SECONDARY PURIFICATION OF ISLETS BY THE USE OF IMMUNOMAGNETIC SEPARATION TECHNIQUES.

by

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THE SECONDARY PURIFICATION OF ISLETS BY THE USE OF IMMUNOMAGNETIC SEPARATION TECHNIQUES.

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Type I diabetes 1.2% of the population in the UK affects. Whilst insulin injections can control diabetes it has been shown that the frequency of secondary complications are only reduced by strict glycemic control. Pancreas transplants have been carried out to reverse diabetes. However, due to the severity, the morbidity and mortality of the procedure, only patients who have already received a kidney transplant and therefore have secondary complications have undergone such a procedure.

Pancreatic islet transplantation is a less invasive procedure. Since the 1980’s, reports of clinical islet transplantation using purified and unpurified islets have been made. Studies in the animal model, recent reports from clinical studies, and work carried out in this thesis, have emphasised the need to purify islets prior to transplantation.

Islets are purified by the use of density gradients. However, due to the overlapping densities of the islet and exocrine tissue, complete separation cannot be achieved. Therefore a more specific method is needed to increase the yield of islets obtained from a cadaver donor pancreas.

This thesis describes the development of a system using immunomagnetic techniques for the purification of islets. Using a quadripolar magnetic field into which the digest is released from a vibrating pipette with the addition of BSA prior to release, islets can be successfully purified with a loss of only 25%. The system can be used as a secondary purification following density dependent purification to increase the yield of islets from a single pancreas, making one donor to one recipient transplants a realistic option.
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 of January 1992 to July 1995, and has not been submitted for another degree at any University.

J.E. Davies.
DEDICATION.

This thesis is dedicated to my Mum and Dad
for all the support and encouragement they have given me
and for always being there.
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<td>Description</td>
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<tr>
<td>ABC</td>
<td>Avidin biotin complex</td>
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<tr>
<td>A.D.</td>
<td>Anno Domini</td>
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<tr>
<td>ALG</td>
<td>Anti lymphocyte globulin.</td>
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<td>ALS</td>
<td>Anti lymphocyte serum.</td>
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<td>ATG</td>
<td>Anti thymocyte globulin.</td>
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<td>B.C.</td>
<td>Before Christ.</td>
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<tr>
<td>BD</td>
<td>Bladder drainage.</td>
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<td>BSA</td>
<td>Bovine serum albumin.</td>
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<tr>
<td>CsA</td>
<td>Cyclosporin A.</td>
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<tr>
<td>15 DSG</td>
<td>15-deoxyspergualin.</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water.</td>
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<tr>
<td>DI</td>
<td>Duct injection of the pancreatic duct.</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco modification of Eagles Medium.</td>
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<tr>
<td>ED</td>
<td>Enteric drainage.</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunoabsorbent assay.</td>
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<tr>
<td>FK506</td>
<td>Fungus from Japan with immunosuppressant properties.</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting.</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum.</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin.</td>
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<tr>
<td>HAT</td>
<td>Hypoxanthine-aminopterin-thymidine supplemented medium.</td>
</tr>
<tr>
<td>HEPES</td>
<td>1M buffer solution pH 7.2-7.4.</td>
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<tr>
<td>HGPRT</td>
<td>Hypoxanthine-guanine-phosphoribosyl transferase.</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen.</td>
</tr>
<tr>
<td>HOC</td>
<td>Hyperosmolar citrate.</td>
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<td>IDDM</td>
<td>Type I insulin dependent diabetes mellitus</td>
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IEq Islet Equivalent (calculated on the basis of the volume of a 150μm diameter spherical islet).

IPTR Islet and Pancreatic Transplant Registry.

LDS no. Leicester Department of Surgery (the LDS no is the identity number designated to a monoclonal antibody).

MEM Minimal Essential Medium with Hank's salts.

MIMS Magnetic immunomicrospheres.

mmol Millimole.

mOsm MilliOsmole/Kg of water.

NCS Newborn Calf Serum.

OKT3 Ortho Klon T3.

PAK Pancreas after kidney transplant.

PBS Phosphate buffered saline.

PEG Polyethylene glycol 1500, 50% w/v HEPES.

PT Pancreas transplant alone.

RBC Red blood cells.

RPMI Roswell Park Memorial Institute-1640 tissue culture medium.

SDS/PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

SPK Simultaneous pancreatic and kidney transplant.

SRBC Sheep red blood cells.

STZ Streptozotocin.

TBS Tris buffered saline.

UD Urether drainage

UV Ultra-violet.

UW ViaspanTM (Belzer UW) cold storage solution.

WAG Wistar Albino Glaxo/Leicester, inbred rat strain.
AIMS

The possibility of reversing diabetes by the transplantation of isolated pancreatic islets led to extensive research being carried out into the purification of islets. Purification of islets based on the physical differences between islet and acinar tissue, using continuous density gradients, has probably been optimised. However, due to the overlapping densities of islets and acinar tissue complete separation will never be achieved. This has led some groups to transplant impure islet preparations. However studies have shown that for reasons of safety, maximising implantation rate and reducing immunogenicity, purified islets only should be transplanted.

The aims of this thesis were to investigate the effect of transplanting purified and unpurified islets intraportally into WAG/Leicester rats and also to develop a system that would enable the secondary purification of islets following density gradient purification.

The effect of allogeneically transplanting islets intraportally into WAG/Leicester rats was examined over a time period to allow assessment of the time for the development of the maximal inflammatory response following transplantation. Purified islets or acinar tissue were transplanted and compared with unpurified islets. The results were assessed using a scoring system to denote the severity of the inflammatory response observed at the different time intervals.

The aim of producing a system to purify islets using immunomagnetic methods was achieved by a series of sequential experiments using commercially available 4.5μm Dynabeads®. The results of one experiment would determine the methodology used in the next.

Immunomagnetic techniques have been previously applied to islet purification using 1.3μm Magnetic immunomicrospheres (MIMS) and an electromagnetic separation system. Therefore a comparison between the efficiency of MIMS and Dynabeads for islet purification was undertaken.
INTRODUCTORY OVERVIEW.

Insulin dependent diabetes mellitus can be controlled by injecting insulin. The list of complications, however, is depressing and is reviewed in Chapter 1. Studies over the last 30 years have shown that good glycemic control can reduce the frequency of these complications. This led to the development of pancreas transplantation. Whole pancreas transplants have been performed successfully since 1966. However, as the procedure is not life saving, the risk of transplantation and subsequent immunosuppression cannot be justified in those patients who may never develop complications from their diabetes. As a result, pancreas transplants have only been carried out in patients already suffering with secondary complications and usually in conjunction with renal transplants.

The transplantation of islets has been shown to be an alternative to whole pancreas transplantation. For successful intraportal transplantation 6,000 IEq/recipient Kg are required. However, the average yield of islets from a donor pancreas is 200,000 making a one donor to one recipient transplant difficult to achieve.

Digest counts following collagenase digestion of the pancreas have indicated that there are often over 1 million islets present. Islets are then purified from this digest by the use of density gradients. However, due to the overlapping densities of the islet and exocrine tissue, complete separation is impossible, accounting for the 5 fold reduction in yield. For this reason, some research centres have chosen to transplant unpurified islets. Chapters 2 and 4 review the literature on unpurified islet transplants in animal models and also clinical studies in the human. The work in Chapter 4 examines the effect of transplanting unpurified islets intraportally in the rat. The results support the findings of other studies that purification is beneficial prior to transplantation. A more specific and reliable method is, therefore, needed to increase the yield from one pancreas to allow a one donor to one recipient transplant.

Chapter 2 reviews the alternative approaches to islet isolation and also methods of cell separation with a view to their application to the purification of islets. Which ever method is chosen, it must minimise the trauma to the islets which have already undergone a period of cold ischaemia and collagenase digestion. The use of immunomagnetic techniques for the purification of islets appeared the most attractive alternative with the technique being rapid, gentle, easy to use on a large scale, with minimal trauma.
A system of negative selection removing the exocrine tissue was chosen. Immunomagnetic beads (Dynabeads®) were coated with anti-exocrine antibodies and bound to the exocrine fragments of the digest. On passage through a magnetic field, the exocrine tissue was retained by the magnet and the islets were allowed to drift through the magnetic field. The production of the monoclonal antibodies raised against the exocrine component of the pancreatic digest is detailed in Chapter 5.

There were two significant limiting factors to using immunomagnetic separation for islet purification. Firstly, due to the large size of the exocrine fragment compared to the beads, any shear forces created during separation causes dislodgement of exocrine fragments from the magnetic beads, thus contaminating the purified islets. Secondly, as the beads enter the magnetic field they become magnetic and cross link with each other, forming a mesh work and trapping islets in the process. Chapter 6 describes the development of a system designed to minimise these problems.

Magnetic immunomicrospheres (MIMS) have been used previously for the purification of islets. A comparison of the use of MIMS with Dynabeads® for the purification of islets was therefore carried out in collaboration with Dr. Supandi Winoto Morbach. This work is detailed in Chapter 7.

The work from this thesis is summarised in Chapter 8 and future work for the application of the system to human islet isolation as a secondary purification technique is discussed.
CHAPTER 1

INTRODUCTION TO INSULIN DEPENDENT DIABETES MELLITUS.

1.1 IDDM.
i. History of Diabetes.
ii. Incidence of IDDM.
iii. Aetiology of Type I diabetes.
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   b. Environmental factors.
   c. Autoimmunity.
iv. Pathogenesis.

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1.4 Conclusion.
1.1. IDDM.

1.1.1 History of Diabetes.

The term diabetes was first introduced by the Phoenician Aretaeus and derives from the Greek word "siphon" referring to water running through the body [1]. The first written description of diabetes, although it was not actually recognised as such, was by the Chinese Emperor (Huang Ti) who wrote about the bladder functioning without restraint causing copious urination. Hindu medical textbooks described diseased flow of urine and honey urine, "diabetic urine is sweet". Early Greeks and Egyptians also noted the phenomenon of sweet tasting urine. Paracelsis a Swiss physician recommended the tasting of urine for sweetness and reported that when urine was boiled 4 ounces of "salt" (although he never tasted it) was recovered.

There was little advance in the dark ages until, in 1682 Johann Brunner performed a pancreatectomy in a dog which went onto develop polyuria and polydipsia although no association was made with diabetes [2]. It was not until 200 years later that Merring and Minkowski performed the same experiment and demonstrated that total pancreatectomy in dogs caused a severe and fatal form of diabetes mellitus [3]. In 1788, Francis Home was the first observer to point out that yeast fermented sugar in diabetic urine. In the same year, Thomas Crawley reported a case history in which diabetes for the first time was diagnosed by demonstrating the presence of sugar in the urine and commented that diabetes might be a result of injury to the pancreas.

The observation that the amount of sugar in the urine varied in diabetic patients in relation to meals was made by Apollinaire Bouchardet. He taught patients to initially test their urine by using unslaked lime and later using a copper reagent [2].

The first description of pancreatic islets was made in 1869 by a medical student, Paul Langerhans [3]. The complete absence of islets in some cases of diabetes was recorded later by Sobolew and Dieckhoft. Opie in 1901, amongst other authors, concluded that islets of Langerhans and insular lesions played an important if not an aetiological role in diabetes.

Perhaps the most important discovery came in 1921, when Banting and Best isolated the blood sugar lowering hormone that many others before had failed to isolate, originally named "isletin" from islets of Langerhans (Figure 1.1). The production of long acting protamine zinc insulin, in 1936 by Hagedorn et al., saw a new phase with the successful treatment of diabetes with insulin.
Figure 1.1. “Isletin” first discovered in 1921.

1.iii. Incidence of IDDM.

The variation in incidence of IDDM world-wide reflects distribution of ethnic populations and demonstrates the importance of different genetic susceptibilities between populations. The highest incidence occurs in people of northern European descent. The disease is less common in racial groups such as Native Americans, blacks and Asians. The incidence ranges from a low of 1 to 2 per 100,000 per year in Japan to a high of 40 per 100,000 per year in parts of Finland [4].

Many studies have examined the changes in the incidence of diabetes and its distribution world-wide with temporal changes reported in over half of the 68 countries with registries. However, few diabetic registries have been in use for more than 20 years so that long term conclusions are tentative. In Leicestershire in the United Kingdom where records have been available for 54 years [5] different trends have been seen in different age groups and in New Zealand an increase was observed amongst the white population but not amongst the Polynesians or Maoris [6]. In northern Europe where populations have been stable and a decrease in prenatal and infant mortality over the last few decades has been seen, there has been a rapid rise in the incidence of Type I diabetes.
One study in Europe highlighted a pronounced north-south gradient in the incidence of IDDM. The prevailing susceptibility genes for IDDM in racially distinct populations was largely responsible for this, but diet and enivironmental factors were also thought to play an important role [7].

In the United Kingdom there is a prevalence of 1.2% and several studies have shown that there has been an overall increase in the incidence of childhood diabetes. In 1951-1960 the incidence was 3.8/100,000 rising in 1971-1980 to 10.6/100,000 per year. This increase was observed in both sexes and in all age groups [5].

Metcalfe and Baum studied the incidence of IDDM in children under 15 years old in 1988 and concluded that IDDM is sporadic over time and geographical area. The lowest incidence rate was seen in Ireland at 6.8/100,000, and the highest in Scotland at 19.8/100,000. Moreover there is a seasonal variation in the development of IDDM with a peak in winter [8].

1.iii. Aetiology of Type I diabetes.
   a. Genetic susceptibility.

   IDDM is a T cell dependent autoimmune disease. Although there are several genes thought to be involved in determining susceptibility, it is estimated that 60-70% of genetic susceptibility is encoded for in the human leucocyte antigen (HLA) region on chromosome 6 in humans and only the remaining 30-40% is encoded for outside this region. The HLA region is divided into 3 subclasses, class I, class II and class III. Primary genetic susceptibility to IDDM appears to lie within the class II region on which the DR, DQ, and DP loci are situated. Each locus is further subdivided into A and B loci which express a class II molecule made up of an α and β chain respectively. Class II molecules are situated on the surface of antigen presenting cells (APC) such as lymphocytes, macrophages and T cells, which present bound foreign or self antigen to CD4+ T cells, in the case of IDDM initiating β cell destruction [9].

   In patients of Caucasian origin with type I diabetes, 95% possess one or both DR3 and DR4 alleles and these are also associated with the disease in other races. However, the DR3 and DR4 alleles cannot be the primary susceptibility determinants, as 60% of non-diabetic Caucasians also possess these alleles [10].

   In 1989, Todd et al. using transracial analysis of populations to study the molecular genetics of diabetes, suggested a primary role for DR4. It has been suggested
by several groups that DQA1 is associated with IDDM, in white Caucasians, Afro-Caribbeans, Negroids, North Indian Asians and Japanese. In linkage disequilibrium with the DQA1 allele is the allele for the DRB1 gene encoding for DR4 specificity [7]. Thus a specific allele (0301) of the DQA1 gene is a potential determinant of susceptibility to type I diabetes and associated susceptibility has also been mapped to a DBQ1 allele.

Wolf et al. found that if DR2 is expressed in white Caucasian populations then protection is given against the disease but, there have been cases reported of diabetics expressing DR2. There are however 2 DQB1 alleles that are found on the DR2 and DR6 haplotypes in all races studied that show protection [9].

In 1990, Neepom proposed a model for the HLA effect in IDDM. He suggested that the products of certain HLA alleles are associated with IDDM due to binding and presentation of specific peptides on the cell surface, inducing an autoimmune response to the β cell [11]. In 1992, Sheeby proposed a model that mirrored Neepom’s [11]. He suggested that instead of the failure of the immune system to maintain tolerance to pancreatic β cells, HLA alleles which were negatively associated with IDDM produced products with high affinity for certain β cell peptides which were needed to maintain tolerance to β cells. The alleles that were common to IDDM, DR3 and DR4, produced products with low affinity for these β cell peptides or which bound in the wrong orientation or configuration failing to establish tolerance.

In summary, IDDM is a complex disorder whose genetic basis remains unclear although DQA1 and DQB1 are increasingly believed to play an important role in the genetic susceptibility.

b. Environmental Factors.

In humans, the development of pancreatic β cell mass occurs rapidly during gestation and infancy. It is thought that the proportion of β cells with the ability to replicate during foetal life is 10%, and this falls to 3% during young adulthood. It has been suggested therefore that the final β cell mass is determined during early life and factors that influence this are important in the subsequent development of diabetes. Poor intra-uterine nutrition in the human has been shown to result in fewer β cells and Hales and Barker [12] have suggested that the factors that influence early growth continue to influence final β cell mass in adulthood. Another suggestion that nutrition is an early trigger for diabetes has been made on the basis that, although the onset of IDDM peaks at around puberty or later, patients are seropositive for islet cell antibodies by the age of 5, thus the speed of onset of IDDM in some individuals is quicker than in others. In
Western Samoans brought up in New Zealand and in the Finnish there has been an association made between cows milk and IDDM. Antibodies to bovine serum albumin (BSA) were present in all patients at the onset of diabetes but only 2.5% of the controls were positive [13].

Secondly, it has been suggested that viral infection may precipitate diabetes via two possible pathways. The first relates to the development of the diabetes many years after the time of the viral infection; congenital rubella syndrome is an example of this. Approximately 20% of patients born with congenital rubella, develop diabetes 5-20 years later [14]. These patients also have a high incidence of thyroiditis and other immunological disorders. It is not known how the virus increases the incidence of IDDM in the patients expressing HLA susceptibility alleles, but it is thought that it may lead to tissue destruction by infecting multiple target tissue such as islets and thyroid. The second pathway is a direct assault on the pancreatic islets during acute viral infections. The virus is thought to trigger a series of complex immunological changes leading to β cell destruction. Mumps, rubella, picornaviruses, cytomegalovirus and retroviruses are all believed to have associations with diabetes [15].

c. Autoimmunity.

Cellular and humoral immune changes characterise the clinical onset of diabetes. Cellular changes were first noted by Gepts in 1965, when he demonstrated that the clinical onset of IDDM is associated with the infiltration of the pancreatic tissues with mononuclear cells. Upon diagnosis of type I diabetes, about 80% of islets contain no β cells and sometimes the islets are seen to be heavily infiltrated with lymphocytes [16]. T-lymphocytes are now thought to play a crucial role in the autoimmune β cell destruction. An increase in activated T-lymphocytes expressing HLA-DR antigens is seen in early lesions of the β cell where there is a predominance of CD4+ cells which are probably preceded by an increase in islet macrophages. With the onset of islet cell destruction, CD8+ cells have been shown to predominate.

In 1982, it was shown that prior to the clinical onset of diabetes, antibodies against the 64kDa glutaric acid decarboxylase (GAD) β cell protein were present. At the time of onset of IDDM, 80-90% of patients had antibodies against this 64kDa protein. GAD is a biosynthetic enzyme for the inhibitory neurotransmitter gamma amino butyric acid expressed in high levels by pancreatic β cells. Circulating antibodies against a 38kDa islet cell protein have also been detected in newly diagnosed IDDM patients [17]. However, the 38kDa protein was only found in HLA DR3 positive patients [18].
A test for islet cell cytoplasmic antibodies together with a test for GAD65 was used to predict the subsequent development of diabetes in 2,805 children [19]. Over a period of 11.5 years, 4 out of 8 that were islet cell antibody positive and 3 that were islet cell antibody negative developed diabetes. Sera from those who were antibody positive and from 100 children randomly selected, were then tested for GAD65 antibodies. Six out of the 8 that were islet cell antibody positive were GAD65 positive (this included the 4 that developed IDDM). One of the 3 islet cell antibody negative individuals who developed IDDM was GAD65 positive. They conclude that a single GAD65 antibody test may have a higher sensitivity for predicting IDDM than a test for islet cell cytoplasmic antibodies. However a combined test for both antibodies would increase the specificity.

The identification of these two autoimmune targets on β cells will enable the measurement of β cell directed autoimmunity in pre clinical subjects [17] and is likely to lead to further advances in our understanding of the early stages of the disease.

