet transplantation could become a routine procedure.

2.7.iv Foetal islet transplantation

The majority of the islet transplants reported by Hering et al. (1988) used foetal islets either of human or animal origin. Data from patients receiving foetal islets included in this report generally appear to have a greater success rate than those who have received adult human islets. The exact reasons for this are difficult to determine due to the differences in patient management. For example, 3 patients in a series of foetal islet transplants in Shanghai have become insulin independent, but as part of the therapy included the

administration of traditional Chinese medicine, it is impossible to assess the effect of this on the islet graft.

One reason for using foetal tissue is that as a proportion of the total pancreas, the islets occupy a greater volume relative to the adult organ, the tissue has a greater capacity for proliferation which is lost in the adult, and the exocrine tissue is less mature, hopefully reducing the problems of contaminating exocrine tissue already discussed (Voss et al. 1989).

Studies by Garvey et al. (1979) however demonstrated that although the foetal tissue is immature, it is still equally able to provoke a rejection response, with foetal and adult tissue being rejected equally rapidly, and a mononuclear cell infiltrate apparent at 48-72 hours. Danilovs et al. (1982) were able to demonstrate dendritic-type DR^+ cells within and in close association with insulin-producing islets, indicating foetal islet tissue is not less immunogenic than adult tissue.

A series of transplants by Farkas et al. (1990) in which patients have been followed-up for up to 7.5 years showed that the mean HbA_{1c} was 6% (within normal range), all patients had detectable C-peptide and the insulin requirement had decreased to approximately 40% of the original dose. Half of these patients with nephropathy had improvement in measurable parameters, although the condition had progressed in the remainder. Also an improvement or stabilisation of retinopathy was noted in most of the eyes with signs of damage.

Foetal islet transplantation in 369 patients in China (Hu et al. 1989) resulted in at least a 25% reduction in insulin requirement by the majority of patients together with enhanced C-peptide levels. No adverse reactions or complications were noted with the exception of wound infections developing in 4 patients.

Lafferty et al. (1989) acheived a 65% reduction in insulin requirement in one patient. They also reported that an improvement is usually seen 3-6 months post-transplant as it takes some time for the foetal tissue to develop. In relation to the development and function of foetal islet tissue, Cuthbertson et al. (1988) and Mullen et al.

(1977) both found that chronic diabetes had a detrimental effect on the graft and that this could be improved either by pretransplant insulin therapy or transplantation of foetal pancreatic tissue 3 weeks before a combined kidney and islet graft in order to reduce the effects of hyperglycaemia on the graft.

Although there appears to have been some success with foetal islet transplantation and suggestions to improve the success rate, there remains one major obstacle to this procedure. This is the ethical dilemma of using foetal tissue for research or clinical purposes. The arguments relating to this problem have been reviewed by Annas and Elias (1989) and Dickson (1989).

2.7.v Xenotransplantation

Another controversial matter in islet transplantation is the use of xenogeneic tissue to transplant into diabetic patients. Arguments for and against the use of xenografts have been put forward by Reemtsma (1990) and Francione (1990) respectively.

Work to develop techniques for treating xenogeneic islets prior to transplantation has been undertaken. Bellgrave and Selawry (1990) found that rats transplanted with hamster islets and given CsA for 30 days then once a week up to 100 days post-transplant tolerated the graft but only if it was placed in the testicular site. Grafts in the renal subcapsular site or injected into the portal vein were rejected.

Lacy et al. (1990) found that 50 rat islets transplanted into mice gave better survival than if 100 islets were used. They suggested that this could have been due to the increased number of passenger leukocytes transplanted due to the increase in the number of islets. They also found that culturing the islets at 24^{o}C or 37^{o}C for 7 days produced better results than if they were only cultured overnight, and also if they were implanted in the renal subcapsular space rather than intraportally. Further reports from the same group (Falqui et al. 1991; Marchetti et al. 1991) have also used 7 day

culture at 24°C to prolong graft survival in conjunction with immunosuppression of the recipient.

Tze et al. (1990) were able to prolong the mean survival of human islets transplanted into rats from 3.7 days to 18.2 days by administration of 5 doses of antithymocyte serum (ATS). Islets in untreated rats became fibrosed and little endocrine tissue was detectable but with the ATS treatment the islet tissue appeared to be well preserved and some human C-peptide could be detected. No human class II molecules could be detected on the cells undergoing rejection.

There is still a lot of work to be undertaken before xenogeneic islet transplants will become an accepted form of therapy for diabetics, but if islet transplantation is to become a regularly used treatment for IDDM patients alternative sources of islets will have to be investigated if the number of transplants required are to be carried out.

2.7.vi Immunomodulation of islets

One of the advantages of working on isolated islets rather than the whole pancreas is that there is more scope for manipulation of the tissue to modify its immunogenicity.

Culturing islets for a period before transplantation has been suggested as a way to reduce the antigenicity of islets. Bowen et al. (1980) and Lacy et al. (1982) both found an increase in graft survival if islets were first allowed to aggregate and then cultured in 95% oxygen for up to 7 days.

Culture temperature has also been suggested to be important. Lacy et al. (1979 and 1980) reported that islets cultured at 24°C for 7 days gave long-term graft survival in recipients treated with antilymphocyte serum (ALS). The grafts survived for longer than if ALS therapy was administered with the implantation of fresh islets. Kneteman et al. (1991) reported the effect of culturing islets at 37°C for 7 days or 7°C for a brief period on the expression of class

I and class II molecules on the islets and the resulting effect on graft survival. The conclusion was that increased class I expression (this was achieved by pretreating the donors with lipopolysaccharide [LPS]) did not affect graft survival but that a decrease in class II expression following a period of culture in either of the conditions described led to delayed rejection of the graft. This data suggests that it is class II expression which is important in affecting graft survival.

Gamma irradiation of islets has been demonstrated to prolong graft survival in conjunction with immunosuppression. James et al. (1989) found that 2.5 Grays of gamma irradiation together with 3 days CsA treatment gave indefinite graft survival of rat islet allografts compared with survival up to 37 days with CsA alone or 6 days with irradiated islets alone. Kenyon et al. (1990) found that UV irradiation of canine islets reduced their immunogenicity and together with a subtherapeutic dose of CsA, allograft survival was prolonged. Lau et al. (1984a and 1984b) also reported extended graft survival of rat allografts following UV irradiation of the islets and CsA treatment on days 0, 1 and 2 post-transplant.

Faustman et al. (1984) used the technique of treating islets with monoclonal antibody against dendritic cells together with complement to achieve prolonged graft survival. It is thought that the presence of antigen presenting cells such as dendritic cells within the graft may be responsible for provoking a rejection response in the recipient (Lafferty et al. 1983; Naji et al. 1987).

An alternative to immunomodulation of islets is immunoisolation. The islets are placed inside a device which allows the passage of insulin, glucose and other essential nutrients but not immunoglobulins, thus shielding the islet from the host's immune system. This aspect of islet transplantation forms the the subject of this thesis and the development of immunoprotective devices will be discussed in detail in the following chapter.

CHAPTER 3

ENCAPSULATION - A REVIEW

3.1 INTRODUCTION

The concept behind the immunoisolation of islets of Langerhans is to provide a transplant system which would remove the need for immunosuppression of the recipient. This is considered important, as immunosuppressive therapy can lead to many problems relating to the toxicity of the drugs available, already discussed in Chapter 2. As the ultimate aim is to transplant newly diagnosed, and therefore quite young diabetic patients, the potential dangers of long-term immunosuppression would be too great, even considering the benefits an islet transplant might provide.

Two approaches to the protection of islets of Langerhans with a physical barrier which would allow the passage of glucose and insulin but prevent immunoglobulins and cells from coming into contact with the islets have been proposed. These are the hybrid artificial pancreas and microencapsulation.

3.2 IMMUNOISOLATION

3.2.i Hybrid artificial pancreas

The hybrid artificial pancreas is defined as "a bionic device constructed of artificial materials that sustain living insulin-secreting cells of pancreatic origin" (Freidman, 1989). This system works on the principle of a vascular shunt. Host blood circuates through the device in contact with a membrane, on the other side of which is an enclosed compartment containing isolated islets. Buchwald et al. (1987), Goosen (1987), and Reach (1989) have all reviewed this subject in some detail. In brief, hollow fibres with islets seeded onto the outside were enclosed in a rigid chamber, and connected to the recipient as a vascular shunt (Tze at al. 1976; Sun et al. 1977). In conjunction with heparin infusion, this device was shown to reverse diabetes in experimental animals for up to six days, with failure occurring due to thrombus formation between the blood vessel and device.

An improvement was made by the use of Amicon fibres seeded with insulinoma cells and implanted into the peritoneal cavity, which

successfully reversed experimental diabetes for over one year (Altman et al. 1986). However, several problems remain with this system, most notably the fragility of this type of fibre, the relatively limited surface area across which diffusion can occur, and the destruction of cells within the fibres after a long period of implantation. The second of these was alleviated by the development of a U-shaped device (Reach et al. 1984). Although the problem of thrombosis remains with devices which are connected to the vascular system, there has been some success. For example, Segawa et al. (1987) found that hollow fibres containing 10,000 islets connected into the systemic circulation, or 14,000 islets connected into the portal circulation were sufficient to maintain normoglycaemia in diabetic dogs, giving a good, although not quite normal, intravenous glucose tolerance test response.

Also, Uhbi et al. (1990) found a decrease in insulin requirement in four out of six dogs following transplantation of hybrid artificial pancreas together with aspirin treatment to prevent clotting. However, the experience of Zekorn et al. (1990) indicates some of the remaining problems: with the development of a maximum of 5um thickness of fibrous tissue around the device, rat islets functioned in streptozotocin-diabetic mice for only 3-8 days, compared with good in vitro function at four weeks. They suggested that islet failure could have been due to several factors, for example, insufficient islet mass, low pO_2 at the implantation site, or a shift in pH due to the accumulation of toxic metabolic products.

The same group (Siebers et al. 1990) reported a difference in the severity of reaction in mice and rats with the latter showing a more marked reaction. However, the reaction remained stable between three weeks and three months. It was also noted that the ultrastructure of the membrane surface affected the severity of reaction to the implant. In contrast with this work, Penfornis et al. (1990) have reported successful transplants in pigs, using an acrylic copolymer membrane, even when the device has become badly fibrosed, and unsuccessful transplants when the device is free of any cellular reaction. They suggested that it is the quality of the grafted material which is of greater importance to the success of the

transplant. In addition to this, less severe fibrosis was noted in the peritoneal cavity than when the implant was placed in the abdominal muscles, and also when implantation sites with portal drainage were used.

3.2.ii Microencapsulation

The second approach to immunoprotection of islets is that of microencapsulation. Rather than placing all the islets inside a membrane, each individual islet is surrounded by a biocompatible substance and a semipermeable membrane, thus allowing transplantation of the islets in a similar manner to free islets, and theoretically solving the problems associated with the hybrid artificial pancreas already discussed (eg. thrombus formation).

The process of encapsulating small volumes of material has been used for many years with many diverse applications having been devised. One of the earliest industrial uses was in the 1930's when the National Cash Register Company developed a process of copying using capsules containing dye which were broken when hit by the typewriter key. This allowed a second copy to be made therefore removing the need for carbon paper (reviewed by Norton et al. 1988). Since that time, the technique of microencapsulation has been applied to the pharmaceutical industry to prolong the release of active drugs, in the photographic industry, in cosmetics and in agriculture. Culture techniques have also benefitted from the process, with encapsulated hybridoma cells producing monoclonal antibodies with a purity, activity and concentration far exceeding that of conventional methods (Lim, 1984; Duff, 1985). A review of the patent briefing and literature search sections published in the Journal of Microencapsulation demonstrates the wide diversity of applications to which the methodology of microencapsulation has been applied.

The first suggestion that microencapsulation could be adapted to replace cells or cell products in vivo was made by Chang (1964), who encapsulated enzymes, suggesting that these capsules could be used to replace enzymes lost due to genetic defects. He based his idea on the naturally-occurring subcellular organelles which contain enzymes

and to the products of the enzymatic reaction. Since this original work by Chang, several applications for microencapsulation technology to provide a substitute for biological defects have been made.

Chang initially worked on producing blood substitutes by developing an artificial red blood cell which was achieved by encapsulating haemoglobin. Subsequent developments have lead to the use of pyridoxylated polyhaemoglobin as a possible blood substitute (see Chang 1988 for a review).

The review by Chang (1988) goes on to discuss further advances in artificial cell development such as: 1) the encapsulation of enzymes, or multienzyme systems, which were devised to act as replacement therapy in individuals lacking the enzyme due to a genetic defect; 2) artificial cells containing adsorbents to remove toxins from the blood stream in cases of kidney failure, poisoning, aluminium or iron overload; 3) removal of toxins in liver failure; 4) removal of antibodies without affecting platelets.

The aspect of entrapping chemicals has also been applied to the potential treatment of diabetes, by incorporating insulin into beads prepared from biodegradable polymers. This has been thoroughly reviewed by Goosen (1987) who concludes that much work needs to be covered to determine the safety of some of the chemicals used, and also points out that there is no feedback to control insulin output from the capsules, thus making them an unsuitable form of therapy in their own right. They may, however, be able to provide a low-level insulin release to help blood glucose control in adult-onset patients.

The other major area of biological application for encapsulation is that of encapsulating cells, or clusters of cells, and it is this aspect, in relation to islets of Langerhans, which forms the text of this thesis.

3.3 MICROENCAPSULATION OF ISLETS OF LANGERHANS

3.3.i History

It was in 1980 that Lim and Sun first reported the method of encapsulating islets of Langerhans as a means of protecting transplanted islets from rejection. The technique involved the suspension of isolated islets in a solution of sodium alginate which was then formed into droplets by spraying it into a dish containing calcium chloride. The droplets were then coated with polylysine and polyethyleneimine to form the semipermeable membrane around the droplets. Subsequent treatment of the droplets with sodium citrate reliquified the inner core of calcium alginate back to sodium alginate.

This initial report included results of perifusion experiments which indicated bi-phasic although slightly delayed insulin release from the islets, and of allograft transplantation experiments which demonstrated an increased period of normoglycaemia in recipient diabetic animals from approximately 4-6 days with free islets to about 20 days with encapsulated islets.

3.3.ii Alginates

Sodium alginate was chosen as the material for encapsulating islets for two major reasons: firstly, it forms a hydrogel which has been calculated to contain 93% water and should, therefore, be highly biocompatible (Goosen et al. 1985), and minimise frictional irritation following implantation due to their high pliability (Goosen, 1987); secondly, the soluble sodium salt solidifies in the presence of most divalent ions, thus allowing the formation of rigid droplets which can subsequently be coated with various layers. Once the appropriate coatings have been deposited, the inner core can be reliquified because the reaction with the divalent ions is reversible (McDowell, 1986). In addition to these aspects, toxicity tests have shown alginates to be very safe when implanted in vivo (Epstein et al. 1970).

Alginate is obtained from brown seaweed (Phaeophyceae) with species of Laminaria, Macrocystis, and Ascophyllum being the most commonly harvested for industrial production (McDowell, 1986). It is usually present as a mixed salt of sodium, potassium, calcium and magnesium, and preparation of the purified sodium salt from freshly harvested seaweed requires a process of washing and milling, precipitation with calcium ions to form the insoluble calcium salt, and treatment with acid followed by addition of sodium carbonate to form the soluble sodium salt.

Alginic acid consists of two components: D-mannuronic acid connected by beta 1-4 linkages and L-guluronic acid connected by alpha 1-4 linkages. Polymannuronic acid has a flat ribbon-like structure (Figure 3.1.i) made stable by hydrogen bonds, and these chains form sheets, again stabilised by hydrogen bonds. Polyguluronic acid is also ribbon-like but is considerably more buckled (Figure 3.1.ii), and this chain is stabilised with hydrogen bonds. The bonding between chains is more complex than for polymannuronic acid, and involves water molecules which in turn form hydrogen bonds.

In a preparation of sodium alginate, the proportion of these two components will vary depending on the species of seaweed used and the part of the plant harvested (e.g. fronds or stipes). The relative proportions present will confer different properties on the alginate. It is the guluronic acid component which binds calcium ions, thus forming a gel, so a preparation of alginate with a high proportion of L-guluronic acid will tend to form a rigid, brittle gel, while a high D-mannuronic acid content will give a more elastic gel.

3.3.iii Method of islet encapsulation

Islets are encapsulated by suspension in a solution of sodium alginate, usually made to a concentration of approximately 1.5% (w/v) in saline. This is then extruded from a syringe by a constant-rate pump through a fine tube which has an outer sheath with oxygen flowing through it. As the alginate is forced through the tubing, the oxygen flow causes it to spray out into droplets which then fall into

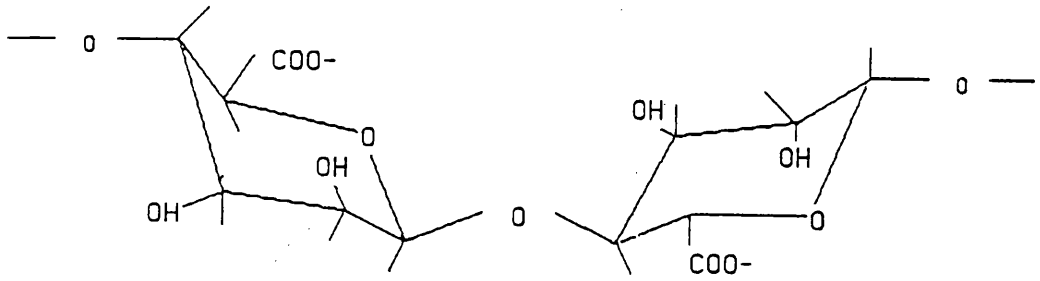


Figure 3.1.i
Structure of mannuronic acid

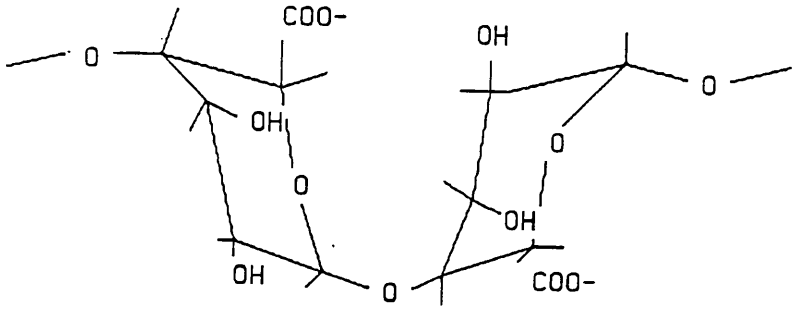


Figure 3.1.ii
Structure of guluronic acid

a solution of calcium chloride where they solidify into spheres, due to exchange of the sodium and calcium ions (Figure 3.2).

Formation of droplets from the alginate solution is dependent on a number of parameters, and because any tails or striations on the surface of the droplet will increase the chance of an adverse reaction to the implant (Goosen et al. 1985), it is essential to ensure that a perfect sphere is obtained. The factors which affect the formation of droplets include the concentration of alginate and calcium chloride, the flow rate of alginate and oxygen, the presence of impurities in the alginate, and the height of the droplet maker above the calcium chloride solution (Goosen et al. 1985). For example, if the distance above the calcium chloride is too great, or the oxygen flow rate too fast, the droplets will hit the surface too hard and will shatter. Conversely, the droplets will become pear-shaped if the oxygen flow rate is too low, or the distance to drop too small, as they will not have chance to round off before coming into contact with the calcium ions. The presence of impurities in the alginate is thought to lead to striations on the surface of the droplets.

Once the droplets have been formed, they are then coated with poly-l-lysine (PLL) which is positively charged and binds to the negatively charged alginate ions by displacing the calcium ions, while maintaining the spherical shape of the droplets. The PLL continues to cross-link with itself to increase the thickness of the coating (Lim, 1988) and thus forms the semi-permeable membrane component of the capsule. Further layers can be added to the droplets, according to the required final composition. The final step is then to re-liquify the inner calcium alginate core by treating the capsules with an agent capable of sequestering the calcium ions, leaving the islet in a liquid inner layer. The most commonly used sequestrant is sodium citrate.

3.3.iv Development of capsule composition

The initial method for encapsulating islets described by Lim and Sun (1980) used a layer of PLL with an outer layer of

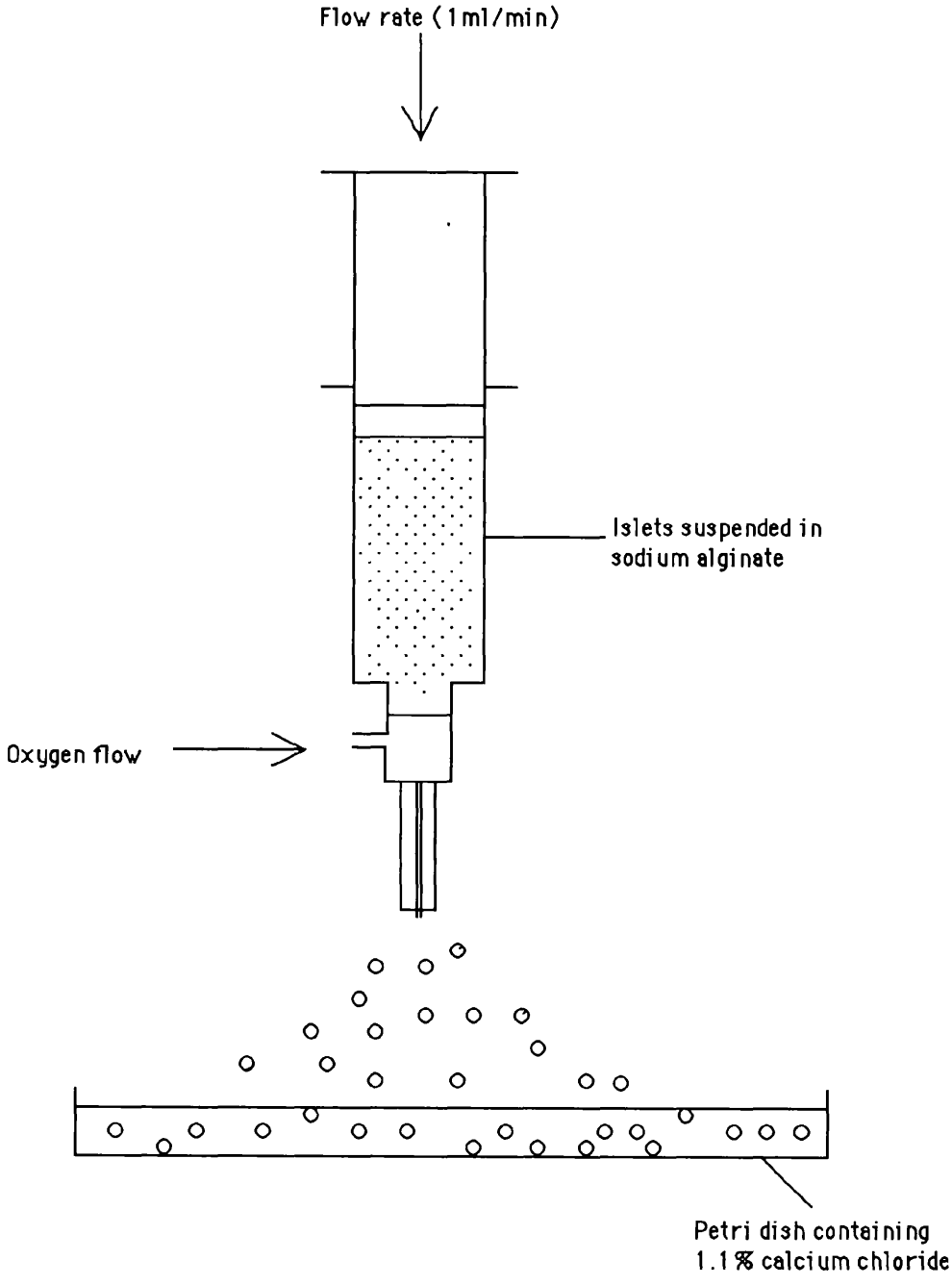


Figure 3.2
Diagram of the islet encapsulation procedure

polyethyleneimine to form the semi-permeable membrane around the capsules. This allowed the passage of insulin and glucose, but not immunoglobulins, thus protecting the islets from the immune system. Some of the earlier authors used polyornithine as an alternative to PLL, but the latter was felt to be more biocompatible and consequently is now used most widely.

Perhaps the most significant change to have been made to the composition of the capsule was described in 1984 by O'Shea et al. They added an extra outer layer of sodium alginate to the capsules, instead of using polyethyleneimine, in order to make them more biocompatible due to the hydrogel properties of the alginate. The outer layer of alginate which is negatively charged binds to any excess uncomplexed amino groups on the PLL layer (Lim, 1988).

The other major modifications made to the encapsulation method have involved the length of time allowed for the layers to be deposited and the concentration of the solutions used. The most important component of the capsule in this respect is the PLL layer, as these factors will determine the size of molecules which are able to pass across the membrane. In the original method, PLL with a molecular weight of 40kD to 60kD was used at a concentration of 0.2%, and the reaction time was 2-4 minutes. A study published by Goosen et al. (1985), however, defined the optimum conditions for deposition of the semi-permeable membrane to utilise PLL with an average molecular weight of 20kD at a concentration of 0.05%, and a reaction time of 6 minutes. This was calculated as giving a molecular weight cut-off of the membrane of approximately 67kD, confirming that insulin (6kD) and glucose could pass across the membrane, while immunoglobulins (lower molecular weight of approximately 150kD; Goosen, 1987) would be excluded. One notable alteration to this protocol appeared in a publication by Fan et al. (1990) in which the reaction time for the PLL layer was increased from 6 to 10 minutes and the concentration was increased to 0.15%. No justification for either of these changes was made. In fact, the report by Goosen et al. (1985) stated that an increase in the concentration of PLL did not have any effect on the thickness of the capsule wall, while an increase in PLL reaction time reduced the flexibility and durability of the capsules.

It is also of interest to note that in the same study to determine the optimum conditions for PLL, Goosen et al. (1985) observed that when the capsules were placed in sodium citrate to reliquify the inner core, they expanded, with the increase in diameter being proportional to the molecular weight of the PLL used. The larger the diameter of the capsule, the longer it will take for glucose and insulin to diffuse across, so a lower molecular weight PLL will aid islet function as well as preventing passage of immunoglobulins across the capsule wall.

Further alternatives to the method of encapsulation were presented at the Third International Congress on Pancreatic and Islet Transplantation in Lyon (1991). Zekorn et al. reported the use of barium ions to solidify the alginate as this reaction is irreversible and should therefore provide more durable capsules. They did not appear to add any further layers to the capsules, and quoted a molecular weight cut-off of 80,000D. The same group also presented data relating to a new method of encapsulation, again with barium alginate, such that the islets were coated with the material thus filling the whole of the capsule space, rather than forming droplets with the islets only occupying part of the internal volume. However, the details of the methodology to produce this type of coating were not presented.

3.3.v Alternative methods of islet encapsulation

Islet encapsulation has also been reported with materials other than alginate. Braun et al. (1985, 1986 and 1987) have utilised cellulose sulphate and poly(dimethyldiallylammoniumchloride) as encapsulation agents. Their results have shown good in vitro insulin release from these islets for up to five weeks in culture. Dupuy et al. (1989) and Gin et al. (1990) have reported results using a membrane of polyacrylamide and agarose. After three weeks in vivo, however, the capsules had become cloudy, presumably due to cell overgrowth, and the islets did not respond to changes in glucose concentration.

Iwata et al. (1986, 1987, 1988, 1989) have also used agarose as a medium for encapsulation, and have shown excellent in vitro insulin release, even after 100 days in culture. Results of their transplant work presented at the Pancreatic and Islet Transplantation Congress (1991) indicated graft survival in NOD mice for over 100 days, with very little evidence of fibrosis when animals were culled at 102 and 192 days post-transplant.

Sefton et al. (1987) and Sugamori and Sefton (1989) have reported the use of Eudragit RL, a polyacrylate. Their results have shown that this material is not sufficiently permeable or biocompatible. Tatarkiewicz et al. (1988) published results of diffusion studies on alginate droplets coated with protamine sulphate and heparin as the semipermeable component. These capsules allowed good diffusion of insulin and glucose while excluding immunoglobulins although no data was reported regarding their biocompatibility.

Clearly, there are several alternative chemicals being investigated as possible encapsulation materials, although most of the published reports appear to relate to alginate/PLL capsules. The studies by Iwata et al. discussed above suggest that agarose is perhaps the most promising alternative to alginate for islet encapsulation.

3.4 RESULTS OF EXPERIMENTAL WORK

3.4.i In vitro experiments with encapsulated islets

Initially, islet function following encapsulation was assessed by both in vitro and in vivo experiments. The in vitro work was usually confined to insulin release studies using either static incubations or perifusion. The latter has the advantage over static incubations of allowing the response time of the islets to glucose stimulation to be determined. This is particularly important for encapsulated islets, as the time taken for the glucose to diffuse into the capsule and the insulin to diffuse out could be an important factor in determining the ultimate success of the ability of encapsulated islets to control blood glucose levels following transplantation. However, it has been suggested that the static

incubation system mimicks more closely the in vivo situation following transplantation into the peritoneal cavity as the solvent drag effect in the perifusion system may act to encourage insulin release from the islets (Chicheportiche et al. 1988).

Results from in vitro experiments have been inconclusive. Lim and Moss (1981), Leung et al. (1983) and Fritschy et al. (1989) all claimed to obtain the same insulin release profiles from encapsulated and free islets. Lim and Sun (1980) and Sun et al. (1987a) showed the total insulin release from the islets to be the same, although the response time for the encapsulated islets was slightly longer due to the time taken for diffusion across the capsule. However, Darquy and Reach (1985) found that islets in a static incubation system had a reduced total insulin release relative to control islets. Lynch et al. (British Diabetic Association Spring Meeting, Brighton, 1991) reported that there was no difference in response time between encapsulated and free islets, although a recovery period of 7 days was required to allow optimum insulin release from the encapsulated islets. Surprisingly, the total insulin release from these encapsulated islets was greater than from the control non-encapsulated islets.

The results presented by Zekorn et al. at the Pancreatic and Islet Transplantion Congress (1991) on the insulin release from islets encapsulated in barium alginate showed a 3 minute delay during perifusion and a total insulin release in static incubations which was 40-60% that of non-encapsulated islets. However, the islets which had a thin coating of barium alginate responded as rapidly and released similar ammounts of insulin as the control islets.

Two factors which may explain the discrepancy in results concerning the response time and the total insulin release respectively are firstly the observation by Chicheportiche and Reach (1988) that islets inside a capsule with a diameter of 350um have a much faster response time to glucose stimulation than islets inside capsules with a diameter of 650um. As many reports do not include details of capsule diameter, it is not possible to make direct comparisons between some of the studies. Secondly, none of these studies have taken islet size into account, a factor which makes

comparison of total insulin release results difficult as it has been shown that there is a linear relationship between insulin release and islet size (Hopcroft et al. 1985).

3.4.ii. Transplantation experiments

Perhaps the most controversial area relating to islet encapsulation is that of the function of the islets following transplantation into diabetic animals, whether they have chemically-induced or spontaneously-occurring diabetes.

Table 3.1 gives a review of the reported transplant experiments, indicating the combination of donors and recipients, the length of time for which the graft functioned and the number of animals transplanted where known. The results of these studies show quite clearly that the length of time for which grafts have functioned varies from as little as 12 days up to nearly 2 years. Direct comparison of the results is difficult as many minor modifications to the encapsulation method have been made, and the combination of donors and recipients varies from isogeneic to discordant xenogeneic transplants.

Other factors which make interpretation of the results difficult are the tendency for some groups to report graft survival times of "up to x days" thus placing the emphasis on the successful transplants while not reporting how quickly the remaining grafts had failed. The fact that many of the publications are in the form of abstracts from conferences adds to the problem of the lack of detailed information on any particular study. In addition to this, it is apparent that some results have been reported in more than one paper, and that in some cases, results have been up-dated in subsequent papers without the fact being highlighted. This gives the overall picture of encapsulated islet transplantation a bias towards the more successful studies, and consequently distorts the true progress of this work.

One interesting aspect of these studies is the use of non obese diabetic (NOD) mice and BioBreeding (BB) rats i.e. the two

Table 3.1

Results of transplant experiments with encapsulated islets, showing the donor and recipient strains/species, and the duration of graft function.

Reference	Donor	Recipient	Graft Function
Lim + Sun (1980)	Wistar	Wistar-Lewis (n=5)	14 days
Araki et al. (1983)	Minced rat pancreas	Wistar (n=4)	12 days
O'Shea et al. (1984)	Wistar	Wistar (n=5)	n=1 >100 days n=2 >1year
Sun et al. (1984)	Wistar	Wistar (n=5)	n=4 >77days (max 310+ days)
Sun + O'Shea (1985a)	Wistar	Wistar (n=8)	1 failed 1 month n=1 648 days
Sun + O'Shea (1985b)	Wistar	Wistar (n=8)	1 failed 1 month n=1 up to 648 d n=5 had 2nd Tp
Calafiore et al. (1986)	Dog	C57/BL6 (n=8) NOD (n=11)	n=6 4+ months n=8 7-10 days n=3 120+ days
Hamaguchi et al. (1986)	Hamster	Wistar	5 days

Table 3.1 (cont.)

Reference	Donor	Recipient	Graft function
O'Shea + Sun (1986)	Wistar	Balb/c (n=22)	28-144 days n=13 had 2nd Tp
Ricker et al. (1986)	RINm5F cells Rat islets	Balb/c NOD	RIN 30 days max rat 65-108 days RIN 14 days max rat 14 days max
Taunton-Rigby (1986)	Wistar	Wistar Lewis	13-84 weeks (n=10)
Calafiore et al. (1987)	Dog	C57BL/6 (n=8) NOD (n=11)	n=5 122+ days {n=8 7-10 days {n=3 60-110+days
Sun et al. (1987b)	Rat	Wistar (n=10) Balb/c (n=8)	n=5 1+ year 50+ days
Wu et al. (1988)	Wistar	Wistar (n=7)	n=4 222+ days
Calafiore et al. (1989a)	Wistar Human	NOD (n=8) NOD (n=5)	n=8 14 days n=4 14 days
Calafiore et al. (1989b)	Pig	Dog (n=4)	insulin required 50-70% down - up to 50 days

Table 3.1 (cont.)