1.1.4. Pathogenesis.

Insulitis refers to inflammatory lesions affecting the islets of Langerhans first noted in 1948 by Von Meyenberg to occur in patients with type I diabetes [20]. It had been noted that lymphocytic infiltration was more frequent in the islets of children with diabetes, but it was not until 1958 that the association of insulitis with juvenile diabetes was made by Le Compte, who found such lesions in 16 out of 23 patients who died within the first six months of the onset of the disease, whereas in those patients who survived one year or more, insulitis was not present [21]. Gepts also reviewed the reported cases of insulitis and noted that in 89% of patients with type I diabetes, where the onset of the disease had occurred before the age of twenty, insulitis had occurred. Insulitis does not affect the islets uniformly and there appears to be a patchy collapse in the framework of the islets, revealing a pattern of cords of small cells. Advanced fibrosis can be seen in some islets and, where β cells have been destroyed, lymphocytes disappear. Gepts has suggested that insulitis is a manifestation of a delayed sensitivity immune reaction and, the observation that insulitis preferentially affects islets containing β cells, is consistent with an immunopathological process specifically directed against these cells [22].

In patients with juvenile diabetes just after the onset of the disease islets can be seen to be regenerating. The newly formed islets are mainly composed of β cells and the number of α and δ cells increases later. However, with time, the regeneration process disappears. Gepts and LeCompte have also suggested that the islet cell architecture that is destroyed by β cell loss and the dispersion of α and δ cells as single
cells in the acinar tissue, may have some significance in the pathogenesis of the disease [23].

Doniach and Morgan [24] from their studies have suggested that the pancreas at the onset of diabetes, appears to stop growing and the pancreata of patients with type I diabetes are often small and reduced in weight. The islets, in type I diabetes, appear to fall into 3 categories. The first are pseudoatrophic islets: these contain small active endocrine cells, with small dense nuclei and thin cytoplasm, the cells form thin cords within an abundant fibrous matrix. The second type are hyperactive islets, found in patients with type I diabetes of short clinical duration. They are made up of cells that are variable in size and have distinct and regular outlines with a large nucleus. The third type of islets found in the diabetic pancreas are PP islets, composed of cells secreting pancreatic polypeptide. Within the cells are central nuclei and the cells are arranged in cylindrical cords.

1.2 Treatment of Diabetes Mellitus by insulin therapy and complications.

1.2.1 Insulin treatment.

a. Intermittent subcutaneous insulin therapy.

A new era in the treatment of IDDM was launched in 1921 with the discovery of insulin (although Meyer in 1909 had already named the internal secretion of the pancreas "insuline") and the first administration in January 1922 of the drug by the Toronto group, Banting, Best, Macleod and Collip [25]. Some patients received insulin once a day others multiple doses. However, with the inconvenience to the patient of multiple doses of porcine and beef insulin, the ability to prolong the action of insulin was investigated. Hagedorn in the 1930's found that the action of insulin was prolonged if protamine was added and the same effect was noted by Scott and Fisher, but, with the addition of zinc instead. These discoveries lead to the production of protamine zinc insulin and isophene insulin. In the mid 1950's lente insulins were introduced, these contained zinc only. Hence a range of quick acting, intermediate and long acting insulins became available [25]. Zinman pointed out in his review of insulin regimes and strategies for IDDM [26] that despite the advancement of insulin research, the purity, improved pharmacokinetics of insulin analogues and the availability of human insulin, achievement of metabolic normalisation remains uncommon. Glycemic control is related to metabolic normalisation and failure to maintain normoglycemia is now accepted as a major factor in the development of the long term complications of diabetes [27].

There have been several reasons for the failure to achieve normoglycemia. Firstly, insulin delivered subcutaneously enters the peripheral venous circulation instead
of the hepatic portal system. Normally half of the insulin is cleared in its first passage through the liver. Subcutaneous injections reduce the exposure of the liver to high insulin concentrations, thus the mode of delivery and action is not entirely physiological [28].

Secondly, it has also been shown that numerous variables such as site and depth of injection, insulin dose, mixture, type of insulin and local heat at injection site influence the absorption of insulin, and thus levels of circulating insulin in the blood [26]. Even in one individual, this may not be constant and approximately 30% of children in the early 70's receiving porcine and bovine insulins, developed lipoatrophy at the injection site. The occurrence of this was reduced by the purification of porcine insulin in the late 70's and, by injecting highly purified porcine or human insulin directly into lipoatrophic skin, lipoatrophy can be reversed [29]. Figure 1.2 shows the different types of insulin available.

Thirdly, the introduction of human insulin in the last 10 years and in 1986 the withdrawal by a manufacturer of short and long acting porcine insulin preparations, forced a massive change over to the use of human insulin [30]. The following year, Teuscher and Berger reported that patients who had switched from porcine to human insulin in this way experienced a loss of warning of hypoglycemia, with a change in hypoglycemic symptoms in 36% of patients, from sweating, tremor, palpitations for example, to those of neuroglycopenia, producing inability to concentrate, speech, headaches, and visual disturbances. Berger et al. in 1989 [31] reported a double blind cross-over trial in which 32 patients received porcine or human insulin for a period of 12 weeks and then crossed over. The patients filled out questionnaires after hypoglycemic episodes and at the end of the trial. They concluded from this study that hypoglycemia developed faster during human insulin administration than with porcine insulin. However, the study has been criticised for not defining hypoglycaemia and the number of hypoglycemic attacks is the highest ever to be reported. A number of questions also arise from this study, such as selection of patients and insulin administration before the study [32,33].

It may be that the tightening of glycemic control and not the type of insulin makes patients prone to hypoglycemia and this has been one of the major problems with the use of pen devices for the administration of insulin. Worries over hypoglycaemia are probably the major factor limiting the achievement of normoglycemia by subcutaneous injections.
b. Insulin pumps.

Waldhausl in 1986 stated that "conventional insulin treatment does not hold any promise for further improvement of the metabolic fate of our patient" [34]. As maintenance of constant normoglycemia in a diabetic patient is seldom achieved by diet or insulin therapy, research in the 70's and 80's began to investigate computerised control systems that would simulate a "normal pancreas". These systems are capable of secreting insulin in response to the normal range of blood glucose preventing deviation beyond physiological limits.

The first report of continuously monitored blood glucose in a feedback controlled insulin delivery closed loop infusion system, was by Kadish in 1963 [35]. The next report came in 1974 by Albisser et al. They reported the use of a microcomputer programmed to respond to the constantly monitored whole blood glucose, by injection of either insulin or glucose to maintain or restore the normal blood sugar [36]. They found that the use of the system in pancreatectemised dogs produced glycaemic control paralleling that of a normal animal. The systems used in both cases were closed loop. However, the extensive clinical applications foreseen by their inventors have not materialised due to the size of the apparatus and the continual
removal of blood from the vein, enforcing immobilisation of the patient and severely limiting the clinical application.

Slama et al. in the same year reported the use of an external pump capable of delivering insulin intravenously, giving better metabolic control than with 3 daily injections [37]. Tamborlane, in 1979, used subcutaneous administration of insulin via a portable infusion pump in patients with brittle juvenile diabetes [38]. The infusion pump did not have a glucose sensor or an intravenous route. It was observed that a greater amount of insulin was required than when using conventional methods. If the same amount of insulin was administered conventionally then the plasma glucose levels were seen to be higher than with the infusion pump. It was also observed that the higher dose failed to reduce maximal fluctuations in plasma glucose and hypoglycemia was noted in 2 patients.

Two types of pumps became commercially available (shown in Figure 1.3). The first is the simple syringe pump, where the plunger is advanced by an electromotor-driven screw, at timed pulses incremental measurements of insulin are delivered. Variation in the time between pulses determines the dose. The second type of pump is the peristaltic pump. These are equipped with miniaturised stepping motors and work on the same principle (the sigma pump principle) as those used in haemodialysis. A high failure rate due to technical problems was encountered with these pumps but they have been successfully used in the evaluation of different delivery routes for insulin: intravenous, intramuscular, subcutaneous and intraperitoneal.

Mecklenburg et al. [39] in 1982, carried out a clinical study of the use of the infusion pump in 100 patients. They found that 93 patients had improved blood sugar control but episodes of ketoacidosis and serious hypoglycemia were both seen. No conclusion could be drawn as to whether the risk of ketoacidosis and hypoglycemia are different in patients using insulin pumps, compared with conventional therapy. However, 10% of patients had skin infections at the injection site, a problem not observed in those patients on conventional therapy. They concluded that the infusion pump could successfully be used clinically, provided there was continual support from the medical staff, as this treatment required a substantial commitment of time and resources.
In 1984, a further 161 patients who used insulin pump therapy demonstrated that although diabetes control improved substantially, 42% of patients experienced complications [40] including, infection at the infusion sites, ketoacidosis and hypoglycemic coma. The data suggested that there was an increase in the risk of ketoacidosis due to unpredictable interruption of the insulin infusion caused by battery failure and tubing defects, but there was no apparent increase in risk of hypoglycemia with insulin pump therapy.

The implantable constant rate pump was developed for use of infusion of heparin in patients with thrombotic problems by Blackshear et al. [37], but it was used for the first time for insulin infusion in 1980 by Buchwald et al. [41]. He implanted a model 400 Infusaid® constant basal rate pump. Such pumps are implanted under the skin and a silicone catheter enters a vein or the peritoneal cavity. The pump produces a continuous flow by the pressure created by freon gas. A reservoir is refilled with insulin by percutaneous injections. The pump remains too large to implant into young children or thin patients but they have been used with long term clinical success. Programmable implantable devices have also been developed but commercially available insulins tend to clog the devices and they have thus had limited use clinically.
A more recent study [42] has compared 3 types of programmable implantable pumps (including the Infusaid®) used by 224 patients for a mean period of 1.5 ± 0.9 years. The major technical problems encountered were, pump flow rate reduction, related to insulin aggregates and 47 catheter obstructions that required either laparoscopic or conventional surgery. Despite these problems only 9 pumps failed. The same group carried out a study investigating catheter survival and the mechanisms involved in obstructed catheter implants [43]. They found catheter obstruction to be a frequent adverse technical event. They concluded if the biocompatibility of the catheter material and the stability of the insulin preparations to prevent immuno-inflammatory reactions and insulin amyloid deposits that appear to be involved in obstructed catheter, then fewer problems may be encountered.

Despite these drawbacks the search for improved pumps will continue, supported by the recent findings of the Diabetes Control and Complications Trial (DCCT) research group who carried out a 6.5 year study assessing the effect of intensive therapy of diabetes on the development and progression of the long term complications. They concluded that intensive therapy, using external insulin pumps or 3 or more insulin injections, with frequent monitoring of blood glucose, delayed the onset of retinopathy and nephropathy, compared to conventional therapy [27].

c. Other routes for insulin administration.

Researchers have investigated other surfaces of the body through which insulin can be absorbed. Saudek in his review of insulin delivery systems discussed the use of nasal delivery of insulin [28]. The first report was in 1935 by Major, and in the 1980's it was observed that if insulin was complexed to surfactant materials then the insulin was able to traverse the nasal mucosa more effectively. However clinical trials have shown that insulin taken nasally is absorbed faster than subcutaneously, but at a lower maximal rate and patient acceptance, and variability must be investigated before nasal administration can become a realistic alternative to subcutaneous injections.

Another promising approach is the delivery of insulin by aerosols. This mode of delivery offers advantages over the use of the nasal mode of delivery because the surface area for adsorption in the lungs is far greater and sufficient amounts of insulin can be absorbed to lower the blood glucose levels in patients with NIDDM.

Since the discovery of insulin, attempts have been made at delivering insulin orally. However, the results have always been disappointing due to the efficiency of the gastrointestinal tract at digesting such proteins.
It seems unlikely that the delivery of exogenous systemic insulin can ever match the minute to minute regulation of a range of metabolic functions exerted by normal pancreatic islets [44], where the response to changes in portal glucose concentration occurs rapidly [45]. This realisation has led to an increasing interest in the transplantation of pancreatic islets either as part of a vascularised pancreas transplant or in the form of either a purified or unpurified islet transplant.

ii Complications.

a. The cause of complications of IDDM.

The DCCT published the results of a large study investigating the effect of intensive treatment on the complications of IDDM [27]. They concluded that intensive therapy is capable of slowing down the progression of complications. Thus glycemic control appears to play an influential role in the development of complications seen in IDDM. The list of possible complications arising from IDDM is depressing, but the British Diabetic Association (BDA) pointed out that studies over the last 30 years have shown that with increased glycemic control the frequency of these complications has reduced [46].

b. Vascular complications.

Late complications of diabetes affect large blood vessels which may become narrowed or blocked. Macrovascular complications affect the vessels to the heart, brain and legs giving rise to heart attacks, strokes and gangrene.

In men, the mortality risk from heart attacks is 1.9 times greater in those with diabetes, including NIDDM, than in non-diabetics and in women the rate is 2.7 times greater. In younger patients however, the increased risk of mortality from heart attacks is disproportionately great. In men under the age of 45, the risk is 5 times and in women the figure is 11.5 times that of the general population. Women with diabetes have a higher risk of dying from a stroke than non-diabetics and in men the risk of death from a stroke is 1.5 times higher. While peripheral vascular disease does not cause as many deaths as strokes and heart attacks, there is a 25 fold increase in the number of deaths due to peripheral vascular disease compared to non-diabetics [46].

The DCCT study of the effect of intensive therapy on the complications of IDDM reported that the overall risk of macrovascular disease with intensive therapy reduced by 41% when compared with conventional treatment [27].
c. Retinopathy.

Retinopathy (damage to the retina) rarely occurs within the first 5 years but there are usually visible signs within 7 years of the onset of IDDM in 50% of patients, and after 20 years, the prevalence is greater than 90%. Figure 1.4 shows an example of retinopathy. In NIDDM at diagnosis there can be visible signs of retinopathy.

At first, retinopathy is slow in progression and does not initially affect vision. It is usually referred to at this stage as background retinopathy. As time progresses, however changes occur which threaten vision and if untreated blindness follows.

Figure 1.4. Retinopathy.

The BDA has reported overall that approximately 30% of patients with diabetes, either IDDM or NIDDM have retinopathy. Each year 1.2% of these develop sight threatening changes. If the correct treatment is given, 70% will retain their vision, but diabetes remains the commonest cause of blindness in the 45-65 age group, and in 1980, patients were being 23 times more likely to have their vision impaired than non-diabetics in the same age group [46].

The results published by the DCCT [27], showed that the incidence of retinopathy with intensive and conventional treatment, during the first 36 months,
followed a similar pattern. However at about 36 months, the pattern altered and the cumulative incidence of retinopathy in the intensive therapy group was 50% less than in the conventional treatment group. It was also found that the adjusted mean risk of retinopathy was reduced by 76% with intensive therapy and this reduction increased with time.

d. Nephropathy.

Diabetic nephropathy can lead to kidney failure and has the highest mortality rate of the diabetic complications. It develops in 35-45% of patients with IDDM and it is less common in NIDDM. As with retinopathy, the onset of nephropathy is later in IDDM than in NIDDM patients (not usually occurring until 5 years after onset of diabetes) [47].

Proteinuria is usually the first indication of the development of diabetic nephropathy resulting in peripheral oedema, followed over the next 5-10 years, by general ill health associated with renal failure. In the absence of dialysis or transplantation the patient deteriorates and dies.

Kidney failure is the commonest cause of death in those who develop diabetes before the age of 30 and the mortality rate from renal failure in those under the age of 50, is 40 times greater than in age matched non-diabetics. In 1985 in the UK, 580 patients developed kidney failure, of those, 450 were thought to be suitable for transplantation but it was only possible to provide transplants or dialysis treatment for 270 patients.

It has been suggested that the progression of nephropathy can be delayed if the dietary protein content and the blood pressure are controlled. The DCCT reported that if nephropathy has not developed by 25-30 years since the onset of the disease, then the risk of developing the complication decreases [27].

e. Neuropathy.

Sixty percent of those with diabetes have some form of neuropathy, but only 20% of them will present with problems. The most common is the development of foot ulceration in those with reduced sensation in the feet (Figure 1.5) Those with diabetes are 50 times more likely to develop foot ulceration than non-diabetics and if peripheral vascular disease is also present, amputation may be the end result. While foot ulceration is often responsive to early treatment, patients with diabetes are still 25 times more likely to have to have a leg amputated than non-diabetics [46].

Mills et al. carried out a study investigating the increase in risk of women giving birth to a malformed infant due to IDDM and how that risk is influenced by metabolic control [48]. They found that even in women with good diabetic control within 21 days of conception, malformations still occurred at twice the rate of the control group (4.9% compared to 2.1%). For those women with poor control entering the study later, 9% of infants born were malformed. From their study alone they were not able to conclude that glycemic control is related to congenital malformation, however if the results are combined with those of Miller et al. [49] then poor glycemic control appears to explain some of the diabetes associated malformations. The Centre for Disease Control also concluded that women with IDDM are at increased risk of stillbirths and obstetric complications as well as congenital malformations [50]. It is clear that there is a need for good glycemic control around the time of conception.
1.3. Alternatives to insulin therapy.

i. Whole pancreas transplantation.

a. A brief review.

Ketoacidosis and death from diabetes can be prevented by the use of exogenous insulin but this does not prevent the secondary complications of the disease from developing (see above). Pancreas transplantation can provide physiological endocrine replacement therapy in type I diabetics, but it is not a life saving operation. Since it is difficult to determine which patients will develop secondary complications, the risk of transplantation and subsequent immunosuppression cannot be justified in those who may never develop complications. As a result pancreas transplants have only been carried out in patients already presenting with secondary complications and usually in conjunction with renal transplants.

In 1966, the first vascularised pancreas transplant was performed at the University of Wisconsin by Kelly et al. [51]. However, the operation did not really gain acceptance until the discovery of a new immunosuppressive agent, cyclosporine and the refinement of the surgical technique.

In the period 1966 to June 1993 a total of 4,799 pancreas transplants have been reported to the International Pancreas Transplant Registry, IPTR [52]. The IPTR reported that during the period October 1987-November 1992, 2061 USA and 809 non USA whole pancreas transplants were performed. Out of 2061 USA transplants, 1749 were carried out simultaneously with a kidney transplant, 173 were transplanted after a kidney transplant, 113 were pancreas alone, 5 were simultaneously with a liver, 3 were transplanted with a liver and kidney, 1 simultaneously with a heart and 1 with a kidney and heart. Outside the USA, 720 of the 809 pancreas transplants reported were simultaneous with a kidney (SPK), 33 were pancreas transplants after a kidney transplant (PAK), 46 were pancreas only transplants (PT) , 6 were simultaneous with a liver and 3 with a heart and kidney.

One of the major technical challenges has been the management of the acinar secretion of the pancreas and in order to remove acinar secretions the pancreas can be transplanted with either, bladder drainage (BD), enteric drainage (ED) or duct injection (DI) of the pancreatic duct. In the USA the majority of transplants have been carried out using bladder drainage, but in non USA transplants only 60% were performed with BD, 31% used DI, 8% used ED and 1% used other techniques such as ureter drainage, (UD) . The overall technical failure was higher outside the USA 18% v 12%, with variations within each recipient category.
At 1 year post-transplant the patient survival rates were virtually identical but there was a difference in the graft survival at 1 year. In the USA the rate for graft survival was 71% and outside the USA the rate was lower, at 66%, with variation in graft survival rates in each recipient category. Comparing simultaneous pancreas and kidney transplants by BD, DI, ED, the IPTR concluded that bladder drainage was associated with the best outcome. It has also been shown using data from 2 other registries that patient and kidney graft survival rates are equivalent if not higher in diabetics receiving a kidney and a pancreas compared to those receiving a kidney alone. With simultaneous pancreas and kidney transplants the pancreas graft survival rate has also been shown to be higher, with the kidneys thought to provide an early marker of pancreas rejection, allowing earlier treatment of such episodes [52].

b. Effect on long term complications.

Sutherland et al. in 1989 presented their data of a 10 year experience with 290 pancreas transplants [53]. Using preliminary data they were able to report that 1 year post transplant, patients with functioning grafts showed an improvement with regard to neuropathy. In some nerves the conduction velocities had improved to above the baseline. Kennedy et al. [54] also examined the effect of pancreas transplants on diabetic neuropathy, 61 patients pre and 12 months post transplant were studied, 27 patients at 24 months and 11 again at 42 months. The control group had similar numbers of patients maintained on insulin. They concluded that the progression of diabetic polyneuropathy could be halted through the restoration of euglycemia following a pancreatic transplant.

In 30% of the patients a deterioration in retinopathy was seen over the initial 3 years, but in patients with functioning grafts for more than 3 years, retinopathy then remained stable. This shows broad agreement with the time scale noted by the DCCT for progression of retinopathy in those with good metabolic control. However Ramsey et al. in 1988 [55] in a study of 22 patients receiving pancreas transplants argued that progression of retinopathy was neither reversed nor halted.

Native kidney biopsies from patients with functioning pancreas grafts showed a decrease in the mesangial matrix volume [53], an observation also made by Bilous et al. [56]. In those with SPK however the recurrence of diabetic nephropathy in kidney allografts can be prevented [57]. In 1993 Fioretto et al. [58] reported the effects of pancreas transplants on the glomerular structure in patients with IDDM with their own kidneys, over a 5 year period. They found that diabetic glomerular lesions were not improved by pancreas transplantation despite 5 years of normoglycemia.
A report on a prospective follow-up of metabolic control, neuropathy, retinopathy and peripheral microcirculation concluded that SPK transplants can improve the quality of life and possibly stabilise some of the advanced secondary complications [59]. But, while pancreas transplants alone can correct severe metabolic instability and improve the quality of life they can not be used for the treatment of pre-existing diabetic nephropathy [58].

c. Complications of pancreas transplants.

In contrast to other solid organs transplants, the pancreas is susceptible to a unique set of complications due to low microcirculatory blood flow and pancreatitis of the acinar elements. Nevertheless with improvements in the technique of pancreas transplantation, including bladder drainage, whole pancreas as opposed to segmental transplantation, application of quadruple immunosuppressive therapy, and the use of improved cold storage solutions, pancreas transplants have become safer and more reliable. However surgical complications remain a major cause of morbidity, particularly those related to the acinar pancreas.

Ozaki et al. carried out a study investigating the surgical complications in solitary and combined pancreas and kidney transplants [60]. SPK transplants had fewer surgical complications when compared to PT alone or PAK. PAK transplants had the highest incidence of surgical complications, with two major problems, pancreatitis and peripancreatic infections. The study revealed that the number of complications with SPK was 29.5%, with PT alone, 33.3% but with PAK 100% had complications thought to be due mainly to recipient selection.

In a review of the results of 200 consecutive SPK transplants during a seven year period at the University of Wisconsin, Sollinger et al. [61] found that with the technical advancement of organ preservation and the refinement of the surgical procedure, rejection became the major cause of graft loss. Out of 200 pancreas transplants, 85.5% patients experienced at least one graft rejection episode, 38% experienced 2 and 16.5% experienced more than 2 episodes. Surgical complications such as small bowel obstruction occurred in 25% of the patients and often required surgical intervention. Infective complications also occurred due to both the surgical procedure and the condition of the diabetic patient. The authors suggested that earlier infections were related to the surgical procedure and later infections to the immunocompromised status of the patient. BD transplants were shown to be associated with a higher risk of urinary tract infections when compared to other methods of pancreas transplantation. Eighty four percent of patients who had BD transplants had infections leading to the death of 3 recipients.
As a result of the high incidence of rejection episodes and the infection rate, 90.5% of patients had to be readmitted to hospital and the highest number of admissions occurred during the first 6 months post transplant.

The surgical technology of pancreas transplantation has probably been optimised. The success rate, morbidity and mortality will now depend on developments in the type of immunosuppression administered and the recipients' condition at the time of transplantation. Whole pancreas transplantation in a patient with complications from a major systemic illness is likely to remain a surgical procedure with a high morbidity and mortality rate. The immunosuppression administered causes further adverse effects, especially when cyclosporine and prednisone are used. As an alternative form of therapy to insulin administration, the financial cost of a pancreas transplant also has to be considered. The procedure itself costs thousands of pounds, more if readmission is required.

The decision to make pancreas transplants available to patients showing fewer chronic complications of IDDM requires careful consideration. An attractive alternative with a dramatically lower surgical risk is islet transplantation.

1.3ii Islet transplantation.
a. A brief review of experimental islet transplantation in animals.
i. History of islet transplantation.

Thirty years ago, Hellerstrom demonstrated that islets could function in vitro following microdissection from a mouse pancreas [62]. Experimental work then began on the isolation and purification of islets with a view to transplantation.

In 1965, Moskalewski developed the technique for isolation of islets in the guinea pig [63]. Relatively large numbers of islets were isolated by crude fragmentation of the pancreas followed by digestion at 37°C with collagenase. Purification was then achieved by sedimentation. This method was modified 2 years later, by injection of balanced salt solution into the pancreatic duct [64]. A further modification was made to the purification procedure by Lindall et al. [65], who first successfully used Ficoll discontinuous density gradients, pioneering the now standard technique for islet isolation. A significant improvement in the efficiency of islet isolation in both the number of islets and reduced contamination with acinar tissue was achieved by the use of other media such as BSA density gradients. The BSA density gradient not only gives a better yield of islets than ficoll but also the islets obtained function better in vitro and in vivo [66].
Most of the experimental islet transplant work has been carried out in the rodent. The use of inbred strains has allowed the technique of transplantation to be assessed independently of immunological factors. The first successful reversal of diabetes in animals was carried out in 1972 by Ballinger and Lacy [67]. They reversed streptozotocin-induced diabetes in the rat by intraperitoneal transplantation of syngeneic islets. Work then began on the effect of transplanting islets at different sites.

ii. Site of transplantation.

There have been numerous studies investigating the different potential sites for islet transplantation. Kemp et al. [68] compared the implantation of islets subcutaneously, intraperitoneally and intraportally in diabetic rats. They concluded that diabetes could be effectively treated by transplanting islets intraportally. However, the severity of diabetes was reduced but not completely reversed following intraperitoneal transplantation and subcutaneous transplantation had no effect on the diabetic state of the animal. The transplantation of islets intraportally was thought to be advantageous because one of the primary sites of action of insulin is the liver [69]. Transplantation into the portal system has theoretical advantages from an immunological viewpoint as well. The humoral and delayed hypersensitivity response to antigens introduced via the portal system are less vigorous than those generated by administration via subcutaneous, intravenous or intraperitoneal routes [70].

Reece-Smith et al. [71] suggested that the kidney capsule was a preferential site to the liver with grafts surviving twice as long. However other studies have failed to confirm this [72,73]. In terms of safety, unpurified dispersed pancreatic digest transplanted intraportally can lead to portal hypertension and even death [74]. On the other hand while the kidney may possibly provide a safer site, the technique involves a full surgical procedure, while intraportal transplants can be carried out less invasively under local anaesthetic using radiological techniques. Studies in the rodent have also shown that less islet tissue is required for reversal of diabetes and an improvement in metabolic control when the intraportal site is used for transplantation [75].

The problem of insufficient islet numbers for reversal of diabetes has been overcome by using multiple isologous donors [76]. From the literature the number of islets required to reverse diabetes varies in rats between 600-1200. London et al. [77] carried out a titration experiment for renal subcapsular transplantation and found that the transplantation of 750 islets successfully and reliably reversed diabetes in the rat.
iii. Effect of transplanting pure and impure islets.

In larger animals sufficient purified islet yields remained difficult to achieve by techniques such as hand picking. The technique of islet isolation therefore had to be altered to try to improve yields in larger animals. One option was not to purify the islets from the dispersed pancreas at all. Mirkovitch and Campiche carried out unpurified intrasplenic autotransplants on 26 dogs. Normoglycemia was achieved in 20 dogs and, after splenectomies in 7 of them, hypoglycemia and death occurred. They concluded that purification of the pancreatic digest was not essential for the endocrine function of the islets [78].

The effect of transplanting unpurified islets has not been extensively examined. There are a few studies in the rodent model [79-81] all of which draw the same conclusion, acinar contamination appears to have a deleterious effect on islet implantation. Histological examination of kidneys after subcapsular transplantation of impure islets revealed that with increasing proportions of acinar contamination, the acinar structure of the acinar tissue was lost and areas of necrosis, foreign body giant cell formation and areas of cystic glandular formation were found [82]. One centre has transplanted unpurified islet tissue, prepared by a non-collagenase method into canines. Although all animals became normoglycemic, this experiment has not successfully been repeated [83].

In humans however, transplantation of unpurified islets resulted in significant complications with portal hypertension [84], sepsis and even death [85]. In addition insufficient numbers of islets were being transplanted, with inadequate immunosuppression leading to rejection [86]. Although the data for transplantation of unpurified islets is sparse, the evidence appears conclusive. Not only is it detrimental to the islet graft survival but also to the patient survival rate thus emphasising the need to purify islets.

Isolation of islet tissue from human pancreata was even less successful than it had been in the dog. It was not until the 1980’s that a successful method for islet isolation was achieved. Gray et al. in 1984 [87] described a method whereby collagenase was injected ductally into the pancreas (in previous methods the pancreas has been distended with saline and then teased apart in the presence of collagenase). Islets were physically separated from acinar tissue thus making effective large scale purification possible.

The human pancreas contains over 1 million islets and therefore handpicking is clearly not a viable option. Isopycnic gradient centrifugation is the separation of digest
using density gradient media equivalent to that of the tissue. Thus the islet and acinar
tissue float to their relevant positions in the gradient allowing rapid large scale
purification of the islets. A large number of density gradient media have been used for
islet purification. Sucrose was initially used [64] and then Ficoll [67] was introduced.
Among others that have been used since are BSA [66], dialysed Ficoll [88] and Euro-
Ficoll [89], which is widely used today. Chapter 2 discusses islet purification using
immunological differences (FACS, lectins, immunomagnetic microspheres), biological
differences (high dose radiation, short term tissue culture and cryopreservation) and
physical differences (electrophoresis and cell density currently the main method)
between the islets and the contaminating acinar tissue.

In 1986 Lacy and Scharp reported the start of the first phase of clinical studies
on human islet transplantation [90]. The initial lack of success did not deter those
involved and over the next 5 years significant progress was made in the isolation and
purification of human pancreatic islets.

b. Immunosuppression.
i. Induction of Immunosuppression.

ALS (antilymphocyte serum) is thought to deplete T helper lymphocytes and
induce suppressor cells when administered to a recipient [91]. It has been shown to
prolong the graft survival when islets are transplanted across a major histocompatibility
barrier [92,93]. Similar findings were observed by Kaufman et al. using ALG
(antilymphocyte globulin) [94]. The timing of ALS administration to the recipient has
been shown to be crucial in producing indefinite survival of crude islet allografts
[95,96].

OKT3 is a murine monoclonal antibody that reacts with a molecule found on the
surface of thymocytes and mature human T cells. The structure recognised by OKT3 is
linked to the T cell antigen receptor and may be vital for functioning human T cells.
OKT3 therefore blocks in vitro, both killing by cytotoxic T cells and initiation of other T

cell functions [97]. It was found that 94% of primary rejection episodes in kidney
allograft recipients could be reversed by administration of OKT3 together with a
reduction in dosage levels of other immunosuppressive drugs being used [97].

In an initial study of 48 transplant patients, OKT3 was shown to be effective at
reversing primary and resistant rejection episodes of kidney, liver, and pancreas
transplants but the rejection rate was high after its prophylactic use in conjunction with
azathioprine and steroids in renal recipients. The viral infection rate was also observed
to be high [98]. Data from the Islet Transplant Registry (ITR) shows that using ALS or
ALG or OKT3 for induction of immunosuppression, increases the success rate of islet transplants in terms of insulin independence [99] (Figure 1.7).

ii. Maintenance of immunosuppression.

Several approaches to the maintenance of recipient immunosuppression have been tried. Pretreating the grafts to minimise the stimulus to the recipient immune system and immunomodulation of the recipient, in an attempt to achieve induction of tolerance, have both been examined. The mainstay of treatment however remains longterm recipient immunosuppression.

Prednisone has for many years been one of the major immunosuppressive agents for long-term immunosuppression and treatment of rejection episodes in solid organ transplants. Morel et al. [100] however confirmed suspicions that prednisone had a deleterious effect on islet cell transplants. They showed that islet function was rapidly and permanently damaged, independent of dose, in dogs undergoing islet transplants. They were unable to determine whether the number of islets transplanted played an important role in the effect of prednisone on islet function, due to the design of their experiment. However they were able to show that established islet transplants were also permanently affected by prednisone. The detrimental effect of prednisone was thought to be due to several factors, including direct β cell toxicity, inhibition of engraftment and peripheral insulin resistance inducing islet exhaustion.

In another study by the same group, Kaufman et al. examined the effects of prednisone, cyclosporine (CsA), azathioprine and RBC absorbed goat anti-dog ALG on islet graft function in pancreatectomised dogs with purified islet transplants [101]. Again they showed that prednisone had a detrimental effect on islet autograft function following transplantation. They also investigated the ability of other immunosuppressive agents to prolong islet allograft functional survival. They compared functional survival of allografts in dogs with CsA/azathioprine and CsA/azathioprine/ALG (with non-immunosuppressed canines as controls). They found a significant prolongation of graft survival with CsA/azathioprine/ALG and concluded that ALG should be considered for use in humans trials of allotransplants.

Other immunosuppressive agents have similarly been shown to have a deleterious effect on islet function. CsA is a known hepatotoxin and nephrotoxin and has also been shown to inhibit glucose-induced insulin secretion [102]. Yagisawa et al. [103] demonstrated a deterioration of glucose metabolism with CsA. They also pointed out that (as Gunnarson et al. had observed) when immunosuppression of a pancreatic transplant was switched from azathioprine to CsA, glucose metabolism deteriorated.
Yagisawa et al. found that a high accumulation of CsA in the pancreas occurred and there was a morphological effect on the islets suggesting a toxic effect of CsA. Similar observations were made by Schilfgaard et al. [104] using canine islets in vivo. CsA has also been shown to inhibit vascularisation of islets following transplantation, although, the use of a calcium antagonist may ameliorate this effect [105].

Scharp et al. [106] carried out a study investigating the effects of prednisone, azathioprine and cyclosporine on islet graft survival in autotransplanted dogs. They found that the group that received autotransplanted islets with no immunosuppression demonstrated long term survival. The groups that received immunosuppression showed adverse effects on islet graft survival.

Gunnarsson et al. [107] suggested that CsA did not adversely affect \( \beta \) cells, but induced insulin resistance. If CsA treatment was discontinued then no further deterioration in the patient was seen.

Controversial evidence was published by Kneteman et al. [108]. They found that intramuscular administration of CsA did not appear to affect islet function. However, with the number of studies that have found CsA detrimental to islet allografts, CsA dosage is generally reduced in human islet transplants.

FK506 is another more recent immunosuppressive agent. It has however been shown to have similar effects to CsA on impairment of insulin secretion [109,110]. Nevertheless, there have been successful islet transplants whereby C-peptide negative recipients have, following transplantation of islets, become C-peptide positive for more than 1 month with FK506 immunosuppression [99].

15-deoxyspergualin (15DSG) is a novel immunosuppressant that has been shown to prolong islet allograft survival in small and large animal models [111]. Sutherland et al. showed that 15DSG was 15 times as potent as CsA in prolonging islet graft survival in the rodent model. They then went on to perform transplantation of unpurified islets using 15-DSG with ALS/ATG for induction immunosuppression in 5 patients. Two of these patients achieved insulin independence. They concluded that 15-DSG is a promising agent for use in human islet transplantation, although, further studies are needed [112].
Islet Transplants From 1974-93 Using Different Induction Immunosuppressants.

![Graph showing islet transplants from 1974-93 Using Different Induction Immunosuppressants.](image)

Figure 1.6 Immunosuppressants used for islet transplants 1974-93.

c. **Human islet allotransplants - summary of transplants up to 1994.**

Kolb and Largiader in 1980 reported the first case of insulin independence in a patient who simultaneously received 2 kidneys and unpurified pancreatic digest into the spleen. No pretransplant insulin levels were reported and the patient became insulin independent 9.5 months post transplant and remarkably remained so even after rejection of both kidneys, until the patient died 20 months post transplant [113].

In 1990, Scharp et al. reported the first case of insulin independence following intraportal purified islet transplantation [114]. A type I diabetic patient with an established kidney transplant received islets from 1.4 pancreata intraportally. The islets were 95% pure and the first islet preparation was a 3 antigen match, the second was a four antigen match. The immunosuppression was not altered significantly from the regime established following the kidney transplant. The patient received one dose of cyclosporine daily. The patient was insulin independent for a period of 25 days. Even though the patient was not insulin independent for a long period of time this was an important landmark for islet transplantation.
Socci et al. in 1991 [115] reported 6 cases of islet transplants in patients, 4 of whom had already received a kidney transplant and the other 2 underwent simultaneous kidney transplant. Triple immunosuppressive therapy consisting of cyclosporine A, azathioprine and prednisone, was given to those receiving a simultaneous kidney and islet transplant. Those who had received a kidney were already undergoing this therapy. Strict metabolic control was maintained after islet transplantation by continuous intravenous insulin administration, to prevent the islets from being exposed to hyperglycaemic stress post-transplant before undergoing revascularisation. Of the 4 patients who received islets after a kidney transplant, only 1 became insulin independent 6 months post transplant. The patient remained off insulin therapy for a period of 5 months. The other 3 patients were not as successful, 1 rejected the islets 15 days post transplant, the other 2 had a decrease in insulin requirement. The patients who received simultaneous kidney transplants did not achieve insulin independence, this may however be due to the relatively low number of islets transplanted.

These two reports demonstrated that islet transplantation was a feasible option for achieving insulin independence in type I diabetes, and numerous reports of clinical trials followed. In 1992, Ricordi [116] reported 22 cases of islet transplants simultaneously with liver transplants. There were 3 groups: the first group underwent combined liver-islet transplantation following upper-abdominal exenteration for cancer, the second group underwent combined liver-islet transplantation for cirrhosis and the third group received simultaneous kidney transplants. From group 1, 6 out of 10 patients achieved insulin independence and one of those patients remained insulin independent for 2 years. The immunosuppressive therapy used was FK506 although patients in groups 2 and 3 received steroids as well. None of the patients in groups 2 or 3 became insulin independent, but there was a reduction in insulin requirements. They concluded that rejection was still a major factor limiting islet transplantation and that the use of steroids might contribute to poor islet engraftment and graft failure.

In 1989 the Islet Transplant Registry (ITR) was transferred to Giessen, Germany. They have reported that between 1990 and 1993, 30 islet transplants per year have been performed at 18 institutions worldwide [99]. In the latest report they highlighted the fact that the principle feasibility of islet transplantation has now been shown with groups in Milan (Socci et al.) and Edmonton (Warnock et al.) having patients insulin independent for 2.6 and 2.3 years respectively. It was also reiterated that (as pointed out by Scharp in 1989 [117]) with the numerous different approaches to transplant site, immunosuppression, and concomitant organs transplanted, cautious analysis of the results was required.
The report summarised the data from 55 islet allografts carried out between 1990 and 1992. All recipients were C-peptide negative pretransplantation. The patient survival rate was 95% and 33% of these had C-peptide levels of >1ng/ml 1 year post transplant. Insulin independence was achieved in 11% of the recipients. The registry highlighted several criteria for success, firstly the islets transplanted into these recipients had been obtained from pancreata with a mean preservation time of less than 8 hours. Secondly, in order to become insulin independent it was concluded that more than 6,000 IEQ/Kg were required to be transplanted intraportally. Lastly, immunosuppressive therapy in the form of ALG/ATG/ALS or OKT3 was administered to patients who became normoglycemic but, in patients who became insulin independent the immunosuppressive therapy given tended to be ALG/ATG/ALS rather than OKT3. If all of the above criteria were fulfilled then 27% of the recipients were insulin independent 1 year post-transplant. However, if one of the criteria was not fulfilled, none of the recipients were insulin independent a year after transplantation. Figure 1.6 shows the graft survival rate for the 55 transplants carried out during 1990-92.