Reference	Donor	Recipient	Graft function
Darquy et al. (1989)	Rat	C57BL/6J	n=8 transient n=9 60 days
		Balb/c	n=4 transient n=6 1+ month
		NOD	Failed
Sun et al. (1989)	Lewis	BB/d (n=12)	up to 230+ days
Weber et al. (1989)	Lewis	C57BL/6J	n=5 up to 80 days
Wu et al. (1989)	Fetal human	Human (n=3)	n=2 insulin required 20-30% down
Calafiore et al. (1990)	Pig	NOD (n=6)	15-50+ days
		Lewis (n=6)	40-80+ days
Chicheportiche et al. (1990a)	Pig	Wistar (n=15)	n=11 failed n=4 42-305 days (STZ reversal in 3)
Fan et al. (1990)	Wistar	BB/d (n=10)	up to 6+ months n=2 had 2nd Tp
Weber et al. (1990)	Lewis Dog	C57BL/6J	rat - 100+ days dog - up to 68 days
		NOD	rat - up to 17 days dog - up to 17 days

Table 3.1 (cont.)

Reference	Donor	Recipient	Graft function
Mazaheri et al. (1991)	Lewis	BB/d	15.8±1.28 days
		+CsA	44.8±7.5 days
		+CsA+Ind	51.4±22.13 days
		+Dex	89.0±23.0 days
Soon Shiong et al. (1991a)	Dog	Dog (+CsA)	115±37 days+
Zekorn et al. (1991)	Lewis	STZ mice	up to 70 days
	Pig	STZ mice	up to 100 days
Weber et al. (1991)	Lewis	NOD	n=10 12.6±1.2 days
		+ UV-B (islets)	n=5 68.2±19.1 days
Lum et al. (1991)	Wistar	NOD (n=16)	n=9 up to 8 mo
			n=5 had 2nd Tp

spontaneously occurring animal models of diabetes, as recipients for the encapsulated islets in some of the more recent studies. The majority of the transplant studies reported have used STZ-diabetic recipients although there is little mention of the possible problem of regeneration of the beta cells following STZ treatment as described by Brekke et al. (1983), Portha and Picon (1982) and Richardt et al. (1984). A more recent study by Chicheportiche et al. (1990a) has in fact demonstrated that the apparently successful reversal of STZ diabetes in rats by the transplantation of encapsulated porcine islets was in fact due to beta cell regeneration in the rat pancreata.

Many of the experiments using NOD mice have shown either failure of the transplant to lower the blood glucose of the animals, or only a small percentage of the animals with grafts which functioned for a relatively long time. Only three of the studies used BB rats, two of which appear to have had greater success than the experiments using NOD mice. However, as in previous reports (Sun and O'Shea, 1985b; O'Shea and Sun, 1986) one of these studies (Fan et al. 1990) described two animals being given a second transplant when their blood glucose started to rise above the normal level, making overall interpretation of the results difficult. It is interesting to note that the most recent study on encapsulated islet transplants into BB/d rats (Mazaheri et al. 1991) shows very poor graft survival - only 15.8 days - and that in order to prolong graft survival the animals had to be treated with immunosuppressive drugs which defeats the major reason for encapsulated islet transplants i.e. the ability to transplant islets without the requirement for immunosuppression.

Studies of encapsulated islet transplant experiments presented at the Pancreatic and Islet Transplantion Congress (1991) were the first which included the use of IVGTT and C-peptide measurements, rather than just daily blood glucose monitoring, to assess the success of the transplants: Soon Shiong et al. (1991a) showed that his 'successful' transplants in dogs gave near-normal IVGTT results, while the C-peptide response, although an improvement on the pre-transplant results, was far from normal. The recipients in these experiments were given CsA treatment for 30 days post-transplant,

however, an ongoing trial suggested that dogs receiving transplants without CsA were equally successful.

A further development was presented by Calafiore (1991) at the same meeting. This was the use of a double-layered vascular graft which incorporates the encapsulated islets between the outer, impermeable layer, and the inner, permeable layer. The decision to use a vascular graft as the vehicle for the encapsulated islets appeared to be due to the poor results of transplants into dogs, relative to those in NOD mice, due to capsular fibrosis. Experiments with empty capsules indicated no problems with fibrosis after 80 days in vivo. In a series of 6 dogs receiving encapsulated porcine islets, 5 had shown a great decrease in their requirement for insulin, while one had become insulin-independent.

The success of these experiments with dogs led to the transplantation of two patients with the grafts. One of these was an insulin-dependent Type 2 diabetic patient who received 150,000 encapsulated islets. At 7 months post-transplant their insulin requirement showed a five-fold decrease, with an improved C-peptide response, and a short period without any exogenous insulin treatment. The other patient was a Type 1 diabetic who received 250,000 encapsulated islets. At 44 days post-transplant they had displayed a decrease in their insulin requirement, an improved mean blood glucose level and some C-peptide response. Even when this early success is considered, many questions must be raised about the safety of using this method of transplantation, especially in the light of the failures of the hybrid artificial pancreas due to thrombosis of the vascular grafts, as already discussed. It is also quite remarkable that such relatively small numbers of islets have resulted in this success when compared to the numbers of non-encapsulated islets implanted in human islet transplant recipients, especially when the numbers of encapsulated islets required to reverse diabetes in experimental animals far exceeds the number of free islets.

3.4.iii Reasons for failure of encapsulated islet grafts

The major factor causing the failure of encapsulated islet grafts is the inflammatory reaction which they provoke. In 1984, two papers from the same group (Sun et al. and O'Shea et al.) described the use of alginate to form the outer layer of the capsules, rather than polyethyleneimine as previously described. The change was made on the hypothesis that the hydrogel properties of the alginate would make it a more biocompatible material for implantation in vivo, and the results of transplants with these capsules indicated much improved graft function as well as prevention of eye cataract development, a condition commonly seen in control animals which had not received transplants.

The failure of some transplants following this change in methodology was explained by the possibility of introducing contaminants during capsule implantation causing the formation of fibrous tissue at the implantation site which engulfed the capsules thus causing them to fail (Sun and O'Shea, 1985a). Also, the same group has suggested that incomplete coating of the capsules with alginate which would allow the PLL layer to provoke a reaction, or variations between alginate batches may be factors contributing to graft failure (Fan et al. 1990). Goosen (1987), in his review of encapsulation, also put forward the theory that graft failure could be a result of using the incorrect molecular weight of PLL which would allow immunoglobulins to pass across the capsule wall and damage the islets.

However, although there has been some apparent success with encapsulated islet transplants, other groups have not shared this success. Many have reported severe inflammatory reactions around the capsules which in some cases has been more marked than the reaction observed against capsules prepared without the islets inside (Ricker et al. 1986; Calafiore et al. 1986, 1987 and 1989a; Weber et al. 1990). Examination of the infiltrating cell types has shown the reaction to be predominantly composed of mononuclear cells, macrophages and fibroblasts (Calafiore's group), although the report by Weber et al. (1990) suggests that the reaction is $CD4^+$ T-cell dependent, as administration of a monoclonal antibody against this T

cell subset increased the success of the grafts. The presence of T-helper cells in the infiltrate around encapsulated islets has been confirmed by Mazaheri et al. (1991), who also noted that up to 100 days post-transplant empty capsules displayed a linear relationship of percentage overgrowth with time. This observation was repeated with encapsulated islet transplants, thus indicating that grafts which survived the longest had the lowest degree of pericapsular fibrosis. The theory that it is the islets inside the capsules which provoke the reaction was strengthened by Weber et al. (1991). They found that pretreatment of the islets with UV-B irradiation reduced their immunogenicity, increasing encapsulated islet xenograft survival from 12.6 $\pm$ 1.2 days without irradiation to 68.2 $\pm$ 19.1 days, using the NOD mouse as the recipient.

The presence of macrophages round the capsules could prove to be highly detrimental to the islets due to the release of cytokines which are toxic to beta cells (Bendtzen et al. 1986; Mandrup-Poulsen et al. 1985, 1986 and 1987; Pukel et al. 1988; Rabinovitch et al. 1988; Sandler et al. 1987 and 1989; Zawalich et al. 1986) and can pass across the capsule membrane due to their relatively low molecular weight of approximately 25kD (Cole et al. 1989a). In addition to this, macrophages are thought to be involved in the activation of fibroblasts (Chvapil, 1981; Jalkanen and Penttinen, 1982; Leibovich and Ross, 1976; Raines et al. 1989). The presence of a layer of cells around the capsule would cut off the flow of nutrients and insulin across the capsule wall, leading to islet death and graft failure, a problem which would be exacerbated by the deposition of collagen by the fibroblasts. A study by Chicheportiche et al. (1990b) demonstrated activation of peritoneal macrophages by alginate-polylysine capsules leading to a decrease in encapsulated islet function, and Darquy et al. (1990) found that complement was activated by capsules when incubated with human serum.

As well as capsule composition, another factor which has been suggested as important in determining the degree of inflammation against the capsules is that of implantation site. All the transplant experiments described use the peritoneal cavity as the implantation site. However, Cole et al. (1989b) described a reduction in reaction to the capsules if they are implanted in the renal subcapsular space.

(Routine use of this site for transplanting encapsulated islets is unlikely due to the limited volume of space available.) The study by Tze and Tai (1982) demonstrated a 7-fold increase in the peritoneal nucleated cell population following implantation of empty microcapsules, a factor indicating that the peritoneal cavity is not favourable site for the implantation of foreign material.

The possibility of directly coating the islets with a layer of barium alginate (described above, Zekorn et al. 1991) could provide an answer to the problem of implantation site. These coated islets are almost the same size as the islets prior to encapsulation, and could therefore be implanted into the renal subcapsular space, or even injected into the liver via the portal vein. The ability to use these implantation sites could help to minimise the problem of capsule fibrosis.

Recent reports by Soon-Shiong et al. (1990 and 1991b) suggest that not only the use of alginate as the outer layer of the capsules is important, but also the alginate preparation may affect the severity of reaction to the capsules. In these studies, two alginate preparations were compared: a high mannuronnic acid alginate and a highly purified, low mannuronic acid alginate. Capsules coated with the former were found to provoke a severe pericapsular reaction of macrophages and fibroblasts, whilst those coated with the purified alginate were mostly free of any reaction. Subsequent testing of the alginates to determine their effect on cytokine release from human monocytes found the purified alginate to have little effect whilst the other preparation caused a dramatic increase in cytokine release. It appears, therefore, that the alginate preparation may be crucial in determining the ultimate success of the transplant. It must be pointed out, however, that in these experiments, the purity of the alginate preparations appeared to differ, thus making it difficult to determine the relative effects of the alginate composition and purity of the preparation.

3.5 SUMMARY

The encapsulation of islets poses many problems which have not yet been solved. Perhaps the most important aspect is that of capsule biocompatibility: until a capsule composition has been developed which can be well tolerated for indefinite periods of time, the whole practice of encapsulation will remain purely experimental. The problem of biocompatibility is linked with the volume of tissue which has to be transplanted to reverse diabetes, as this limits the sites available for implantation. The peritoneal cavity provides the only acceptable location but increases the chances of graft failure due to the cell population present.

An additional problem is the provision of sufficient numbers of islets to reverse diabetes when the peritoneal cavity is used. Many of the experiments described have transplanted 4,500-5000 encapsulated islets into rats to achieve success. This far exceeds the number of 750 free islets required to reverse diabetes in rats when the renal subcapsular site is used (Loftus et al. 1991), and has obvious implications for the source of donor islets.

However, even taking these problems into account, work on encapsulated islet transplantation has been continued due to the possible rewards of this technique. These include the use of xenogeneic islets which would solve the problem of supply of donor islets. In addition, the availability of a transplant system which does not require immunosuppression of the recipient is very attractive, especially as the ultimate goal would be to transplant newly diagnosed, and therefore often very young, diabetic patients prior to the possible development of complications.

CHAPTER 4

THE EFFECT OF CAPSULE COMPOSITION ON THE *IN VITRO* VIABILITY OF ENCAPSULATED ISLETS

4.1 INTRODUCTION

In 1984, as already discussed in the previous chapter, O'Shea et al. substituted the outer capsule layer of polyethyleneimine for a layer of alginate, on the assumption that this would make the capsules more biocompatible. However, there were no details of experiments undertaken to support this hypothesis. The first two parts of this project were designed, therefore, to assess capsule composition in relationship to both their in vitro function and biocompatibility. The ultimate aim was to determine the optimum capsule composition for future transplantation experiments. The work on capsule biocompatibility will be covered in Chapter 5.

Capsules were prepared with an outer layer of sodium alginate, or with an outer layer of PLL. Two different preparations of alginate were tested. One had a relatively high mannuronic acid content and the other had a relatively high guluronic acid content. The choice of these two alginate preparations allowed the effect of the higher degree of calcium binding with the high guluronic acid alginate to be assessed.

In vitro islet viability was assessed by two methods. The first of these was a membrane integrity assay using fluorescent dyes. The second method was to determine glucose stimulated insulin release from the islets in a perifusion system. WAG/Ola rats were used as islet donors for these experiments.

4.2 VIABILITY ASSAYS

4.2.i Microfluorometry

Many stains for the assessment of cell viability have been described, although most of these are not appropriate for use with islets. Neutral red (Sawicki et al. 1967) and trypan blue (Evans and Schulemann, 1914) are both commonly used stains for single cells, but do not allow assessment of all the cells in an islet simultaneously (Bank, 1987; Gray and Morris, 1987). Diphenylthiocarbazone (dithizone) has been demonstrated to selectively stain islets (Maske, 1957), but cannot be used as an indication of viability as islets

will continue to stain with dithizone for up to 20 hours after cell death (London et al. 1989a). Consequently, the method described by London et al. (1989a) which uses the fluorescent dyes fluorescein diacetate (FDA) and propidium iodide (PI) was chosen, as this allowed all the cells within the islet to be assessed.

FDA is a non-polar ester which can pass across plasma membranes where it becomes hydrolysed by intracellular esterases producing free fluorescein. Fluorescein is polar and cannot pass back across the intact membrane of living cells, so it accumulates inside the cell causing the cytoplasm to fluoresce green under appropriate conditions (Rotman and Papermaster, 1966). Conversely, PI is excluded from living cells, but can cross the membranes of dead or damaged cells where it binds to the nucleic acids producing a discrete spot of bright red fluorescence (Corliss and White, 1981).

Therefore, by using a combination of these two dyes and the appropriate conditions for fluorescence, it is possible to assess the overall viability of an islet. The details will be described in section 4.4.iv.

4.2.ii Perifusions

Although the FDA/PI staining allows a quick assessment to be made on the viability of an islet in terms of membrane integrity, it does not provide any information on the ability of the islet to release insulin in response to changes in glucose concentration. Therefore, islet function was assessed by perifusion, using a method first described in 1972 by Lacy et al. This involves placing islets in a chamber connected to a system through which medium can flow over the islets and be collected for analysis of insulin content. Two reservoirs of medium are connected to the system, one containing high glucose medium and the other low glucose medium. By changing the flow from one reservoir to the other, the ability of the islets to respond to the change in glucose concentration can be determined. Determination of the functional viability of the islets was considered important to this project as it was not known whether the encapsulation procedure would cause damage to the islets.

The decision was taken to determine insulin release in a perifusion system, rather than by static incubation, to allow the time taken by the islets to respond to an increase in glucose concentration to be calculated. Due to the diffusion time of glucose and insulin in and out of the capsule, it was possible that the response time of the islets could be delayed. It was considered important for this factor to be determined as a delay in release of insulin in vivo could affect the efficiency of an encapsulated islet transplant. Although perifusions offer many advantages, the suggestion that the solvent drag could affect insulin release (Chicheportiche et al. 1988) has already been highlighted in Chapter 3.

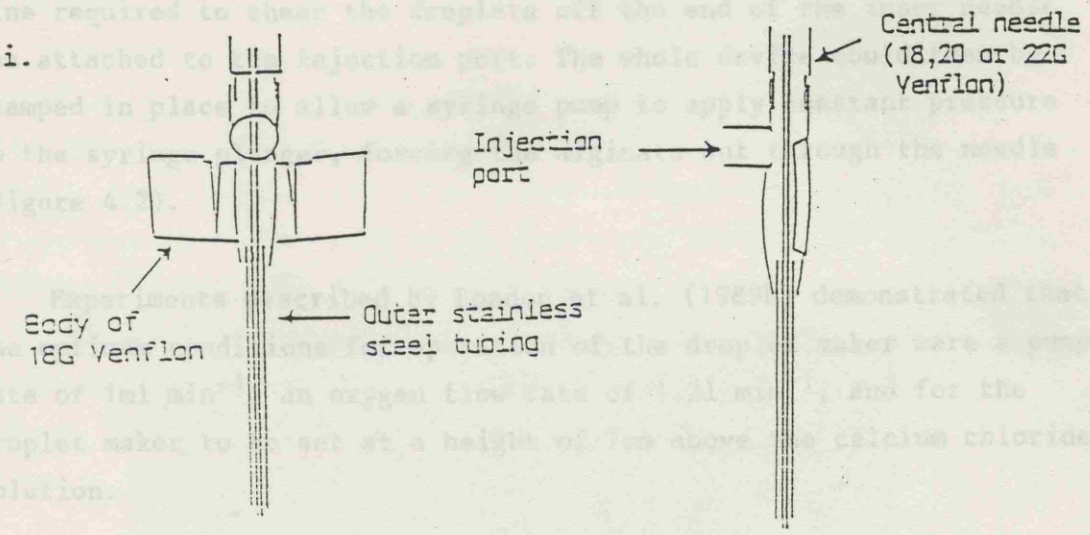
In addition to measuring insulin release from the islets, their DNA content was also determined and the insulin release results corrected for this. In 1985, Hopcroft et al. highlighted the importance of expressing insulin release per ng DNA rather than per islet, as the size of an islet (and therefore the DNA content) up to 300um is strongly correlated with the amount of insulin released.

4.3 DEVELOPMENT OF THE DROPLET MAKER

The earlier papers describing the process of islet encapsulation used droplet makers previously developed for other purposes (Ennis and James, 1950; Sparks et al. 1969). The major problem with these devices is that because they are made of glass they are very fragile which makes cleaning them a problem. A droplet maker was developed in the Department of Surgery, University of Leicester, which was sturdy, easy to clean and sterilisible either by autoclave or by immersion in industrial methylated spirit (IMS) (London et al. 1989b).

The droplet maker was constructed from two Venflon (Viggio, Sweden) intravenous cannulae (Figure 4.1.i and 4.1.ii). The outer section was formed from an 18G cannula with the inner needle and polypropylene tubing removed, while the inner section was the central needle of a 22G cannula. The tubes were glued to the body of the cannula with epoxy resin. It was then possible to attach a syringe

i.



ii.

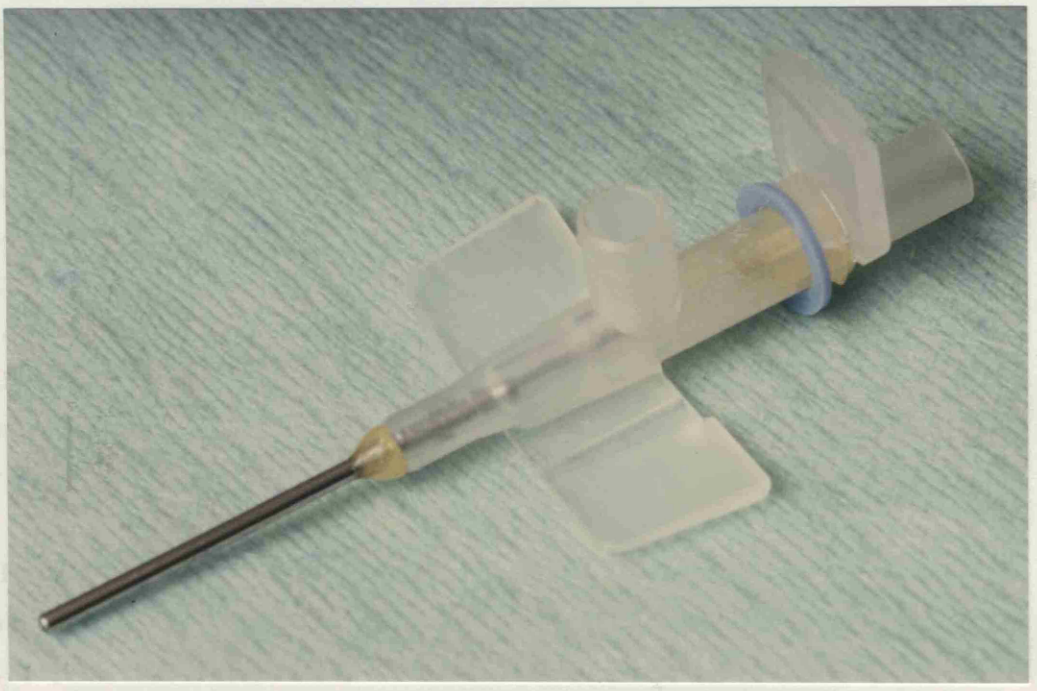


Figure 4.1
Diagram (i) and photograph (x2) (ii) of the droplet maker.

containing alginate to the body of the cannula, and the oxygen supply line required to shear the droplets off the end of the inner needle was attached to the injection port. The whole device could then be clamped in place to allow a syringe pump to apply constant pressure to the syringe plunger, forcing the alginate out through the needle (Figure 4.2).

Experiments described by London et al. (1989b) demonstrated that the optimum conditions for operation of the droplet maker were a pump rate of 1ml min^{-1}, an oxygen flow rate of 1.21 min^{-1}, and for the droplet maker to be set at a height of 7cm above the calcium chloride solution.

4.4 MATERIALS AND METHODS

4.4.i Rat islet isolation

Islets were prepared from WAG/Ola rats of either sex (150-200g), under terminal fluothane anaesthesia. The pancreas was exposed by displacement of the liver and intestines, the common bile duct occluded at the duodenal end, and the animal exanguinated by severing the aorta. Ice-cold collagenase (1337U ml^{-1}, Type XI Lot 48F-6808, Sigma, Poole, U.K.; or 1mg ml^{-1}, Lot 03021C, Serva Feinbiochemica GmbH & Co., Heidelberg) in minimum essential medium (MEM, containing 10ml DNase and 1.5ml 1M calcium chloride per 100ml) was used to distend the pancreas by injection via the common bile duct as described by Sutton et al. (1986).

Following resection, the pancreas was incubated in pre-warmed MEM at 37°C for 21 minutes, disrupted by vigorous shaking for 1 minute, passed through a 500um mesh, and washed several times with MEM containing 0.5% bovine serum albumin (BSA). The islets were purified on a discontinuous BSA gradient as described by Lake et al. (1986): The pellet was suspended in 300mOsmolar BSA (Applied Protein Products, Brierley Hill, U.K.) with a density of 1.085g cm^{-3}, allowing 3ml BSA per pancreas, and added to a 10 ml tube. 3ml of 1.069g cm^{-3} BSA were layered on top of the suspension, with a further 3ml layer of 1.056g cm^{-3} BSA added to complete the gradient. The tubes were centrifuged at 800g for 20 minutes (no brake), and the

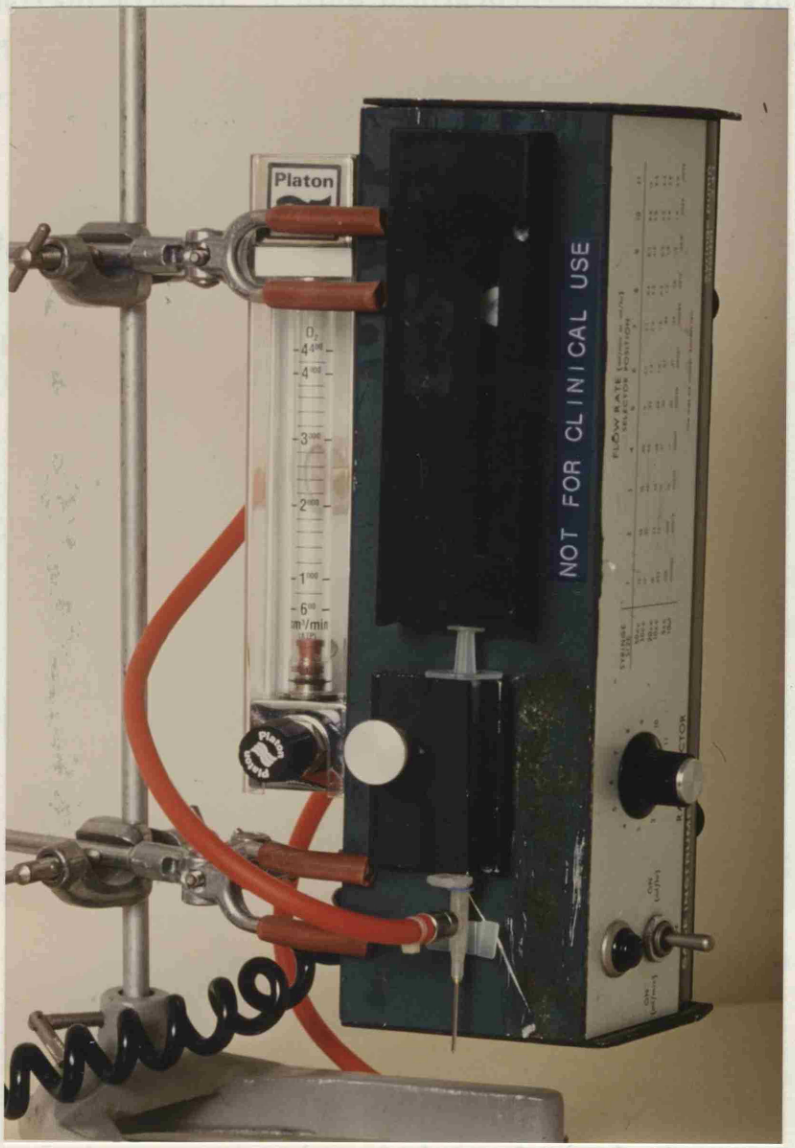


Figure 4.2
The encapsulation apparatus (x0.5).

islets harvested from the interface between the top and middle layers using a Pasteur pipette. The average purity of the islets was 90-95%.

The islets were washed with MEM containing 0.5% BSA, then cultured in petri dishes in an atmosphere of 95% air:5% CO_2, at 37°C in RPMI 1640 culture medium (Flow Laboratories, Rickmansworth, U.K.) containing 10% foetal calf serum (Andersson and Hellerstrom, 1980), 100U ml^{-1} penicillin, 100U ml^{-1} streptomycin, 1mM sodium pyruvate, 0.1mM 2-mercaptoethanol, 0.01uM hydrocortisone, 2mM L-glutamine, 0.02M HEPES. Figure 4.3 shows a photograph of purified rat islets stained with dithizone.

4.4.ii Alginates used for encapsulation

Two preparations of sodium alginate were used in this study: Keltone LV and A7C618. Both were supplied by Kelco Division, Merck + Co. Inc., San Diego, California.

Keltone LV: mannuronic acid content approximately 61%
guluronic acid content approximately 39%
viscosity of 1% solution at 20°C, 50mPa.s

This will be referred to as M-alginate.

A7C618: mannuronic acid content approximately 31%
guluronic acid content approximately 69%
viscosity of 1% solution at 20°C, 34mPa.s

This will be referred to as G-alginate.

Both of these preparations had a relatively low viscosity which was to allow formation of regularly formed droplets.

Figure 4.3
Dithizone-stained islets (x100).

4.4.iii Encapsulation of islets

All solutions used were filter sterilised using a 0.22um filter. Islets were cultured for 48 hours after isolation before being encapsulated. The method of encapsulation was essentially that described by Sun (1988). Figure 4.4 shows a flow diagram of the encapsulation method.

The islets were washed several times in 0.9% saline in order to remove all traces of calcium from the culture medium, then suspended in a 1.5% solution of M-alginate in 0.9% saline. The suspension was drawn into a 2ml syringe which was then attached to the droplet maker, and the solution extruded under pressure into a dish containing 1.1% calcium chloride, using the conditions described above.

The droplets were transferred into a 50ml conical tube, the supernatant pipetted off, and the droplets washed with 0.55% calcium chloride, 0.27% calcium chloride, and 0.9% saline. They were then washed for 3 minutes in 0.1% CHES (2-[N-cyclohexylamino]ethane-sulfonic acid; Sigma) in 0.9% saline, pH 8.2, suspended in 0.05% PLL hydrochloride (molecular weight approximately 20,000D, Sigma) in 0.9% saline for 6 minutes, washed again in 0.1% CHES, and in 0.9% saline.

The capsules which were to have an outer layer of alginate were suspended for 4 minutes in 0.15% alginate in 0.9% saline, then washed with saline. Capsules coated with M-alginate and with G-alginate were prepared.

The final step for all capsules was to be suspended in 55mM sodium citrate for 6 minutes to reliquify the inner alginate layer, followed by washing with saline and culture medium before being cultured using the conditions described above (Section 4.4.i). Figures 4.5.i and 4.5.ii show schematic diagrams of the different capsule compositions. The viability experiments were carried out over a period of four weeks and during this time, the culture medium was changed every 3 to 4 days. Figure 4.6 shows a photograph of encapsulated islets.

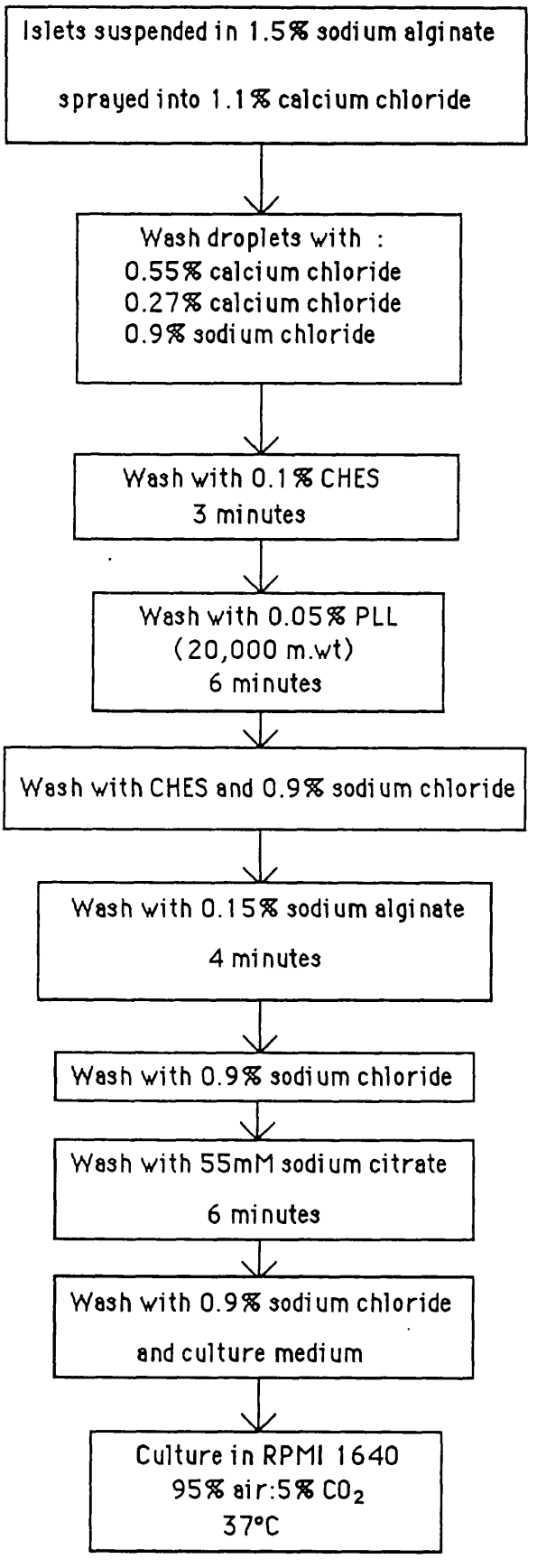
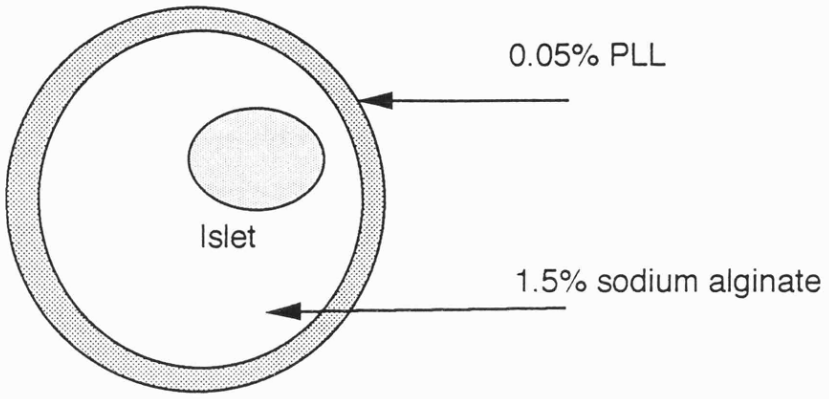
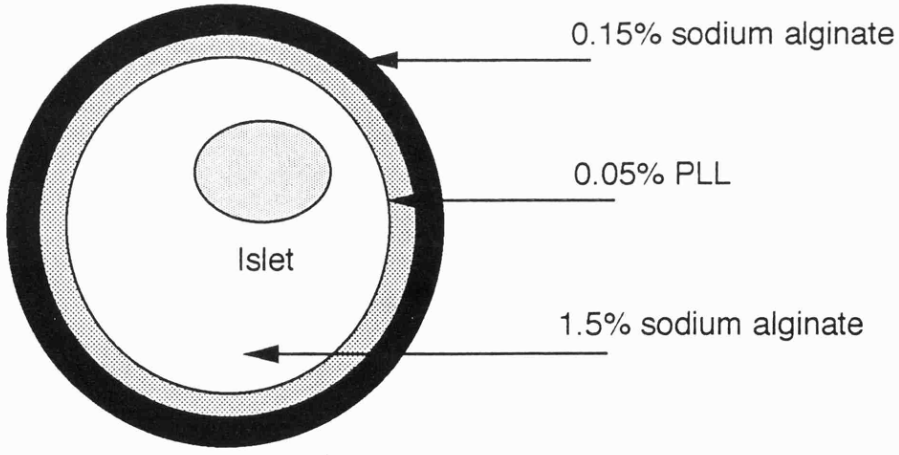


Figure 4.4
Flow diagram of the encapsulation method



i)



ii)

Figure 4.5
Schematic diagram of capsules with outer layer of i), PLL and ii), sodium alginate

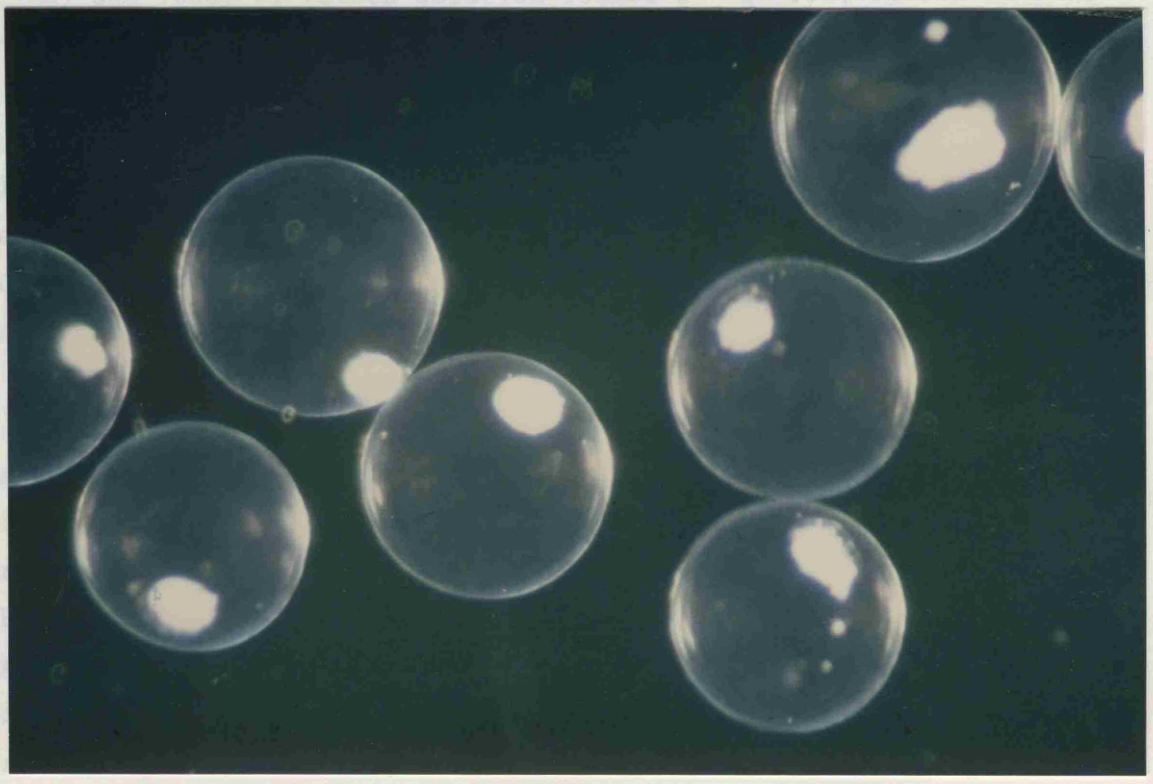


Figure 4.6
Encapsulated islets (x70).