The ITR report went on to point out that patients who achieved insulin independence had daily insulin requirements pretransplant of between 0.6 and 1.2U/Kg. Patients who received cryopreserved islets alone did not become insulin independent. The results from the 55 transplants have suggested that the underlying peripheral insulin resistance is one of the major obstacles to achieving insulin independence rather than steroid-induced insulin resistance. A further factor is glucagon, produced by α cells in the pancreas, activates glycogenolysis. This is the breakdown of glycogen to increase levels of glucose in cells. As a result it is frequently called the hyperglycaemic factor.

Portal hypertension was noted as being a potentially life-threatening risk if unpurified digest was transplanted intraportally, even though analysis of recent results have suggested that purity may not affect allograft survival. The report concluded that until more islet transplants have been carried out with standardised criteria for islet assessment and patient monitoring, it is not possible to ascertain whether islet transplants improve quality of life in patients with diabetes, or results in stabilisation or regression of the complications.
Graft Survival Rate Over 1 Year In 55 Recipients Between 1990-92

Figure 1.7 Graft survival rates.

**iii. Alternative methods to pancreas and islet transplants.**

Human organ shortage severely limits the application of whole organ transplants and islet transplants are more severely limited by the inefficiency of islet isolation, with more than one donor being required per recipient. Alternative methods for reversing insulin dependence in type I diabetics will therefore continue to be explored.

**b. Xenotransplantation.**

An alternative source of islets for transplantation has enormous potential in islet transplantation but with transplantation across a species barrier the graft must be immunoisolated for successful xenografting to occur. This either involves transplantation into an immunologically privileged site or the use of some form of bioencapsulation.

There are now two accepted immunologically privileged sites which are able to confer some degree of immunoisolation on the islet xenografts. They are the brain/intrathecal space and the testes. Both sites pose potential problems if xenotransplantation is to become practical.
Bioencapsulation offers the most promising approach to immunoisolation. However, the technique is not without problems, with rupturing of isolation membranes, proteinaceous deposition on the membrane and attachment of immune cells to it.

Historically, the first ‘islet’ xenotransplantation occurred over 100 years ago, in 1893 when a diabetic was treated with a xenograft of minced sheep pancreas. A century later clinical success in humans has yet to be achieved.

The choice of donor species to provide islets that, once isolated are viable and pure, is clearly a prerequisite to human xenotransplantation. The islets should preferably be from a non-primate source in which there are fewer ethical constraints on obtaining pancreatic tissue [118]. There have been numerous experimental xenotransplantations carried out using different combinations of donors and recipients which have been reviewed by Auchincloss [119], and by Stegall and Hardy [120].

One promising source of islet xenografts has been the pig [118]. The pig is already a source of insulin for human diabetics and the porcine pancreas can be readily obtained when the animal is slaughtered for food. Many groups can now routinely isolate porcine islets [121-123] and the results of a clinical trial with pig-to human islet transplants were reported by Tibell et al. in 1993 [124]. All 8 grafts failed, however there were detectable levels of porcine C-peptide in some of the patients and the longest and highest secretion of C-peptide (from day 58 - 347 post transplant) was seen in a patient who had been treated with ATG. Groth et al. [125] recently transplanted fetal pig islet-like clusters into 10 insulin dependent kidney transplant patients. C-peptide was not detectable in the plasma, but 4 of the patients excreted C-peptide in the urine. Three of the patients had been treated with 15-DSG, the fourth with ATG. They concluded that porcine islets could be transplanted safely intraportally or under the kidney capsule, although the benefits to the patient are clearly questionable.

An alternative is graft immunomodulation. Prolongation of transplant survival with maintenance of normoglycemia was achieved when rat islets were cultured for 7 days at 24°C prior to transplantation [126]. Human islets have also been shown to be less immunogenic after culture at 24°C for less than 5 days. The islets retained their functional capacity equivalent to that of 37°C cultures [127]. UV-B irradiation has been shown to preserve endocrine function but reduce the immunogenicity of human islets by suppressing ICAM 1 induction [128,129]. Cryopreservation has also been investigated for immunoalteration of donor islets. Rat islets were transplanted into mice
immediately after isolation or after cryopreservation. All of the fresh islets by day 13 had rejected, but 37% of the cryopreserved islets were still functioning. It is thought that cryopreservation destroys passenger leukocytes [130].

Xenotransplantation raises ethical issues which must be considered. The exploitation of nonhuman animals in xenotransplantation for direct human benefit is for some a basic moral issue. Articles by Francione [131] and Reemtsma [132] discuss the ethical issues of xenotransplantation. Francione felt that we need to decide if the animals have rights; if they do, xenotransplantation is morally wrong. Reetsma on the other hand concluded that if the biological barrier is overcome then the success of xenotransplantation will overcome the ethical issues. Before xenotransplantation can become clinical practice, these issues must be clarified.

CONCLUSION.

Islet transplantation offers the hope of normalising blood glucose control in patients with diabetes preventing the progression of complications without the morbidity associated with whole pancreas transplantation. In order for islet transplantation to succeed, however the yield of islets obtained from one pancreas has to be improved to allow a reliable one donor to one recipient transplant. Over 1 million islets are present in a pancreas but with current techniques on average only 200,000 islets are isolated. As pointed out in a review by London et al. [133] introduction of a further 50 density gradient media for separation will not alter the fact that islet and acinar tissue have overlapping densities. Alternative approaches to islet purification are therefore needed and are discussed in Chapter 2.
A REVIEW OF CELL SEPARATION TECHNIQUES.

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2.4. Immunomagnetic techniques and Islet purification.

i. Application of immunomagnetic separation to islet purification.

ii. The use of immunomagnetic separation for islet purification.
2.1 Islet purification.

2.1.1 Why bother?

Since 1980, the serious risk involved with the intraportal transplant of unpurified islet transplants has been recognised. In 1980 a case of disseminated intravascular coagulation and portal hypertension following auto transplantation [84] was reported. The patient did survive after 39 units of blood. Results from animal and clinical studies carried out by this group, suggested that thromboplastins are released from injured cells during the preparation of pancreatic tissue for auto transplantation. The thromboplastin results in thrombi being formed in portal venous radicles that are at least partially responsible for portal venous pressure elevations that follow. These changes can be moderated or prevented by the use of heparin.

Traverso et al. in 1981 reported 4 cases where patients receiving an auto transplant experienced an immediate drop in blood pressure and a rise in portal pressure. For this reason, only 1 of the patients received the entire graft [134].

One year later, a case of acute portal hypertension was reported following portal infusion of unpurified islets, the patient developed hepatic infarction and died due to liver failure. The post-mortem showed thromboemboli containing pancreatic acinar and islet tissue in the intrahepatic portal venous radicles and widespread ischaemic infarcts with periportal sparing [135].

A patient 2 years after an intraportal islet transplant presented with massive bleeding from gastroesophageal varices associated with portal vein thrombosis and required a mesocaval H graft [85]. The portal hypertension was due to portal vein thrombosis caused by portal vein infusion of pancreatic homogenate, which is a serious complication of this procedure. This was the first report of the development of bleeding esophageal varices, caused by portal vein thrombosis.

Despite these initial problems, autotransplants of unpurified islets have continued and without serious complications [136]. However Shapiro et al. [137] carried out a combined liver and islet transplant, the islets were purified but supplemented with less pure islets to achieve an optimal islet engraftment mass of more than 10,000 IE/Kg. The liver failed to function and evidence of portal vein thrombosis was seen. The patient under went a second liver transplant which was successful. The first liver was sectioned and the left branch of the portal vein was found to be obliterated completely by fresh thrombus, also thrombus was found within the right portal vein. Extensive zonal hepatic necrosis was found and also portal emboli of pancreatic tissue throughout the liver was seen on histological examination. Liver
failure was attributed to hepatic necrosis following extensive portal vein thrombosis, with the underlying cause being the islet embolisation procedure. Shaprio et al. felt that the use of less pure tissue contributed to the portal vein thrombosis. They advised extreme caution in transplanting unpurified islets and recommended that when carrying out combined liver/islet allografts to delay the infusion of the islets until the liver function is well established.

These findings together with those from animal models (Chapter 4) can emphasise the need for the purification of islets prior to transplantation.

2.1ii Possible techniques.

Current methods for islet isolation are based on the automated method described by Ricordi et al., whereby the pancreas was intraductally distended with collagenase and then incubated at 39°C in a chamber with stainless steel balls and a mesh across the outlet. Media was circulated through the chamber using a peristaltic pump; when cleaved islets were seen, the circuit was opened and the islets collected. Ficoll density gradients were used to purify islets on the basis of the differences in density between the islet and acinar tissue. Modifications of this method have improved islet yield and islet viability by the use of continuous density gradients [138]. However, separation based on the physical differences between the acinar and endocrine pancreatic tissue will not produce reliable high yields of islets due to the overlapping densities.

Some of the alternative approaches that have been used for islet purification include the use of a fluorescence activated cell sorter (FACS). Crude preparations of pancreatic digest are stained with neutral red and then islets (high fluorescence with neutral red) are identified by the use of flow cytometry [139,140]. The advantages and disadvantages of the use of the FACS are discussed in Section 2.2iii.

Laser photodynamic therapy has been used to destroy selectively stained acinar tissue [141]. This alternative approach has been experimented with in the rat model and in the human. Acinar tissue was stained using a fluorescein tagged lectin and then destroyed by the use of an argon laser. Islet viability and functional integrity were preserved.

The amylase content of pancreatic tissue has been shown to decrease whilst the insulin content remains unchanged when the pancreatic digest is subjected to cryopreservation [142]. This method of purification resulted in an increase of greater than three-fold in the insulin/amylase ratio, whilst histologically the islets remained intact.
Short term tissue culture has also been investigated as a method for the destruction of acinar tissue [143]. Acinar enzymes are depleted by short term tissue culture whilst islets remain intact.

Radiation has also been examined as a tool for the purification of islets [144]. A 7-8 fold increase in the insulin amylase ratio was observed when a high dose of 5000 rads was administered to a graft. However, this increase may not have been entirely due to the radiation as the preparative process prior to radiation may have a destructive effect on the acinar tissue. In islets neoplasia can be induced by radiation, therefore further experiments are required.

These alternative approaches to islet purification show potential although further work is needed before they can be considered for clinical transplantation. The following section discusses the different methods of cell separation with a view to their application to islet purification.

2.2 Methods of cell separation.

2.2i Using physical differences.

Techniques based purely on the physical differences between cells have been the most commonly used methods for the separation of different cells populations. Cells can be separated according to differences in cell density, size and surface charge. Methods of separating populations on the basis of these differences are discussed below. The literature on physical separation of populations of cells is vast. There are however several review articles that discuss the practical applications of the various techniques [145-149].


Velocity sedimentation refers to sedimentation through a medium that occurs before cells arrive at their buoyant densities. A cell moving through a uniform medium under gravitational or centrifugal forces, reaches a constant velocity that is a balance between the applied field and the resistance to movement through the medium. When the cell is moving under a centrifugal field, the position of the cell is varied and the equation of motion is given by an adaptation of Stokes Law:

\[
V = \frac{\delta x}{\delta t} = \frac{r^2(p - p_0)\omega^2 x}{18\eta}.
\]
Where $V$ is the final velocity, $x$ is the radial position at time $t$, $\omega$ is the angular velocity, $\rho$ is the density of the cell, $\rho_0$ is the density of the medium, $r$ is the radius of the cell and $\eta$ is the viscosity of the medium at radial position $x$ [147].

It can be seen from this equation that the rate of sedimentation depends on particle size and density. There are 2 types of sedimentation separation: with and without the use of a density gradient.

i. Differential centrifugation (without a gradient).

This is commonly and successfully used where sedimentation involves the separation of particles where the sedimentation rates differ by order of magnitudes, e.g. the separation of nuclei and ribosomes. This generally yields only one pure fraction but can be repeated to produce a series of pure fractions.

ii. Gradient differential centrifugation.

This involves the layering of the mixture to be separated over a gradient with a small range of densities followed by centrifugation to separate the mixture into zones or bands. The particles do not reach their isopycnic (equal density) point and, if centrifugation is prolonged, then pelleting of particles occurs.

b. Cell density

The separation of a population of cells, purely on the basis of the difference in their density by sedimentation, to a point where the density of the medium is equivalent to the cells own, is referred to as density dependent or, isopycnic centrifugation. Isopycnic centrifugation as with velocity sedimentation, can be carried out with and without the use of a gradient.

i. Isopycnic centrifugation (without a gradient).

The commonest form of this method involves the use of a neutral density, a density medium that is in between those of the particles or cells undergoing separation. The cells are separated on the basis of whether they float or sink.

ii. Isopycnic gradient centrifugation.

Isopycnic gradient centrifugation can be further divided, into the use of discontinuous or continuous density gradients. In the former, a series of discrete layers of medium of different density are used, with the range of densities being dictated by the required fractions. The gradient is then centrifuged to allow the cells or particles to reach their isopycnic points [148]. The second method of isopycnic centrifugation is
where the density of the medium changes continuously and the cells band at their buoyant densities. This second approach is 'true' isopycnic gradient separation [147].

Pretlow and Pretlow [146] reviewed the literature on the comparison of velocity and isopycnic sedimentation. The two methods are not mutually exclusive and can be carried out independently or in sequence [150-152]. From their analysis of the data, they concluded that sequential use of velocity and isopycnic sedimentation is occasionally useful but velocity sedimentation for the separation of mammalian cells is superior in terms of purification of the cells. It was highlighted that in a few instances isopycnic sedimentation was more effective. This was generally seen when the densities of the cells being separated were outside the normal range of cell densities, and included pancreatic cells. Discontinuous density gradients have been described by de Duve [153] as a way of generating artificial bands of cells. This maybe a convenient way of compressing together certain fractions for preparative purposes, but he went on to caution that discontinuous centrifugation created the illusion of a clear cut separation where none existed. Density gradient purification in a continuous gradient was described as the analytical method "par excellence".

The phenomenon that pancreatic cells could be purified by isopycnic centrifugation was also noted by Blackmon et al. [154] who purified pancreatic acinar cells on the basis of them being more dense than most mammalian cells.

As mentioned in Chapter 1, BSA discontinuous gradients [66] and Euro-Ficoll gradients [89] are widely used today for the purification of islets. However, over the last few years developments in large scale continuous density gradients have been of major importance in the success of islet purification [138,155] and have become the method of choice, although remaining far from perfect.

c. Physical differences in the cell surface.
   i. Phase partition.

   The separation of molecules on the basis of their differential solubility in two immiscible liquids has been a standard procedure in chemistry for decades. Albertsson [156,157] applied this principle to the separation of cells and developed a series of aqueous polymer phase systems for the separation of macromolecules, cell particles and whole cells.

   When concentrated aqueous solutions of certain polymers (e.g. dextran and polyethylene glycol) are mixed together, they will separate into 2 distinct phases. The phases are very similar in their chemical composition, they have similar pH and salt
compositions, they are both aqueous and have identical osmotic activities. The notable difference is in the concentration of the “incompatible polymers”. When particles are introduced into such a system their distribution is described by the Bronsted formula 

\[ K = e^{M/RT} \]

Where \( K \) is the partition coefficient, the ratio of the concentration of the substance in the upper phase to the concentration of the lower phase, \( M \) is the molecular weight, or the surface area for larger particles, \( R \) is the Boltzmann constant, \( \lambda \) is a constant characteristic of the system, dependent on the characteristics of the two phases and is also dependent on the chemical nature of the substance.

Walters et al. have demonstrated the usefulness of such a system for the separation of erythrocytes of different ages, the resolution of 2 populations of reticulocytes and the separation of red and white blood cells [158-160]. However, as pointed out by Shortman [147] the system if it is to work, must be delicately balanced. If the molecular weight is high, only a narrow range of conditions will give \( K \) values in the range required in order for this extraction technique to be useful. Also, if \( M \) is high, small changes in \( \lambda \) caused by small differences in the chemical composition, will produce marked changes in \( K \). While this system can cause experimental problems due to the sensitivity, it is a powerful system that will enable the separation of cells on the basis of small differences in their surface composition.

ii. Electrophoresis.

The technique of separating cells and micro-organisms by the use of an electric field has been used for more than half a century [161] and there have been many reviews of the subject [147,149,162]. Most animal cells carry a net negative charge with the intensity of this charge varying according to the type of cell. The variation in surface charge allows the separation of cells on the basis of their migration rate in an electric field. There are two methods which have been used, cells can be electrophoresed in a medium which is stationary and thus their electrophoretic mobility is described by the following equation;

\[ v = \frac{\varepsilon E}{6\pi \eta} \]
where \( v \) is the electrophoretic mobility of the cell, \( \zeta \) is the electrokinetic potential of the cell, \( \varepsilon \) is the dielectric constant, \( E \) is the field strength and \( \eta \) is the viscosity of the medium.

The second method introduces cells into a medium which is flowing vertically down through a horizontal electrical field. The cells are separated due to the different paths taken in the electric field by cells of differing electrophoretic mobility, and are collected continuously at the bottom of the chamber. This method of continuous-deflection electrophoresis was first described by Hannig in 1964 and is reviewed by Shortman [147]. The electrophoretic mobility of the cell in this situation is related to the angle of deflection from the flow \( \alpha \);

\[
\tan \alpha = \frac{vi}{qow}
\]

where \( v \) is the electrophoretic mobility of the cell, \( i \) is the current, \( q \) is the cross section of the chamber, \( \sigma \) is the specific conductivity of the medium and \( w \) is the velocity of flow of the belt of fluid in the chamber.

Proteolytic enzymes, altering the pH, and virus infections altering the functional cell surface groups have been used to manipulate the electrophoretic mobility of cells. The application of electrophoresis to islet purification (although it has successfully been used for canine islet purification [163]) has limited use due to the excessive influence on the process of the sedimentation rate of the islet and acinar tissue.

2.2ii. Using functional/biological differences.

a. Physical damage.

Physical damage inflicted on populations of cells by methods such as cryopreservation [164], tissue culture [165], ionising irradiation [166], hyperthermic killing [167] and the use of laser photoradiation [168] have been employed to selectively destroy sensitive cell types in populations of tumour cells and lymphocytes, in order to enrich or deplete them. Cryopreservation [169], tissue culture [170] and high doses of irradiation [171] and warm ischaemia [172], have been used concurrently with other methods for the purification of pancreatic islets.

b. Non-specific adherence.

The use of differences in cell adhesiveness have been employed to separate the different types of white blood cells. Glass nylon wool columns have been used to
purify lymphocytes [173], while simple attachment to plastic petri dish surfaces enables the separation of macrophages from lymphocytes [174]. In 1958, Widley and Ridge reported the separation of white cells from red by an initial sedimentation step followed by adherence in wide vessels. Glass beads were introduced as an alternative to glass wool with the advantage of uniformity in packing the columns [175]. Modifications have been made to the columns and large volumes of blood can be separated into three distinct leucocyte fractions, but separation of lymphocytes from red blood cells is not possible [176].

c. Phagocytosis.

Phagocytosis has been used as a method for the separation of blood cells. Neutrophils will phagocytose iron particles, this increases the density of the neutrophils thereby allowing separation by sedimentation [177]. A comparison of the different methods employed to separate macrophages and lymphocytes was carried out by Vetvicka et al. [178]. They found that phagocytosis of silica particles was the most efficient method and produced uniform results.

2.2iii. The use of immunological techniques.

A major breakthrough in the resolution and discriminating power of experimentally-produced antibodies was provided in 1975 by the Nobel prize winners, Kohler and Milstein who developed a method for the production of monoclonal antibodies [179]. The ability to produce homogeneous populations of antibody molecules, in which all the molecules are identical and of the same precise specificity for a given epitope, revolutionised the application of immune-mediated techniques to cell separation and a variety of techniques have developed.

The following section will discuss;

a) The use of specific absorption of cells onto a solid phase which has been coated with antibodies or antigens for cell separation.

b) The use of antibody mediated cytotoxicity for the elimination of specific cell types.

c) Alteration of the physical characteristics of cell populations by attachment of markers using antibody / antigen interactions, thus allowing separation by a second specific procedure.

a. Specific absorption of cells on a solid phase.

It has been difficult to establish an optimal balance between stability, specificity, inertness and ease of elution in such systems. The problems of non-specific interactions between cells and the matrix and the elution of the cells bound to beads have not been
adequately resolved, despite the variety of substances that have been used as a matrix for cell separation, including polyacrylamide beads [180], polystyrene [181], glass beads [182], polyurethane foam [183], agarose [184], cellulose [185], gelatin [186], Sepharose [187], collagen [188], rayon [189] and nylon [190].

A number of configurations have also been used in an attempt to purify cells using this procedure. Most are derived from the principle of affinity chromatography, such as the use of digestible [188] or meltable [191] immunoabsorption columns, however, other techniques have also been employed eg. fibres [190], films [192] and test-tubes [193]. The two methods that are most commonly used are, affinity chromatography whereby non-adherent cells are allowed to drift through the column under gravity, and the use of a flat immunoabsorbent matrix where the non-adherent cells are removed by decanting and washing.

i. Affinity chromatography.

Campbell et al. in 1951 first applied the methodology for affinity chromatography to antibody purification using immobilised antigen and subsequent specific elution with a hapten [185]. This technology was first used in 1969 for the depletion of B cells from suspensions of lymph nodes or spleen by passage over matrices coated with anti-Ig antibody [182]. The technology has proved remarkably adaptable and has been successfully used for the separation of various lymphocyte sub-populations including, T cells and B cells [194,195], the removal of tumour cells from bone marrow [196], the positive selection of CD34 antigen positive stem cells [197,198] and complement receptor bearing cells [199].

ii. Panning.

Flat surfaces coated with antigens and antibodies have been used for the separation of specific cell populations by adherence. Macrophages and fibroblasts have been used as target cell monolayers to remove sensitized lymphocyte populations. Poly-L-lysine has enabled spleen cells [200], tumour cells [201], lymphoid and non-lymphoid cells to be used as immunoabsorbent antigens by attaching cell monolayers to plastic tissue culture plates [202].

Polystyrene petri dishes (100mm in diameter) have been used for the separation of immunoglobulin positive (Ig+) populations. Antibodies can be adsorbed onto the polystyrene surface, thus Ig+ cells adhere and non-adherent cells can be decanted off. The use of this technique enables the separation of up to 2x10^8 cells, purifying 90% or more Ig+ cells [181,203].
One method that remains popular for positive cell selection involves the use of hapten groups. Gelatin is used as an immunoadsorbent to coat petri dishes for the separation of lymphocyte cells. Haptens are able to covalently bind to gelatin without affecting its ability to form an insoluble gel at low temperatures. Cells then bind to the insoluble matrix and can be recovered by incubation at 37°C, at which temperature the gelatin melts [188,204].

b. Cytotoxicity.

i. Complement Mediated Lysis.

Antisera can bind to specific cell populations and are capable of initiating the classical pathway of the complement system, resulting in cell lysis. The technique of complement mediated lysis is widely used for the purification of sub-populations of lymphocytes and also extensively in bone marrow purging for the removal of myeloma [205], lymphoma [206] lymphoid [207], malignant myeloid [208] and epithelial [209] tumour cells.

The antisera used in complement mediated lysis must be specific and have a high titre. The source of non-toxic complement usually used is guinea-pig or rabbit serum. However, it has been suggested that complement mediated lysis may not be as efficient as originally thought. Using PCR, Gribben et al. found that only 50% of patients had no detectable lymphoma cells following such purging. Reinfusion of patients with lymphoma cells may be one of the contributing factors to relapse [210]. Another disadvantage of complement mediated lysis is that the eliminated population of cells cannot be recovered.

ii. Immunotoxins and “suicide methods”.

An alternative method to cell lysis used for bone marrow purging, involves the use of immunotoxins. Antibodies are covalently coupled to a naturally-derived toxin mainly from plants but also from bacteria. Ricin is an example of a two chain toxin. The A chain of the toxin is the active chain, it inhibits protein synthesis on ribosomes and the B chain binds to galactose residues of the cell membrane. The B chain therefore helps the A chain to penetrate into the cytoplasm to reach the ribosomes. Therefore, an antibody-ricin conjugate involves only the A chain. The toxin is inactive during transport but, through its concentration on the target cell by the antibody, it is capable of penetrating the cell membrane. Protein synthesis is inhibited leading eventually to cell death [211]. Other toxins that have been used include, pokeweed antiviral protein [212], saporin [213] and abrin [214].
The inactivation of specific antigen binding lymphocytes by selective incorporation of radioisotope of high activity, is a further modification. Lymphocytes when incubated in vitro with radio-iodinated antigen of high specific activity, have reduced capacity to respond to the antigen on transfer to an irradiated recipient. Transfer of immunity to other antigens is not affected [215]. The use of ionizing radiation can kill cells some distance from the radiation source, and because of this targeting, requirements for many isotopic immunoconjugates are not as specific as those requiring internalisation. This however can become a problem, as the energy and path length of the radiation emitted increases, target selectivity may decrease. Alpha particles that have a range of 20-80µm and Auger electron emitters with a range of less than 5µm may offer potential advantages in targeted therapy over those with longer ranges [216].

Cell destruction by photosensitivity, involves the coupling of antibody to liposomes containing light sensitive dyes. Bound cells on exposure to light are destroyed. This has also been studied as a potential for bone marrow purging [217].

The application of cytotoxic methods to islet purification have been experimented with [141,218]. However, due to the potential damage to islets by acinar enzymes released on destruction, research has not progressed in this area.

c. Labelling of specific cell populations.
   i. Fluorescence activated cell sorting.

The fluorescence activated cell sorter (FACS) is an instrument that is capable of combining analytical capacity with the ability to sort cells, and has been used extensively for immunological research. It is now used in clinical laboratory immunology for the analysis of T, B and other lymphoid cells. A single cell suspension, isolated from tissue or blood, is labelled with fluorescent-labelled antibody or a fluorochrome dye such as ethidium bromide (this stains DNA). Cells are forced in single file through a nozzle in a liquid jet surrounded by a sheath of saline or water. The stream of liquid is caused by vibration of the nozzle to break up into droplets. The size of the droplets can be altered so that one drop will contain one cell. A monochrome laser beam illuminates the droplets and fluorescent detectors monitors them. Droplets that emit the correct fluorescent signal are electrically charged in a high voltage field between deflection plates and are then sorted for collection. The cells remain sterile and viable during this procedure [219].

The FACS requires single cell suspensions, however extensive modifications to one type of FACS sorter (PARTEC), which uses a piezoelectric valve for sorting, has enabled its use for the separation of islets [139]. The cell sorter is able to produce 90%
pure islets (up to 300μm in diameter) that are intact and viable. Another advantage is that uncleaved islets can be separated. The disadvantages are, that the equipment is expensive, requires expertise for running and maintenance, and the procedure is also slow, although it has been reported that 100,000 islets can be purified in 1 hour [220].

ii. Rosetting.

Lymphocytes from several species upon reaction with native or specific antibody-coated erythrocytes form structures known as rosettes, a single lymphocyte surrounded by a number of attached erythrocytes. Rosetting is often used to alter the physical characteristics of cells in a population to enable purification based on the increase in density and size of rosetted lymphocytes [221,222]. In the early 70's, it was observed that unsensitized sheep erythrocytes and non-immune human lymphocytes formed spontaneous rosettes [223,224]. Further studies revealed that this phenomenon is specific to T lymphocytes [225,226]. The technique has now been modified to allow the purification of human B lymphocytes, human lymphocytes bearing Fc receptors, and separation of lymphocyte sub-populations bearing complement receptors [215].

iii. Immunomicrospheres.

Microspheres with various characteristics have been used for enhancing cell separation by altering the physical characteristics of the cells or giving them new characteristics, such as magnetic susceptibility and fluorescence [227]. The matrix of microspheres can be made of either inorganic, organic or proteinaceous materials, or even a combination of these. Microspheres can be solid or semipermeable and have a ligand or optically active molecule attached to the surface. Through chemical modification the density, surface charge, hydrophilicity and chemical reactivity can also be altered.

If the difference in density and size of cell populations are small or overlap, it is not always possible to separate the cells on the basis of physical differences. However, microspheres can be used to increase the density of some cells. An example of this is the separation of specific lymphocyte populations using lymphoprep with low speed centrifugation [228]. This technique can be applied to preparative scale separation of cells using using differential and density gradient centrifugation.

The labelling of cells with microspheres has been shown to reduce their electrophoretic mobility. Again this is a particularly useful technique when the electrophoretic mobilities of cell populations overlap. The separation of specific populations of erythrocytes [229] and lymphocytes [230] have been carried out using this technique.
One of the advantages of microspheres over the use of immobile surfaces for cell separation, is that non-specific adherence to surfaces and dislodgement of cells due to shear forces are reduced. The shear forces between microspheres and cells are smaller than between immobile surfaces and cells and also several microspheres can be bound to one cell making removal of all of them less likely.

Microspheres have been applied to the sorting of populations with a low number of cell surface antigens. Binding of 50-70 fluorescent labelled microspheres, coupled with antigen, per cell generates an intense fluorescent signal that enables successful FACS [231]. However, the technique of FACS in conjunction with fluorescent microspheres has mainly been used for analytical [232] or small scale separations due to the limited rate of the process.

Islets have been purified by the adaptation of some of the above methods. Using density dependent purification the number of islets purified from one pancreas is at best 200,000. However, there are estimated to be over 1 million islets in the pancreas. With accepted protocols requiring 6,000 islet equivalents per recipient kilogram, it can be seen that in order to carry out a one donor to one recipient transplant, a more specific and reliable method of purification is required. Separation based purely on the physical and biological differences of islets and acinar tissue is unlikely to produce reliable purification to the level required for transplantation.

Populations of cells have been separated on the basis of physical differences (such as differences in density, cell size), functional differences (the use of cryopreservation, irradiation) and also on the basis of immunological differences (cytotoxicity, rosetting and immunomicrospheres), all of which have been discussed in section 2.2. Immunomagnetic separation techniques have been employed in research for the separation of peripheral blood cells, non-haematological cells [233], suborganelles [234] and more recently for allotransplantation of parathyroid cells [235]. They have also been used for HLA typing[236]. Immunomagnetic techniques are specific, rapid, highly reproducible and relatively inexpensive.

2.3. The Use of Magnetism.

2.3.1. The history of magnetism.

One of the abundant minerals of the earth is a useful ore of iron called, magnetite. It has the composition Fe₂O₄, is a crystalline mineral, dark in colour with a specific gravity equal to about five-sevenths of that of iron (≈5000kg/m²). Occasionally pieces of magnetite that are permanently magnetised are found in nature,
called Lodestones. The name of the ore is said to be derived from the Greek province of Magnesia in Thessaly where Lodestones were discovered [237].

The description of the application of the influence of magnetism goes back to 2637 BC, when it is documented that, the troops of the Chinese Emperor, Huang-Ti, in pursuit of the rebellious prince, Tcheyeou, lost their way in the fog. The emperor constructed a chariot on which stood erect a prominent female figure, which indicated the four cardinal points and always turned to the south which ever way the chariot went [238].

The simple device, the compass, made from a Lodestone buoyant on a disk of cork, was thought to have been invented by the Italians or Arabics. William Gilbert's work, De Magnete, Magneticae corporibus, et de Magno Magnete Tellure, was a significant mark in the history of magnetism. It not only summarised all knowledge on magnetism, but it also presented experiments that he had carried out. Through these he introduced the concepts of magnetic poles, lines of force and concluded that the earth was one huge magnet with magnetic poles situated near the geographical poles.

The different types of magnetic materials can be classified on the basis of their susceptibility (χ) or permeability (μ). The first group of materials are known as the diamagnetics, their susceptibility is small and negative, χ = -10^-5. The magnetic response of this group opposes the applied magnetic field. Examples of materials in this group are copper, silver, gold and beryllium.

Paramagnetics are the second group of materials in which χ is small but positive and ranges between 10^-3 and 10^-5. In this group, the magnetisation is weak but it is aligned parallel with the direction of the magnetic field. Aluminium, platinum and manganese are materials in this group.

The third and most recognised group is the ferromagnetic solids. χ is positive and greater than 1, and the typical range is 50 to 10,000. Iron, cobalt, nickel and several rare earth metals and their alloys are included in this group.

2.3ii. Separation of cells by magnetic fields.
The use of magnetic fields for the separation of cells was first reported in 1934 by Rous and Beard [239], who found that Kupffer cells phagocytosed iron particles making them magnetically susceptible. The use of magnetic microspheres for cell separation was first reported in 1977 by Molday et al. [240] for the separation of mouse spleen T and B lymphocytes. The magnetic microspheres were prepared by a complex
procedure and they tended to form aggregates. Other disadvantages of the method, were the time period that the cells were left for in the magnetic field (2 hours) and only $1 \times 10^6$ lymphocytes were processed at one time.

It became apparent from initial experiments that magnetic microspheres to be used in cell separation have to fulfil certain criteria. They should be stable in the media or isotonic buffers being used for the separation. They should not aggregate in physiological media, and should allow satisfactory physical separation of the cells labelled with the particles from the unlabelled cells. The microspheres should bind relatively strongly to immunoglobulins or appropriate ligands, either by adsorption or by covalent bonding. Finally, non-specific binding to cells should not occur [241].

Since 1977 many different types of microspheres have been used for the separation of cells. There are four basic types of microspheres used for cell separation: proteins, liposomes, various organic polymers and colloidal metals. The range of sizes of microspheres varies between 0.03-10$\mu$m and Table 1 summarises the type of microsphere matrix used, the method of antibody attachment, the ligands used, the type of cell being targeted and from what population and also the separation technique used.
Table 1. Summarises information relating to the use of magnetic microspheres for cell separation, compiled from Molday et al.²²⁷, Plaisoucas ²³⁷.

<table>
<thead>
<tr>
<th>Microsphere matrix</th>
<th>Band diameter</th>
<th>Polymerisation method</th>
<th>Method of antibody attachment</th>
<th>Ligand on microsphere Primary</th>
<th>Ligand on microsphere Secondary</th>
<th>Target cells</th>
<th>Technique of separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Serum albumin</td>
<td>0.2 - 1.5μm</td>
<td>Emulsion polymerisation</td>
<td>Entrapment</td>
<td>Staphylococcal protein A</td>
<td>anti-rat Ig</td>
<td>Ig⁺ cells</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Human Serum albumin</td>
<td></td>
<td></td>
<td>Entrapment</td>
<td>Staphylococcal protein A</td>
<td>HLA-BW6 MAB</td>
<td>HLA-BWA6⁺ cells</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Human Serum albumin</td>
<td></td>
<td>Emulsion polymerisation</td>
<td>Entrapment</td>
<td>Staphylococcal protein A</td>
<td>anti-chicken RBC</td>
<td>chicken RBC</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Albumin</td>
<td></td>
<td>coupling with SPDP</td>
<td></td>
<td>anti-Ig</td>
<td></td>
<td>chicken RBC</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Dextran</td>
<td>30-40nm</td>
<td>Precipitation</td>
<td>reaction with aldehyde group</td>
<td>Staphylococcal protein A</td>
<td></td>
<td>human RBC</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Polyhydroxyethyl methacrylate</td>
<td>30-50nm</td>
<td>⁶⁰C gamma irradiation</td>
<td>reaction with aldehyde group</td>
<td>anti-mouse Ig</td>
<td></td>
<td>Mouse thymocytes prelabelled with microspheres</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Polyglutaraldehyde</td>
<td>0.1-0.5μm</td>
<td>Chemical polymerisation of glutaraldehyde at high pH</td>
<td>reaction with aldehyde group</td>
<td>anti-IgG</td>
<td></td>
<td>human RBC prelabelled with microspheres</td>
<td>Magnetic</td>
</tr>
<tr>
<td>Microsphere matrix</td>
<td>Bead diameter</td>
<td>Polymerisation method</td>
<td>Method of antibody attachment</td>
<td>Ligand on microsphere</td>
<td>Target cells</td>
<td>Technique of separation</td>
<td></td>
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<tr>
<td>Polyacrylamide</td>
<td>1-5μm</td>
<td>emulsion polymerisation</td>
<td>Entrapment</td>
<td>anti-human lymphocyte</td>
<td>T cells</td>
<td>Density centrifugation</td>
<td></td>
</tr>
<tr>
<td>Polystyrene</td>
<td>0.783μm</td>
<td>coupling with carboimide</td>
<td>Myeloma protein GPC-8</td>
<td></td>
<td>Ig</td>
<td>FACS</td>
<td></td>
</tr>
<tr>
<td>Polyglutaraldehyde</td>
<td>0.2μm</td>
<td>alkaline polymerisation</td>
<td>reaction with aldehyde group</td>
<td>anti-IgG</td>
<td>human RBC</td>
<td>Flow electrophoresis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>reaction with aldehyde group</td>
<td></td>
<td>anti human-Ig</td>
<td>Ig+ cells</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Polyacrolein</td>
<td>4μm or other sizes</td>
<td>Anionic polymerisation of acrolein at pH 10.5</td>
<td>reaction with aldehyde group</td>
<td>anti-IgG</td>
<td>human RBC</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Polystyrene</td>
<td></td>
<td>reaction with aldehyde group</td>
<td>anti-mouse myosin</td>
<td></td>
<td>dead mouse myocytes</td>
<td>FACS</td>
<td></td>
</tr>
<tr>
<td>Microsphere matrix</td>
<td>Bead diameter</td>
<td>Polymerisation method</td>
<td>Method of antibody attachment</td>
<td>Ligand on microsphere</td>
<td>Target cells</td>
<td>Technique of separation</td>
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<tr>
<td>Polyacrylamide agrose hybrid</td>
<td></td>
<td></td>
<td>reaction with aldehyde group</td>
<td>anti-mouse Ig</td>
<td>04 antigen</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Polystyrene polyacrylatein hybrid</td>
<td>Various</td>
<td>60Co gamma irradiation</td>
<td>reaction with aldehyde group</td>
<td>anti-sheep RBC</td>
<td>sheep RBC</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Polystyrene</td>
<td>3mm</td>
<td>Chemical</td>
<td>Non-specific adsorption</td>
<td>anti-mouse Ig</td>
<td>human neoblastoma</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td></td>
<td></td>
<td>Non-specific adsorption</td>
<td>anti-mouse IgG</td>
<td>OKT3 antigen</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Polystyrene polyacrylatein hybrid</td>
<td>10μm</td>
<td>60Co gamma irradiation</td>
<td>reaction with aldehyde group</td>
<td>anti-mouse Ig</td>
<td>human neoblastoma</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Metallic colloid colloid Colloidal gold</td>
<td>150mm or 20-60nm</td>
<td>Precipitation or reduction</td>
<td>Non-specific adsorption</td>
<td>anti-mouse Ig</td>
<td>CALL antigen</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>anti-mouse IgG</td>
<td>OKT3 antigen</td>
<td>Density centrifugation</td>
<td></td>
</tr>
<tr>
<td>Microsphere matrix</td>
<td>Bead diameter</td>
<td>Polymerisation method</td>
<td>Method of antibody attachment</td>
<td>Ligand on microsphere</td>
<td>Target cells</td>
<td>Technique of separation</td>
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<tr>
<td>Hydrogel</td>
<td>&gt;50nm</td>
<td>Redox polymerisation system</td>
<td>reaction with aldehyde group</td>
<td>cholera gen</td>
<td>high titre GMI C1300 neoblastoma</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Hydrogel</td>
<td></td>
<td></td>
<td>reaction with aldehyde group</td>
<td>Ricin</td>
<td>HBLA wild type</td>
<td>Magnetic</td>
<td></td>
</tr>
<tr>
<td>Distearoyl-or-dipalmitoyl-phosphatidyl-choline liposome</td>
<td></td>
<td></td>
<td>Entrapment</td>
<td>anti-human plasma fibronectin</td>
<td>mouse embry fibroblasts prelabelled with microspheres and carmine dye</td>
<td>Magnetic</td>
<td></td>
</tr>
</tbody>
</table>
It can be seen from table 1 that one of the major difference between matrices is the method of attachment of the ligand. The magnetic system most commonly used involved the use of a permanent magnet. Figure 2.1 shows a schematic cell separation. The permanent magnet creates a magnetic field or flux gradient pulling the sphere towards the magnetic pole.

a. Method of antibody attachment.