4.4.iv Microfluorometry

This assay was performed on control non-encapsulated islets and on islets encapsulated in the three different types of capsule at 4-5 day intervals for a period of 4 weeks. The assay method used was described by London et al. (1989a).

The solution used to stain the islets was prepared by adding together 4190 ul phosphate-buffered saline (PBS), 125ul FDA (stock solution 24umol l^{-1} in acetone, stored at -20°C, in covered bottle; Sigma) and 188ul PI (stock solution 95umol l^{-1} in PBS, stored at +4°C in covered bottle; Sigma).

10 islets (5 per slide) from each group were hand-picked onto a glass cavity microscope slide (circular cavity 15mm diameter, 0.8mm depth, white glass; Richardsons, Leicester, U.K.) and 50ul of the FDA/PI solution added to each. The slides were kept at room temperature in a darkened box for 45-60 minutes.

The islets were examined using an Olympus (Olympus, Tokyo, Japan) BH2 RFL microscope equipped for epi-illumination fluorescence microscopy. The ultraviolet light source was provided by a 100W high-pressure mercury burner. The microscope was fitted with a blue-green (B-G) dichroic mirror, DM-500 (0-515) - DM-580 (0-590). Blue light excitation was provided by a 490nm filter (BP-490) with supplementary filter (EY 455) reducing the lower wavelength excitation. Green light excitation was provided by a 545nm filter (BP-545). A 520nm (B-460) barrier filter was used with blue light excitation, a 610nm (R-610) barrier filter with green light excitation. The shutter slider was set at the empty aperture position for blue light excitation and at the neutral-density (ND) filter position for green light excitation. The light path selector was set at the half-way (CV) position. All fluorescence microscopy was performed with a 10x objective (A10xPL) and 10x eyepiece. Prior to fluorescence microscopy the cavity slide containing the islets was removed from the darkened box, placed on the microscope stage and the islet centred in the field of view using a low intensity halogen light source.

Live cells within the islets appeared bright green under blue light excitation, whilst dead and damaged cells showed up as red spots under green light excitation. A scoring system similar to that described by Gray and Morris (1987b) was used to assess the viability of the islets. Each islet was viewed as a sphere with three concentric layers and the number of red spots i.e. dead cells in each of the layers was scored on a scale of 0 to 5 as the microscope was focussed through the whole islet, with a score of 0 indicating no red spots and 5 indicating that all the cells were fluorescing red with no green fluorescence visible under blue light excitation. By adding all the scores together the viability of each islet was assessed. Islets with an overall score of 0 were considered to be fully viable while those with a score of 15 were totally dead. Figure 4.7 shows an islet under both blue and green light excitation.

4.4.v Perifusion

Perifusion experiments were undertaken in duplicate at weekly intervals over a period of four weeks, with the first experiment taking place seven days after islet isolation (i.e. 5 days after encapsulation). 50 islets from each group (encapsulated and non-encapsulated controls) were hand picked and placed in perifusion chambers on loosely-packed glass wool (Figure 4.8). The perifusion medium was Gey and Gey solution (0.11M NaCl, 0.27M $NaHCO_3$, 5mM KCl, 0.1mM $MgCl_2.6H_2O$, 0.28mM $MgSO_4.7H_2O$, 0.2mM KH_2PO_4, 0.8mM Na_2HPO_4; Gey and Gey, 1936) containing 0.1% BSA (RIA grade, lot 86F-07091, Sigma). The pH was maintained at 7.4 by continuous gassing with 95% air:5% CO_2. The chambers and perifusion solutions were maintained at 37°C in a water bath. The pump rate was set at 1ml min^{-1}.

The islets were perifused for one hour with low glucose medium i.e. 1.7mM glucose to allow a stable baseline insulin release to be attained. Samples were then collected for one minute every ten minutes during perifusion with low glucose medium for 20 minutes, high glucose medium (25mM glucose) for one hour, and low glucose medium for a further hour. Additional samples were collected at 1, 3, 5, 7 and 9 minutes after the change from low to high glucose (Warnock

i.

ii.

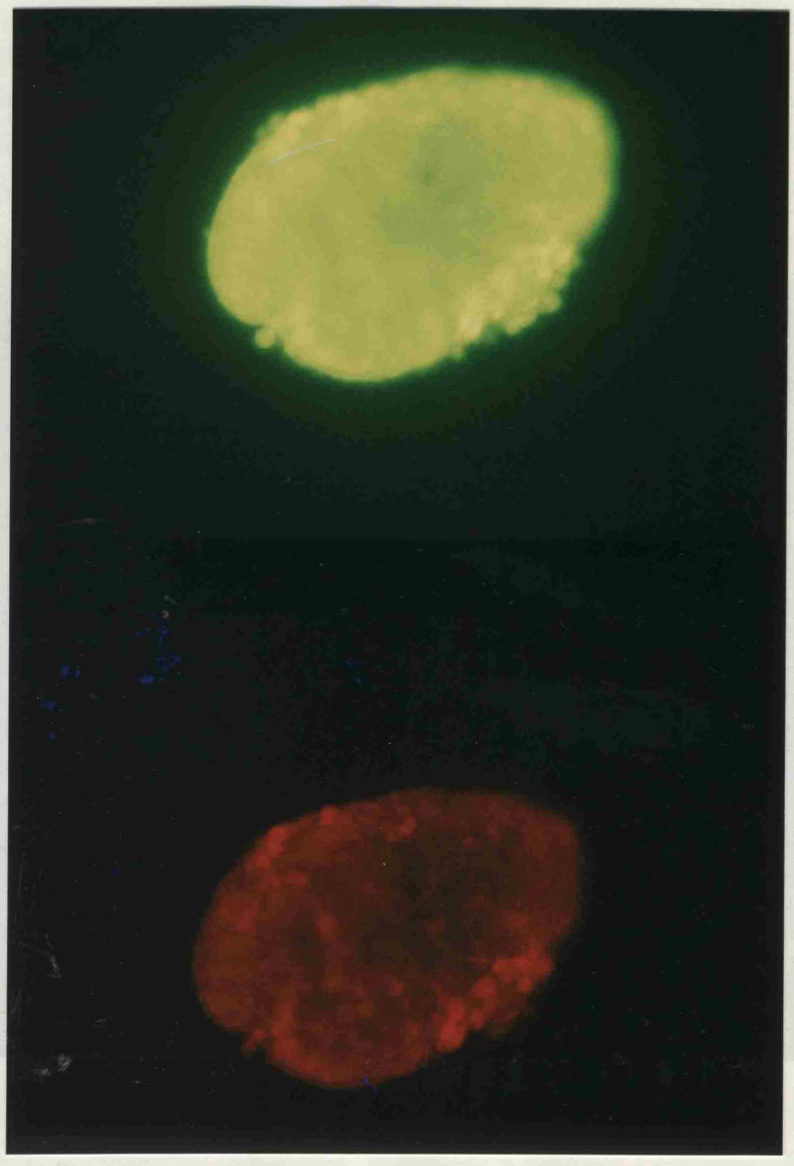


Figure 4.7
Live islet stained with FDA and PI under blue light (i) and green light (ii) excitation (x250).

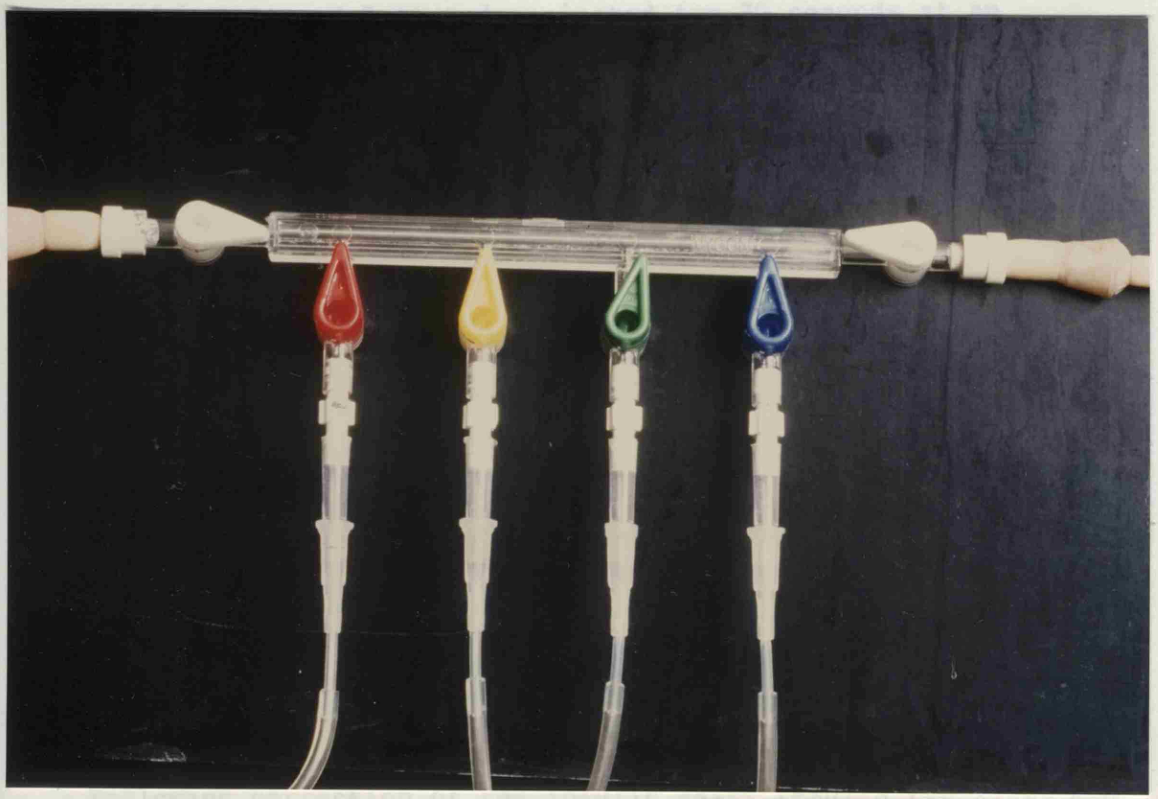


Figure 4.8
Perifusion chambers (x0.6).

et al. 1988). Samples were frozen at -20°C until assayed for insulin content. The insulin assay method is described in Appendix 1.

4.4.vi DNA assay

Following perifusion, the islets were recovered from the chambers, and the encapsulated islets treated with 5% sodium hexametaphosphate to dissolve the capsules. The islets were placed in an eppendorf tube with 1.5 ml DNA assay buffer (2M NaCl, 0.05M Na_2HPO_4, 2mM EDTA, pH 7.4) and sonicated for 20 seconds at an amplitude of 14 microns (Soniprep 150, MSE, Crawley, Sussex, U.K.). Aliquots were stored at -20°C until assayed for DNA content. The DNA assay is described in Appendix 1.

In order to determine whether the treatment of islets with sodium hexametaphosphate affected the islet DNA content, the following experiment was undertaken: 20 goups of 20 islets were handpicked into eppendorf tubes. 10 groups were untreated while the other 10 were vortexed with sodium hexametaphosphate and centrifuged twice to mimick the treatment of the encapsulated islets. The islets were then sonicated and aliquots frozen.

4.4.vii Analysis of perifusion data

Following correction of the insulin assay results for islet DNA content, the perifusion results were expressed as total insulin release by calculating the area under the perifusion curve (i.e. insulin release plotted against time, Kneteman et al. 1989), using a simple Fortran program. It was also possible to calculate the total insulin release during the low and high glucose perifusion periods using the same program, from which the stimulation increase and stimulation index could be calculated.

The stimulation increase (expressed as uU insulin/ng DNA/min) was defined as the mean insulin release during both low glucose periods subtracted from the insulin release during the high glucose period. This allows assessment of the responsiveness of the islets to

high glucose challenge to be determined in terms of the absolute insulin release. The stimulation index assesses the proportionate increase in insulin release and provides an indication of islet damage. This was calculated by dividing the insulin release during the high glucose period by the mean insulin release during the two low glucose periods (McKay and Karow, 1983).

The response time was defined as the time taken for the insulin release to reach twice the mean basal release following the change to high glucose perifusion medium. For this calculation, the mean basal insulin release was determined from the three readings in the first 20 minutes of low glucose perifusion. The lag time of the system (4 minutes) was taken into account in this calculation.

4.4.viii Statistical Analysis

The results from the experiment to determine whether sodium hexametaphosphate treatment of the encapsulated islets affected their DNA content were analysed by a Students t test.

4.5 RESULTS

4.5.i Microfluorometry

Figure 4.9 shows a photograph of an encapsulated islet stained with FDA/PI under blue light excitation.

Table 4.1 has the results of the scores for each group of islets. The numbers quoted are the median (range) scores for the 10 islets studied in each group at each time interval. No statistical analysis was undertaken on this data.

4.5.ii Perifusion

Figure 4.10 shows a typical example of an insulin release profile during perifusion in these experiments.

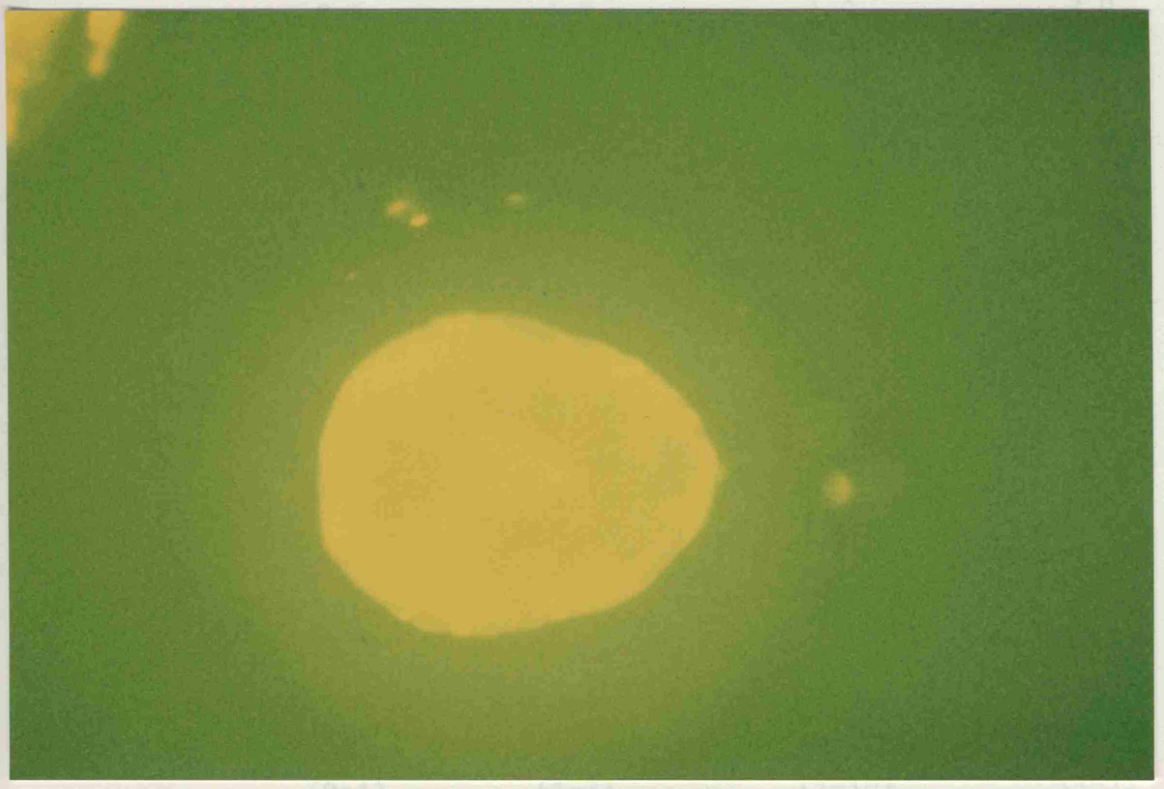


Figure 4.9
Encapsulated islet stained with FDA and PI under blue light excitation (x200).

Table 4.1

Results of the microfluorometric assay. The scores shown are the median (range) of 10 islets. (0=fully live; 15=fully dead.)

Days in culture	Free islets	PLL capsules	G-alginate capsules	M-alginate capsules
4	2.0 (1-4)	1.5 (1-2)	1.0 (1-4)	2.0 (1-4)
8	1.5 (1-4)	2.0 (2-4)	2.0 (1-3)	2.0 (1-4)
11	1.0 (0-2)	1.5 (0-3)	2.0 (1-4)	2.0 (1-3)
15	1.0 (1-2)	2.0 (1-3)	1.0 (1-2)	1.0 (1-3)
19	1.5 (1-2)	1.0 (0-3)	1.0 (0-3)	1.0 (1-3)
22	1.0 (0-1)	2.0 (1-3)	2.0 (1-10)	1.0 (1-3)
26	1.0 (1-3)	1.0 (1-2)	1.0 (1-2)	2.0 (1-3)
29	2.0 (1-5)	1.0 (0-2)	1.5 (1-5)	1.0 (1-5)

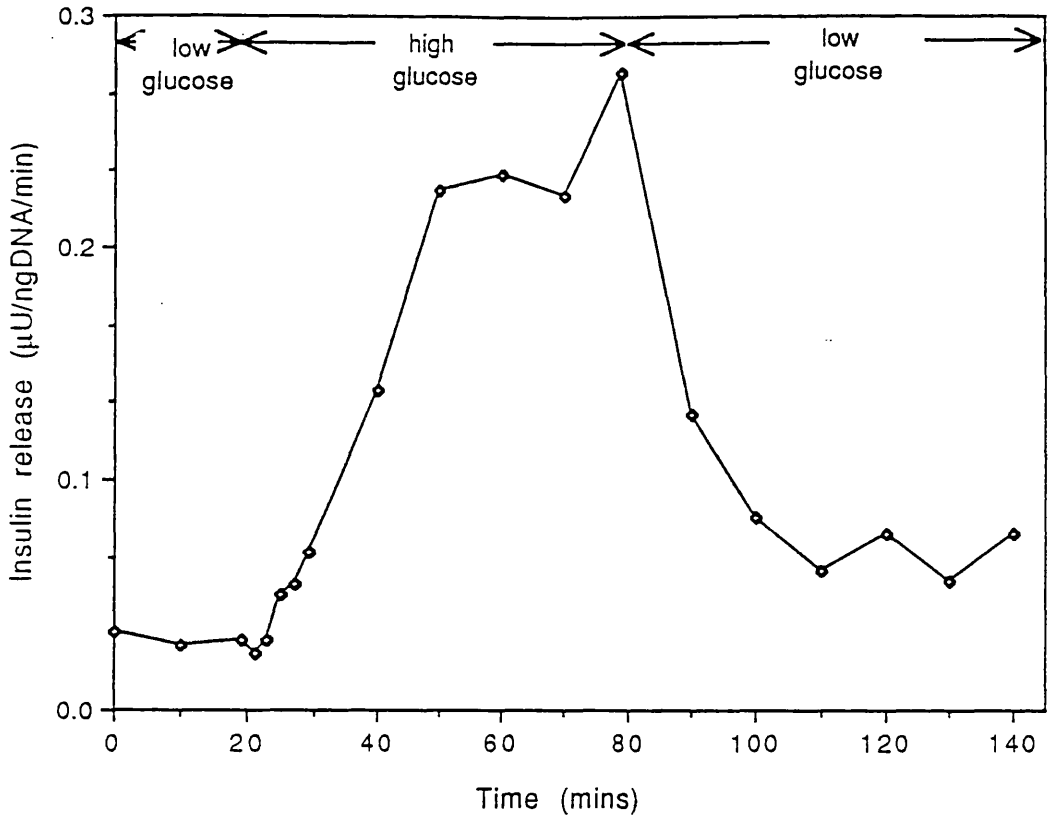


Figure 4.10
Example of a typical perifusion curve (M-alginate outer layer, week 4)

Figures 4.11 and 4.12 show the stimulation increase and stimulation index data respectively. The perifusions were undertaken in duplicate, and this data represents the mean of the two experiments.

Table 4.2 includes the response time of each group of islets for each perifusion experiment. Due to the degree of variation apparent in some of the duplicate sets of results, the mean response time was not calculated.

The data in Table 4.3 relates to the experiment which was undertaken to determine whether the sodium hexametaphosphate treatment of the encapsulated islets to remove the capsule prior to sonication affected the results of the DNA assay. Statistical analysis of this data using the Students t test showed that there was no significant difference between the amount of DNA in each group of islets ($p=0.11$).

4.6 DISCUSSION

4.6.i Microfluorometry

The results of the microfluorometric assay (Table 4.1) show that there was very little difference in the scores when all the groups were considered. Due to the nature of the data, statistical analysis was not considered appropriate. However, as the median value was never higher than 2 and only one islet had a score above 5 (G-alginate coated capsule, day 22), it was concluded that the presence of the capsule and its composition was not adversely affecting the membrane integrity of the islets as assessed by the microfluorometric assay.

4.6.ii Perifusion

The stimulation increase data (Figure 4.11) showed that the response of the non-encapsulated islets to an increase in glucose concentration was more marked than for the encapsulated islets. This means that the control islets were releasing greater absolute amounts

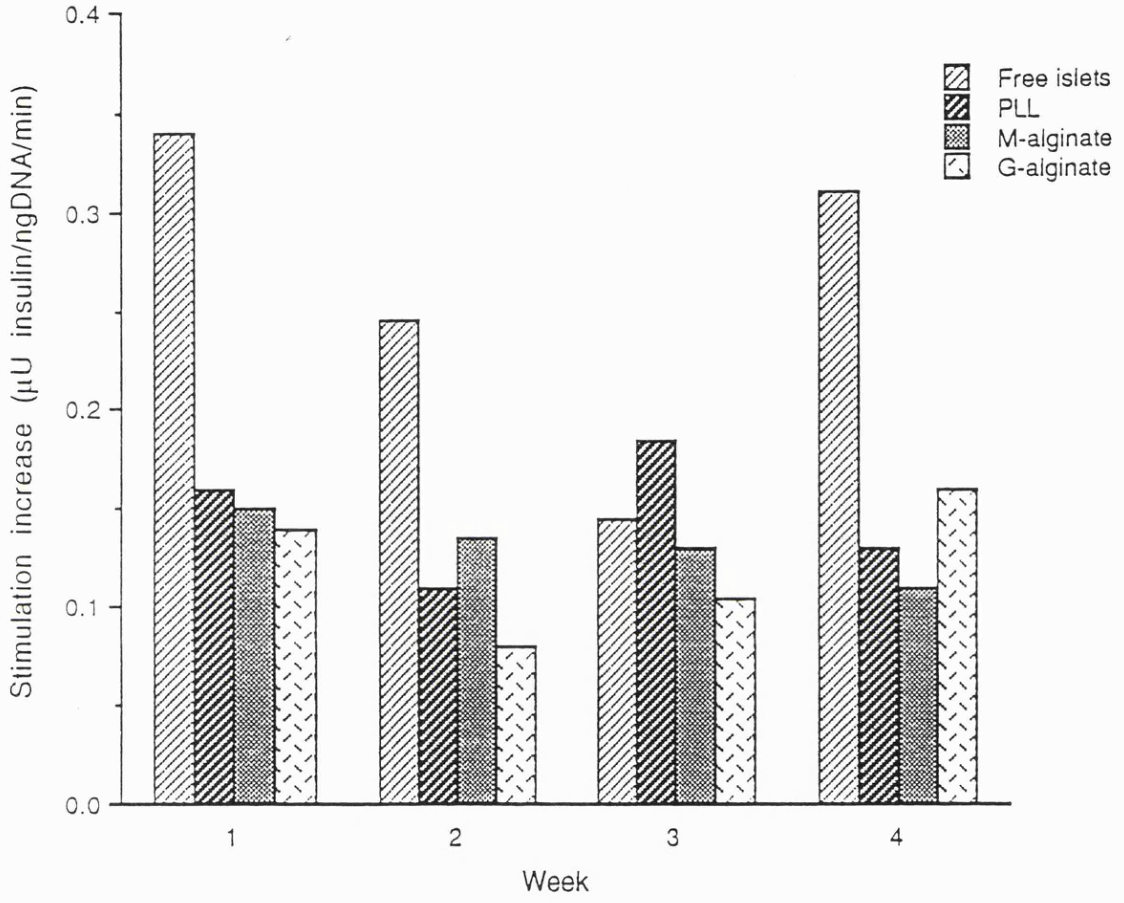


Figure 4.11
Mean stimulation increase data.

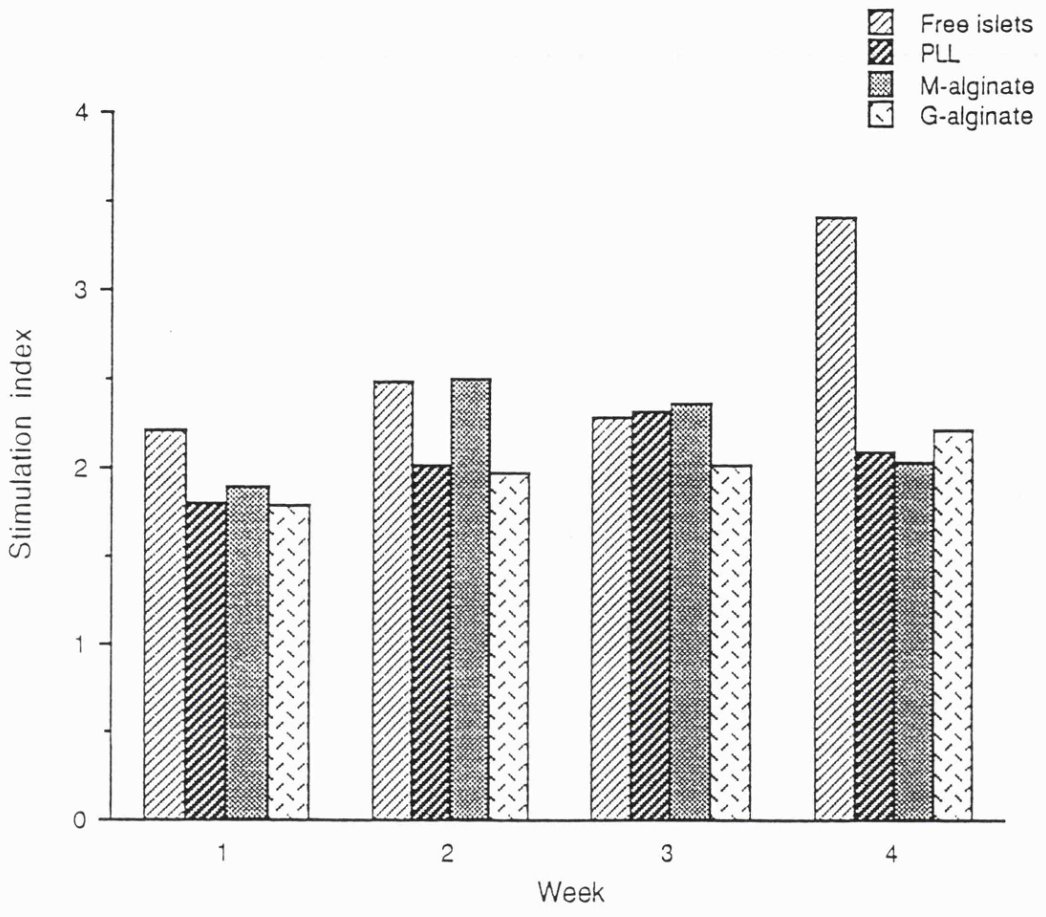


Figure 4.12
Mean stimulation index data

Table 4.2

Results of the time (in minutes) taken for the islets to respond to glucose stimulation during perifusion. Data from the duplicate experiments are shown separately as experiments 1 and 2.

	----Experiment 1---				---Experiment 2----			
Week	1	2	3	4	1	2	3	4
Free islets	18	26	7	6	7.5	5	3	0.5
PLL capsules	4.5	9.5	30	6.5	1	5	3	9.5
G-alginate capsules	3.5	1	2.5	5.5	0.5	6	4.5	11.5
M-alginate capsules	3.5	4.5	8.5	13.5	2	9	3.5	6.5

Table 4.3

Results of the experiment undertaken to determine whether sodium hexametaphosphate treatment to remove the capsule following perifusion affected the DNA content of the islets.(Results quoted as ng DNA per islet.)

Sample	Treated	Untreated	
1	4.2	8.1	
2	8.9	4.7	
3	4.7	12.8	
4	1.9	7.5	
5	6.6	7.0	
6	12.7	16.9	
7	8.9	9.4	
8	7.9	10.3	
9	10.8	15.0	
10	10.3	12.7	
Mean	153.7	208.5	(p=0.11)

of insulin than the encapsulated islets, and this was apparent after only one week in culture. Capsule composition did not appear to affect the results from the three groups of encapsulated islets. Due to the fact that all the insulin release results have been corrected for islet DNA content, and the results in Table 4.3 show that treatment to remove the capsules is unlikely to have affected the results of the DNA assay, the difference in total insulin release is considered to be a true reflection of the function of the islets and that encapsulation has caused a reduction in the amount of insulin the islets are able to release.

In contrast to the stimulation increase data, the stimulation indices (Figure 4.12) demonstrated similar results for all the groups involved, with the exception of the control islets on week 4 which suddenly showed an increase in this parameter. The reason for this increase is unknown. The similarity in stimulation index results demonstrates that although the encapsulated islets are unable to respond to an increase in glucose concentration by releasing the same absolute amount of insulin as the control islets, their ability to respond when considered as a proportion of the basal release is unaffected. These results also indicate that the apparent reduction in insulin release from the encapsulated islets is a reflection of the insulin output during both basal and stimulated release, and does not relate only to a decreased ability to respond to the glucose challenge.

These results suggest that the islets have not been damaged by the encapsulation process, as this would be reflected in the stimulation index due to the inability of damaged islets to return to a basal level of insulin secretion following a return to low glucose perifusion medium (McKay and Karow, 1983). The reason for the decreased absolute insulin release is unknown. Any loss of cells from the islets during encapsulation leading to a decrease in islet size and therefore insulin release (Hopcroft et al. 1985), would be accounted for by correction of the results for DNA content. The implication of these results, however, is that the number of encapsulated islets required to reverse diabetes would be considerably greater than the number of unencapsulated islets. The reports on encapsulated islet transplants (see Table 3.1) have tended

to use 4500-5000 encapsulated islets which is considerably greater than the numbers quoted for free islets (for example, 750 islets required to reverse diabetes when transplanted in the renal subcapsular space; Loftus et al. 1991). However, a major consideration is that very few studies on islet transplantation have used the peritoneal cavity as the implantation site, so a direct comparison of the numbers of islets required to reverse diabetes when transplanted under the kidney capsule or intraportally could not be made with transplantation into the peritoneal cavity. One recent study (Fritschy et al. 1991) determined that 1400-1500 islets when implanted into the peritoneal cavity were able to maintain normoglycaemia and normal growth in diabetic rats. However, even if the number of islets transplanted was increased to 3000, none of the recipients displayed a normal response to IVGTT.