The following section will discuss the non-specific adsorption, specific adsorption, direct and indirect coupling of antibodies to microspheres.

i. Non-specific adsorption

Hydrophobic particles can be readily coated with immunoglobulins, even though hydrophilic particles are thought to be more suitable for cell targeting. Immunoglobulins will bind to the surface of such hydrophobic microspheres, by van der Waals-London forces. Moreover they tend to bind via the Fc portion, leaving the Fab immunoreactive site free to interact with the antigen. There is a lower limit for the concentration of antibody incubated with the microsphere, as total saturation appears to be important in the prevention of antibody denaturation.

Non-specific adsorption appears to have some disadvantages. This method of antibody binding may not be efficient and the microspheres may be coated in a mixture of active and inactive antibodies due to their orientation. However, systems using this
method of attachment of antibodies to microspheres have been able to achieve 97-100% removal of target cells.

**ii. Specific adsorption.**

By use of a ligand, such as Protein A or Avidin antibodies can be non-covalently adsorbed onto the surface of microspheres. The ligand interacts specifically with the intact or modified antibody (a process known as entrapment).

This method has been used by coupling of *Staphylococcus aureus* protein A to human serum albumin microspheres [242]. Protein A is able to bind with high affinity to the Fc portion of the majority of IgG subclasses. The target cell removal has been greater than 99%. The limitation of using such a system *in vivo* may be that protein A binding to certain subclasses of IgG activates the complement system.

Avidin has four high affinity binding sites for biotin. When biotin is conjugated to proteins, avidin will form irreversible complexes with the biotinylated proteins. Kaplan *et al.* [243] utilised this to couple biotinylated monoclonal antibody to polymethacrylate microspheres. Non-specific binding to cells by avidin was reduced by the use of acetylated avidin.

**iii. Direct coupling.**

Microspheres with functional aldehyde groups on the surface, enable direct linking of antibodies. Examples of such microspheres are given in Table 1. Hydrophilic polyglutaraldehyde when bound, has long extending chains from the microspheres. This assists the binding of the proteins [244]. The polymer contains repeating units of conjugated aldehyde groups which form stable bonds with primary amino groups in the protein (Figure 2.2). Proteins can be coupled to reactive aldehyde groups by coupling in neutral phosphate buffer for several hours [245]. The pH influences the rate of reaction between the aldehyde group and the amino group. At a lower pH the reactivity of the aldehyde group towards NH₂ will be enhanced, but the NH₂ will also be converted into NH₃⁺, the unreactive form. Therefore the maximum reaction rate will occur at a moderate pH.
iv. *Indirect coupling.*

If direct coupling is not possible because the microsphere’s surface does not carry the requisite groups for attachment of the antibody, then alternative methods have to be employed. There are several of these, some of which will be discussed below.

*a. Carbodiimde method.*

Carboxyl groups on the surface of microspheres can be activated by the use of a water soluble carbodiimde derivative. The amino acid, on the antibody, is coupled to the carboxyl group via an amide link or the carboxylic acid on the antibody can be coupled to an amino acid group on the microsphere. This method has been used for the coupling of antibodies to latex microspheres [246,247]. In order to prevent proteination of the amine at low pH, and decomposition of the carboxyl group at a higher pH, the pH has to be kept neutral. A disadvantage of this method is that proteins contain both amino acid and carboxyl, therefore there will be intra and inter molecule cross linking occuring in the antibody.
b. Cyanogen bromide method.

Cyanogen bromide is used to activate the hydroxyl groups on the surface of microspheres at an alkaline pH. The hydroxyl groups are then coupled to amino groups on the antibody at a pH range of 7-10. As with carbodiimide and aldehyde coupling methods, the reaction is pH dependent and at a neutral pH it is very slow. Another disadvantage of this method is the toxicity of the cyanogen bromide.
c. *Glutaraldehyde method.*

This method enables the linkage of amino groups on microspheres to be linked to amino groups on the antibody. Microspheres can also be coated with diamino groups, but the carbodiimide method has to be employed before the glutaraldehyde method. The actual mechanism of the glutaraldehyde reaction is not fully understood, but a proposed model is shown in Figure 2.5.

Proteins have been linked to methyl methacrylate microspheres, using the two step process [248]. The two step reaction procedure has been found to be the most effective method, compared to the carbodiimide and cyanogen bromide methods [249]. The two step reaction process also reduces the possibility of intra and inter cross linking of antibodies occurring.

![Figure 2.5. Glutaraldehyde method of coupling antibodies](image)

v. Other methods for coupling antibodies to microspheres.

The EDDQ method involves the coupling of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinolinone (EDDQ). This method is also a two step procedure reaction. Microspheres are initially activated with EDDQ to form a mixed carbonic anhydride, which is then able to react with amino groups on a protein. This method has been used to couple enzymes to sephadex CM-50 and cellulose-CM.
Proteins can be coupled to polymers using the Woodward's reagent K method. The carboxyl groups on the polymer are reacted with N-ethyl-5-phenylisoxazolium 3'-sulfonate. Active ester derivatives are formed which can then react with protein amines to form amide bonds.

b. Positive or negative selection?

When planning any immunomagnetic cell separation, in addition to type of magnetic field, microsphere and method of antibody binding, several other variables must be considered for example positive or negative selection and direct or indirect binding.

Positive selection, is the removal of the required population of cells. This process has been successfully used for the selection of haematological cells [250], including antigen specific cells [251,252], and non-haematological cells [253]. Negative selection, the removal of populations of cells not required, has also been used successfully for the purification of haematological cells, [254-256]. Using the negative selection procedure, the cells which are required are not damaged and do not have beads attached. The removal of beads during positive selection adds a further step to the purification process.

c. Direct or indirect labelling methods?

Cells can be labelled by direct or indirect methods when using immunomagnetic separation techniques. Figure 2.6 shows a schematic representation for both methods. The direct method uses microspheres coated with antibodies or ligands which are reacted directly with the cells. The antibodies recognize cell surface antigens or structures specifically expressed on the cell surface of cells being selected. The indirect method involves the coating of the population of cells being selected with antibodies recognizing cell surface antigens. The coated cells are then conjugated with microspheres previously coated with antibodies against the antibody coating on the cells (eg. cells are coated with mouse IgG and the microspheres are coated with anti-mouse IgG). It is also possible to coat the beads with a secondary polyclonal antibody which acts as a spacer to improve the orientation of a monoclonal antibody.

It has now been shown that efficacy of direct-bead-to-cell binding depends on factors such as the concentration of the antibody, incubation medium, temperature and the time of incubation of the beads and cells [257]. All of these must be optimised to ensure maximal binding of beads and minimal non-specific binding.
Figure 2.6 Direct and indirect methods of immunomagnetic separation
2.3iii. The M-450 Dynabead, its development and application.

There are several immunomagnetic microspheres with different surface properties and of different sizes available on the commercial market. However, the most widely used immunomagnetic microsphere is the Dynabead®. The Dynabead® is a monosized and monodispersed magnetic microsphere, produced by the polymerization of styrene divinylbenzene and the magnetic material is precipitated inside from iron salt solutions (a two step swelling process) [258]. The polymer particles are made magnetisable in the second step of the process, by the formation of magnetite or other magnetic iron compounds. This in situ process enables the deposition of up to 35% iron in the form of magnetic oxides in the pores of the monodispersed particles. The porous magnetic particles have a very large surface area of between 50-100m²/g. The original beads were 3μm in size with a surface area of 100m²/g. This large surface area required a lot of antibody, which became embedded in the pores and thus inactivated. The 3μm beads also showed non-specific binding. By filling the pores with polymeric compounds, not only was non-specific binding reduced but the surface area was also reduced to 3-5m²/g. The M-450 Dynabead® (Figure 2.7) is 4.5μm in size and contains 20% of iron as magnetite oxides [259].

![Figure 2.7 M450-Dynabeads](image)

The smooth hydrophobic surface of Dynabeads facilitates non-specific antibody adsorption (section 2ai) while surface hydroxyl groups provide the means for covalent
chemical coupling to proteins and monoclonal antibodies (tosylactivated M-450). IgM antibodies can be physically adsorbed onto uncoated non-activated M-450 Dynabeads, whilst IgG antibodies should be used in conjunction with secondary antibody coated M-450 Dynabeads. This ensures correct spatial orientation of the Fab region for cell attachment. Dynal have produced a smaller bead 2.8μm in size which is designed for the isolation of prokaryotic cells.

a. The number of Dynabeads required for cell separation.

Several parameters determine the ideal bead to target cell ratio. They are:
1. The type of cell isolation, positive or negative.
2. The ratio of the number of target cells to the total cell population.
3. The total number of cells present.
4. The affinity or avidity of the primary antibody used.

A relatively low number of beads to cell can be used for positive selection e.g. 3:1 whereas negative selection requires a higher ratio in the range of 10:1-40:1. Too few beads is not efficient in the removal of all target cells and too many beads causes trapping of cells, non-specific binding and blockage of the antigen binding sites. It has been estimated that a large particle such as an acinar fragment will require 1000 beads to one particle [260].

b. The separation device.

For immunomagnetic cell separation using Dynabeads®, Dynal produce magnetic particle concentrators. These contain neodymium-iron-boron magnets, which are either designed to hold, a single test-tube of various diameters, the MPC®,-1, six separate tubes, the MPC®-6 or six eppendorf tubes, the MPC®-E (Figure 2.8). The neodymium-iron-boron magnet has been successfully used in many isolation studies, including epidermal Langerhans cells[261], biliary epithelial cells [262], human sperm without autoantibodies bound [263], human megakaryocytes [264] and human endometrial stromal and glandular cells [265].

2.3iv. Immunomagnetic separation techniques applied to cell separation.

a. Established uses for cell separation techniques using magnetic microspheres.

The time efficiency and the capacity to handle large numbers of cells gives immunomagnetic separation techniques advantages over other cell separation techniques. Immunomagnetic separation techniques have been used for the removal of tumour cells from bone marrow in autologous bone marrow transplants (bone marrow purging) and also for the removal of T cells from human bone marrow in allogenic bone marrow transplants (T cell purging).
Figure 2.8. The MPC®-1, MPC®-6, MPC®-E.

i. Bone marrow purging.

Patients with leukemia, lymphoma, neuroblastoma, testicular carcinoma, ovarian and other cancers, can now be treated by protocols including autologous bone marrow transplantation, using bone marrow purging. Most cancers exhibit a dose related response to chemotherapy and irradiation, eradication of all tumour cells can be achieved by using high levels. There is a limit however on the dosage of chemotherapy and irradiation because the patients hemopoietic system will fail resulting in death of the patient. Bone marrow purging offers a solution to this problem. The bone marrow is removed, all malignant cells purged and the marrow reinfused into the patient following treatment to reconstitute the destroyed hemopoietic system [241].

It has been suggested that failure to remove all of the tumour cells could increase the chances of relapse. Section 2.3ivb discusses some of the methods that have been employed for bone marrow purging.

The first description of bone marrow purging using immunomagnetic particles was in 1983 by Poynton et al. [266]. Cobalt magnetite albumin immunomagnetic colloid particles were used to remove leukaemic cells. Remission of three months and longer was observed in three out of four patients. The fourth patient died due to
infection. 3μm polystyrene beads and a panel of monoclonal antibodies were used successfully for purging in patients with neuroblastoma. A three log depletion of tumour cells and a recovery of 65% of bone marrow cells was achieved [267].

A variety of magnetic materials with immunoglobulins covalently linked such as magnetic alloys, and ferromagnetic microspheres of varying sizes, have been compared with Dynal M-450 microspheres to determine if they are better for cell depletion and enrichment [268]. The M-450 microsphere remained the superior material. However, Kemshead et al. did point out that the M-450 microspheres have been optimised over several years and that conditions used in the evaluation may not have been ideal for other ferromagnetic materials. The M-450 microsphere is now routinely used for immunomagnetic bone marrow purging of leukaemic cells [269] neuroblasts [270], myeloma [271] and lymphoma cells [272].

**ii. T cell depletion in allogenic bone marrow transplants.**

It has been shown that graft versus host disease does not occur in patients who receive bone marrow containing less than 10^5-10^6 T cells/kg body weight. Immunomagnetic techniques have been employed for the depletion of T cells as an alternative to methods involving the use of immunotoxins and high doses of complement.

Contaminating T cells (20-30%) were removed from the population of bone marrow cells by the use of Dynabeads. In less than 40 minutes the population had less than 0.02% contaminating T cells. The recovery of non-T cells was 43-74% which were 99% viable and the method used did not disturb the growth potential of stem cells [273].

### 2.4 Islet purification

#### i. Application of immunomagnetic separation to islet purification.

The size of the particles undergoing separation is a major consideration in the application of immunomagnetic techniques of cell separation to islet purification. The average islet is 150μm (range of 50-300μm) in diameter and the size of acinar particles varies greatly up to about 500μm in diameter. Due to the large size of the particles, they do not remain in suspension during the magnetic separation and the islet and acinar tissue (the digest) will only be mobile in the magnetic field for a limited period of time. Calculation of the force on a particle in a magnetic field and the consequent velocity of the particle is given in Appendix 1. Equation 9 states that the velocity on a particle is given by;
where \( V \) is the volume of the acinar particle with beads attached, \( V = \frac{4}{3} \pi a^3 \), \( M \) is the magnetic moment of the spheres (Gauss or emu/cm\(^3\)), \( a \) is the radius of the acinar particle with beads attached, \( \eta \) is the viscosity of the medium and \( \frac{dH(R)}{dR} \) is the flux gradient (in units of Gauss/cm).

If we take 4.5\( \mu \)m beads (M-450 Dynabead\(^\circledR\), one of the largest beads available on the market) which have a magnetic moment of 10 gauss each, 50 of these label an acinar particle of 150\( \mu \)m in diameter (0.015 cm). Therefore, the total diameter \( D_c \) of 1 acinar particle with 50 beads attached will be:

\[
D_c = (D_c^3 + nD_b^3)^{1/3}
\]

where \( D_c \) is the diameter of the acinar particle, \( D_b \) is the diameter of the Dynabead and \( n \) is the number of dynabeads attached to 1 acinar particle. Therefore, from this formula the diameter of the acinar bead complex is:

\[
D_c = (0.015^3 + 50 \times 0.0045^3)^{1/3} = 0.01992\text{cm}
\]

Thus the radius of the complex is 0.00996cm, the contribution of the acinar fragment in this figure is relatively massive. This will be used to calculate the drag force \( F_d \) (see Appendix 1 Equation 7). The volume of one Dynabead is,

\[
V = \frac{4}{3} \pi a^3 = \frac{4}{3} \pi (2.25 \times 10^{-3})^3 = 4.7 \times 10^{-8}\text{cm}^3
\]

The velocity can therefore be calculated using Equation 9 in the appendix 1. If the labelled particle is then allowed to drift under gravity in a medium with a viscosity \( \eta \) of 0.01P (\( \eta \) of water at 20\( ^\circ \)C), through a magnetic field with a flux gradient of 1500 gauss/cm (using the MPC\(^\circledR\)-6 magnet, the field generated is assumed to be a sphere at a distance of 1.2cm from the centre of the magnet) then the velocity can be calculated.

(Personal communication A.T.Skjeltorp, Institutt fur Energiteknikk, N-2007 Kjeller, Norway). Equation 5 calculates the velocity of an acinar particle with 50 beads attached to it;
If smaller beads (such as MACS® beads 100nm in size, Miltenyl Biotec GmbH, Berglach Gladbach, Germany) are used then the speed of the particle through the field decreases. The use of a flat side pull magnet has been employed for purification [273], but the necessary exposure time to the magnet is in the order of several minutes. During this period the digest will settle under gravity before the magnetic field has had time to act. The Dynabead has therefore been chosen for studies on the purification of islets.

2.4ii. The use of immunomagnetic separation for islet purification.

The acinar and endocrine tissue of the pancreas express antigens that differentiate them [274]. Monoclonal antibodies have been raised against islet antigens in an attempt to identify antigens involved in the autoimmune aetiology of diabetes [275]. The use of such antibodies for a system of positive selection would enable the removal of all islets cleaved and uncleaved. However, the need to remove all the Dynabeads with Detachabeads® (a goat antimouse F'ab antiserum which facilitates the removal of positively isolated cells from primary coated Dynabeads®) prior to transplantation adds an extra step to the process and makes it less favourable than negative selection. There is also the possibility of an alteration in antigenic expression, and therefore immunogenicity, due to islet antigen-antibody interactions.

A negative selection system therefore requires monoclonal antibodies to the acinar component of the pancreas. There are antibodies that have been shown to react to the acinar tissue but these were initially developed for the use in other tissues such as pancreatic islet [276], breast tissue [277] and ovarian tumours [278]. It would be preferential to use a single cross-reacting antibody or lectin for such a negative selection system. However, the pancreas is not solely made up of islet and acinar tissue, pancreatic ductal tissue, vessel and lymph nodes are present in the digest and are thought to play a role in rejection [80]. It has been shown in bone marrow purging that a cocktail of monoclonal antibodies is successful [267]. Therefore, a panel of monoclonal antibodies against the non-islet components of the pancreas could be very effective at removing all the non-islet components of the pancreatic digest.

The use of magnetic immunomicrospheres for islet purification were first described in 1987 by Muller-Ruchholtz et al. [279]. Using a modification of the method described by Kandzia et al. [280] they used magnetic albumin microspheres (size 0.2-1.5μm) coupled to lectins. Yields of 50-75% rat islets with an average purity of 93%
were obtained by the use of an electromagnet and re-exposure of the digest several
times to the magnetic field.

Dynabeads® have been used for rat islet purification by Soon-Shiong [281] and
Fujioka [282]. Blood reactive monoclonal antibodies which cross reacted with the
acinar but not the endocrine components of the pancreas were coupled to Dynabeads®. With exposure of the digest to the electromagnetic field several times they obtained
yields of 72% with a purity of 84%.

From these studies it has been shown that it is possible to adapt
immunomagnetic techniques to islet purification to allow reasonable yields of relatively
high purity to be isolated. However, both studies did not investigate optimising the
magnetic separation technique.

The aim of this thesis was to demonstrate the necessity for islet purification
prior to transplantation. Using negative selection, thereby avoiding the transplantation
of beads, purification of islets by the use of immunomagnetic techniques with M-450
Dynabeads® was investigated. The system would then be used as a secondary
purification procedure after density dependent methods in islet purification.
CHAPTER 3.

METHODS.

3.1. Introduction.
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      b. IgM ELISA.
      c. Polyacrylamide gel electrophoresis.
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3.5. Coating of M-450 Dynabeads with antibodies.
3.1 Introduction.

The following Chapter describes the methods used throughout this work which have been established in the Department of Surgery, University of Leicester, describing the purification of rat and human islets, the transplantation of rat islets and the production and purification of monoclonal antibodies for coating M-450 Dynabeads®. The methods used for immunomagnetic separation of islets will be described as they were developed in Chapter 6.

3.2 Methods involved in Rodent work.

i. Rat islet isolation.

Rat islets and acinar tissue were prepared using a combination of intraductal collagenase digestion [283] and Bovine serum albumin (BSA) density gradient purification [284] for the evaluation of the immunomagnetic separation techniques being developed. Under terminal halothane anaesthesia, pancreata were harvested from inbred WAG/Leiceser rats of either sex, weighing 200-250g.

![Figure 3.1 Distension of the rat pancreas by intraductal injection of collagenase.](image)

A midline incision was made and the liver was displaced in order to expose the pancreas. The common bile duct was occluded by clipping it at the duodenal end of the duct, the animal was then exanguinated. The pancreas was distended (Figure 3.1) by injecting 5mls of ice cold (4°C) minimal essential medium (MEM) containing
collagenase (Serva lot 03092C or lot 13073C 1mg/ml), 1.5mM calcium chloride and 0.02mg/ml DNase (Serva DN-25).

After careful resection of the distended pancreas, the gland was incubated with 10mls of pre-warmed MEM at 37°C for 13 minutes to activate the collagenase. The digestion process was arrested by pouring off the MEM and replacing it with ice-cold medium. After vigorous shaking for 1 minute to disperse the pancreas, the digest was poured through a 500μm mesh and washed with MEM containing 0.5% BSA.

The digest was resuspended in high density BSA (1.085g/cm³ 300mOsm BSA) using 6ml/pancreas and 3mls of the suspension was layered at the bottom of each of two 10ml test tubes. The suspension was then carefully overlaid with 3mls BSA of density 1.069g/cm³, a layer of BSA of density 1.056g/cm³ and finally a layer of MEM. The discontinuous density gradients were centrifuged at 800g for 20 minutes at 4°C with no brake.

![Diagram showing the discontinuous density gradients used to purify rat islets.](image)

**Figure 3.2** Diagram showing the discontinuous density gradients used to purify rat islets.

The islets were removed from the middle interface using a pasteur pipette, washed with MEM and placed into a petri dish in RPMI 1640 supplemented with penicillin, streptomycin, fetal calf serum, hydrocortisone, 2 mercaptoethanol and L-glutamine and cultured at 37°C in a CO₂ incubator. The acinar tissue from both the lower interface and the pellet of the density gradient was also removed and cultured under the same conditions for use in subsequent immunomagnetic purification studies and for transplantation.
A system of "adding back" has been used throughout this work whereby a defined number of pure islets have been hand counted following BSA density purification and added to a known quantity of acinar particles to prepare standard aliquots and allow comparative separations to be performed.

ii. Induction of diabetes.

In order to assess the inflammatory response of transplanted syngeneic islets and acinar tissue in diabetic rats, WAG/Leicester rats were injected with streptozotocin to induce diabetes. Streptozotocin is a nitrosoare antineoplastic agent which induces DNA lesions in islets [285].

WAG/Leics rats of either sex weighing 150-190g were anaesthetised using halothane. The femoral vein was exposed by a small inguinal incision. Streptozotocin (STZ, Sigma No.S-0130) was then administered at a concentration of 55mg/kg in the form of an intravenous injection within one minute of dissolving in 1ml citrate buffer pH 4.2. The non-fasting blood glucose was then monitored every 48 hours using BM Test 1-44 (Boehringer Mannheim, Mannheim, Germany) glucose test strip. Once the blood glucose exceeded 20mmol/l, this was defined as the onset of diabetes, and the animals were given daily doses of insulin subcutaneously (Ultralente heat treated bovine insulin, Novo Industri A/S.). Food and water were freely available.

3.3. Human islet production.

i. Donor retrieval.

With appropriate consent, human pancreata were removed from brain dead heart-beating organ donors. The procedure was usually part of a multi-organ donation with the retrieval of the heart, lungs and liver. The kidneys were always removed for transplantation. In order to minimise the period of warm ischaemia the systemic circulation was perfused with hyperosmolar citrate (HOC) at 4°C following the initial vascularised dissection of the organs. If the liver was to be retrieved, the portal system was perfused with University of Wisconsin solution (UW) at 4°C. The heart and ventilation were then arrested. Once the liver and kidneys had been removed the pancreas was dissected carefully in order not to rupture the capsule. The head of the pancreas was dissected from the duodenum and all extrapancreatic blood vessels and any fat was removed. The pancreas was then transported at 4°C in HOC to the laboratory.

ii. Distension and digestion of the pancreas with collagenase.

All subsequent procedures were carried out in a Class II Microbiological Safety Cabinet (Medical Air Technology LTD, Manchester UK.). After transportation of the
pancreas to the laboratory, a sample of the transport fluid was taken for microbiological analysis. The pancreas was transected to expose the ducts and the head and tail of the pancreas were weighed. The main ducts of the head and tail were cannulated using an Abbocath®-T cannula (Abbott Ireland Ltd, Sligo, Republic of Ireland) of an appropriate gauge (14-20G) secured by suture.

Collagenase (Serva Feinbiochemica, Heidelberg, Germany) at a concentration of 3mg/g pancreas, dissolved in Hanks Balanced Salt solution 2ml/g pancreas (Gibco), was intraductally injected at a temperature of 22°C into the pancreas (Figure 3.3). The cannula was slowly drawn back during injection of collagenase to ensure the whole of the pancreas became distended. The cannula was then removed and digestion accomplished using an automated method modified from that described by Ricordi [286]. Briefly the head and tail of the pancreas were placed in a stainless steel chamber with 10 stainless steel balls of diameter 1cm and a 500μm mesh was placed across the outlet (Figure 3.4). The digestion of the pancreas was in 3 phases;

**Phase 1:** Using a peristaltic pump (503S, Watson-Marlow Ltd, Falmouth, UK) MEM (see appendix 2) was circulated through the chamber at a temperature of 37-38°C (to maximise activity of the collagenase enzyme) at a flow rate of 96ml/min. Digestion of the pancreas was aided by gentle shaking and inversion of the chamber. Regular sampling of the outflow from the chamber enabled the digestion process to be monitored. Staining with dithizone (see appendix 2) enabled accurate identification of cleaved islets.

**Phase 2:** When cleaved islets were seen, the circuit was opened to allow collection and fresh MEM at 37-38°C was pumped through the circuit at an increased rate of 130ml/min. The outflow from the digestion chamber was then collected into 1 litre sterile bottles containing 100mls New born calf serum (NCS). Phase 2 enabled the continuation of the digestion process within the chamber whilst removing the already digested islets and acinar fragments. This prevented fragmentation of the islets due to continued collagenase activity.

**Phase 3:** If islets were seen to fragment during monitoring of phase 2, then the temperature of the MEM was lowered to 22°C to stop further digestion. The collected digest in MEM was centrifuged at 200g for 2 minutes the supernatant removed and the pellet containing the digest was then resuspended in UW solution (University of Wisconsin solution) and stored at 4°C.
Figure 3.3 Distension of the human pancreas by intraductal injection of collagenase.

Figure 3.4 The chamber used for digestion containing the pancreas.
iii. The use of the COBE 2991 for large scale density gradient purification of islets.

The COBE 2991 cell processor was used for the isolation of human islets using a system of linear continuous density gradients [287]. A gradient maker shown on top of the COBE 2991 in Figure 3.5 was set up with 275ml of high density gradient medium 1.108g/cm³ in the high density chamber and 150ml of low density medium 1.072g/cm³ in the low density chamber. 125ml of high density medium was pumped onto the COBE whilst stationary. Once air had been removed from the COBE bag, the COBE was spun at 1200rpm and the high and low density gradient media mixed (on a magnetic stirrer with a magnetic flea) whilst being pumped into the COBE bag. This produced a linear density gradient within the bag decreasing in density from 1.108 at the bottom to 1.072 at the top. Once the density medium was loaded into the bag, the digest suspended in 100ml UW solution, was then pumped on. All the digest was flushed on with 50ml UW solution and the COBE spun at 1500rpm for 20 minutes to allow digest to reach its isopycnic point on the gradient. Using the “supernatant out” mode on the COBE the first 150ml of UW was pumped off and thereafter 12 x 30ml fractions down the gradient were collected in 50ml tubes. Samples of each fraction were stained with dithizone and the fractions containing purified islets were pooled and incubated at 28°C in RPMI supplemented as described in the rat islet isolation section, 100μl samples of acinar tissue were also incubated at 28°C in RPMI for use later with Dynabeads.

3.4. Monoclonal Antibody Production.

To produce antibodies, an antigen is administered to a recipient to elicit an antibody response. Some substances can be antigens without being immunogenic, and generally only macromolecules that have a relatively complex structure which are recognised as being foreign by the recipient are immunogenic. In order to raise monoclonal antibodies against the non-islet components of the pancreas, pure acinar tissue was injected into mice to elicit an immune response.

Using the methods described in Sections 3.2 and 3.3 acinar tissue from rat and human pancreata, was produced and frozen in 100μl aliquots suspended in 0.5ml MEM and stored at -20°C. Pig acinar and pancreatic islets were separated by a similar method to that used for the isolation of human tissue.
Figure 3.5 The COBE 2991 cell processor with gradient maker.
i. Immunisation of mice.

Balb/c mice 8-10 weeks old were pre-bled (to provide negative control serum in subsequent screening) and injected with 100μl of antigen (acinar tissue) and adjuvant (Titremax® Vaccel, TM Inc. Norcross, Georgia, USA.) into the peritoneal cavity. After 3 weeks, a further 100μl of antigen in PBS was administered intraperitoneally. The mice were then bled 7 days after this and the serum assayed by immunohistological techniques (see section 3.4iii). If the results were positive, showing anti-acinar antibody production, the mice were again boosted with 100μl of antigen 4 days prior to the removal of the spleen for the fusion.

ii. Fusion.

Antibody producing spleen cells, were mixed with a selected immortal cell line of myeloma cells, and exposed to an agent, polyethylene glycol, to promote the formation of hybrid cells. To encourage growth of the hybrids, feeder cells were introduced to the mixture.

The fusions and cloning of the monoclonal antibodies were performed by Audrey Larkins in the Department of Surgery. The immunological screening of the hybridomas was carried out by myself. The following method describes the production of monoclonal antibody producing cell lines.

Under halothane anaesthesia the immunised BALB/c mice were injected with a lethal dose of barbiturate and a cardiac puncture performed to collect serum. In order to obtain peritoneal macrophages for use as feeder cells, 5mls of 50:50 medium (RPME:DME) was injected into the peritoneal cavity and then aspirated. The cells were later irradiated and used as feeder cells during hybridoma growth. Following this the spleen was removed. The subsequent techniques were carried out in a class II hood.

A single cell suspension was produced by passage of the spleen through a 125μm mesh. Following lysis of red blood cells with 0.75% tris ammonium chloride (pH 7.5) the number of viable cells were counted using an Improved Neubauer Haemocytometer (Weber Scientific International Ltd, Lancing, UK). The spleen cells were then mixed at ratio of 4:1 with mouse myeloma cells (NS-O). The fusion of splenocytes and the myeloma cells was accomplished by the addition of 800μl of a fusing agent, polyethylene glycol (PEG 1500, 50% w/v HEPES, Boehringer Mannheim) over 1 minute. The cells were then washed by the addition of 10mls of 50:50 medium and pelleted by centrifugation. Using a 24 well Lindro plate, cells were plated out at a ratio of 2x10⁶ spleen cells per well in 0.5ml of final suspension medium (Appendix 2). Figure 3.6 summarises the production of hybridomas.
In order to remove the unfused immortal NS-O cells and select for the spleen-myeloma cell hybrids, the ability of the hybrid cells to utilise a salvage pathway for the production of guanosine was exploited. The salvage pathway requires the presence of the hypoxanthine phosphoribosyl transferase enzyme (HGPRT). If the main biosynthetic pathway has been blocked (by aminopterin or amethopterin antibiotics in HAT medium), cells that are lacking the enzyme (HGPRT⁻) ie. myeloma cells, will only grow if fused with HGPRT⁺ cells ie. spleen cells.

After 24 hours, 1ml of the medium was removed and 1ml of HAT medium (hypoxanthine, aminopterin, thymidine) was added. The medium was changed every 3 days and on day 9 following the fusion, 1ml of supernatant was removed from each well for screening, using immunohistology, 1ml of HA medium (Appendix 2) was then added. Once positive wells had been identified, the cells were cloned in HA medium to ensure that no residual aminopterin remained in the wells.

iii. Immunological screening of hybridomas.

In order to screen for production of antibodies to pancreatic acinar tissue, samples of supernatants were taken from each well containing hybridoma cells for
immunohistological staining on sections of human, rat or pig pancreata. The
hybridomas were screened for IgM anti acinar antibodies as these can simply be
adsorbed onto uncoated non-activated Dynabeads® (see Chapter 2 section 2.2iii)

Samples of fresh pancreas were cut into oblong sections and then mounted onto
cork blocks by embedding the samples in Tissue-Tek O.C.T. (Miles Scientific,
Naperville, Illinois, USA.). The blocks were frozen using isopentane in liquid nitrogen
and stored in liquid nitrogen. Sections 5μm thick were cut from these blocks using a
cryostat (2800, Frigocut E, Reichert-Jung GmbH, Heidelberg, Germany.) and
mounted onto gelatinised multispot slides (Appendix 2). The slides were then frozen
and stored at -20°C. Before use, slides were thawed slowly by being placed at 4°C for
1 hour, then at room temperature for another hour, then fixed in acetone for 10 minutes.

100μl samples from the supernatants of hybridomas were placed onto individual
pancreas sections. Monoclonal antibodies produced within the Department were
routinely used as positive controls during the staining process. Fluorescence medium
(Appendix 2) and Tris-buffered saline (TBS) (appendix 2) were used as negative
controls to determine the background staining intensity.

The sections were incubated at room temperature for a minimum period of 40
minutes and then washed with TBS, 100 μl of the secondary layer, goat μ-specific
anti-mouse IgM (Sigma B-9265) diluted 1:1000 was added to the sections and
incubated for 40 minutes at room temperature. Following incubation, the secondary
layer was washed off with TBS and 100μl of the tertiary layer, extravidin alkaline
phosphatase (Sigma E2636) diluted to 1:400 in TBS was added and incubated for 30
minutes at room temperature. Again the sections were washed with TBS after
incubation and 100μl of chromogenic substrate (Appendix 2) was added for 15 minutes.
The sections were then washed in double-distilled H2O (ddH2O) and counter-stained
with acid haematoxylin solution (Sigma 285-2) for 5 minutes. After a final wash in
ddH2O, the sections were dried and mounted with a glass cover slip using glycerol
gelatin (Sigma GG-1). Figure 3.7 illustrates this method.

Microscopic examination using a light microscope at x150 magnification,
enabled identification of wells containing hybridomas producing monoclonal antibodies
to the acinar components of the pancreas. Positive staining developed as a red colour,
due to the interaction of the alkaline phosphatase, against the blue counter stain.

Cloning is used to isolate single clones of specific antibody-secreting cells. A prerequisite to cloning is a medium in which hybridoma cells can be grown from low concentrations. High cell dilutions can inhibit growth of the hybrids; however, this potential problem can be overcome by the addition of feeder cells, peritoneal macrophages, to the medium before cloning. Feeder cells were produced by the following method.

An adult Balb/c mouse was culled by dislocation of the neck. Two mls of RPMI medium was injected into the peritoneal cavity. After gentle massaging of the abdomen the fluid was aspirated, and then the cells spun at 400g for 1 minute, irradiated with 3000 rads to prevent cell replication, then resuspended in RPMI medium ready for use.

Following immunohistological staining, positive hybridomas were harvested and a sample was taken for counting. The cells were diluted in RPMI +10% FCS to a final concentration of 100 cells in 10ml. To each well of a 96-well plate, 100μl was added giving a ratio of 1 cell per well. The supernatants were re-assessed by immunohistology 1 week later and positive wells were recloned to ensure monoclonality (Figure 3.8). Cells from positive wells were then harvested and counted, before being used to produce antibody or cryopreserved. For cryopreservation, the cells (2x10^6) were suspended in vials of freezing mix (Appendix 2) and placed at 4°C for 1
hour, then -20°C for 1 hour, before being transferred to -70°C overnight and finally to -196°C for storage in liquid nitrogen.

v. Production of antibody in ascitic fluid.

In order to produce high concentrations of antibody quickly, cells can be injected into the peritoneal cavity of pristane treated animals. Pristane is the plasmacytogenic component of mineral oils which is known to induce plasmacytomases when injected into the peritoneal cavity of mice. It also acts by depressing the normal immunological function of the mouse [288,289].

Hybrids were grown in vivo. Balb/c mice were injected with 0.5ml pristane (2,6,10,14 tetramethylpentadecane, Sigma, T7640) 5-7days prior to injection of the antibody producing cells. Cells (2-5x10⁶) were injected into the peritoneal cavity and after about 1-2 weeks, ascitic fluid with a very high antibody concentration was produced and removed with a syringe and needle once the animal had been culled.

**Figure 3.8** Illustration of the cloning of hybridomas.
vi. Assessment of monoclonal antibody purification by IgM ELISA and polyacrylamide gel electrophoresis.

As described above, ascitic fluid was produced to give large quantities of monoclonal antibodies. Although the ascitic fluid contained high titres of the IgM antibody required, it also contained smaller native protein molecules from the host mouse in large amounts. These contaminating proteins could potentially block binding sites on M-450 Dynabeads® immunomicrospheres during coating with the relevant antibody. Therefore the ascites was purified (see Figure 3.9).

![Flow diagram for the purification of ascites](image)

Figure 3.9 Flow diagram for the purification of ascites.

a. Purification of IgM monoclonal antibodies.

As mentioned above, the IgM antibody was fractionated from the components of the ascitic fluid by the use of gel chromatography. Gel chromatography enables the separation of proteins on the basis of their molecular dimensions using porous gels of
polyacrylamide, cross linked dextran or agarose. Between the liquid phase around the beads an equilibrium is set up and molecules can diffuse between the two phases according to the size of the pores in the gel. Therefore, molecules are able to move down the column at a rate dependent on their molecular size. Larger molecules pass through the column and do not enter the beads due to exclusion from the gel pores. However, smaller molecules enter the beads and have to pass through the liquid surrounding the beads as well. The sample moves down the column as a band due to the solute molecules maintaining a concentration equilibrium with the gel and liquid phase. The number of beads in the column also determines the rate the sample passes down the column. Large molecules therefore leave the column first with the solute in the 'void volume' $V_0$, the small molecules enter the gel thus entering the 'included volume', $V_i$, and therefore passage through the column is dependent on size.

The total bed volume, $V_t = V_0 + V_i$

Molecules of intermediate size are eluted from the column in a volume between the $V_0$ and $V_t$ known as the eluted volume, $V_e$.

IgM has a molecular weight of $9 \times 10^5$ kD and therefore sepharose 4B with a separation range of molecular weights between $6 \times 10^4$ and $20 \times 10^5$ kD (agarose gel Pharmacia, Uppsala, Sweden) was chosen to fractionate the ascitic fluid.

Using an LKB chromatography column (Figure 3.10) with a 65cm x 2.6cm internal diameter, the gel was loaded and allowed to settle overnight at room temperature. Ethanol, used as a preservative for the gel, was removed along with any air bubbles by washing with TBS for 24 hours. After this process, the column was transferred to the cold room at 4°C ready for use. The column was calibrated using proteins (BSA MW 67,000 and Ferritin MW 440,000) dissolved in 2mls of TBS. Samples were fractionated in an upward direction at a flow rate of 0.5ml/min using an LKB vanoperpex II pump. A flow-through UV analyser (LKB Uvicord SII) continually analysed the UV absorbance of the fractions and this was recorded on an LKB 2210 chart recorder before collection (using an LKG 2211 Superac) into 150x2ml fractions. The protein content of each fraction could be approximately calculated by the use of the following equation:

$$\text{Protein concentration mg/ml} = \text{UV absorption at 280nm} \times 0.741.$$
b. IgM ELISA.

Using the IgM ELISA (Enzyme-linked immunosorbent assay) method established in the Department of Surgery, the concentration of IgM in fractions could be determined. 100µl of polyclonal mouse IgM at a concentration of 50mg/ml in 0.3M Na2CO3 pH9.5 was used to coat wells of a 96 well ELISA plate (Linbra® EIA Microtitration plate, Flow Laboratories, Irvine UK.). The first row was left blank in order to assess background binding of anti-mouse IgM (negative control). The last four wells of the last row on the plate were the positive controls: after washing, the enzyme-labelled anti-mouse IgM and the phosphatase substrate only were added to these 4 wells. The plates were sealed and incubated at 4°C overnight.