The response time data (Table 4.2) was quite variable amongst all the groups. This variability meant that calculation of the mean response times from the duplicate experiments was inappropriate and consequently statistical analysis was not performed on the data. It is worth noting, however, that on the occasions when a group of islets took a particularly long time to respond to the increase in glucose concentration, the response time of the islets in the duplicate experiment was much shorter. For example, the control islets in the first experiment on week 2 took 26 minutes to respond, but in the duplicate experiment this was cut to only 5 minutes. Similarly, the PLL coated encapsulated islets in experiment 1, week 3 showed a response time of 30 minutes compared with 3 minutes in the second experiment.

It was concluded from these results therefore, that there was not any consistent difference in response times between the four groups of islets, and that the occasional differences between duplicate experiments were a result of the problems inherent in using biological tissue in experimental systems. All the islets had been treated in the same manner, and as all the groups were perifused at the same time due to the use of four chambers in the perifusion system, differences in the way in which the islets had been handled were unlikely to have occurred. However, air bubbles occasionally lodged in the perifusion chambers, and these could have affected

insulin release from the islets leading to an increase in the time taken to reach twice the basal insulin release following perifusion with high glucose medium.

It is of great interest to note that the presence of the capsule around the islet does not appear to have caused an increase in the response time of the islets to glucose challenge. This result is encouraging for the success of future transplant experiments, as the ability of islets to respond immediately to changes in blood glucose is essential to the successful function of an islet graft. The use of intravenous glucose tolerance tests following islet transplantation should confirm the ability of encapsulated islets to respond rapidly to increased blood glucose levels, and thus establish their efficacy as a treatment for diabetes mellitus.

4.7 SUMMARY

The microfluorometric assay and perifusion results have demonstrated excellent in vitro viability of both the non-encapsulated and encapsulated islets for up to four weeks after isolation. Capsule composition does not appear to affect any of the results assessed.

The FDA/PI stain is a rapid method of assessing islet membrane integrity, however the extra information gained from the perifusion experiments is considerable, and has highlighted the decreased absolute insulin release from the islets following encapsulation, a factor which could not have been determined from viability staining.

It is interesting to note the differences apparent in reports of insulin release studies which have shown encapsulated islets to release the same amounts of insulin with the same response times (Lim and Moss, 1981; Leung et al. 1983; Fritschy et al. 1989), the same amount of insulin with a delayed response time (Lim and Sun, 1980; Sun et al. 1987), or the same response time with an increase in insulin release (Lynch et al. 1991). No other reports have described a reduction in the amount of insulin released together with the same response time, however the results from the other studies were not

corrected for islet DNA content. Also, only the study reported by Lynch et al. (1991) determined the effect of time in culture on the insulin release from the islets. The results of encapsulated islet transplants should help to elucidate the problems of relative insulin release by allowing comparison of the numbers of non-encapsulated and encapsulated islets needed to reverse diabetes, and response times of non-encapsulated and encapsulated islets by performing intravenous glucose tolerance tests (IVGTT).

CHAPTER 5

THE BIOCOMPATIBILITY OF ALGINATE - POLY-L-LYSINE CAPSULES

5.1 INTRODUCTION

The results obtained from the in vitro experiments described in the previous chapter were encouraging in that the encapsulated islets remained viable for up to four weeks in culture. The presence of the capsule affected the total amount of insulin released from the islets but not the response time of the islets or the proportionate increase in insulin released in response to an increase in glucose concentration. In addition, there were no differences in the results from the islets encapsulated in the three capsule compositions.

Biocompatibility has been defined as the "ability of a material to perform within an appropriate host response in a specific application" (Williams, 1987). Having determined the excellent in vitro insulin release of encapsulated islets the next stage of the project was to assess the biocompatibility of the capsules. The information required was whether the capsules could survive in the host or whether they provoked an adverse reaction, and to determine whether capsule compostion affected their biocompatibility as has been suggested by O'Shea et al. (1984).

5.2 MATERIALS AND METHODS

5.2.i. Capsule compositions

Previous reports have shown that capsules containing islets provoke a more severe response than empty capsules (Weber et al. 1990; Calafiore et al. 1989). Therefore, the studies to assess the biocompatibility of the different capsule compositions were undertaken with empty capsules rather than with capsules containing islets to prevent the presence of the islets from affecting the results.

The same three capsule compositions were used as for the in vitro studies i.e. an inner core of M-alginate coated with PLL alone, PLL with an outer layer of G-alginate, or PLL with an outer layer of M-alginate.

5.2.ii Transplant recipients

Four strains of rat were used as recipients. These were the Wistar-derived WAG/Ola rat (supplied by Biomedical Services Unit, University of Leicester), the PVG nude rat (supplied by National Institute for Medical Research, Mill Hill, U.K.), the diabetes-prone BioBreeding (BB/d) rat and the non-diabetes prone BioBreeding (BB/ndp) rat (both supplied by Dr. P. Thibert, Ottawa, Ontario, Canada, and maintained by Biomedical Services Unit, University of Leicester).

The WAG/Ola rat was used to determine the response to the capsules in a standard laboratory rat, acting as a control for the other strains. The nude rat is athymic and is unable to mount a T cell mediated response (Festing, 1981), although it is capable of mounting a classical foreign body reaction. Implantation of capsules into these animals would determine whether T cells were involved in a reaction mounted against the capsules. The BB/d rats, which were all confirmed to be diabetic before implantation of the capsules, were used because this strain would ultimately be used as recipients for encapsulated islet transplants. The BB/ndp rats were used as a control for the BB/d rats to determine whether the diabetic state of these animals and the autoimmune nature of the condition had any affect on reaction to the capsules, and also because some of the initial transplant work would be undertaken in the BB/ndp rats. The details of the BioBreeding strain of rats has already been discussed in Chapter 2.

5.2.iii Capsule transplantation and retrieval

The initial capsule biocompatibility study was undertaken using the renal subcapsular space as this is one of the preferred sites for islet transplantation as already discussed in Chapter 2, and also allows histology to be easily carried out as the whole graft can be removed by nephrectomy. The other commonly-used site for islet transplantation is the liver via the portal vein. However, this was not used for encapsulated islet transplantation as this would almost

certainly lead to occlusion of the small vessels and to hepatic infarction due to the size of the capsules, and so was not considered as a possible implantation site.

The recipients were anaesthetised with fluothane, the kidney exposed, and a scalpel blade used to gently incise the kidney capsule. Approximately 50 capsules were placed in the renal subcapsular space. A plasma clot, using plasma from the recipient strain, was formed around the capsules in order to make them easier to implant. Some animals received just a plasma clot i.e. the negative control, while some received a thick paste of talc in saline to act as the positive control, as talc is known to provoke a severe, classical foreign body reaction (Postlethwait et al. 1949). Two animals of each strain were implanted with each type of capsule, plasma clot or talc.

While this work was being undertaken, it became apparent that the renal subcapsular space would not provide a sufficiently large volume to implant enough encapsulated islets to reverse diabetes. It was possible to place approximately 200 capsules under each kidney without causing too much damage to the capsule. Even if both kidneys were used, 400 islets would not be sufficient to reverse diabetes as this is below the minimum number of free islets required to reverse diabetes (Loftus et al. 1991). In addition, from the projected increase in the number of encapsulated islets relative to non-encapsulated islets which would have to be transplanted, it was apparent that an alternative site would have to be sought. The site considered to provide sufficient space to transplant encapsulated islets was the peritoneal cavity, the only site successfully used by other groups for encapsulated islet transplants (see Table 3.1).

In order to determine the biocompatibility of the capsules in the peritoneal cavity, approximately 1000 capsules suspended in saline were implanted via a small midline incision into the four strains of rat described above, while under fluothane anaesthesia. A thick paste of talc was also placed between the liver and diaphragm to act as the positive control. Two animals of each strain were transplanted with each type of capsule and with talc.

After three weeks had elapsed, the animals were sacrificed. The kidney from the animals which had received capsules, talc or a plasma clot into the renal subcapsular space were removed and fixed in formalin. The intraperitoneal capsules and talc were also removed and placed in formalin. Some of these capsules were still free-floating and were washed out with saline, whilst others had adhered to the internal organs and these had to be resected. The samples were processed by the Department of Pathology, University of Leicester. Processing of the free-floating capsules involved placing them in warm agar, allowing the capsules to sink, then sectioning the agar and capsules when the agar had set. All sections were stained with haematoxylin and eosin.

5.2.iv Immunohistochemistry

In order to determine the nature of the reaction to the capsules and talc, samples from the peritoneal cavity of the BB/d rats were taken for staining with monoclonal antibodies. Samples were embedded in Tissue-Tek O.C.T. compound (Miles Scientific, Naperville, Illinois), then frozen and stored in liquid nitrogen.

Five micrometer sections were cut and mounted onto gelatin-coated glass microscope slides. The samples were fixed in acetone for 10 minutes, dried, and 100ul of mouse anti-rat monoclonal antibody (diluted 1:50 with fluorescence medium; MEM containing 10% foetal calf serum and 0.02% sodium azide) added to each section. Sections of rat spleen were routinely stained with the panel of antibodies as a positive control for the staining process. Negative control sections were incubated with fluorescence medium alone to determine the intensity of the background staining. The following mouse anti-rat IgG antibodies were used:

OX33	B lymphocytes,	(Serotec, Oxford, U.K.)
OX34	$CD2^+$ T lymphocytes/thymocytes	" " "
OX18	MHC Class I	" " "
ED1	Macrophages/monocytes	" " "
ED2	Tissue macrophages, mature macrophages in the peritoneum	" " "
ED3	Lymphoid organ macrophages	" " "
OX19	$CD5^+$ T lymphocytes/thymocytes	(Seralab, U.K.)
OX8	$CD8^+$ T lymphocytes/thymocytes	" "
OX4	MHC Class II	" "

The sections were incubated overnight at 4°C in a flat box containing damp tissues to prevent the sections from drying out. They were then washed with tris buffered saline (TBS) and 100ul of the secondary layer of anti-mouse IgG biotin conjugate (diluted 1:1000 with TBS; Sigma) added for 45 minutes. This was washed off with TBS and 100ul of the tertiary layer of extravidin alkaline phosphatase conjugate (diluted 1:400 with TBS; Sigma) added for 20 minutes. Following a further wash with TBS, 100ul of chromogenic substrate (10mg naphthol AS-BI phosphate, 10mg fast red, 100ul levamisole, 10ml tris buffer) were added for 15 minutes and washed off with water. The sections were counter-stained with acid haematoxylin (Sigma) for 5 minutes, rinsed with water, dried and mounted with glycerol gelatin and a glass cover slip. The detection system used caused positive cells to stain red against a blue counter-stain.

5.2.v Electron microscopy

In addition to the immunohistochemistry, samples were fixed and processed for electron microscopy to help determine the nature of the reaction to the capsules. All these samples were processed by the Electron Microscopy Department, University of Leicester.

5.2.vi Assessment of the pericapsular reaction

It was possible to directly measure the thickness of the cellular reaction to the capsules placed in the renal subcapsular space. Serial sections of these samples were stained, and measurements (um) were made on section numbers 1, 2, 5, 7 and 10, in at least two places around each capsule present on the section. The mean of these measurements was calculated.

Samples collected from the peritoneal cavity posed a greater problem, as many of the capsules had been broken or had collapsed, making direct measurement of the infiltrate thickness difficult and potentially unrepresentative of the situation. Consequently a scoring system was devised to determine the severity of the reaction. A scale of 0-10 was used in which 0 indicated no cellular reaction and 10 indicated severe pericapsular fibrosis, possibly with signs of capsule rupture.

All the sections were examined independently by two observers who were unaware of the capsule composition or the strain of rat on each section. In cases where several sections were assessed for one animal, the mean of the scores was calculated.

In order to verify this method as a suitable means of assessing the degree of fibrosis, the sections from the renal subcapsular samples were also scored "blind" by the same individuals, and the mean measurements analysed using the Spearman rank correlation test to determine how closely the visual scores and physical measurements correlated.

5.3 RESULTS

5.3.i Reaction to renal subcapsular samples

Figure 5.1 shows the mean thickness of the pericapsular reaction to the capsules placed in the renal subcapsular space. The corresponding scores are shown in Table 5.1. The results for the talc samples are also shown in this table.

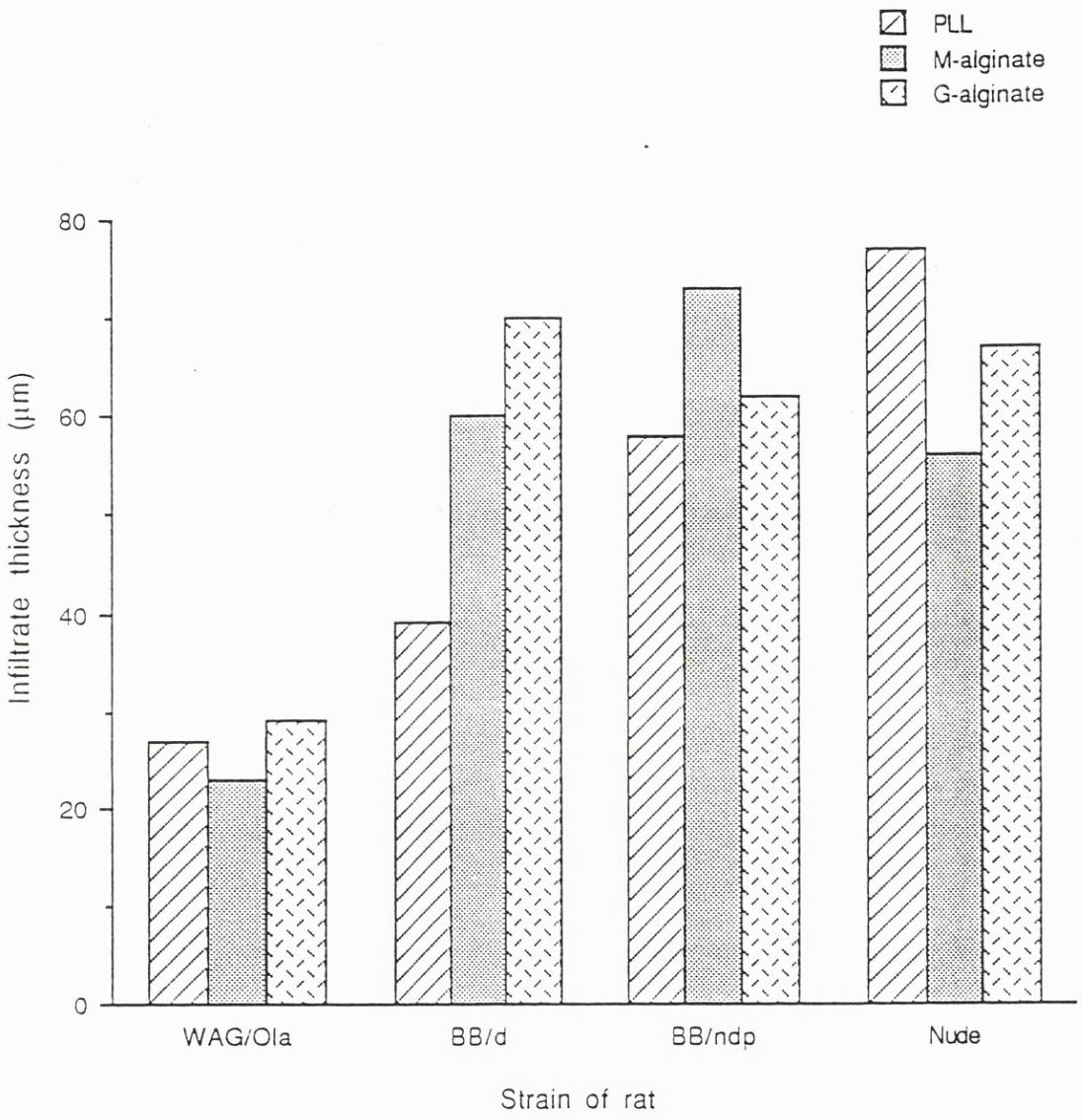


Figure 5.1
Thickness (µm) of pericapsular reaction around capsules implanted in the renal subcapsular space

Table 5.1

Results of the scoring system for the renal subcapsular and intraperitoneal samples. The mean scores allocated by both observers (1 and 2) are shown together with the overall mean. The score for the reaction to talc is also shown. (0 = no inflammation; 10 = maximal inflammation.)

Strain of rat	Capsule Composition	Kidney			Peritoneum		
		1	2	Mean	1	2	Mean
WAG/Ola	PLL	2	1.5	1.75	3.5	6	4.75
	G-alginate	1	1.5	1.25	6	1	3.5
	M-alginate	1	1	1	1	0	0.5
	Talc	4	4	4	9.5	9.5	9.5
BB/d	PLL	5	5	5	8.5	10	9.25
	G-alginate	6	5	5.5	7.5	10	8.75
	M-alginate	4	6	5	4.5	7.5	6
	Talc	5	4	4.5	10	10	10
BB/ndp	PLL	2.5	5	3.75	4	2	3
	G-alginate	5.5	5	5.25	3	2.5	2.75
	M-alginate	7	9	8	1.5	0.5	1
	Talc	5	5	5	9.5	10	9.75
Nude	PLL	5	6	5.5	7	5	6
	G-alginate	7.5	4.5	6	6.5	6.5	6.5
	M-alginate	6.5	4.5	5.5	5.5	7	6.25
	Talc	8	6	7	10	9.5	9.75

The results of the correlation between the measured infiltrate thickness and the scores indicated a highly significant correlation (DF=10, r=0.87, $p<0.002$).

Figures 5.2.i and 5.2.ii show photographs of the haematoxylin and eosin stained sections of the reaction to the renal subcapsular capsules coated with M-alginate in the WAG/Ola and BB/d rats respectively.

5.3.ii Reaction to intraperitoneal capsules

The results of the scores for the capsules from the peritoneum are shown in Table 5.1. This table includes the results of the scores given to the talc samples.

Figure 5.3 is a photograph of capsules which had been washed out of the peritoneal cavity with saline.

Figures 5.4.i and 5.4.ii show photographs of the haematoxylin and eosin stained sections of M-alginate coated capsules retrieved from the peritoneal cavity of the WAG/Ola and BB/d rats respectively.

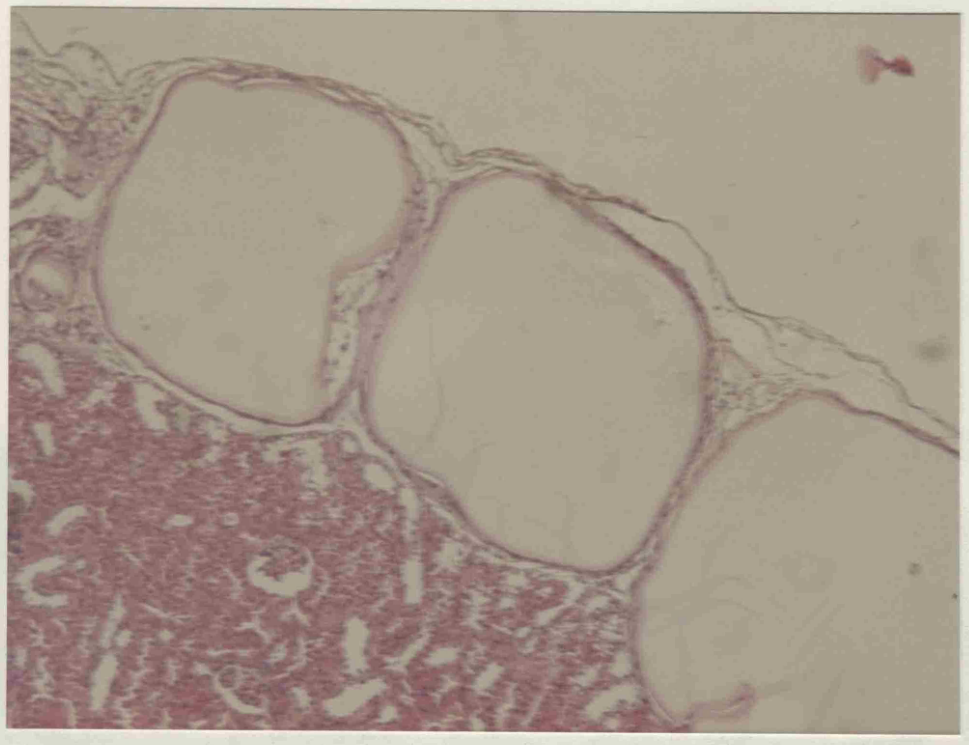
5.3.iii Immunohistochemistry

Table 5.2 contains the results of staining frozen sections of both capsules and talc from the peritoneal cavity of the BB/d rat with a panel of monoclonal antibodies. Positive results were gained with the ED1 and ED2 antibodies. Figures 5.5.i and 5.5.ii show photographs of capsules stained with these two antibodies.

5.3.iv Electron microscopy

Figures 5.6.i and 5.6.ii are electron micrographs of the samples taken from the peritoneal cavity of BB/d rats. These indicate the presence of macrophages and fibroblasts.

i.



ii.

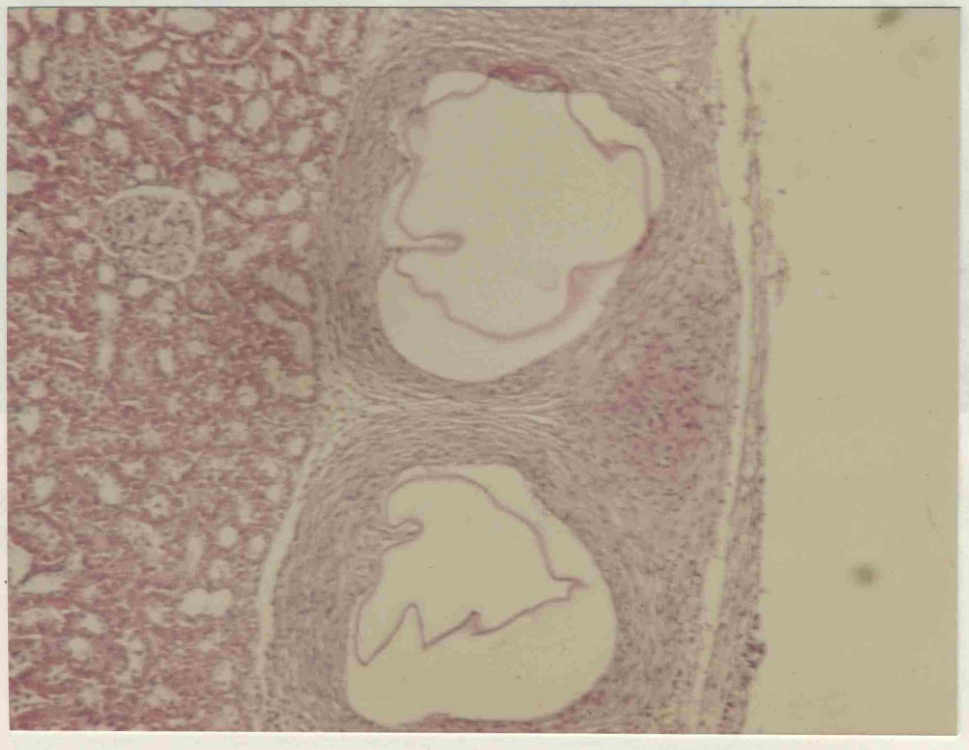


Figure 5.2
M-alginate coated capsules in the renal subcapsular space of the WAG/Ola rat (i) and the BB/d rat (ii) after three weeks (H and E) (x100).

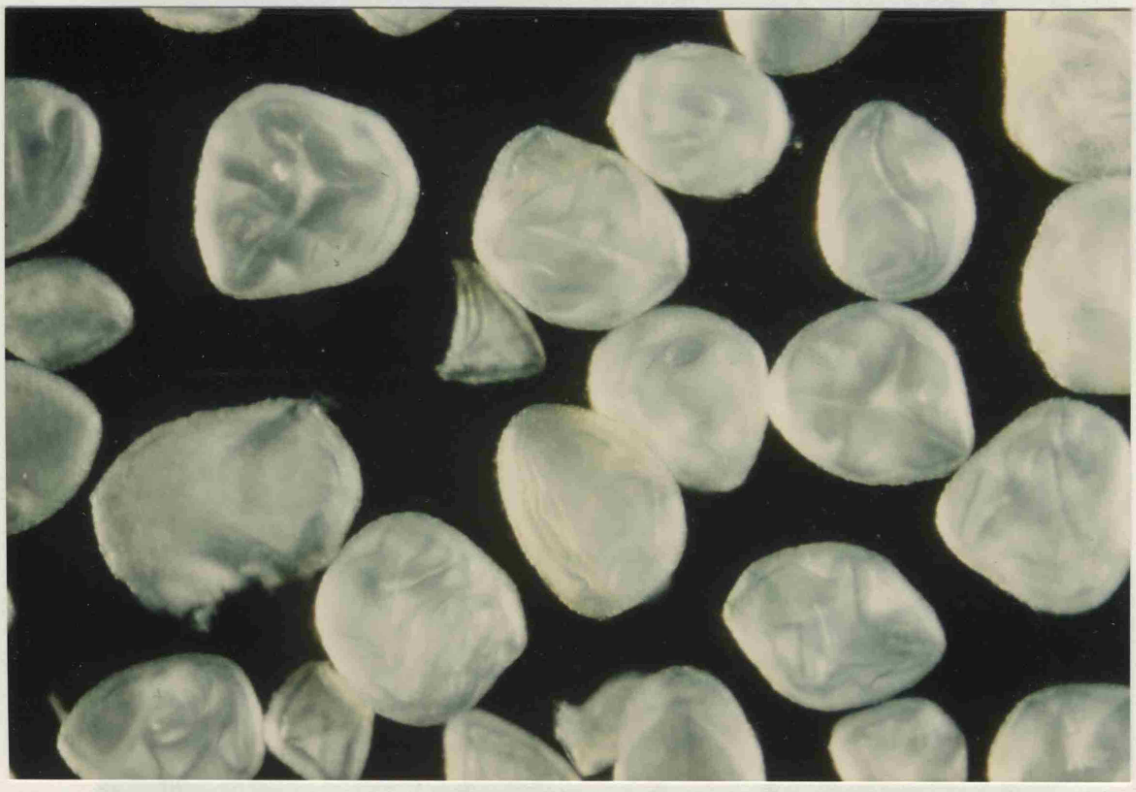
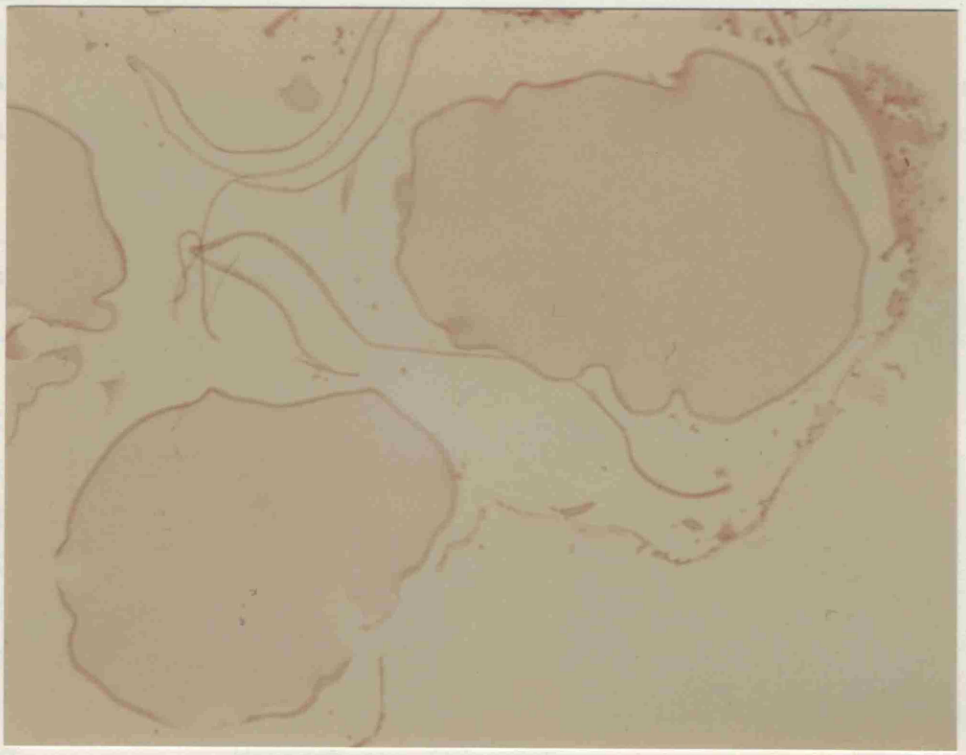


Figure 5.3
Capsules washed out of the peritoneal cavity.

i.



ii.

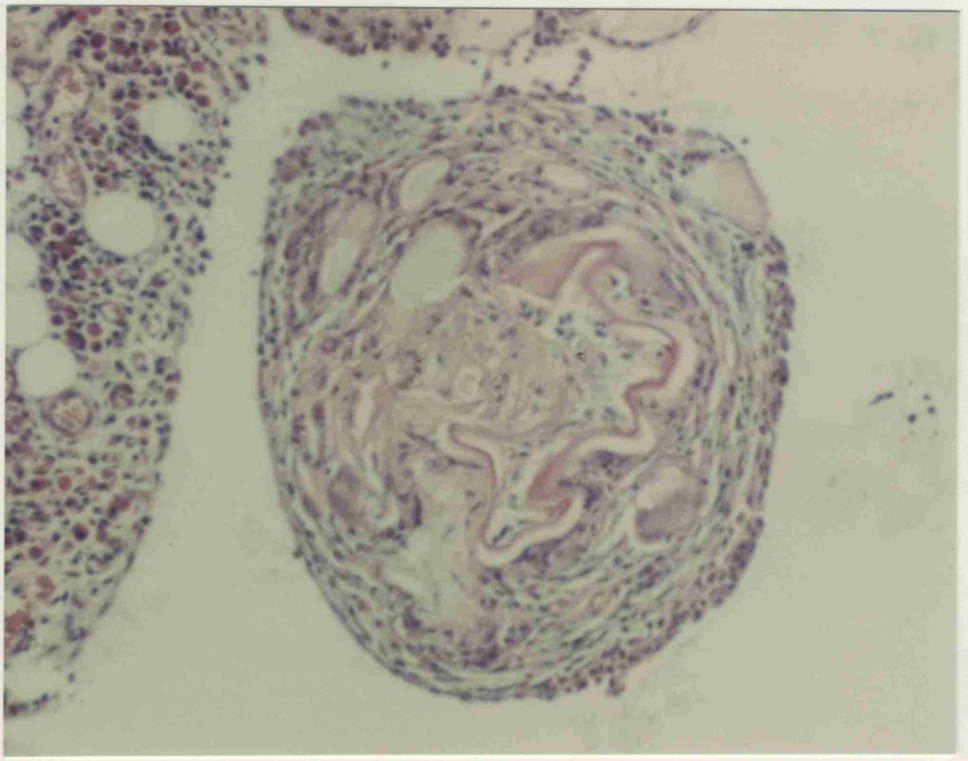


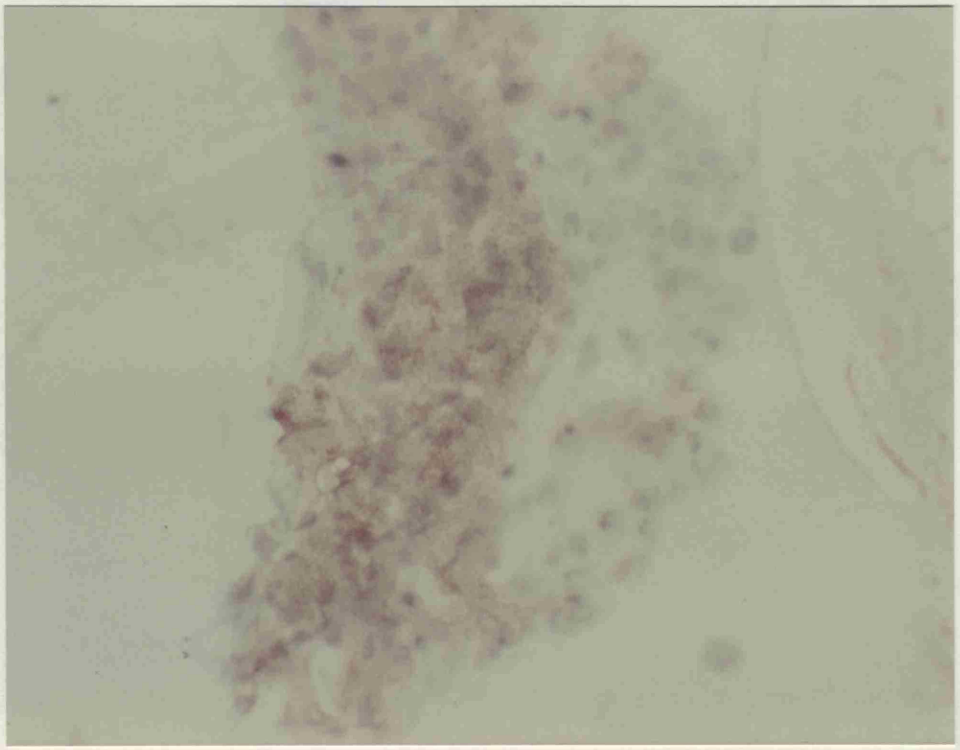
Figure 5.4
M-alginate coated capsules from the peritoneal cavity of the WAG/Ola rat (i) and BB/d rat (ii) after three weeks (H and E) (x100).

Table 5.2

Results of the immunohistochemistry of the capsule and talc sections from the BB/d rat. (+ denotes a positive stain; - denotes no staining.)

Antibody	Specificity	Capsules	Talc
OX 18	MHC class I	-	-
OX 4	MHC class II	+	+
OX 8	$CD8^+$ T cells	-	-
OX 19	$CD5^+$ T cells	-	-
OX 34	$CD2^+$ T cells	-	-
OX 33	B lymphocytes	-	-
ED1	Monocytes/macrophages	+	+
ED2	Macrophages (tissue/ peritoneum)	+	+
ED3	Macrophages (lymphoid organs)	-	-

i.



ii.

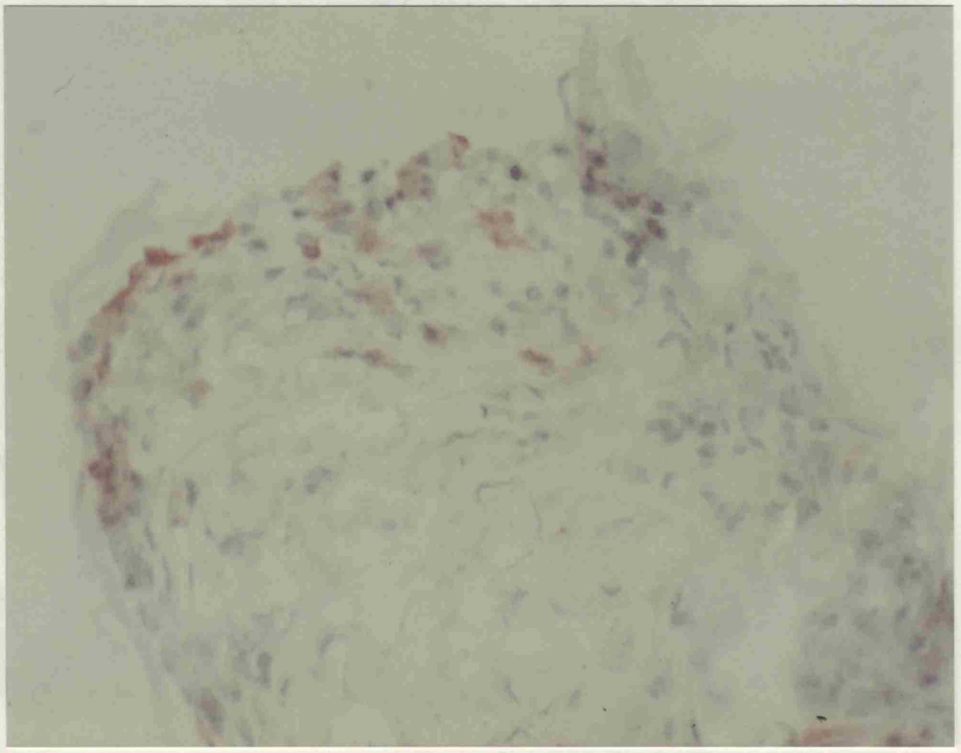


Figure 5.5
M-alginate coated capsules from the peritoneal cavity of the BB/d rat stained with the ED1 (i) and ED2 (ii) monoclonal antibodies (x800).

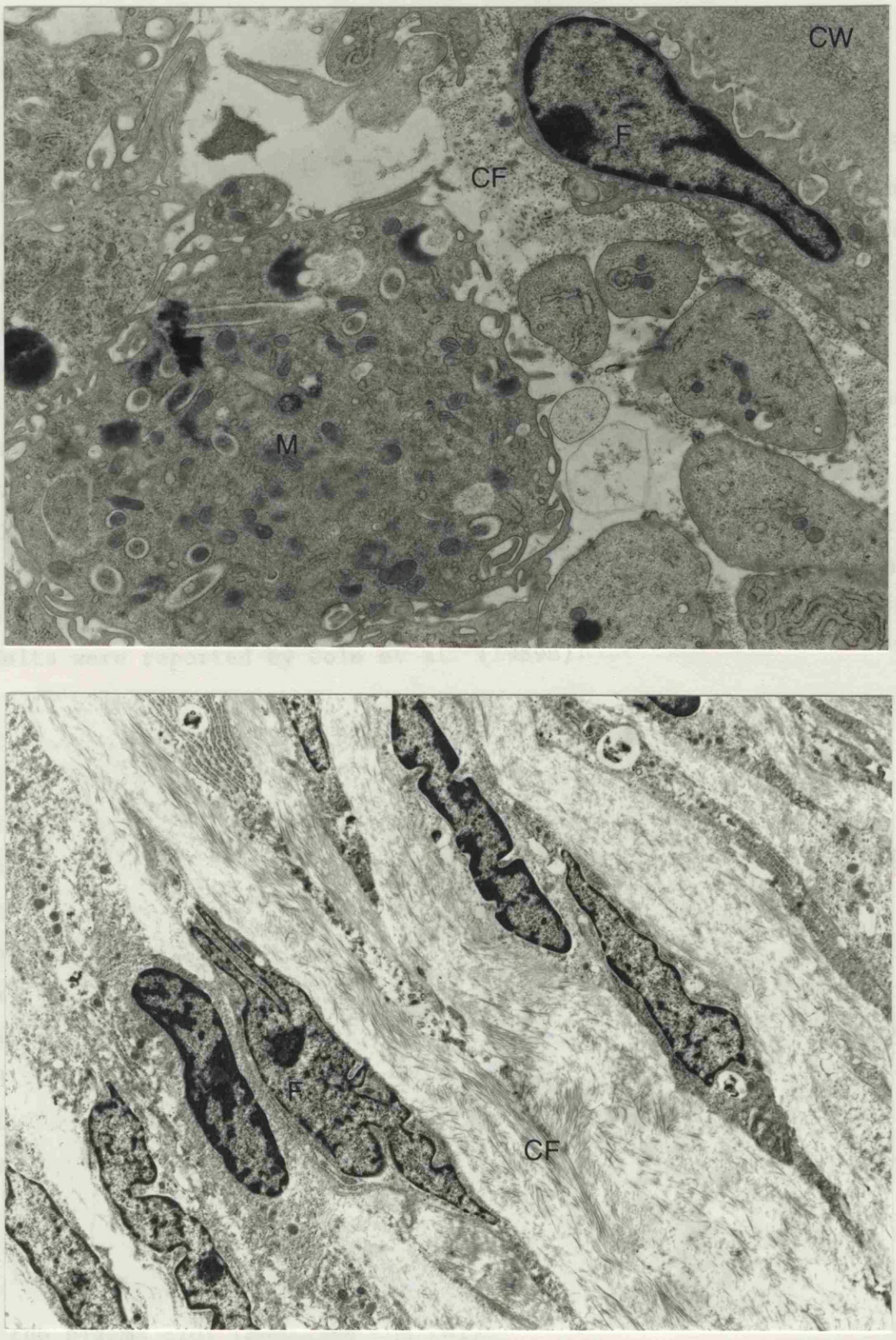


Figure 5.6
Electron micrographs (x6500) of capsules from the peritoneal cavity of the BB/d rat. (M = macrophage; F = fibroblast; CF = collagen fibres; CW = capsule wall).

5.4 DISCUSSION

5.4.i Pericapsular fibrosis of the capsules

a. Renal subcapsular space

The results of the reaction to the capsules placed in the renal subcapsular space (Figure 5.1 and Table 5.1) indicated that there was a difference in the response to the capsules mounted by the different strains of rat, with the WAG/Ola rats displaying the weakest response, and the other three strains all showing a similar, more pronounced reaction. This was interesting as both the WAG/Ola rats and the BB rats are derived from the Wistar strain, and as the BB/ndp animals do not suffer from the autoimmune diabetic state, such a notable difference was unexpected. It also suggested that the diabetic condition of the BB/d animals was not affecting the severity of the reaction to the capsules, as there was no difference between the reaction to the capsules in the two strains of BB rat. Similar results were reported by Cole et al. (1989b).

Several other features were apparent from these results. Firstly, the nude rats displayed a marked reaction to the capsules indicating that the reaction was a classical foreign body reaction (FBR) and was not a T cell mediated reaction. Secondly, the results have shown that when implanted in the renal subcapsular space, the capsule composition did not appear to affect the severity of the reaction. Thirdly, the highly significant correlation between the measured infiltrate thickness around the renal subcapsular capsules and the corresponding scores indicated that the scoring system was a valid method to assess reaction to the capsules. The validity was also confirmed by the similarity in the independently-awarded scores of the two observers.

No reaction was detected in any of the animals to the presence of the plasma clot (negative control).

b. Peritoneal cavity

The results of the reaction to the intraperitoneal capsules indicated some fundamental differences from the reaction provoked by

the capsules placed in the renal subcapsular space. With the exception of the BB/ndp rats, the reaction in the peritoneal cavity was more severe than the corresponding capsule composition in the renal site; there was a considerable difference in the reaction between the BB/ndp and the BB/d rats; capsule composition did affect the severity of reaction.

The increased reaction to capsules in the peritoneal cavity relative to the renal subcapsular space was also observed by Cole et al. (1989b) and was perhaps not surprising as macrophages comprise a large proportion of the cell population in the peritoneal cavity (Kubica and Olszewski, 1989; Puntis, 1989) and these cells play a prominent role in the inflammatory response (Lord, 1986; Pizzoferrato et al. 1986). In 1982, Jalkanen and Penttinen described the release of a factor from macrophages which enhances collagen synthesis from fibroblasts. A chain of reactions such as this would obviously lead to fibrosis of the capsules once the macrophages had been stimulated by their presence. The increased response in the peritoneal cavity was also demonstrated by the reaction to talc, indicating that material placed in the peritoneal cavity will provoke a more severe reaction relative to the same material placed in the renal subcapsular space. The reason for implanting capsules in the peritoneal cavity has already been discussed, and unless the biocompatibility of the capsules could be improved to prevent such severe fibrosis, the relatively hostile cell population of the peritoneal cavity will remain an obstacle.

One factor which should be considered when interpreting these results is the report by Siebers et al. (1990) in which it was noted that when artificial membranes were implanted into mice and rats, the response in mice was weaker, indicating that a species variation in response to the capsules may exist. This factor was not investigated in this study.

The increased severity in reaction to the capsules in the BB/d rats relative to the BB/ndp rats was in contrast to the results from the renal samples, and suggested that a factor related to the diabetic condition of these animals may have caused the increased reaction. It is not known whether this difference in response was due

to the diabetic condition of the animals, the autoimmune nature of the diabetes, the T lymphopaenic state of the BB/d rats (Poussier et al. 1982), or a combination of these factors.

The severe nature of the reaction in the nude rats suggested that T cells were not involved in the response, possibly indicating that the T lymphopaenia present in the BB/d rats would not affect their reaction to the capsules. However, it is possible that the cells involved in the FBR to the capsules are normally regulated by T cell subsets which are under-represented in the BB/d animals, thus leading to the increased response.

One factor related to the diabetic state of the BB/d animals which could have affected the severity of the reaction to the capsules is the changes apparent in the regulation of haemostasis. Diabetic patients have been demonstrated to have an increased susceptibility to thrombosis due to changes in clotting factors, increased platelet aggregation and inhibition of fibrinolysis (Ambrus et al. 1979; Brownlee et al. 1983; Brownlee et al. 1984; Ceriello et al. 1989; Ceriello et al. 1990; Hamet et al. 1983; Jones, 1985; Sandler et al. 1988). A similar situation has also been detected in BB/d rats and in Wistar rats with streptozotocin-induced diabetes (Hamet et al. 1983). This situation could, in part, be a factor leading to the increased fibrosis in the BB/d rats. A comprehensive review of the changes in the haemostatic system of diabetic patients has been written by Ostermann and van de Loo (1986).

The reason for the difference in response to the capsules in the peritoneal cavity between the BB/d and BB/ndp rats, when reaction to the capsules in the renal subcapsular space was the same is unknown. The generally increased reaction may have caused any minor differences to become highlighted in this site. It is also interesting to note that the reactions were similar for both the BB/ndp and the WAG/Ola rats, a situation which might also have been expected in the renal subcapsular samples due to the fact that both strains are derived from the Wistar rat.

Perhaps the most striking feature of these results relative to those from the renal subcapsular samples was that the composition of

the capsules did affect the severity of the FBR. All of the strains of rat demonstrated a severe response to the presence of talc, indicating that they were all capable of mounting a classical FBR (Morson, 1978). In each of the strains of rat, the mean score allocated for each type of capsule was always lower than that for talc, indicating that the capsules were more biocompatible than the talc. However, particularly in the nude rat and the BB/d rat, the capsules scored quite highly, indicating that a considerable improvement is required to increase their biocompatibility.

The BB/d, BB/ndp and WAG/Ola rats all displayed reactions related to the capsule outer layer, with the M-alginate coating provoking the weakest response. This confirmed the hypothesis of O'Shea et al. (1984) that alginate-coated capsules are more biocompatible than PLL-coated capsules. Increased biocompatibility of alginate over polyethyleneimine has also been demonstrated in vitro by Al-Shamkhani et al. (1991). It is notable that with the exception of the nude rat, both types of alginate increased the biocompatibility of the capsules relative to PLL, with the presence of the outer layer of alginate and the composition of the alginate appearing to be important in determining the biocompatibility of the capsules. It is possible that the purity of the two different alginate preparations may be different, and this factor could have accounted for the difference in the reaction provoked by the capsules rather than the difference in chemical composition of the alginates.

It is interesting to note that other groups already mentioned (Weber et al. 1990a; Calafiore et al. 1989a) did not detect a significant pericapsular reaction to empty capsules although pericapsular fibrosis was present when the capsules contained islets. However, a more recent study by Mazaheri et al. (1991) reported an intense pericapsular infiltrate around empty capsules with the percentage of capsules overgrown with cells increasing in a linear relationship with time. The results of their encapsulated islet transplants were also very poor due to the problem of pericapsular fibrosis, with a mean graft survival of only 14 days.

In the light of these studies, the results of the biocompatibility assessment of the three types of capsule were not

encouraging for the success of encapsulated islet transplants as the capsules did not fit the definition of biocompatibility quoted above. This problem may be further increased if the pericapsular fibrosis becomes more severe when the capsules contain islets.

5.4.ii Immunohistochemistry and electron microscopy

The results of the immunohistochemistry and electron microscopy confirmed that the fibrosis was a classical foreign body reaction, as suggested by the response to the capsules in the nude rat.

Table 5.2 shows the results of the immunohistochemical staining of the pericapsular infiltrate and the reaction to the talc samples. The sections of the capsules were difficult to cut. Even when frozen, the capsules were soft, and the cells attached to the capsules tended to fall away from the capsule wall during processing. Consequently the frozen sections appeared disrupted relative to the formalin-fixed sections. However, as negative control sections were always processed at the same time, there was never any doubt about the specificity of the staining.

Figures 5.5.i and 5.5.ii both show staining with the macrophage and monocyte markers (ED1 and ED2). The MHC class II marker (OX4) also gave a weak positive stain. Similar staining patterns were present in the talc samples.

The results from the electron microscopy processing (Figures 5.6.i and 5.6.ii) showed clearly the presence of macrophages, fibroblasts and collagen fibres, confirming that the reaction was a classical FBR.

5.5 TESTING ALTERNATIVE ALGINATE PREPARATIONS

5.5.i Alternative alginates available

After the in vitro and in vivo experiments on capsule composition had been completed using the alginates supplied by Kelco International, three alternative alginate preparations became

available from Protan Limited (Alton, Hampshire). Two of these had been filtered through 0.2um filters and one through a 0.1um filter and were claimed to be of greater purity than the standard alginate preparations available, such as those used in this study so far. Following the results of the biocompatibility testing described above, it was considered important to test these alginates in a similar manner in order to determine whether pericapsular fibrosis could be minimised.

The three preparations were as follows:

Pronova LVM sodium alginate
filtered through 0.2um filter
approximately 65% mannuronic acid content
viscosity of 1% solution at 20°C, 20cps

This will be referred to as **LVM alginate**

Pronova LVG sodium alginate
filtered through 0.2um filter
at least 65% guluronic acid content
viscosity of 1% solution at 20°C, 200cps

This will be referred to as **LVG(0.2) alginate**

Pronova LVG sodium alginate
filtered through 0.1um filter
at least 65% guluronic acid content
viscosity of 1% solution at 20°C, 28cps

This will be referred to as **LVG(0.1) alginate**

(N.B. It should be noted that there is a slight difference in the methods used by Kelco International and Protan Ltd. to determine the viscosity of the alginate preparations. Kelco use the Brookfield viscometer at a spindle speed of 60rpm and Protan use a spindle speed of 20 rpm.)

5.5.ii Experiments undertaken with Protan alginates

a. Intraperitoneal implants.

Empty capsules were prepared as already described, using LVM alginate as the inner core for all the capsules, and each of the three new alginates as the outer layers. Approximately 1000 capsules were placed in the peritoneal cavity of BB/d, BB/ndp and WAG/Ola rats, retrieved after three weeks and processed as already described for light microscopy, immunohistochemistry and electron microscopy. Assessment of severity of the pericapsular fibrosis was undertaken using the scoring system described in Section 5.2.vi. Nude rats were not implanted with these capsules as the experiments with the Kelco alginate had already demonstrated the nature of the reaction to the capsules, and use of this strain of rat was unlikely to provide any extra information about the biocompatibility of the new alginates.

Capsules were not implanted into the renal subcapsular space because by the time these experiments were undertaken, it had become apparent that the peritoneal cavity would be the site used for encapsulated islet transplants.

b. Microfluorometry.

When the results from the biocompatibility study were available, in vitro studies were undertaken on islets encapsulated in the alginate which had given the most favourable biocompatibility results. Control non-encapsulated islets were also assessed for membrane integrity.

The protocol used was the same as that described in Section 4.4.iv, i.e. islets were encapsulated then cultured for a period of four weeks during which islets were stained with FDA/PI every 3-4 days to determine membrane integrity of the islets.

c. Perifusion.

In addition to the microfluorometry, encapsulated islet function was also assessed by perifusion as before (Section 4.4.v). Experiments were carried out in duplicate at weekly intervals for four weeks, and the DNA-corrected insulin release from the islets determined. Control non-encapsulated islets were also perifused.

5.5.iii Results

a. Reaction to the capsules.

The results of the scores of the reaction to the capsules are shown in Table 5.3. The mean scores for the capsules coated with the two Kelco alginates are also included to allow easy comparison of the results. The appearance of the capsules by light and electron microscopy, and the results of staining the frozen sections with monoclonal antibodies were the same as for the capsules prepared from the Kelco alginates. Consequently, photographs of the Protan alginate capsules have not been included.

b. Microfluorometry.

The results of the microfluorometric assay on the encapsulated and control islets are in Table 5.4.

c. Perifusion.

The stimulation increase and stimulation index data from the perifusion experiments are shown in Figures 5.7 and 5.8 respectively. The response time data is presented in Table 5.5.

5.5.iv. Discussion.

The results in Table 5.3 indicated that the capsules coated with the new alginate preparations tended to provoke a more severe

Table 5.3

Results of the scoring system for the Protan alginate capsules retreived from the peritoneal cavity. The scores of the individual observers (1 and 2) and the mean of these scores are shown. (The mean scores of the Kelco alginate capsules have also been incuded).

Strain of rat	Capsule composition	Scores		
		1	2	Mean
WAG/Ola	LVG(0.2) alginate	5.5	7.25	6.38
	LVG(0.1) alginate	2.25	2.0	2.13
	LVM alginate	4.5	7.5	6.0
	(M-alginate			0.5)
	(G-alginate			3.5)
BB/ndp	LVG(0.2) alginate	5.5	7.75	6.63
	LVG(0.1) alginate	4.75	6.5	5.63
	LVM alginate	4.5	6.5	5.5
	(M-alginate			1.0)
	(G-alginate			2.75)
BB/d	LVG(0.2) alginate	2.75	4.5	3.63
	LVG(0.1) alginate	6.25	7.5	6.88
	LVM alginate	6.25	7.0	6.63
	(M-alginate			6.0)
	(G-alginate			8.75)

Table 5.4

Results (median, range) of the microfluorometric assay data from the islets encapsulated with LVG(0.2) alginate, and the control non-encapsulated islets.

Days in culture	Free islets	LVG(0.2) encapsulated islets
1	1.5 (1 - 4)	-
5	1.5 (1 - 5)	1 (0 - 2)
8	1 (1 - 5)	1 (0 - 2)
12	4 (1 - 10)	1 (0 - 5)
15	1.5 (1 - 3)	0.5 (0 - 1)
19	1 (0 - 2)	0.5 (0 - 2)
22	1 (0 - 1)	0 (0 - 2)
26	1 (0 - 2)	1 (0 - 5)

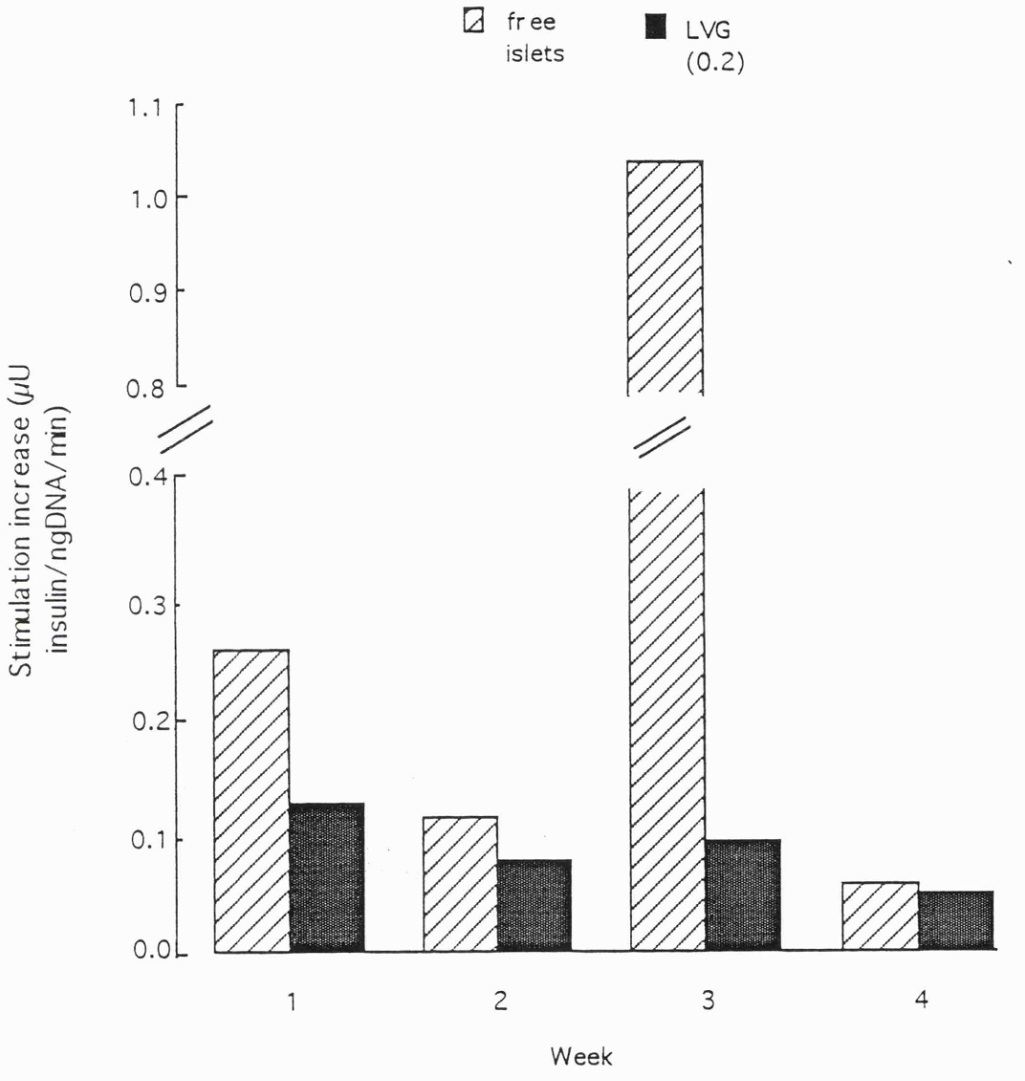


Figure 5.7
Stimulation increase data from the perifusion of islets encapsulated in LVG(0.2) alginate

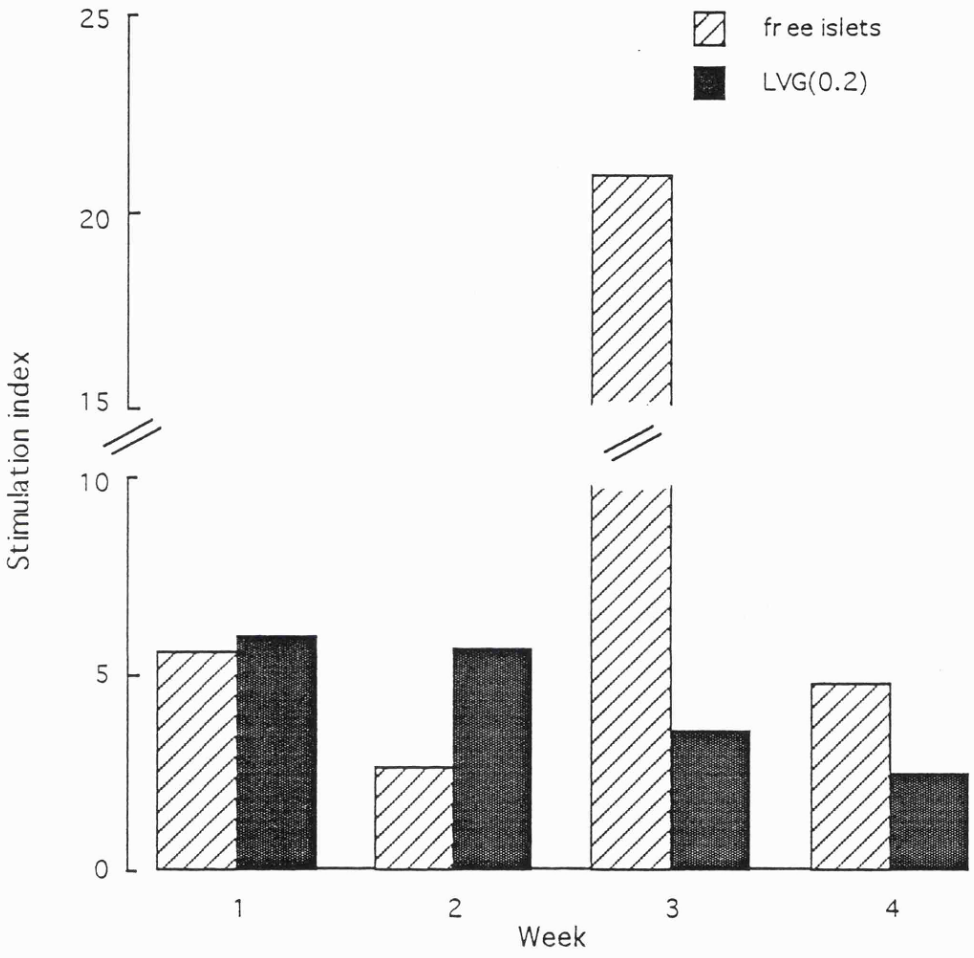


Figure 5.8
Stimulation index data from the perifusion of islets encapsulated in LVG(0.2) alginate

Table 5.5

Results of the time (in minutes) taken for the islets to respond to glucose stimulation during perifusion. Data from the duplicate experiments are shown separately as experiments 1 and 2.

	Free Islets		Encapsulated Islets	
	Exp 1	Exp 2	Exp 1	Exp 2
Week 1	0	0	0	1
Week 2	26	0	0	1.5
Week 3	0	0	0	0
Week 4	0	0	0.5	1

response than the alginates already tested. The LVG(0.1) alginate which was supposed to be more pure than the other preparations provoked a severe reaction in both the BB/d and BB/ndp rats, although the response was much weaker in the WAG/Ola rat.

The interesting feature to note, however, is the response of the BB/d rats to the capsules coated with LVG(0.2) alginate. These capsules had a score of 3.6 which was considerably less than for any of the other alginate preparations in the BB/d rat. The problem of increased fibrosis in the BB/d rat has already been discussed, so the finding that one of the alginate preparations did reduce the severity of the pericapsular reaction was of great importance. Consequently, the decision was taken to use LVG(0.2) alginate to coat capsules for the transplantation experiments.

Although the preliminary encapsulated islet transplant experiments were to be carried out in the BB/ndp rats, and the LVG(0.2) alginate was not the most favourable preparation in this strain, it was considered important to take measures to minimise pericapsular fibrosis of transplants in the BB/d rats. The experiments in the BB/ndp rats were to be the controls for the BB/d rat experiments, so LVG(0.2) alginate-coated capsules were used to allow valid comparisons to be made.

The results of the immunohistochemistry were the same as for the Kelco alginates, confirming that the reaction was a typical FBR.

The severity of reaction to the three different Protan alginate preparations demonstrated that their chemical composition did not have such a definite effect on the in vivo response as the Kelco alginates. Data presented by Soon-Shiong et al. (1991b) suggested that alginates with a high proportion of mannuronic acid are more likely to provoke a severe fibrotic reaction. However, the data from this study suggested that it was the purity of the preparations which determined their biocompatibility rather than the proportions of mannuronic and guluronic acids present in the alginates.

The results of the microfluorometric assay (Table 5.4) showed that the encapsulated islets had not been damaged by the

encapsulation procedure, and that the new alginate preparation did not affect the membrane integrity of the islets for up to 4 weeks in culture.

The perifusion results reflect those already discussed in Chapter 4 relating to the Kelco alginate capsules i.e. that the stimulation increase (Fig. 5.7) is reduced in the encapsulated islets, but the stimulation index (Fig. 5.8) is similar in the control and encapsulated islets. The stimulation index did show a considerable variation over the four week period for both groups of islets, but neither group demonstrated a consistently higher value than the other. The reason for the increase in insulin release from the control islets in the third week is unknown, and because it was not repeated on other weeks, cannot be regarded as a general reflection of greater viability of the control islets. There appears to be more variation in the results than those from the perifusions undertaken on the Kelco capsules, although the results show improved insulin release profiles. Variation in results is an inherent problem with perifusion systems and although steps were taken to minimise the presence of air bubbles in the system, one of the major causes of variable results, it was not possible to completely prevent the generation of some bubbles. The improvement in the results could be a reflection of increased experience in the technique. In addition, a different batch of collagenase was used to isolate the islets from that used for the previous perifusion experiments, and this might have affected the viability of the islets. A study by McShane et al. (1989) reported considerable differences in the ability of different batches of collagenase to digest the pancreas, although they did not assess the affect on islet viability.

The response time data (Table 5.5) also shows a major improvement on the previous series of perifusions, with only one group of islets showing a response time of greater than 1.5 minutes (control islets, week 2, experiment 1) and this was due to a very high basal insulin release detected in the first sample, probably due to disturbance of the islets prior to commencement of sample collection. These results confirm the previous findings that the presence of a capsule did not adversely affect the ability of the islets to respond to the increase in glucose concentration.

5.6 SUMMARY

In conclusion, the results discussed here have demonstrated a major problem with pericapsular fibrosis which was more pronounced in the peritoneal cavity relative to the renal subcapsular space. A considerable variation in response to the capsules was apparent between the strains of rat used, and the severe reaction present in the BB/d rat was not encouraging for the success of encapsulated islet transplant experiments.

Capsule composition did affect their biocompatibility, and these results have shown that by testing different alginate preparations, the severity of the pericapsular reaction can be minimised.

The results suggest that it was the purity of individual alginate preparations, rather than their chemical composition, which determined their biocompatibility.

CHAPTER 6

THE TRANSPLANTATION OF NON-ENCAPSULATED AND ENCAPSULATED ISLETS INTO THE BB/ndp RAT

6.1 INTRODUCTION

The experiments undertaken to determine the biocompatibility of the various capsule compositions demonstrated that to provide the volume of space required for the encapsulated islets, the peritoneal cavity would have to be used as the site of implantation. Some of the early studies on islet transplantation used the peritoneal cavity (Ballinger and Lacy, 1972; Kemp et al. 1973; Reckard et al. 1973; Gray and Watkins, 1976). These demonstrated that the number of islets required to reverse diabetes was greater than for other studies using alternative implantation sites. Even with an increase in the number of islets transplanted to give normal random non-fasting blood glucose readings, intravenous glucose tolerance tests in these studies were abnormal. However, all of these studies used supraoptimal numbers of islets, and the minimum number of islets required to just reverse diabetes when transplanted into the peritoneal cavity is not known. One very recent study (Fritschy et al. 1991), has demonstrated that transplantation of 1500 islets into the peritoneal cavity of diabetic rats will maintain normoglycaemia, but abnormal IVGTT results were obtained even after the transplantation of 3000 islets.

Therefore, the first part of the transplantation study was to determine the number of non-encapsulated islets required to reverse diabetes when transplanted into the peritoneal cavity and to compare this with the number of encapsulated islets needed to reverse diabetes.

This work had to be carried out using BB/ndp rats as donors and streptozotocin (STZ)-diabetic recipients because BB/ndp islets transplanted into BB/d rats are destroyed by the autoimmune disease process (Prowse et al. 1986; Woehrle et al. 1986). Renal subcapsular transplants were undertaken between our colony of BB/ndp and BB/d rats to confirm that autoimmune islet destruction did occur between the strains of rat. Figure 6.1 shows a photograph of the destroyed islets. Similarly, BB/ndp islets were also transplanted into STZ-diabetic BB/ndp rats to confirm that there was no sign of allograft rejection, indicating that the colony was inbred.

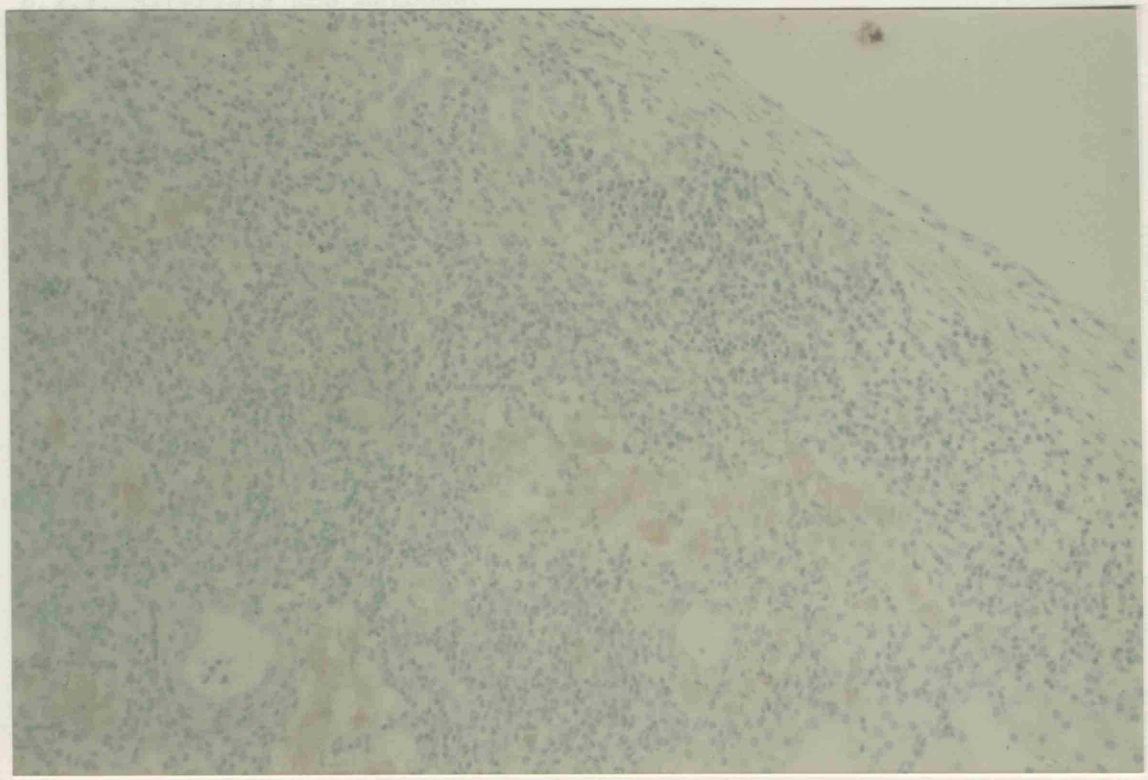


Figure 6.1
BB/ndp rat islets transplanted into the renal subcapsular space of the BB/d rat. (Stained for insulin) (x600).

Once the exact number of encapsulated islets required to reverse diabetes had been determined, transplants into BB/d rats could be performed with the number of islets known to be sufficient to reverse diabetes, and any graft failure would therefore be due to failure of the capsule to protect against autoimmune damage, poor capsule biocompatibility or problems with diffusion across the capsule, rather than the transplantation of too few islets.

6.2 INTRAPERITONEAL TRANSPLANTATION OF NON-ENCAPSULATED ISLETS

6.2.i. Materials and methods.

a.Induction of diabetes.

BB/ndp rats of either sex weighing between 180-200g were anaesthetised with fluothane and a small inguinal incision made to expose the femoral vein. An intravenous injection of 65mg kg^{-1} streptozotocin (STZ, Sigma) in citrate buffer, pH4.2, was given and the animals were recovered. The non-fasting blood glucose (BG) of the animals was monitored for one week (BM-Test 1-44 glucose test strips, Boehringer Mannheim GmbH, Mannheim) to determine the onset of diabetes, defined as greater than 20mmol l^{-1}. The following week (i.e. for the second week after the injection), the animals were given daily insulin injections (Ultralente heat-treated bovine insulin, Novo Industri A/S, Copenhagen). Food and water were available ad libitum.

b. Islet isolation.

The procedure for islet isolation has already been described (Section 4.4.i). The donors were BB/ndp rats weighing 200-250g. Serva collagenase was used for these experiments. The densities of the BSA gradient had to be modified slightly to optimise the number of islets obtained from these rats. Densities of 1.081g cm^{-3}, 1.067g cm^{-3} and 1.056g cm^{-3} were used for the three layers.

c. Experimental groups.

Four groups of six animals received islet transplants of 1000, 1500, 2000 and 3000 islets respectively.

In addition to these four groups, there was a group of six animals which had been rendered diabetic and remained on insulin injections without an islet transplant, but had their injection omitted for one day each week. These will be referred to as STZ controls. These animals were maintained for 100 days, culled and the pancreas resected, sectioned and stained at several different levels for the presence of insulin to detect any signs of beta cell recovery from the STZ treatment. Serial sections were also stained with glucagon and somatostatin to aid localisation of the islets.

d. Islet transplantation.

Islets were transplanted following 24 hours in culture, and all the islets were hand-picked in the size range 100-200um to ensure the exact number were transplanted. The recipients, now weighing 190-220g, were anaesthetised and a small midline incision made into the peritoneal cavity. The islets were placed in the peritoneal cavity in a small volume (approximately 1ml) of saline. The animals were recovered and their BG monitored every other day for the first 2 weeks and twice each week thereafter. Transplants were considered to be successful if the BG remained below 10mmol l^{-1}. Animals with two consecutive BG readings above 20mmol l^{-1} were culled as these transplants were considered to have failed.

e. IVGTT and glucose assay

Recipients which still had functioning grafts at 28 days post-transplant underwent an intravenous glucose tolerance test (IVGTT). In addition six normal controls and six STZ-diabetic animals underwent IVGTT's.

The animals were fasted overnight, anaesthetised and given a femoral vein injection of glucose (0.5g kg^{-1}). Blood samples were collected from the tail vein into fluoride oxalate tubes immediately before the injection, then at 5, 10, 15, 20, 30 and 40 minutes afterwards. 100ul of blood was deproteinised in 1ml 5% trichloroacetic acid, centrifuged, and the supernatant assayed for glucose content. A GOD-Perid kit (Boehringer Mannheim GmbH, Mannheim, Germany) was used to analyse the glucose concentration of the samples.

Animals were recovered from anaesthesia and maintained for a maximum of 100 days post-transplant, or until the graft failed. A second IVGTT was performed at 100 days post-transplant, after which the animals were culled, and the pancreas removed from those which had successful long-term grafts so that they could be stained for the presence of insulin, glucagon and somatostatin as described above.

The data gained from the IVGTT's was assessed by calculating the area under the curve when BG was plotted against time. The results were compared using the Mann Whitney U test to determine statistical significance.

6.2.ii Results.

a. STZ controls.

Figure 6.2 shows the mean BG readings for the group of STZ controls for the experimental period. These results demonstrate that the BG rose on the day after omission of the insulin injection on every occasion.

The sections of pancreas which were stained for the presence of insulin all indicated negligible, if any, insulin. Figure 6.3.i shows an islet from a normal animal stained for insulin, Figure 6.3.ii shows an islet from one of the experimental animals culled at 100 days showing no regeneration, and Figure 6.3.iii shows an islet from an animal culled at 100 days post-transplant with a very weak stain indicating the presence of some insulin.

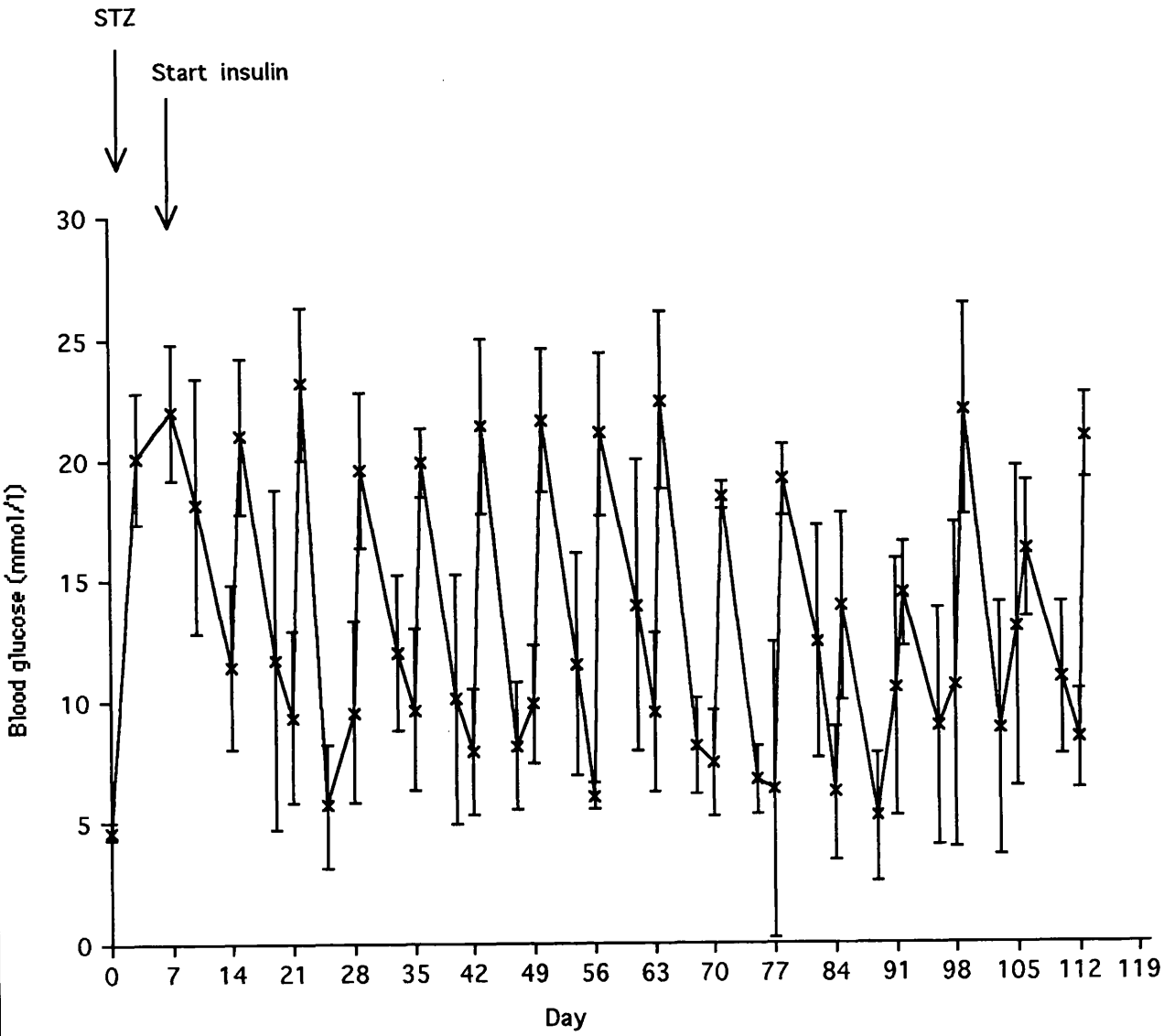
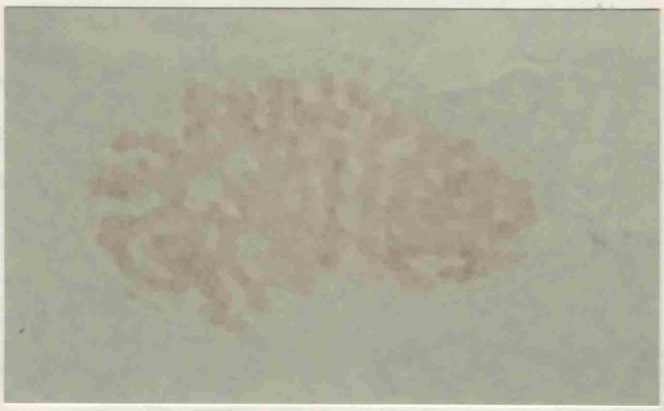


Figure 6.2
Mean blood glucose profiles of the STZ control group (n=6).

i.



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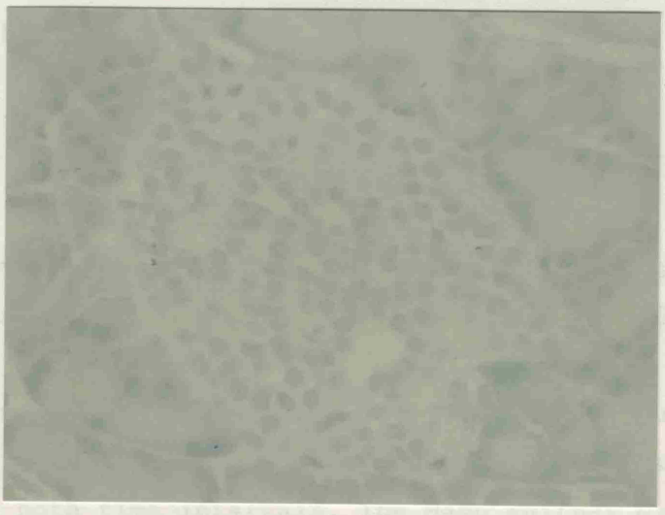

iii.

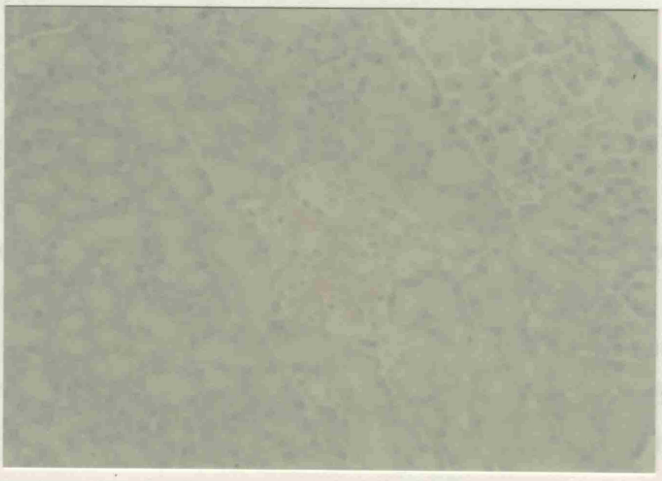

Figure 6.3
Islets stained for insulin from (i) normal control (x275),
(ii) STZ-diabetic rat with no recovery of beta cells (x450),
(iii) STZ-diabetic rat with partial recovery of beta cells (x150).

b. Transplant recipient blood glucose data.

Figure 6.4 shows a graph of the mean BG readings of the four islet transplant groups. The standard error of the data has only been included for the animals receiving 3000 islets to avoid confusion. The actual length of graft survival for the four groups are shown in Table 6.1.

The weights of the animals receiving 3000 islets all increased from the range of 195-220g at transplant to 250-280g at 100 days post-transplant.

c. IVGTT

Figure 6.5 shows the IVGTT curves for the normal and diabetic controls and the 3000 islet recipients at 28 and 100 days post-transplant. The area under the curve data is shown in Table 6.2. There was no significant difference when the area under the curve of the normal controls was compared with the data from the transplanted animals at both time intervals. The Mann Whitney U test was used for this analysis.

6.2.iii Discussion

The results of the transplantation of non-encapsulated islets into STZ-BB/ndp rats have shown that 1000, 1500 or 2000 islets when implanted in the peritoneal cavity were insufficient to reverse diabetes, but that 3000 islets routinely reversed diabetes in these animals (Figure 6.4). This figure greatly exceeds the number of 750 islets reported by Loftus et al. (1991) to reverse STZ-diabetes when transplanted into the renal subcapsular space, and highlights the inefficiency of the peritoneal cavity as a site for islet transplantation. The results of the IVGTT's performed at both 28 and 100 days post-transplant in the 3000 islet recipients demonstrated that good blood glucose control had been achieved in these animals (Figure 6.5). This is in contrast with the results reported by

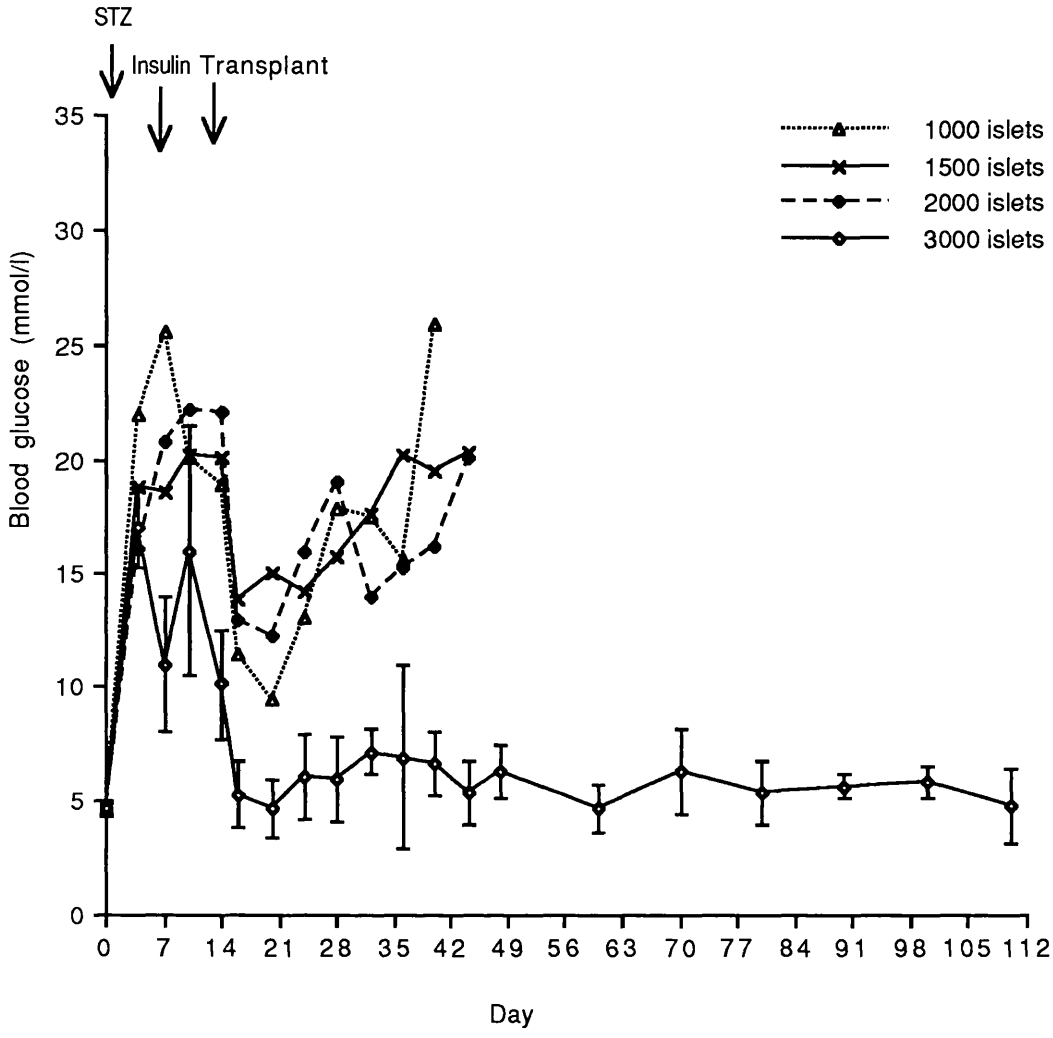

Figure 6.4
Mean blood glucose profiles of the four groups of animals transplanted with non-encapsulated islets (n=6 in all groups).

Table 6.1

Graft survival of the encapsulated and non-encapsulated BB/ndp islet transplants into the peritoneal cavity of STZ-BB/ndp rats. (0 indicates that the graft did not lower the blood glucose below 10mmol l^{-1}.)

Transplant group	Graft survival (days)
1000 islets	12, 6, 0, 11, 15, 49
1500 islets	10, 0, 12, 85, 11, 0
2000 islets	9, 0, 3, 11, 63, 11
3000 islets	100, 100, 100, 100, 100, 100
3000 encapsulated islets	0, 4, 6, 0, 14
5000 encapsulated islets	0, 50, 32, 0, 60

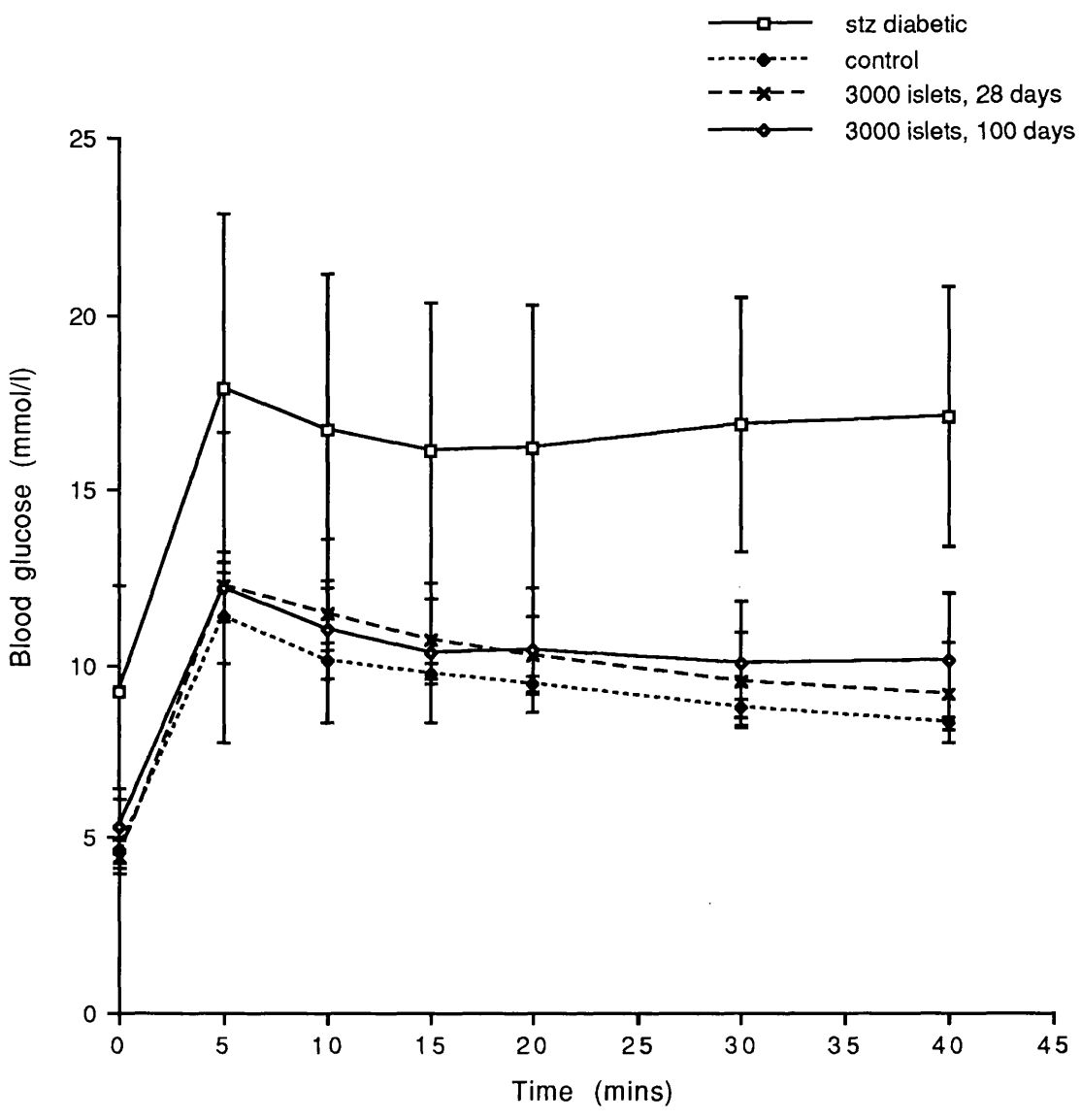

Figure 6.5
IVGTT data from the diabetic and normal control animals, and the animals transplanted with 3000 non-encapsulated islets at 28 and 100 days post transplant (n=6 in all groups).

Table 6.2

Area under the curve of the IVGTT's from normal controls (n=6), STZ-diabetics (n=6) and from the transplanted animals.

Transplant groups	Median	Range
STZ-diabetics	636	444 - 934
Normal controls	367	348 - 382
3000 free islets, 28 days (n=6)	401	343 - 455
3000 free islets, 100 days (n=6)	426	303 - 528
5000 encapsulated islets (n=3)	430	357 - 470

Fritschy et al. (1991) which demonstrated abnormal IVGTT results following the transplantation of 3000 islets. It should be noted, however, that the recipients in this study weighed between 300-350g at the time of diabetes induction, compared with 180-200g in the present study.

The IVGTT data was assessed by calculating the area under the curve, a method previously described as an alternative to calculating the k value (Koivisto et al. 1989; Kenyon et al. 1990). Alejandro et al. (1989) verified the method when they determined that the area under the curve paralleled the responses demonstrated by the k values. It should be noted that the unexpectedly low area under the IVGTT curve of the STZ-diabetic group was due to them having insulin injections up to the day immediately before the IVGTT. The long-acting insulin preparation used to inject these animals could have had a residual effect leading to the relatively low values.

Several factors in these experiments were standardised in order to allow valid comparison of the results from the different groups of animals receiving different numbers of islets. Firstly, the weight of the recipients was within the range of 180-200g when diabetes was induced so that an equal number of islets per gram of body weight could be given to the recipients. Secondly, all the islets were hand-picked within the size range of 100-200um thus reducing the possibility of transplanting different masses of islets per gram of body weight. Some studies have shown that islet mass relative to body weight can affect the success of transplants (Hayek et al. 1990; Koncz et al. 1976). Also, it has been highlighted that a simple count of the number of islets to be transplanted is meaningless if the size of the islets is not taken into account, as a small increase in diameter is translated into a much larger increase in islet mass which can cause a major source of error if only islet numbers are quoted (Gray and Morris, 1987a).

Thirdly, the treatment of the recipients with insulin for a week between the onset of diabetes and transplantation allowed the diabetic state of all the recipients to be standardised. In addition, it has been demonstrated that islets transplanted into hyperglycaemic recipients become degranulated and show an abnormal response to

glucose challenge if the recipient is not given exogenous insulin treatment post-transplant (Gray et al. 1986; Korsgren et al. 1989). Although insulin therapy was not maintained after transplant, the residual effect of the insulin, and the low BG of the animals at transplantation as a result of the insulin therapy meant that all the recipients received the islets whilst in a similar state.

A major problem with the use of chemically-induced diabetic recipients for islet transplantation is monitoring the pancreas for evidence of recovery of the beta cells from the toxic effect of the chemical (Brekke et al. 1983; Chicheportiche et al. 1990). When the renal subcapsular site is used for islet implantation, it is possible to perform a nephrectomy to remove the graft and then to monitor the BG to determine whether beta cell recovery has taken place (Lake et al. 1989). However, removal of the graft is not possible when the islets are dispersed within the peritoneal cavity.

In order to monitor the pancreas for evidence of reversal of the chemical treatment, the pancreas was removed from both the STZ controls and from the animals with succesful transplants at 100 days, sectioned at 4 levels and stained for the presence of insulin. Serial sections were stained to detect the presence of glucagon and somatostatin to help localise the islets. Examination of these sections showed that the majority of the islets did not contain any insulin (Fig. 6.3.ii), and only a very weak stain was detected in the few islets which were positive for insulin (Fig. 6.3.iii). The increase in BG of the STZ controls on the days following omission of the insulin injection indicated that the animals were still diabetic. In addition, the failure of the grafts of 1000, 1500 and 2000 islets showed that recovery from the STZ treatment had not occurred in these animals. These factors all indicated that STZ reversal had not occurred and was, therefore, unlikely to have been a significant factor in the successfully transplanted recipients.

In conclusion, these experiments have demonstrated that 3000 islets were required to reverse STZ-induced diabetes in BB/ndp rats weighing 190-220g when transplanted into the peritoneal cavity.

6.3 INTRAPERITONEAL TRANSPLANTATION OF ENCAPSULATED ISLETS

6.3.i Introduction

Once the non-encapsulated islet transplants had been completed, the next stage in the transplantation experiments was to transplant encapsulated islets into STZ-diabetic BB/ndp rats to determine the number required to reverse diabetes.

Although there have been reports of encapsulated islet transplants (see Table 3.1), relatively few of these have used rats as the recipients, and only 4 used BB/d rats. The studies using STZ-diabetic rats have quoted the need for between 4000 to 5000 encapsulated islets to reverse diabetes, using the peritoneal cavity as the implantation site (O'Shea et al. 1984 and Taunton-Rigby 1986 used 4000; Wu et al. 1988 used 4-4500; Sun et al. 1987 used 4500-5000). One possible source of error with these studies is that there is no mention of how the islets were counted suggesting that they were not hand-picked, and therefore sampling errors could have be large due to the presence of empty capsules or to capsules containing more than one islet. In addition, it should be noted that there is a considerable variation in the weights of the recipients quoted in some of these studies. For example, Wu et al. (1988) used animals weighing between 180-250g, Sun et al. (1987) used 250-350g rats, and Fan et al. (1990) used rats weighing 207-310g. Also, none of the studies refer to any audit on the regeneration of beta cells following the STZ treatment.

Studies using the BB/d rat as the recipient have used between 4000 and 5000 encapsulated islets to reverse diabetes (Sun et al. 1989; Fan et al. 1989; Mazaheri et al. 1991), although the report by Fan et al. (1990) initially used 4-5000 islets followed by a second transplant of 2500-3500 islets into two out of ten animals which became diabetic again. There have been no reports to date which have used the BB/ndp rat as the recipient, although the studies already mentioned suggested that approximately 5000 encapsulated islets would be required to reverse diabetes.

6.3.ii Materials and methods

Diabetes was induced in 180-200g BB/ndp rats using STZ and the same protocol of BG monitoring and insulin therapy was followed as described above. Islets were isolated and encapsulated using LVG(0.2) alginate after 48 hours in culture using the method described in Section 4.2.iii.

Two groups of five animals were transplanted intraperitoneally with 3000 or 5000 encapsulated islets respectively. The islets were hand-picked so the exact number of islets was transplanted.

The animals were monitored as above by non-fasting BG readings and IVGTT's at 28 days post-transplant. Animals with failed grafts were culled and samples of the graft taken for routine histology, for immunohistochemistry of frozen sections (see Section 5.2.iv), and for electron microscopy to determine the nature of the reaction to the capsules.

6.3.iii Results

a. Transplant recipient blood glucose data

Figures 6.6 and 6.7 show the BG data for each individual animal in the group receiving 3000 and 5000 encapsulated islets respectively. The graft survival times have been included in Table 6.1.

Only three animals which received 5000 encapsulated islets had grafts which functioned for over 28 days and of these animals, two showed a weight loss of 10g from the time of transplant (grafts functioned for 50 and 35 days) while the third gained 15g in the 60 days for which the graft functioned.

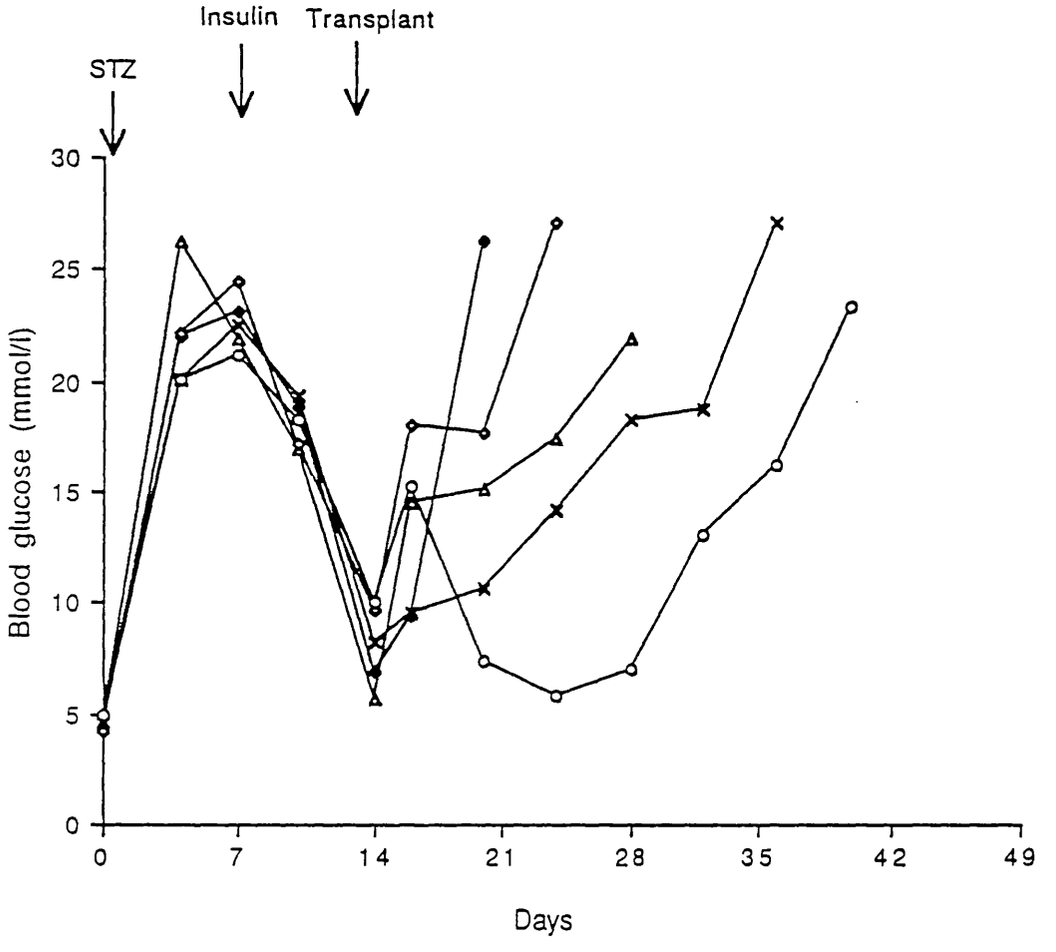

Figure 6.6
Blood glucose profiles of the animals transplanted with 3000 encapsulated islets.

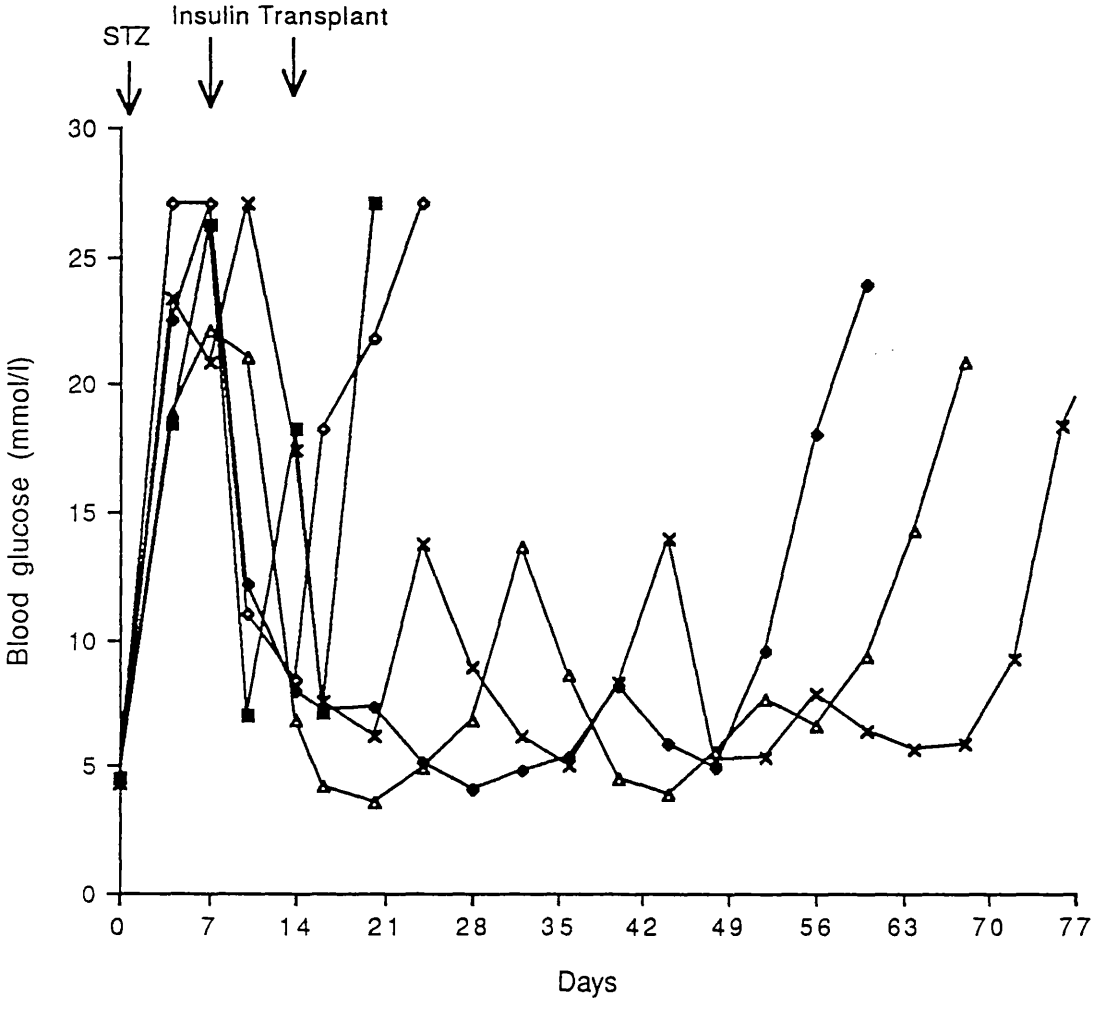

Figure 6.7
Blood glucose profiles of the animals transplanted with 5000 encapsulated islets

b. IVGTT

Figure 6.8 shows the IVGTT curves of the STZ-diabetic controls the normal controls and the 3 surviving 5000 encapsulated islet recipients which still had functioning grafts at 28 days post transplant. The area under the curve data for this group has been included in Table 6.2.

c. Histology

Figure 6.9 shows a photograph of a haematoxylin and eosin stained section of the encapsulated islets retrieved from an animal following graft failure.

Immunohistochemistry and electron microscopy showed the presence of macrophages and fibroblasts around the capsules.

6.4.iv Discussion

The results have demonstrated that more encapsulated islets were required to reverse diabetes than non-encapsulated islets as out of the five animals with 3000 encapsulated islet grafts, only one functioned for over one week (Figure 6.6). Three of the animals which received 5000 encapsulated islets had functioning grafts at 35, 50 and 60 days respectively (Figure 6.7), and this is the number of encapsulated islets required to reverse diabetes quoted by most groups (O'Shea et al. 1984; Sun et al. 1987; Taunton-Rigby, 1986; Wu et al. 1988).

It is noteable that only one study reported to date has determined islet function by IVGTT (Soon-Shiong 1991a), with all the other groups relying upon daily BG monitoring to determine graft function. Non-fasting BG readings can be misleading, depending for example on when the animal last ate, and it is possible to obtain normal BG readings and an abnormal IVGTT result from the same animal (Moorhouse et al. 1964). An animal with an islet graft might be expected to have better control over BG than one just receiving

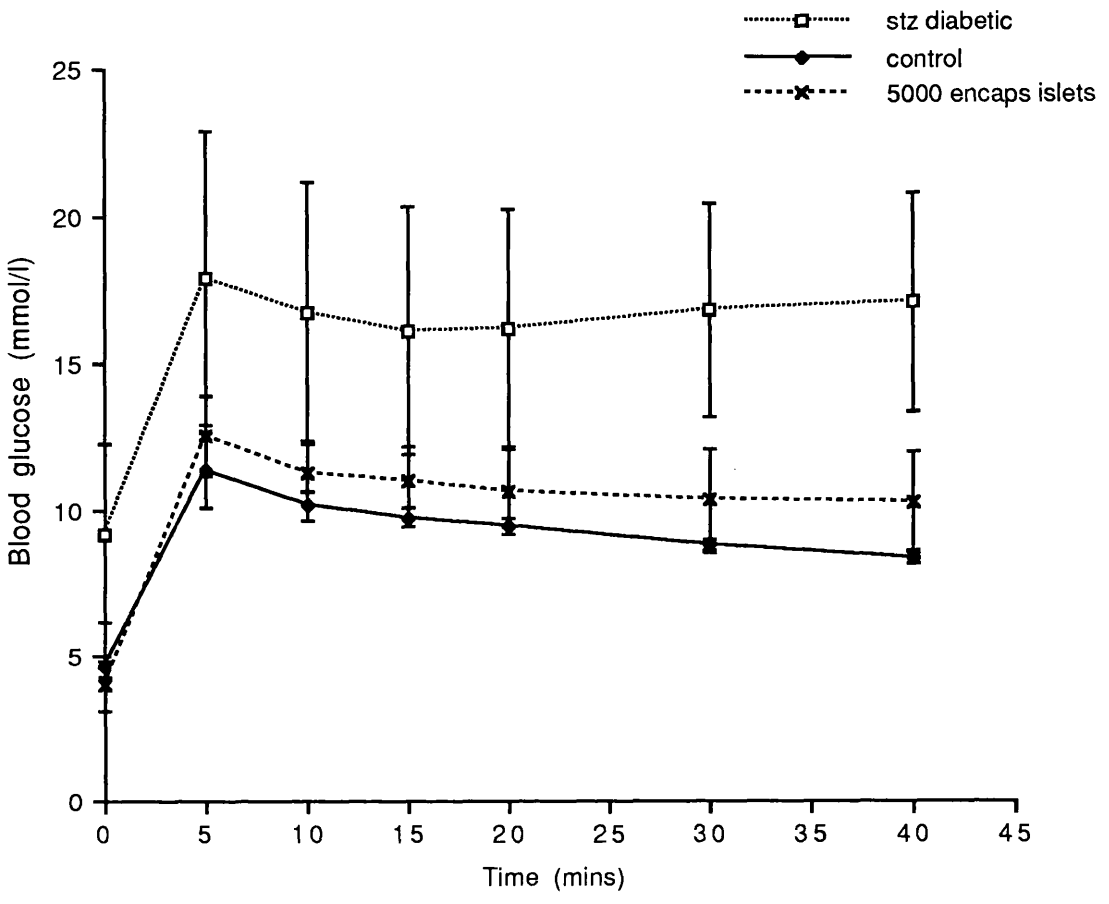

Figure 6.8
IVGTT data from the diabetic and normal control animals (n=6), and the animals transplanted with 5000 encapsulated islets (n=3) at 28 days post transplant.

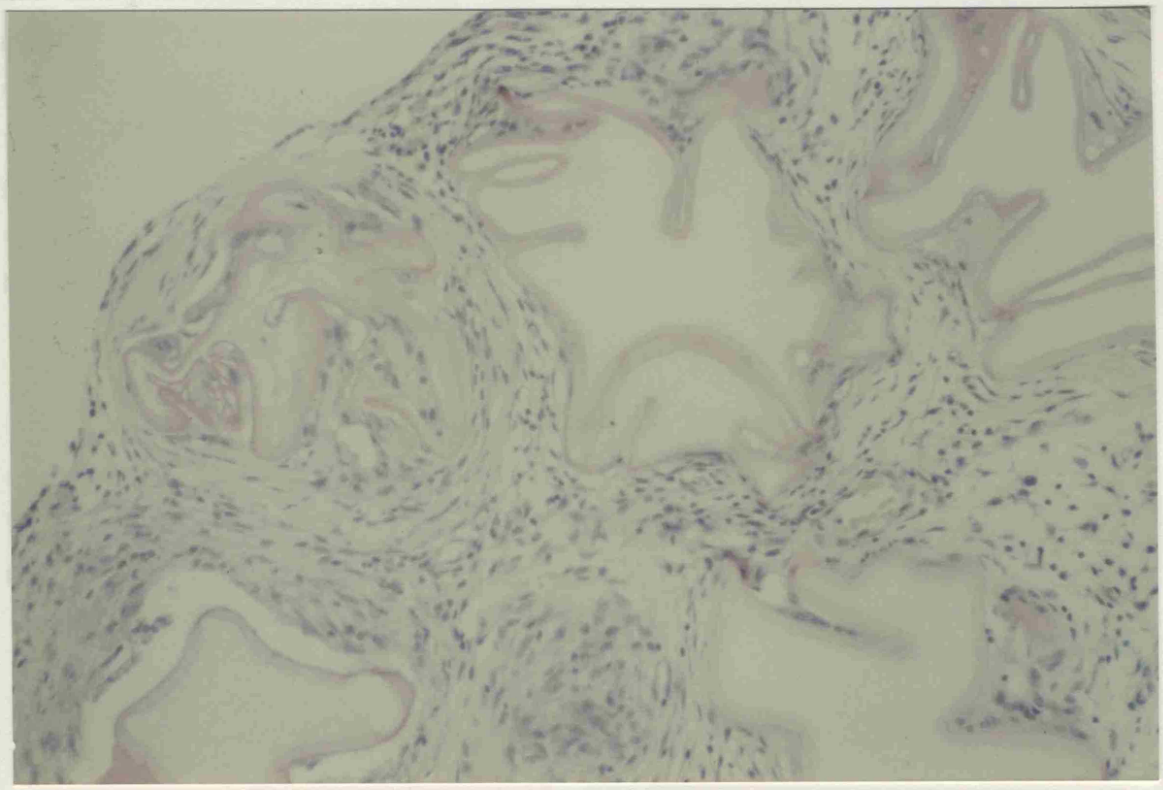

Figure 6.9
Failed encapsulated islet transplant (H and E) (x100).

insulin injections, but the control may not be very fine if insufficient islets had been transplanted, and this loss of exact control may be sufficient to lead to the complications associated with diabetes (Sun et al. 1987). Consequently, IVGTT results provide more comprehensive data about the graft function.

The results of the IVGTT study (Figure 6.8) showed that there was no significant difference between the animals which had received 5000 encapsulated islets and both the normal controls and the 3000 non-encapsulated islet recipients (data from 3000 islet recipients not included on graph to avoid confusion) indicating good control over BG following an injection of glucose. However, it must be remembered that only 3 animals had functioning encapsulated islet grafts at 28 days post-transplant and these results have been compared with data from 6 animals in each of the other two goups. More data would be required from other 5000 encapsulated islet transplant recipients to validate these results.

The other important factor to note from these results is that all the transplants eventually failed. Firstly, this indicated that beta cell regeneration had not taken place in these animals, but also more importantly that there was a problem with biocompatibility of the capsules leading to pericapsular fibrosis and graft failure (Figure 6.9).

The results of the immunohistochemistry and electron microscopy to determine the nature of the reaction to the capsules demonstrated that the same cell types were involved as with the empty capsules i.e. macrophages and fibroblasts. Other studies which have assessed the reaction to capsules have shown the presence of $CD4^+$ T cells in NOD mice (Weber et al. 1990a), T-helper cells and Ia^+ cells in BB rats (Mazaheri et al. 1991) or mononuclear cells, macrophages and immature giant cells in NOD mice (Calafiore et al. 1987). The study by Weber et al. (1990a) concluded that the reaction was against the islets inside the capsules rather than the capsules which were biocompatible. Calafiore et al. (1987) have also demonstrated a more severe reaction to capsules containing islets relative to empty capsules, although the study by Mazaheri et al. (1991) did show

intense pericapsular fibrosis of empty capsules which became more severe with time.

The failure of the encapsulated islet transplants demonstrated that poor biocompatibility of the capsules was a major problem and that further transplant studies would be meaningless until this had been resolved as they would all ultimately fail and would not provide any information on the viability of encapsulated islet function in vivo.

It must be highlighted at this stage that the work by Sun et al. (1987), Wu et al. (1988) and Fan et al. (1990), although appearing to be successful, does show the failure of some transplants as early as 22 days (Wu et al. 1988) and up to 92 days (Fan et al. 1990). In the latter study, the failure of the transplant was countered by giving the recipient a second transplant of 2500-3500 encapsulated islets. This resulted in a fall in BG for between 84 and 114 days. These results are an improvement on those in the present study, although they indicate that the current materials available for encapsulation do not yet fulfill the biocompatibility requirements.

It is interesting to note, however, that Fan et al. (1990) considered their results to be successful: "encapsulation of islets appears to provide total protection from the host immune system while eliminating the need for immunosuppression". They also add that: "because no biomaterial is 100% biocompatible, it is inevitable that some capsules will eventually attract cell overgrowth".

The discussion in their paper goes on to consider factors which may vary between preparations of encapsulated islets which could lead to the more rapid failure of some transplants relative to others. For example, capsule strength (related to alginate viscosity and reaction time between the alginate and PLL) would determine their durability. It is, therefore, interesting to note that in this paper, the concentration of PLL has been increased from 0.05% to 0.15%, and the PLL reaction time was increased from 6 to 10 minutes. This contradicts the study by Goosen et al. (1985) which states that PLL up to a concentration of 0.1% and a reaction time of between 6 and 9 minutes produces durable and flexible capsules, but increasing the

reaction time to 9, 12 or 20 minutes reduces the durability of the capsules.

They also quote the smoothness of the capsules and the sterility and composition of the alginate as important factors affecting capsule survival. In the present study, alginate composition has already been discussed and it has been stressed that all solutions were filtered through a 0.22um filter before use. Obviously, sphericity of the capsules will vary between preparations and between capsules in the same preparation, however the effect of capsule shape on the failure of the current transplants should have been minimised as all the capsules were hand-picked allowing the opportunity to discard any obviously distorted ones.

Another factor which Fan et al. quote as a possible trigger of pericapsular fibrosis is incomplete coating of the PLL layer with alginate. However, in this respect, the protocol used in the current study was the same as in their paper (0.15% algiante for 4 minutes), and therefore unlikely to be of any great significance. It is interesting that in one of the animals described by Fan et al. approximately 30% of the capsules were either adhered to the internal organs or overgrown with cells, demonstrating that although they do have successful transplants, they also have some capsules failing due to pericapsular fibrosis.

6.4 SUMMARY

In summary, this study has shown that, when transplanted into the peritoneal cavity, 3000 non-encapsulated islets were sufficient to achieve normoglycaemia and give normal IVGTT results up to 100 days post-transplant. Following encapsulation, this number was increased to 5000 islets. However, the poor biocompatibility of the capsules led to the failure of the grafts and until this could be improved, further transplant experiments would not be worthwhile as the grafts would almost certainly fail due to pericapsular fibrosis.

CHAPTER 7

THE PURIFICATION OF SODIUM ALGINATE

7.1 INTRODUCTION

The final part of the study on islet encapsulation was due to involve encapsulated islet transplantation into BB/d rats once the number of encapsulated islets required to reverse diabetes in the STZ-BB/ndp rat had been determined. The series of transplants into the BB/d rat were to include syngeneic (BB/ndp) and allogeneic donors to determine whether the capsules could protect against both autoimmune destruction and allograft rejection. In addition, experiments were planned using pig and human islets to assess capsule function with xenogeneic islets and to determine the long-term in vivo function of these islets, an important factor if encapsulated pig islet transplants are to become a viable form of treatment in IDDM patients.

However, due to the failure of the encapsulated islet transplants in the STZ-BB/ndp rats as a result of severe pericapsular fibrosis, further encapsulated islet transplants would be meaningless until a more biocompatible preparation of alginate became available to allow long-term capsule function to be assessed. Therefore, the next stage of the study was designed to determine the factor or factors present in the alginate responsible for provoking pericapsular fibrosis, and to attempt to remove this material in order to improve its biocompatibility.

7.2 POTENTIAL PROMOTERS OF PERICAPSULAR FIBROSIS

7.2.i Introduction

The alginates supplied by Protan Ltd. were from their range of PRONOVA^{TM} alginates and some of the information supplied by the company stated that their "unique ultrapurification facilities create products with unequalled purity and compatibility with living tissue". However, as already described the tests undertaken with these preparations had demonstrated that the biocompatibility of the alginates was not sufficient for the purposes of this project.

In addition, Protan supplied analytical certificates with each alginate preparation and it was the information on these certificates

which highlighted two potential sources of impurity capable of provoking pericapsular fibrosis. These were endotoxins and protein.

7.2.ii Endotoxins

Endotoxins have been described as "lipopolysaccharide-protein complexes contained in cell walls of gram-negative bacteria including non-infectious gram-negatives" (Morrison and Ulevitch, 1978). They are composed of lipid A bound to a core polysaccharide which is in turn bound to O-specific polysaccharides (Rietschel et al. 1982) and are located in the outer membrane of bacteria (Russell, 1976). Endotoxins were thought to be released in association with cell destruction and autolysis, although further work has demonstrated the release of endotoxin in culture fluid from various strains of bacteria (see Russell, 1976, for a review of this work).

The nature of action of endotoxin has been extensively reviewed (Bennett and Beeson, 1950; Greisman and Hornick, 1972; Vaheri et al. 1973; Russell, 1976; Morrison and Ulevitch, 1978; Reitschel et al. 1982; Nowotny, 1983). In brief, it is the lipid A part of the structure which is the active agent of endotoxin and this has several actions which have been listed in Table 7.1. The extensive range of effects attributed to endotoxin are relevant to this project as the stimulation of macrophages by endotoxin could be an important factor in the development of pericapsular fibrosis. Jeannin et al. (1985) demonstrated the induction of cytolytic activity in rat resident peritoneal macrophages by endotoxin. Only one of the three Protan alginate preparations, LVG(0.1), had been tested for endotoxin content and this was present at a level of 396 EU g^{-1} (endotoxin units per gram). Therefore, it was decided to determine the endotoxin content of all of the alginate preparations in order to assess whether this factor could be related to the development of fibrosis. The tests were carried out commercially as the specialised equipment necessary for the tests was not available in the department. The results are shown in Table 7.2.

Although demonstrating the presence of endotoxin in all the alginates, the results did not relate to the severity of fibrosis

Table 7.1.

A list of actions known or thought to be a result of the presence of endotoxin.

1. Complement and alternative complement activation.
2. Formation of fibrin thrombi due to action on the coagulation system.
3. Aggregation of platelets.
4. Release of secretory and lytic agents from platelets.
5. Leukocytosis and leukopaenia due to action on neutrophils.
6. Release of procoagulant, collagenases and prostaglandins from macrophages and monocytes in response to very low levels of endotoxin.
7. Breakdown of endothelium.
8. Possible action on mast cells.

Table 7.2

Results of the endotoxin assay on the five alginate preparations, and the saline used to dissolve the solid sodium alginate. (The conversion from ng ml^{-1} to EU g^{-1} calculated according to Piotrowicz 1988.)

Alginate	Endotoxin Content ng ml^{-1}	EU g^{-1}
M-alginate	2.9	193
G-alginate	51.7	3441
LVM	17.5	1167
LVG(0.1)	2.1	140
LVG(0.2)	4.3	287
Saline	<0.025	<1.7

provoked by the samples. The effects of endotoxins already described indicate that it would be preferential to reduce the amount of endotoxin present, or if possible to completely remove it. However, because it is notoriously difficult to remove endotoxin from substances (Bennett and Beeson, 1950), it would not have been feasible to purify the alginate by removal of the endotoxin.

7.2.iii Protein

The other source of contamination considered to be a potential promoter of fibrosis was protein. The data provided by Protan indicated that protein was present in their preparations at the following levels:

LVG(0.2)	0.24%
LVG(0.1)	0.14%
LVM	0.12%

Foreign proteins introduced into the body, or in contact with body tissues in an in vitro system, have been shown to have poor biocompatibility. In 1991, Al-Shamkhani et al. demonstrated that polyethyleneimine (a polypeptide) had a similar effect on the lysis of erythrocytes to that of Triton-X-100 (the positive control), leading to 100% lysis within one hour. This was thought to be due to electrostatic binding of the polypeptide to the negatively charged erythrocyte membrane. Similarly, Steadman et al. (1990) demonstrated the ability of poly-l-lysine to change the net surface charge of polymorphonuclear cells, confirming that electrostatic forces can play a role in the selective uptake of charged particles by phagocytosis. This factor could have great importance in this project. Firstly, if contaminating protein was present in the alginate, this would explain the severity of pericapsular fibrosis apparent around the capsules. The results of the biocompatibility of the different capsule compositions in Section 5.3 confirm the increased severity of fibrosis when capsules were coated with protein (i.e. PLL) in comparison with an outer layer of alginate. Secondly, incomplete coverage of the PLL layer by the alginate, as suggested by Fan et al. (1990) would increase the possibility of severe

pericapsular fibrosis due to the polypeptide layer being partially "visible" to the cells in the peritoneal cavity.

It was decided, therefore, to assess the protein content of all the alginate preparations and to attempt to remove the protein from the alginate to determine whether this would increase the biocompatibility of the material.

7.3 DETECTION OF PROTEIN IN THE ALGINATE

7.3.1 Introduction

There are several well-defined methods for the detection of protein and for its removal from substances, however, it soon became apparent that the removal of protein from the alginate was not going to be a simple task. This was due to the two properties of alginate which make it such a good material for encapsulation: the viscosity of sodium alginate and the exchange of divalent ions with sodium ions to form an insoluble alginate salt from soluble sodium alginate.

These factors meant that the standard methods of protein quantification were inappropriate for use with sodium alginate as they all ultimately rely on the use of solutions usually containing copper ions which cause the precipitation of alginate. This meant that the standard methods described by Bradford (1976), Peterson (1977), Markwell et al. (1978), Markwell et al. (1981) and Smith et al. (1985) could not be used. The viscosity of the alginate also meant that purification on a column system would be difficult, especially to provide the quantity of purified alginate which would be required for encapsulating islets for transplantation.

The common way to avoid this problem would have been to precipitate the protein present in the alginate with trichloroacetic acid (TCA) and to assay the protein precipitate using a standard protein assay method, but as TCA also precipitates alginate this was not an answer to the problem. A method had been described, however, which used TCA to precipitate both the protein and alginate, followed by treatment of the precipitate with a saturated solution of magnesium sulphate which redissolved only the alginate (Bundesen and

Martinek, 1954). The same study gave a method for re-extracting the alginate from the magnesium sulphate solution, and this was investigated as a possible method for purifying the alginate.

In practice, however, this was not possible as there was considerable variation in the amount of protein extracted from the alginate each time the method was attempted, indicating that it could not be used routinely to remove protein from the alginate. In addition, the alginate did not dissolve in the magnesium sulphate as readily as suggested by the authors, so considerable volumes were required to fully dissolve the precipitate. This in turn meant that large volumes of ethanol were required to re-extract the alginate from the magnesium sulphate solution providing the opportunity for introducing other contaminants into the alginate. Also, the resulting alginate solution was very dilute and using the uronic acid assay method described by Blumenkrantz and Asboe-Hansen (1973) alginate was undetectable in the final extract. Consequently, it was not considered to be a suitable method for protein assessment or alginate purification.

7.3.ii Ultraviolet absorption method of protein detection

An alternative method for protein detection which has been described and does not rely on the addition reagents to the solution being studied is that of measuring UV absorption at 260nm and 280nm (Johnstone and Thorpe, 1982). Once the absorbance at the two wavelengths has been determined, the concentration of protein in the solutions can be calculated using the formula:

$$\text{Protein (mg ml}^{-1}) = (1.55 \times \text{Abs at 280nm}) - (0.77 \times \text{Abs at 260nm})$$

However, this method is only accurate if the absorbance ratio 260nm:280nm is below 0.6. This was not the case when the alginate samples were assessed indicating that other factors present in the solutions were interfering with the test and that it could not be used to provide an accurate assessment of the protein present in the alginate samples.

7.3.iii Polyacrylamide gel electrophoresis

The next step undertaken to determine the presence of protein in the alginates was to analyse the samples by polyacrylamide gel electrophoresis (PAGE). This method also allowed the molecular weight of the contaminant to be determined, thus providing important information relating to the choice of method for purification.

Initially, it was decided to study two alginate preparations by PAGE as only 10 samples could be loaded onto each gel, and as molecular weight markers and control samples had to be included, it was not practical to study all of the alginates.

The alginates which were chosen to be studied were the G-alginate from Kelco International and LVG(0.2) from Protan Ltd. These two alginate preparations were chosen because they provoked the strongest and weakest response in the BB/d rat respectively, and should, therefore, have provided the most complete indication of the protein contamination.

The full methods used to prepare and stain the gels are provided in Appendix 1.

The first gel was made as a gradient from 5% acrylamide at the top to 12.5% at the bottom, and 2-mercaptoethanol (2-ME) was used as the reducing agent in the sample buffer. Alginate solutions at a final concentration of 0.05% were applied to the gel (prior to addition of the sample buffer the alginates were prepared at a concentration of 0.15%), together with molecular weight markers and a negative control (sample buffer). Silver staining was used to determine the presence of protein and a band was detected in all the lanes at approximately 45-50kD. However, previous experience suggested that this was an artefact resulting from the use of 2-ME.

The second gel was a repeat of the conditions used for the first, but dithiothreitol (DTT) was used as the reducing agent in the sample buffer. This succeeded in removing the 45-50kD band seen when 2-ME was used, and in addition, very faint bands were visible in both

alginate preparations with a molecular weight of less than 15.4kD (i.e. the lower limit of the molecular weight markers used) (Figure 7.1). As this band did not appear in any of the control samples, it was concluded that these bands represented the contaminating protein in the alginates. It was noteable that the band was stronger in the G-alginate than the LVG(0.2) alginate suggesting that protein was present at a higher concentration in this alginate.

7.4 REMOVAL OF PROTEIN FROM THE ALGINATE

7.4.i Trypsinisation of sodium alginate

Once the presence of the protein contaminant had been confirmed and its approximate molecular weight determined, the next step was to try to remove it, and the first method used was to treat the alginate with trypsin.

Equal volumes of a 2% solution of trypsin and a 1.5% solution of sodium alginate were added to each other, giving final concentrations of 1% and 0.75% respectively. The mixture was then placed on a roller table in a 37^{o}C incubator for 24 hours, and the resulting sample run on a gel using the same conditions as described for the second gel. The samples were loaded in duplicate so that the gel could be cut in half and stained for carbohydrate as well as protein to confirm that the carbohydrate molecules had not been affected by the trypsinisation.

The carbohydrate stain confirmed that the alginate had not been affected by the trypsin treatment (data not shown), but also the low molecular weight band was still present in the treated alginate indicating that the trypsinisation had not removed the protein (Figure 7.1).

The next step in attempting to remove the protein was either to continue with the trypsin treatment and attempt to optimise the conditions to determine whether this would remove the protein, or to try alternative methods. The decision was taken to assess alternative methods, as ultimately the addition of trypsin to the alginate was another potential source of contamination.

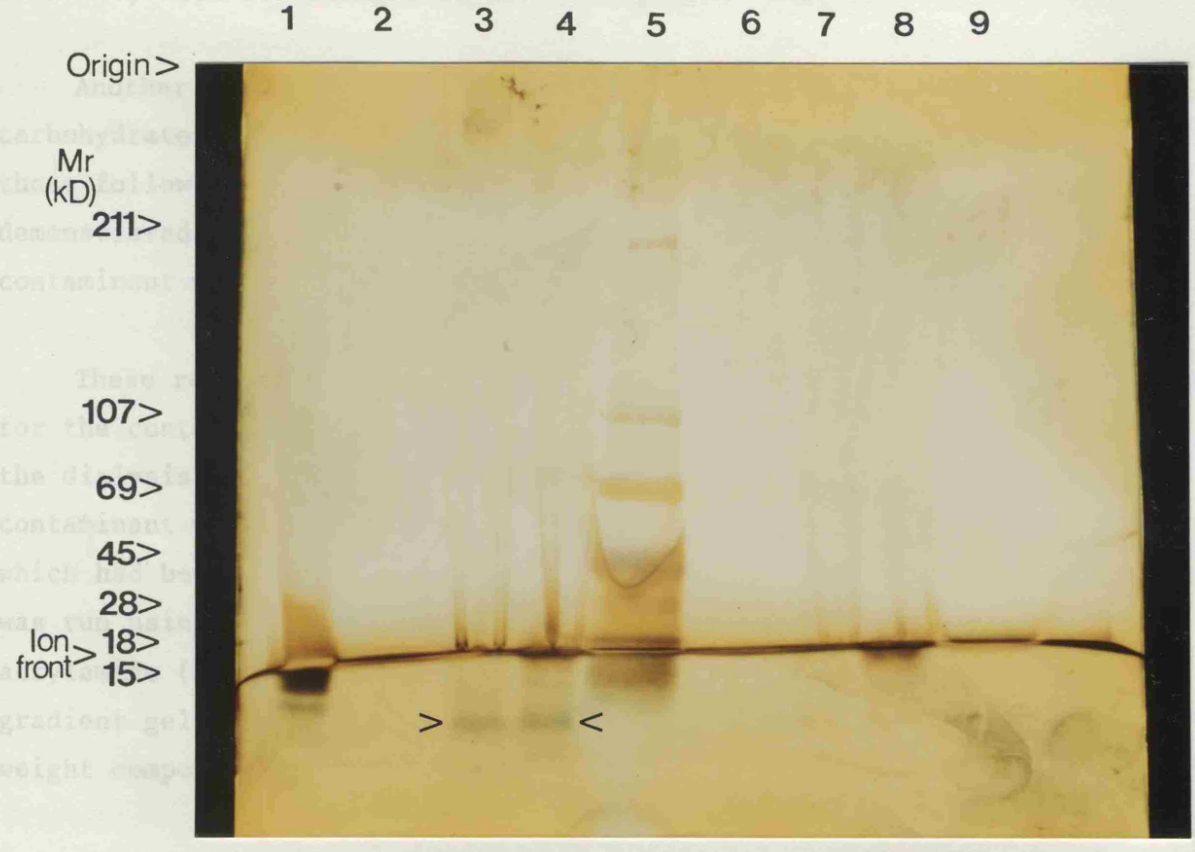

Figure 7.1
Polyacrylamide gradient gel stained for protein (x0.7).
(Lane 1=trypsin; 2=loading buffer + 2ME; 3=G-alginate;
4=G-alginate + trypsin; 5=molecular weight standards;
6=loading buffer; 7=LVG(0.2); 8=LVG(0.2) + typsin; 9=loading
buffer + DTT).
Arrows indicate the protein contaminant.

7.4.i Dialysis of sodium alginate

The next method used to try to purify the alginate was dialysis as low molecular weight proteins can be removed relatively easily by this method.

The alginate was placed in a dialysis sac (No. 250-11, Sigma) which would retain molecules with a molecular weight of over 12kD, and dialysed against phosphate-buffered saline (PBS) for a total of 75 hours, with two changes of PBS during that time.

Another gel was run using the dialysed alginate, and again both carbohydrate and protein stains used. The results were the same as those following the trypsinisation i.e. the carbohydrate stain demonstrated that the alginate had not been affected, but the protein contaminant was still present.

These results were unexpected as the molecular weight determined for the contaminant indicated that it should have been removed using the dialysis conditions detailed above. In order to confirm that the contaminant was of a low molecular weight, and not a larger molecule which had been reduced by the DTT in the sample buffer, another gel was run using a non-reducing sample buffer. In addition a 15% acrylamide (single concentration) gel was prepared, rather than a gradient gel to achieve a better separation of the lower molecular weight components.

The results of this gel confirmed that the contaminating protein did have a very low molecular weight as indicated by the other gels, and by using low molecular weight standards, the protein was determined to have a molecular weight of approximately 3kD.

A problem which had been encountered when running alginate samples on gels was that the appearance of the protein band was variable as it only stained very weakly with the silver stain even though this method of protein detection is more sensitive than using Coomassie blue (Switzer et al. 1979). Therefore, instead of using a 0.15% solution of alginate, it was decided to prepare a 1.5% solution to try to acheive a more reproducible result. This created another

problem, however, as the viscosity of the higher alginate concentration caused the lanes in the gel to become distorted and the alginate tended to spread into the adjacent lanes thus reducing the number of samples which could be applied to the gel. Consequently, it was only possible to study one alginate preparation on each gel so the decision was taken to continue the purification work with only the G-alginate as the protein band was more reproducible than with the LVG(0.2) alginate. The use of the higher concentration of alginate did improve the resolution of the silver staining.

The unexpected results from the dialysis suggested that the protein might have been bound to the alginate preventing its removal by a method commonly used for the removal of low molecular weight proteins from solutions. The next step, therefore, was to dialyse the alginate again but to add a reducing agent to the dialysis buffer to try to break down the disulphide bonds thought to be linking the protein to the alginate, thus allowing it to be removed.

DTT was used as the reducing agent at a concentration of 0.6mg ml^{-1} in PBS. The alginate was dialysed against PBS containing DTT for four hours with fresh dialysis buffer added after two hours, then for a further 75 hours against PBS alone to remove the DTT and any protein which had been separated from the alginate. During this time the PBS was changed after 1 hour and 72 hours.

Another gel was then prepared using a gradient of 5% to 12.5% acrylamide and the dialysed alginate applied in reducing sample buffer. Silver staining of the gel demonstrated that the protein contaminant had been removed from the dialysed alginate (Figure 7.2).

7.5 BIOCOMPATIBILITY TESTING OF THE DIALYSED ALGINATE

The result of the last gel indicated that the protein contaminant had been removed, and consequently the next stage was to test the dialysed alginate in vivo to determine whether treatment of the alginate had increased the biocompatibility.

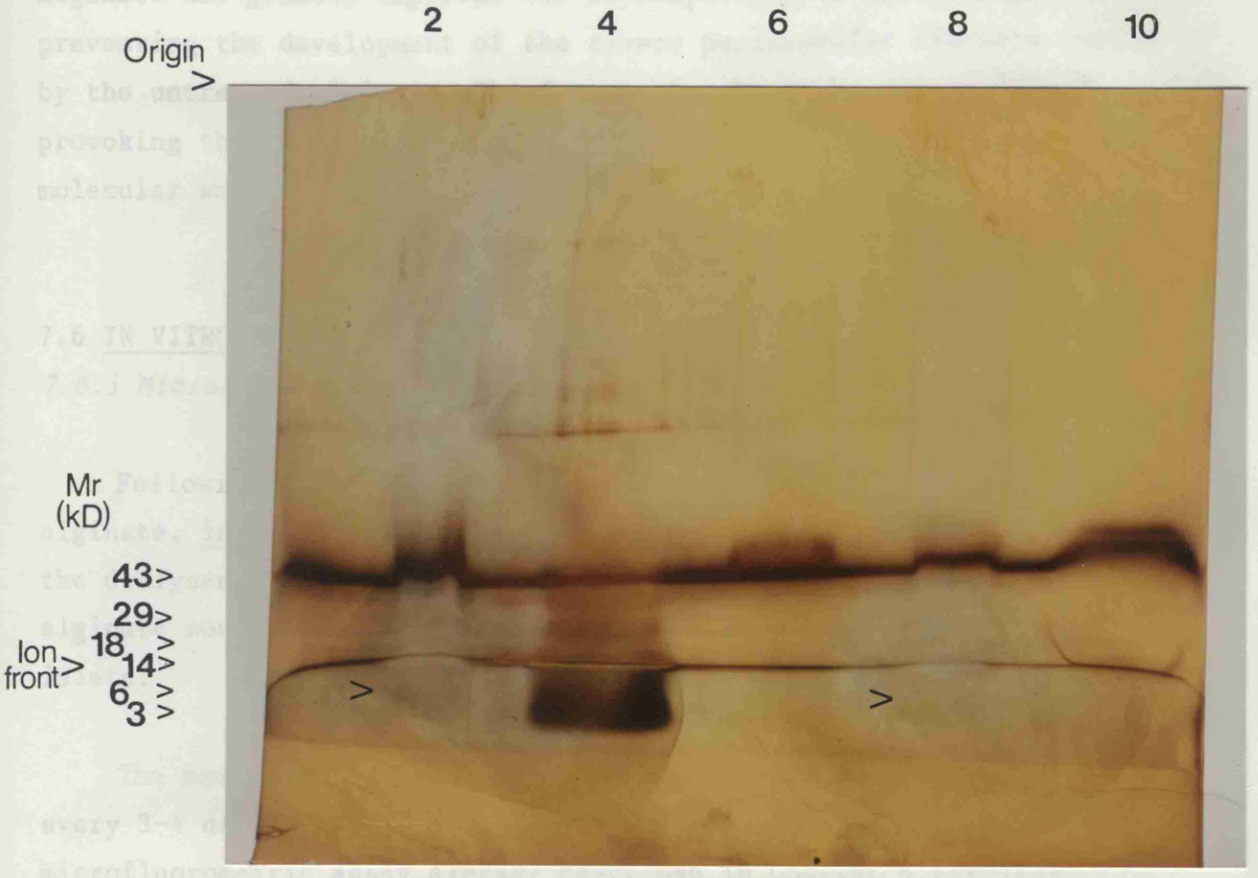

Figure 7.2
Polyacrylamide gradient gel stained for protein (x0.7).
Arrows indicate the protein contaminant (lane 2) and the absence of this band in the dialysed alginate (lane 8).
(Lane 2=G-alginate; 4=low molecular weight markers;
6=PBS + DTT; 8=dialysed G-alginate; 10=loading buffer).

This testing was undertaken as already described in Chapter 5 (Section 5.2.iii). Empty capsules coated with the dialysed alginate were implanted in the peritoneal cavity of BB/d, BB/ndp and WAG/Ola rats. The capsules were retrieved after three weeks and examined histologically using haematoxylin and eosin stained sections.

Figure 7.3 shows the results of the histology on capsules retrieved from a BB/d rat and is representative of the reaction to the capsules in all the strains of rat studied. This photograph when compared with Figure 5.4.ii clearly demonstrated that dialysis of the alginate had greatly improved the biocompatibility of the alginate preventing the development of the severe pericapsular fibrosis caused by the untreated alginate. The factor thought to be responsible for provoking the fibrotic reaction was assumed to be the very low molecular weight protein detected by PAGE.

7.6 IN VITRO VIABILITY STUDIES

7.6.i Microfluorometry

Following the excellent biocompatibility results of the dialysed alginate, in vitro assays were undertaken with islets encapsulated in the dialysed alginate in order to determine whether treatment of the alginate would lead to any adverse effects on the encapsulated islets.

The membrane integrity of the encapsulated islets was assessed every 3-4 days over a period of four weeks using the microfluorometric assay already described in Chapter 4 (section 4.4.iv).

The results shown in Table 7.3 demonstrated that the membrane integrity of the islets was good over the study period, although the scores were not as evenly clustered around the 0-2 level as had been the case with islets previously assessed by this manner. However, from day 8 onwards only one islet had a score of over 5 indicating that the membrane integrity of these islets was excellent.

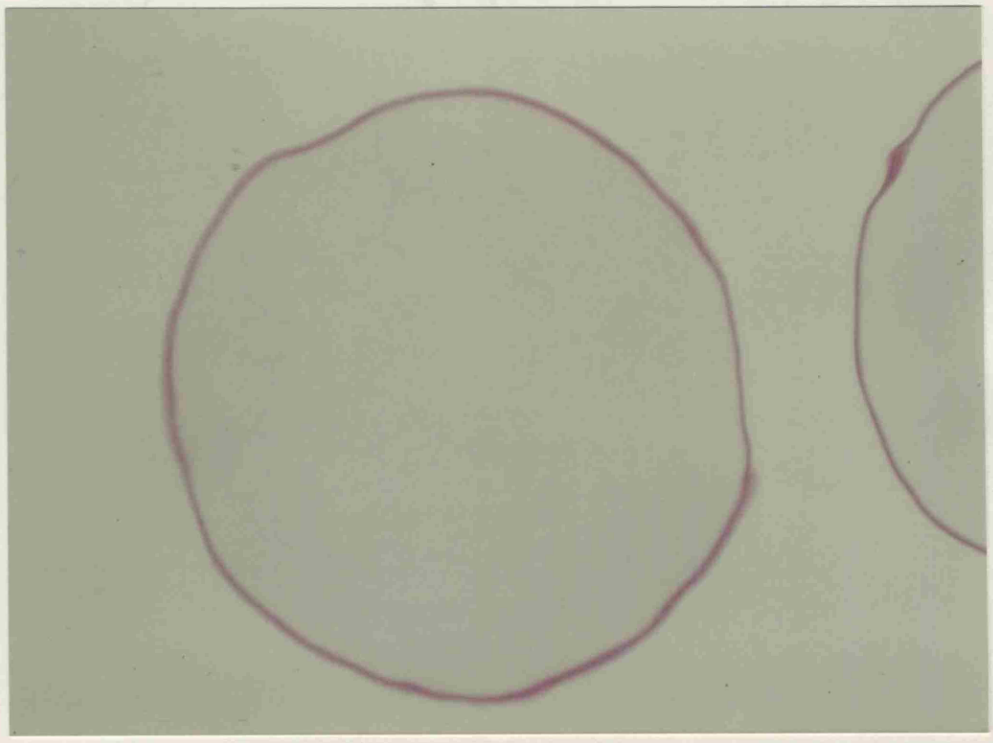

Figure 7.3
Dialysed alginate coated capsules from the peritoneal cavity of the BB/d rat after 3 weeks (H and E) (x180).

Table 7.3

Results (median, range) of the microfluorometric assay from islets encapsulated with dialysed alginate and control non-encapsulated islets.

Days in culture	Free islets	Encapsulated islets
1	1.5 (1 - 4)	-
5	1.5 (1 - 5)	7 (5 - 10)
8	1 (1 - 5)	3 (1 - 10)
12	4 (1 - 10)	0.5 (0 - 5)
15	1.5 (1 - 3)	1.5 (0 - 5)
19	1 (0 - 2)	0 (0 - 3)
22	1 (0 - 1)	1 (0 - 3)
26	1 (0 - 2)	1 (0 - 5)

7.6.ii Perifusion

The DNA-corrected insulin release from islets encapsulated in the dialysed alginate was determined by perifusion as described in Chapter 4 (Section 4.4.v). These experiments were undertaken at the same time as those with the islets encapsulated in Protan LVG(0.2) alginate (Chapter 5), and consequently, the non-encapsulated islets acted as controls for both groups of encapsulated islets.

Figures 7.4 and 7.5 show the mean stimulation increase and stimulation index data respectively, and Table 7.4 contains the response time data.

These results showed that the encapsulated islets had excellent response times, a factor which has been observed in all the perifusions undertaken with encapsulated islets in this project. The stimulation increase and stimulation index results reflected the trend which has previously been noted with the encapsulated islets i.e. there was a reduction in the absolute amount of insulin released from these islets relative to controls, but the proportionate increase in insulin release was similar for both the encapsulated and non-encapsulated islets. The possible reasons for the variability in this data have already been discussed in Chapter 5.

7.7 DISCUSSION

The results obtained in this part of the study have demonstrated that a protein contaminant was present in the alginate samples and that this could be removed by dialysis using DTT as an additive in the dialysis buffer.

The biocompatibility tests on the capsules coated with dialysed alginate demonstrated the absence of pericapsular fibrosis after implantation in the peritoneal cavity of WAG/Ola, BB/d and BB/ndp rats for three weeks, a significant improvement on the severe pericapsular fibrosis which developed when the untreated alginate was used.

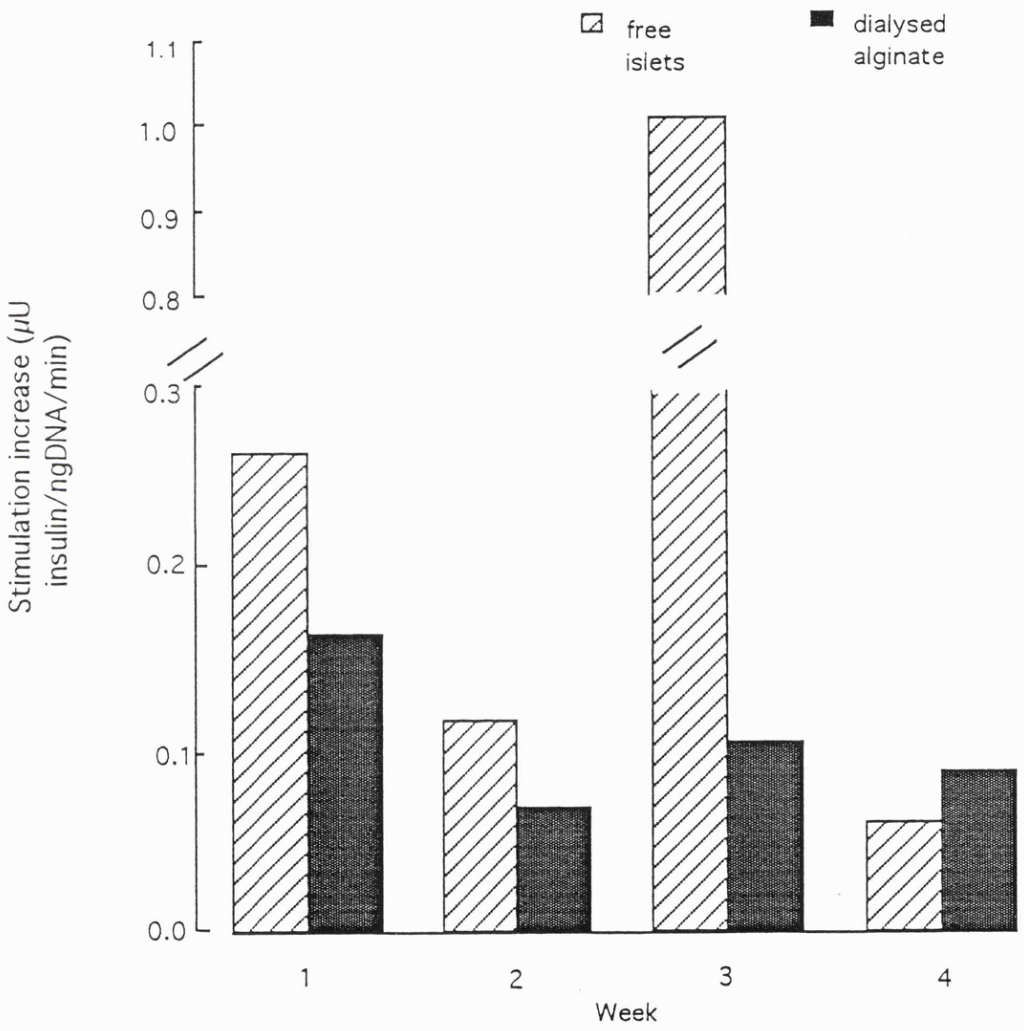

Figure 7.4

Stimulation increase data from the perifusion of islets encapsulated in dialysed alginate

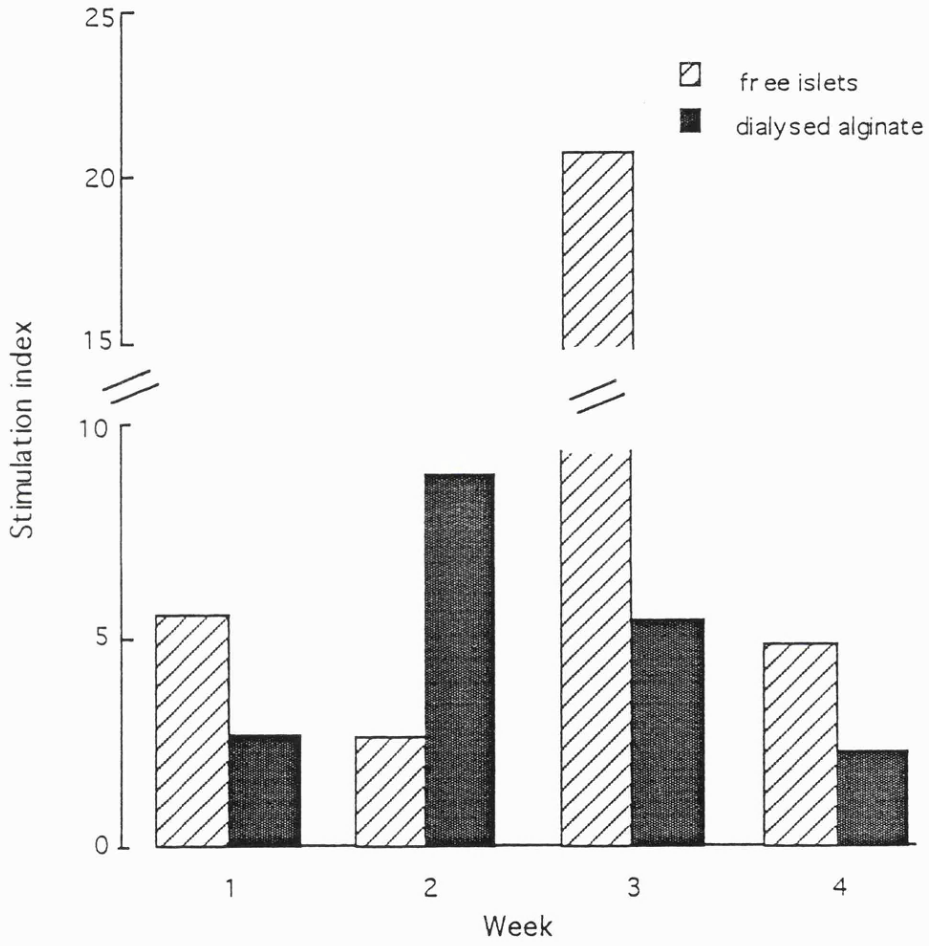

Figure 7.5
Stimulation index data from the perifusion of islets encapsulated in dialysed alginate

Table 7.4

Results of the time (in minutes) taken for islets to respond to glucose stimulation during perifusion. Data from duplicate experiments are shown separately as experiments 1 and 2.

	Free islets		Encapsulated islets	
	Exp 1	Exp 2	Exp 1	Exp 2
Week 1	0	0	0.5	0
Week 2	26	0	0	0
Week 3	0	0	0	0
Week 4	0	0	0	0

In addition, this method of treating the alginate did not have any detrimental affect on the viability of encapsulated islets, with excellent results from both microfluorometric assays and perifusions over a period of four weeks in culture.

7.8 SUMMARY

The results outlined in this chapter have demonstrated that the preparations of alginate which had been used to coat capsules for experiments detailed in the earlier chapters were contaminated with a low molecular weight protein. Once the source of contamination had been determined it was possible to purify the sodium alginate by dialysis provided that DTT was present in the dialysis buffer.

When the biocompatibility of the dialysed alginate was tested by implantation in the peritoneal cavity for three weeks, capsules were completely free of any pericapsular fibrosis even in the BB/d rat which had mounted an intense pericapsular fibrotic reaction to capsules coated with untreated alginate. In addition, treatment of the alginate did not have any adverse affect on the <u>in vitro</u> viability of islets encapsulated in the dialysed alginate, when studied over a period of four weeks using microfluorometry and perifusion.

CHAPTER 8

FUTURE PROSPECTS

The results from the final set of experiments were very encouraging. Experiments which now need to be undertaken include a long-term study of the in vivo response to empty capsules made with the dialysed alginate to assess whether a fibrotic reaction would eventually develop after implantation in the peritoneal cavity, and therefore to determine whether any further modifications to the purification method are required. This is particularly important in the BB/d rat as this strain developed the most severe response to capsules coated with untreated alginate, especially as this could have been a result of the autoimmune diabetic condition of these animals.

Transplantation experiments also need to be undertaken to determine whether the presence of islets in the capsules causes an increase in the risk of pericapsular fibrosis. In addition experiments are required to assess the ability of the capsules to protect islets against autoimmune disease destruction and allograft and xenograft rejection by using various combinations of donors and recipients. These combinations include:

1. BB/ndp rat islets into STZ-BB/ndp recipients to assess long-term function of encapsulated islets in a syngeneic situation.

2. WAG/Ola rat islets into STZ-BB/ndp recipients to determine whether the capsules can protect against allograft rejection, and whether the presence of islets in the capsules provokes a fibrotic reaction not seen when empty capsules are used.

3. BB/ndp rat islets into BB/d recipients to determine whether the capsules can protect islets from autoimmune destruction.

4. WAG/Ola rat, pig and human islets into BB/d recipients to assess the long-term function of allogeneic and xenogeneic islets in an autoimmune spontaneously-occurring model of diabetes.

The results of the transplantation experiments would determine any further studies which may need to be undertaken. For example, if empty capsules did not provoke a fibrotic reaction, but capsules containing islets did become fibrosed, this would suggest that the presence of islets was causing a response, possibly due to the release of substances small enough to cross the capsule wall. Also, if syngeneic transplants were successful but allografts failed due to damaged islets rather than as a result of pericapsular fibrosis, this would imply that antigens were being released by the islets which in turn were provoking the release of cytotoxic factors by the recipient. Experiments could then be undertaken to alter the pore size of the PLL layer to prevent passage across the capsule wall of the molecules causing damage to the islets. This could be achieved by increasing the reaction time of the PLL during capsule formation, increasing the concentration of the PLL, or using a lower molecular weight PLL preparation. This would provide the opportunity to develop an in vitro assay system to assess the capsule membrane properties when different conditions were used to form the PLL layer.

In addition to the transplantation experiments, another factor which needs to be assessed for encapsulated islet transplantation is the possiblity of reducing the size of the capsules allowing alternative implantation sites to be investigated. If the capsule size could be sufficiently reduced, it might be possible to use the preferred sites for islet implantation such as the renal subcapsular space or the portal vein, rather than using the peritoneal cavity. This would have the potential advantage of fewer encapsulated islets being needed to reverse diabetes. Also, the reduced pericapsular fibrosis noted in the renal subcapsular space relative to the peritoneal cavity might have a major impact on the success of a transplant.

APPENDIX 1

METHODS

A. Insulin assay
B. DNA assay
C. Polyacrylamide gel electrophoresis
D. Staining gels for presence of protein (Silver stain)
E. Staining gels for presence of carbohydrate (Periodic acid Schiff stain)

A. INSULIN ASSAY

This method was modified from that described by Hales and Randle (1963).

1. Perifusion samples were diluted if necessary with assay buffer (PBS containing 0.05% BSA, lot 127F-0384, Sigma), and 50ul added to each tube in duplicate.

2. Duplicate samples of blanks (assay buffer alone), control rat serum, and rat insulin standards (0, 0.25, 0.5, 1, 2, 4 and 8ng ml^{-1}, Novo Biolabs Ltd, Cambridge, UK) were also set up.

3. 50ul guinea pig anti-bovine serum (1:20,000 dilution in assay buffer; ICN Biomedicals Ltd, High Wycombe, UK) were added to each tube with the exception of the blanks, the tubes vortexed, and stored at $+4^{o}C$ for 4-6 hours.

4. 50ul ^{125}I-insulin (approximately 20-30,000 counts per tube, Amersham International Ltd., Amersham, UK) were added to each tube, the tubes vortexed and stored at $+4^{o}C$ overnight.

5. 50ul Sac Cel (solid phase second antibody coated cellulose suspension; IDS, Washington, UK) diluted 1:2 with PBS were added to each tube, the tubes vortexed and kept at room temperature for 30 minutes.

6. 1ml water was added to each tube, the tubes centrifuged at 3500g for 6 minutes (no brake), and the supernatant aspirated.

7. The samples were counted in a 1280 Ultrogamma (LKB, South Croydon, UK), the standard curve plotted, and the insulin content of the samples calculated from the graph, or in a 1282 Compugamma CS (LKB) which calculated the standard curve and the insulin content of the samples using the MultiCalc data management package (Pharmacia, Milton Keynes, UK). Figure A.1 shows the coefficient of variation graph of the insulin assay.

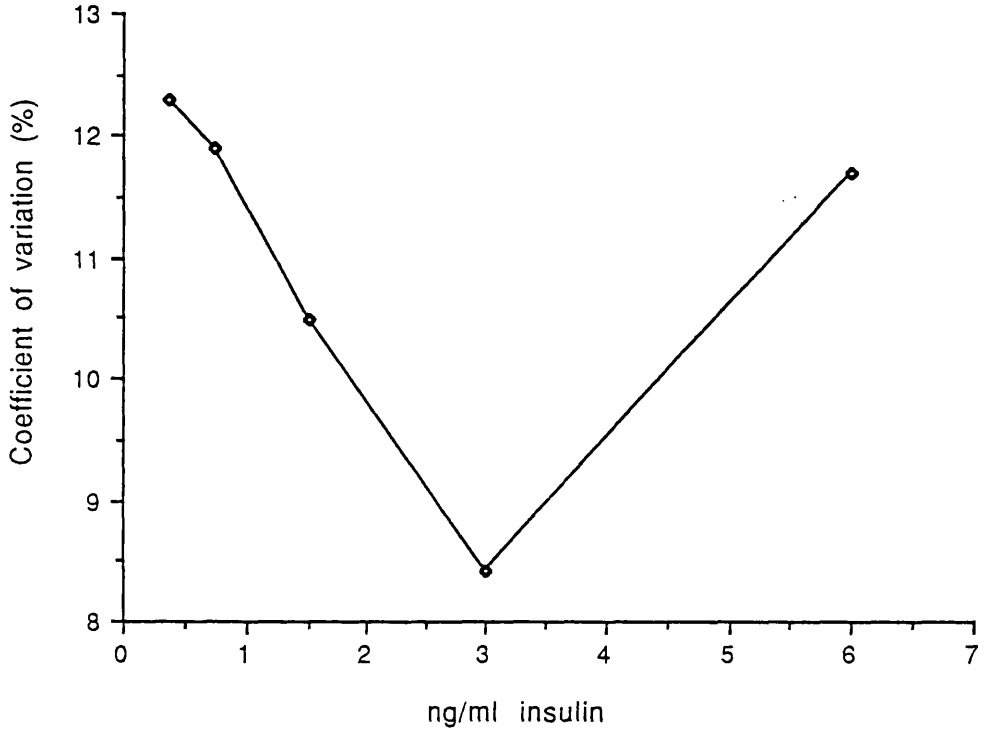

Figure A.1
Graph of the coefficient of variation of the insulin assay

B. DNA ASSAY

This method is based on the methods described by Labarca and Paigen, (1980) and Hopcroft et al. (1985).

1. Following perifusion, islets were recovered from the perifusion chambers. Encapsulated islets were treated with 5% sodium hexametaphosphate to remove the capsule.

2. 1.5ml DNA assay buffer (2M NaCl, 0.05M Na_2HPO_4, 2mM EDTA, pH 7.4) was added to each group of islets which were sonicated for 20 seconds at an amplitude of 14 microns (Soniprep 150, MSE, Crawley, Sussex, UK). Aliquots were stored at -20°C.

3. A stock solution of calf thymus DNA (Sigma) at a concentration of 50ug ml^{-1} was stored at -20°C, and used to prepare a standard curve of 0, 100, 200, 300, 400 and 500ng DNA.

4. The fluochrome reagent was bisbenzimide (Hoechst 33258, Sigma) made to a working concentration of 0.1ug ml^{-1} in NTE buffer (100mM NaCl, 10mM tris-HCl, 10mM EDTA, pH 7.4).

5. 2.6ml fluochrome reagent were added to 400ul of the standards and samples in duplicate, and incubated at 30°C for between 15 and 60 minutes.

6. The contents of each tube were vortexed, added to a fluorimeter cell (No. 67.755 acryl cuvettes, Sarstedt, Germany) and placed in the holder of an LS-5B luminescence spectrometer (Perkin Elmer, Beaconsfield, UK) set at excitation wavelength 356nm and emission wavelength 448nm.

7. The fluorescence of each sample was read, the standard curve plotted, and the amount of DNA in the islet samples calculated from the standard curve. Figure A.2 shows the coefficient of variation graph of the DNA assay.

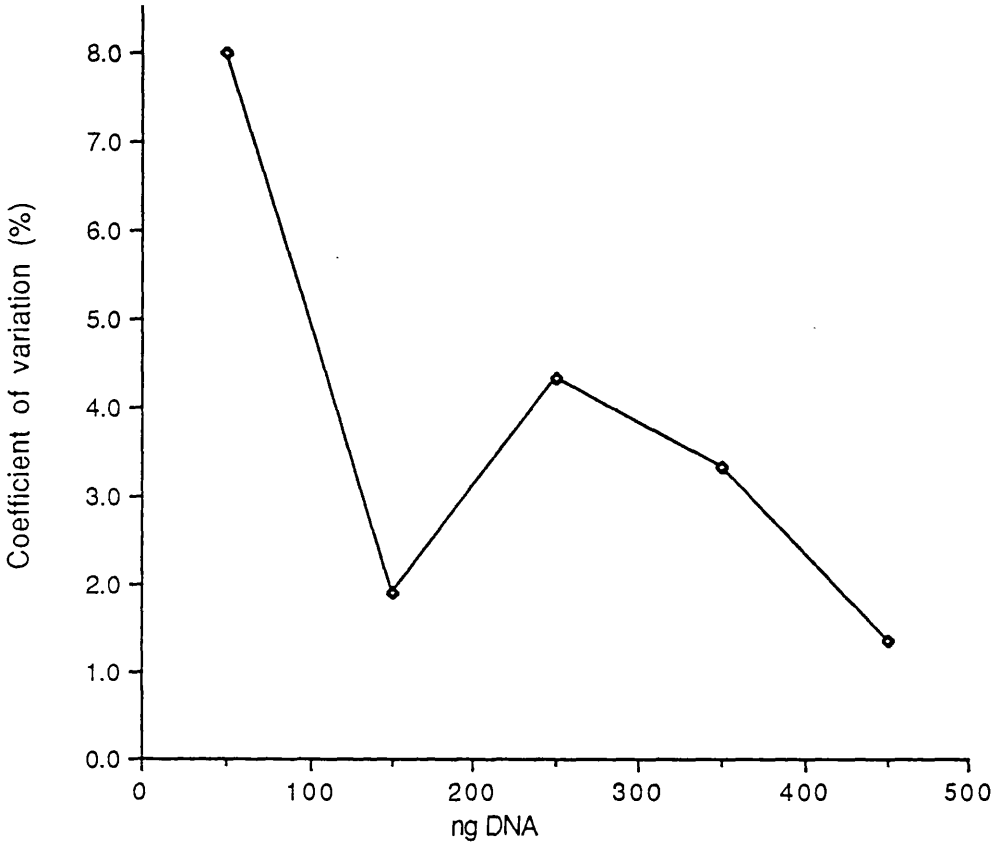

Figure A.2
Graph of the coefficient of variation of the DNA assay

C. POLYACRYLAMIDE GEL ELECTROPHORESIS

Solutions required to prepare gels:

50% acrylamide: 50g acrylamide + 1.3g bisacrylamide made up to 100ml with water.

Stacking buffer: 1M tris-HCl, pH 6.8

Resolving buffer: 1M tris-base, pH 8.8

Composition of resolving gels:

(Volumes in ml to give a total of 30ml)

	--Gradient--		
	5% +	12.5%	15%
50% acrylamide	1.5	3.25	9
Resolving buffer	5.625	5.625	11.25
Water	7.39	5.14	8.78
10% SDS	0.15	0.15	0.3
TEMED (Sigma)	0.01	0.01	0.02
1.5% ammonium persulphate	0.34	0.34	0.68

Composition of stacking gels:

(Volumes in ml to give a total of 10 ml)

	3%	5%
50% acrylamide	0.6	1.0
Stacking buffer	1.25	1.25
Water	7.7	7.3
10% SDS	0.1	0.1
TEMED	0.01	0.01
1.5% ammonium persulphate	0.35	0.35

Sample buffer:

50% (w/v) sucrose + 0.005% (w/v) bromophenol blue made up in 125mM tris-HCl, pH 6.8.

18mg ml^{-1} DTT added to make reducing sample buffer.

Use 2 volumes of sample buffer with 1 volume of sample.

Running buffer:

25mM tris-base, 190mM glycine, 3.5mM SDS, pH 8.3.

1. Resolving gels were prepared either as gradients of 5% to 12.5% acrylamide using a gradient maker, with a 3% stacking gel, or as a single concentration of 15% acrylamide with a 5% stacking gel. The sample wells were filled with running buffer.

2. The samples were diluted with twice their volume of sample buffer, boiled for 5 minutes, and 50ul loaded into the wells of the stacking gel. The gel apparatus was assembled, and the current applied. The gels were usually run slowly overnight to minimise any distortion of the gel due to the viscosity of the alginate samples.

3. When the bromophenol blue had run to within approximately 1cm of the end, the current was switched off and the gel fixed for staining. The solutions used for fixing the gels depended on whether they were to be stained for protein or carbohydrate.

D. STAINING GELS FOR THE PRESENCE OF PROTEIN (SILVER STAIN).

This method was adapted from those described by Switzer et al. (1979) and Oakley et al. (1980).

1. The gel was fixed in 50% methanol + 12% glacial acetic acid for a minimum of 20-30 minutes then washed three times for 10 minutes with 10% ethanol + 5% acetic acid.

2. The gel was then washed for 5 minutes with 200ml 0.0034M potassium dichromate + 0.0032M nitric acid, then with distilled water four times for 30 seconds each.

3. 200ml 0.012M silver nitrate was added to the gel and left for 5 minutes on a light box, and a further 25 minutes unilluminated.

4. 0.28M sodium carbonate containing 0.5ml l^{-1} formalin was added to the gel which was kept moving until the solution turned black. The solution was decanted, the wash repeated, and the solution left until bands in the gel developed, indicating the presence of protein.

5. Once the bands were fully developed, the gel was fixed with 1% acetic acid, then washed with distilled water.

E. STAINING GELS FOR THE PRESENCE OF CARBOHYDRATE (PERIODIC ACID SCHIFF STAIN).

This method was described by Fairbanks et al. (1971).

1. The gel was fixed with 25% isopropanol + 10% glacial acetic acid overnight, then washed with 10% isopropanol + 10% acetic acid for 6-9 hours, followed by an overnight wash with 10% acetic acid, then approximately 4 hours also in 10% acetic acid.

2. The gel was washed with 0.5% periodic acid for 2 hours, 0.5% sodium arsenite + 5% acetic acid for 30-60 minutes, twice with 0.1% sodium arsenite + 5% acetic acid for 20 minutes each, and acetic acid for 10-20 minutes.

3. It was then left in Schiff reagent overnight and washed several times with 0.1% sodium metabisulphite + 0.01M HCl, until the rinse solution failed to turn pink on the addition of formaldehyde.

APPENDIX 2

Publications arising from the work in this thesis.

Clayton HA, London NJM, Colloby PS, Bell PRF, James RFL.
A study of the effect of capsule composition on the viability of cultured alginate/poly-l-lysine - encapsulated islets.
Diabetes Research, 1990; 14: 127-132.

Clayton HA, London NJM, Colloby P, Bell PRF, James RFL.
The effect of capsule composition on the insulin release characteristics of poly-l-lysine/alginate encapsulated islets. (Abstract)
Diabetes Medicine, 1990; 7 (Suppl.1): A18

Clayton HA, London NJM, Colloby PS, Bell PRF, James RFL.
The effect of capsule composition on the biocompatibility of alginate - poly-l-lysine capsules.
Journal of Microencapsulation, 1991; 8: 221-233.

Clayton HA, London NJM, Colloby PS, Bell PRF, James RFL.
Effect of capsule composition on the in vivo response to alginate/poly-l-lysine capsules. (Abstract)
Gut, 1991; 32: A343.

Clayton HA, London NJM, Bell PRF, James RFL.
The transplantation of encapsulated islets of Langerhans into the peritoneal cavity of the Biobreeding rat.
Transplantation, in press.

Clayton HA, London NJM, Colloby PS, James RFL, Bell PRF.
The effect of capsule composition on the viability and biocompatibility of sodium alginate/poly-l-lysine encapsulated islets.
Transplantation Proceedings, in press.

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