Figure 3.10 The LKB chromatography column with a coloured solvent front traversing up the column.
Excess coating buffer was removed by washing the plates three times with PBS (Appendix 2) the next day. To each well, 200µl of blocking buffer (5% BSA in PBS) was added and incubated at room temperature for 30 minutes. The plates were then washed three times with PBS.

Serial dilutions of polyclonal IgM were made using 0.1% gel/PBS to give a range of standards from 50 to 0.78mg/ml. Fifty µl of the IgM standards and samples from each fraction from the column were added to the appropriate wells, and 50µl of the competing alkaline phosphatase anti-mouse IgM (Sigma A 7784) diluted 1:1000 in 0.1%gel/PBS was added to all wells except the positive controls. The plate was sealed and incubated at room temperature for 90 minutes.

Following incubation, the plates were again washed 3 times in PBS. To all wells, except the negative controls 100µl of phosphatase substrate (P-Nitrophenyl phosphate disodium, Sigma 104-0) at 1mg/ml in glycine buffer (Appendix 2) was added. 50µl of the alkaline phosphatase was then added to each of the positive control wells.

The plates were incubated at room temperature until the colour developed by the substrate could be measured using a Titertek Multiscan MCC/340, (EFLAB, Finland) (usually at around 2 hours). The greater the colour developed, the lower the concentration of IgM in the sample. A plot of the readings obtained from the standards were plotted against the log of the concentration. This then enabled the concentration of the samples from the column to be calculated.

c. Use of membrane filtration for concentration of IgM.

Membrane filtration [290] utilises a physically strengthened semipermeable membrane filter. There are a range of such filters with different molecular weight cut-offs available. Pressure is applied by compressed nitrogen gas to a solution on one side of the membrane, this drives smaller molecules through the filter to the other side retaining molecules of the desired molecular weight or above. Stirring the solute prevents accumulation on the membrane surface which decreases the efficiency of the filter.

In order to concentrate the purified but dilute IgM from the relevant fractions following gel chromatography, the fractions were pooled into batches of 5, 66-70, 71-75 etc. An Amicon 8200 stirred cell unit (Amicon Ltd, Stonehouse UK) was used (Figure 3.11), with a YM10 Diaflo ultrafilter (Amicon) which has a molecular weight cut off of 10,000kDa. The stirred cell unit was pressurised at 15psi with compressed nitrogen gas. The protein content for the unfiltered, IgM containing fraction and the
ultrafiltrate produced by the stirred cell unit, was measured, to ensure that no loss of IgM into the ultrafiltrate had occurred.

Figure 3.11 The Amicon 8200 stirred cell unit used for membrane ultrafiltration.

**d. Polyacrylamide gel electrophoresis.**

Electrophoresis is the movement of a molecule with a net charge in an electric field. This phenomenon enables the separation of proteins. The velocity of migration of the protein depends not only on the net charge of the protein but also the strength of the electric field and the frictional coefficient. The frictional coefficient depends on the mass and shape of the migrating molecule and the viscosity of the medium. The use of gels for electrophoresis enables molecular sieving to occur, where molecules that are small compared to the pores in the gel are able to move faster through the gel than molecules that are larger than the pore size. The use of different concentrations of acrylamide for the gel controls the pore size.

Following concentration by ultrafiltration, the purity of the concentrated pooled IgM fractions (5 fractions from LKB 2211 Superac were pooled at once eg.60-65,66-70) were assessed using gel electrophoresis.

A resolving gel made of 7.5% acrylamide (Appendix 2) was allowed to set between 2 glass plates clamped with perspex spacers in between (Figure 3.12).
stacking gel, 7.7% acrylamide (Appendix 2) was run onto the top and a 10 slot perspex comb was inserted into the stacking gel before it set. Samples were loaded onto the gel after boiling for 5 minutes (to denature the protein) in 10µl aliquots, with 10µl of sample buffer and 30µl of H2O. The molecular weight markers used were BRL heavy prestained molecular weight markers of 200kD, 97.4kD, 68kD, 43kD, 29kD, 18.4kD, and 14.3kD. A sample of ascitic fluid before purification was also run on the gel for comparison.

The gel was placed in a gel tank filled with running buffer (Appendix 2) and using a voltage of 25mV and a current of 11mA, the gel was left to run overnight. Once the sample dye had almost run to the bottom of the gel the current was turned off and the gel placed in Coomassie brilliant blue for staining (Appendix 2).

After 5-6 hours on a gentle rocker, the staining buffer was rinsed off briefly in water and then placed in destain (Appendix 2) to remove any background staining. The gel was then analysed to ascertain which fractions of IgM did not contain any impurities. The pure fractions of IgM were then pooled to a known final concentration and filter sterilised using an acrodisc 0.2µm filter (Acrodisc, Gelman Sciences UK.). After final estimation of the IgM concentration, the concentrate was stored at 4°C in 1ml aliquots.

3.5 Coating of M-450 Dynabeads® with antibodies.

Throughout this experimental work, commercially available M-450 uncoated Dynabeads® (Dynal, Oslo, Norway) have been used. From the manufacturers information it is known that 1ml of the Dynabead solution contains 4x10⁸ beads or 30mg/ml. The M-450 Dynabeads® were coated by non-specific adsorption with monoclonal antibodies raised against acinar tissue.

Before the beads were coated with the required antibody, the Dynabeads® were vortexed to provide a homogeneous suspension. A known number of beads were pipetted into a sterile eppendorf and using the MPC®-E magnetic particle separator the preservative solution was pipetted off after 30 seconds (Figure 3.12 ).
For optimal binding determined in prior experiments, M-450 Dynabeads® were coated with antibody at a concentration of 8μg/mg of beads. The antibody was diluted in borate buffer (Appendix 2) at pH9.5 to give a ratio of 50mg beads/ml of IgM antibody solution.

The beads were incubated with the IgM solution overnight at 4°C on a spiramix. Following incubation, the antibody supernatant was removed using the MPC®-E magnetic particle separator. The beads were then washed 4 times for 5 minutes each time using 0.5% BSA in 0.01M PBS (Appendix 2) and then suspended in 0.1% BSA in 0.1M PBS and stored at 4°C overnight. Before long term storage at 4°C a preservative, sodium azide (0.02%), was added. Figure 3.13 shows Dynabeads® coated with the monoclonal antibody LDS 10 binding to rat acinar leaving the dithizone stained islet unlabelled.
Figure 3.13 Dynabeads® coated in LDS 10 monoclonal antibody labelling the rat exocrine tissue, an unlabelled dithizone stained islet can be seen.
CHAPTER 4.

THE INFLAMMATORY RESPONSE TO THE ISOGENEIC TRANSPLANTATION OF PURE AND IMPURE ISLETS IN WAG/LEICESTER RATS.

4.1 Introduction.
4.2 Methods.
4.3 Results.
4.4 Conclusions.
4.1 Introduction.

The effect of transplanting unpurified islets reviewed in Section 1.4iii has not been extensively studied. It is thought that acinar tissue contamination may affect islet transplantation by the induction of harmful systemic affects such as disseminated intravascular coagulation (DIC) and possibly increasing the immunogenicity of the transplanted tissue [82].

There have been only a few studies examining the effect of acinar contamination on islet implantation [291]. Gray et al. in 1988 examined the insulin content of islets when transplanted with acinar tissue under the kidney capsule of non-diabetic rats. They found that if islets of 50% or less purity were transplanted, then the islet implantation was reduced to 50% and, histologically there was a deleterious affect. They did not comment on the nature of the histological effect.

Another study by Gotoh et al. investigated the effect of contaminating lymph nodes, ductal and vascular tissue as well as acinar tissue on islet graft survival [80]. They found that lymph nodes, (and to a lesser extent ductal and vascular tissue) played a major role in inducing an immune response which resulted in acute rejection of islets. Although no difference in survival was found between purified and unpurified islet allografts contaminated with acinar tissue, an early loss of graft function was found in the latter group. They suggested that the acinar tissue did not directly act as a source of immunising donor antigen, but caused adverse effects, such as inflammation around the islet grafts, which contributed to the rejection process and to their functional loss.

Gores et al. carried out a study in a similar model to that used by Gotoh et al. and they found that partially purified islets rejected at the same rate as purified islets. In both preparations however, contaminating lymph nodes were present.

The use of the liver as a site for islet transplantation has caused problems when large quantities of contaminating acinar tissue have been infused into the portal vein. A rise in portal vein pressure has been observed in animal models and also in humans [84,292]. The only published study to have investigated the effect of acinar contamination on the function of islets transplanted intraportally was carried out by Downing et al. [81]. They compared the effect of transplanting handpicked islets, ficoll purified islets, ficoll purified islets contaminated with acinar obtained from the pellet of the ficoll gradients, the pellet alone and dispersed pancreas. The function of isolated islets grafts, reflected by the restoration of normoglycemia, normal urine volumes and weight gain, was not affected by contamination of the graft with increasing amounts of pellet debris. However, the insulin content at 12 weeks was negatively correlated to the
amylase content of the tissue. All of the animals transplanted with dispersed pancreatic
digest died within 24 hours of the operation. Gross portal venous congestion was
observed during autopsy.

Acinar contamination may cause 1) immune rejection problems, 2) an
inflammatory reaction, 3) systemic upset eg DIC, 4) obstruction of the portal vein. This
study was therefore designed to investigate 2&4, examining the histological effect of
transplanting unpurified islet tissue isogeneically into the portal vein of WAG/Leicester
rats.

The principal of the inflammatory reaction is the delivery of phagocytic cells to
sites of trauma or immunological stimulation. Lymphocytes can be activated by contact
with antigen. Activated lymphocytes produce lymphokines and one of these, γ-
interferon, is a major stimulator of monocytes and macrophages. Products of
monocytes and macrophages, monokines, activate lymphocytes which produce
inflammatory mediators setting the stage for persistence of the inflammatory response.

The function of the inflammatory reaction is to eliminate particulate matter, such
as cell debris, through the process of phagocytosis. During the inflammatory reaction,
the circulating phagocytes respond to chemotactic stimuli and selectively migrate into the
inflamed tissue.

The predominant cell in the early stages of an acute inflammatory reaction is the
neutrophil, while in the later stages the macrophage predominates. The macrophage is
part of the mononuclear phagocyte system, which consists of closely related cells of
bone marrow origin, including blood monocytes and tissue macrophages. Tissue
macrophages have a half life of several months and have the potential for being
activated. They increase in cell size, the levels of lysosomal enzymes increase and the
metabolism of the cell increases facilitating a greater ability to phagocytose. Following
activation, macrophages secrete a wide variety of biologically active products which are
important mediators of the tissue destruction and fibrosis characteristic of chronic
inflammation.

This study examined the inflammatory reaction which occurred following the
isogenic intraportal transplantation of either pure islets, pure acinar, a mixture of islets
and acinar tissue, and acinar tissue immunomagnetically labelled with Dynabeads® at
various time intervals. Initially the response was assessed in 2 recipient rats in each
group at each time interval, 4,8,12,24 and 72 hours and 7 days following
transplantation. Four further transplants were carried out and assessed at 12 hours (the
maximal response time) to allow comparison of the maximal response between the four
groups.

4.2 Method.

Rat islets were prepared the day before transplantation using the method of
collagenase digestion and purification using BSA gradients (Chapter 3.2i). The islets
were incubated at 37°C overnight in RPMI 10% FCS. On the day of the transplant,
1000 rat islets were hand picked and resuspended in 0.5ml RPMI for intraportal
transplantation.

Acinar tissue was prepared using the same method but the digestion time was
reduced from 13 minutes to 8 minutes to produce acinar fragments that were of a similar
size to islets and also resembled the acinar tissue produced in human islet isolation.

In this study there were four recipient groups with 2 recipients in each group,
Group 1 received 1000 islets alone intraportally.
Group 2 received 1000 acinar fragments alone.
Group 3 received 1000 islets and 1000 acinar fragments.
Group 4 received 1000 acinar fragments with Dynabeads® attached.

In the fourth group, which received acinar fragments with Dynabeads®
attached, the acinar fragments were incubated with Dynabeads® (coated with LDS 10)
for 20 minutes prior to transplantation.

Following hand picking, the islet and acinar fragments were centrifuged for 2
minutes at 1200rpm at 4°C, then resuspended in 0.5ml RPMI in a 1ml syringe with a
22 gauge needle. The recipients were anaesthetised using halothane and a small midline
incision was made. The portal vein was exposed and the islets and acinar tissue were
dispersed in the syringe prior to injection. Following injection (Figure 4.1), the syringe
was washed out by drawing it back twice ensuring no tissue was left in the syringe.
Haemostasis was secured by an absorbable haemostatic gauze (oxidised cellulose B.P.
Becton Dickinson, Oxford, UK) and the wound was sutured. On recovery from the
anaesthetic, the recipient was administered 0.1ml of the analgesic, Temgesic (National
Vetinary Supplies, Stoke On Trent, UK.).
After a set period of time of either 4, 8, 12, 24, 72 hours or 7 days, the livers were removed from the recipients and the animals culled under anaesthetic. The livers were fixed in 10% formalin embedded in paraffin and sectioned by the Histology Department for histological examination. Sections from different levels of the liver were cut and all sections were stained with the Haemotoxylin and Eosin method (Appendix 3). ABC staining using the primary antibodies LDS 10 (anti acinar) and HB124 (anti insulin) was performed on each of the relevant sections (Appendix 3) to confirm the presence of islets and or acinar. The sections were then examined for signs of inflammation by an experienced Consultant Histopathologist. Neither inter or intra-observer variation were assessed.

Initially, a comparison between STZ induced diabetic and non diabetic rats was set up. However, no significant difference between animals in these two groups was found (Wilcoxon matched-pairs signed ranks test achieved confidence 94.5% CI 0-0.5), and the remainder of the study was carried out using non-diabetic rats.

The following scoring system was used to assess the inflammatory response in each section of liver for each animal;
H&E staining.
1 1-3 small foci of inflammation not associated with pancreatic tissue or foreign tissue or a minor inflammatory infiltrate around transplanted tissue.
2 1-3 prominent foci of inflammation easily visible on low power examination.
3 4 or more prominent foci of inflammation easily visible on low power examination.

4.3. Results.
All the animals survived and the results are shown in the tables below. The median score for the inflammation seen using H & E staining from the different levels from each recipient.

Table 4.1. Inflammatory score for transplanted pure islets.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Median for H&amp;E 1</th>
<th>Median for H&amp;E 2</th>
<th>Overall Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours</td>
<td>0</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>8 hours</td>
<td>0</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>12 hours</td>
<td>0</td>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>72 hours</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7 days</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.2. Inflammatory score for transplanted acinar tissue.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Median for H&amp;E 1</th>
<th>Median for H&amp;E 2</th>
<th>Overall Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours</td>
<td>2</td>
<td>0</td>
<td>0-2</td>
</tr>
<tr>
<td>8 hours</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12 hours</td>
<td>0</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>72 hours</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7 days</td>
<td>0</td>
<td>0</td>
<td>0-2</td>
</tr>
</tbody>
</table>
The inflammatory response observed with groups 1 and 2 (islets alone and acinar alone) indicated that in the majority of the time intervals examined there was little or no inflammation. However, a maximum score of 2 was seen at 12 and 24 hours.

Table 3 shows that the inflammatory response observed when islets and acinar were transplanted together was greater than that seen with the other groups. The maximum score of 3 was observed at all time intervals up to 24 hours, with the 12 hour time interval giving the highest response. In this group, the response at 30 days was also examined to see if there was still acinar present and if there were signs of inflammation. The results showed that there was still an inflammatory reaction.

Table 4 shows the results from the transplantation of acinar bound with beads. As in groups 1 and 2 there is minimal response, however the highest response is observed at 12 hours with the maximum score of 3.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Median for H&amp;E 1</th>
<th>Median for H&amp;E 2</th>
<th>Overall Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours</td>
<td>2</td>
<td>0</td>
<td>0-3</td>
</tr>
<tr>
<td>8 hours</td>
<td>2</td>
<td>3</td>
<td>0-3</td>
</tr>
<tr>
<td>12 hours</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>24 hours</td>
<td>2</td>
<td>1.5</td>
<td>0-3</td>
</tr>
<tr>
<td>72 hours</td>
<td>0.5</td>
<td>1.5</td>
<td>0-2</td>
</tr>
<tr>
<td>7 days</td>
<td>0</td>
<td>0</td>
<td>0-2</td>
</tr>
<tr>
<td>30 days</td>
<td>0.5</td>
<td>0</td>
<td>0-2</td>
</tr>
</tbody>
</table>

Table 4.3. Inflammatory score for transplanted islets and acinar tissue.

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Median for H&amp;E 1</th>
<th>Median for H&amp;E 2</th>
<th>Overall Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 hours</td>
<td>1</td>
<td>0.5</td>
<td>0-2</td>
</tr>
<tr>
<td>8 hours</td>
<td>1</td>
<td>2</td>
<td>0-2</td>
</tr>
<tr>
<td>12 hours</td>
<td>3</td>
<td>0</td>
<td>0-3</td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>0</td>
<td>0-2</td>
</tr>
<tr>
<td>72 hours</td>
<td>0</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>7 days</td>
<td>0</td>
<td>0</td>
<td>0-2</td>
</tr>
<tr>
<td>30 days</td>
<td>0</td>
<td>0</td>
<td>0-1</td>
</tr>
</tbody>
</table>

Table 4.4. Inflammatory score for transplanted acinar tissue and beads.
In all of the 4 groups the highest inflammatory score was observed at 12 and 24 hours. For this reason the number of animals in each of the 4 groups at the 12 hour time interval was expanded to 6. Tables 4.5-4.8 show the results (the median score) of the sections for each animal in the expanded groups. The inflammatory response for each group was statistically compared using the Mann Whitney U test, the p values and the 95% confidence intervals are shown in Table 4.9.

Table 4.5. Inflammatory score at 12 hours for transplanted pure islets.

<table>
<thead>
<tr>
<th>Wag</th>
<th>Median for H&amp;E</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0-2</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1-2</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0-2</td>
</tr>
<tr>
<td>Overall</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.6. Inflammatory score at 12 hours for transplanted acinar.

<table>
<thead>
<tr>
<th>Wag</th>
<th>Median for H&amp;E</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>0-3</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0-1</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td>Overall</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

The inflammatory response observed at 12 hours in groups 1 (islets transplanted alone) and 2 (acinar transplanted alone) was minimal. Although the overall median for group 1 was slightly higher than group 2 the range in group 1 less than in group 2 where the maximum score of 3 was obtained in some sections from the livers of half the animals in this group.
Table 4.7. Inflammatory score at 12 hours for transplanted islets and acinar tissue.

<table>
<thead>
<tr>
<th>Wag</th>
<th>Median for H&amp;E</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2-3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1-2</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>1-2</td>
</tr>
<tr>
<td>Overall</td>
<td>2.5</td>
<td>1-2</td>
</tr>
</tbody>
</table>

The results from group 3 showed a higher inflammatory response had occurred following the transplantation of islets together with acinar. The overall median for this group was 2.5 and in 4 of the animals in this group, the maximum score of 3 was obtained.

H&E staining of the sections from group 3 (islets and acinar tissue) illustrated the problems of transplanting unpurified islets. Necrosis of the islets and acinar tissue was observed, but also necrosis of the surrounding tissue was seen. Portal vein thrombosis and necrosis around the hepatic artery occurred in some of the livers. This may have arisen due to the volume of tissue transplanted intraportally.

Table 4.8. Inflammatory score at 12 hours for transplanted acinar and beads.

<table>
<thead>
<tr>
<th>Wag</th>
<th>Median for H&amp;E</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>2-3</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0-3</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0-2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1-3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0-3</td>
</tr>
<tr>
<td>Overall</td>
<td>2</td>
<td>1-3</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>1-3</td>
</tr>
</tbody>
</table>
Group 4 also had a higher inflammatory response to the transplantation of acinar tissue bound with beads. The overall median was 2. The overall median for group 4 was higher than in group 2 and this may have been due to the presence of the Dynabeads® initiating a foreign body response.

Table 4.9 shows the p values, 95% confidence intervals and the w score, obtained using the Mann Whitney U test. The asterisked p values indicates that the transplanted tissue in the column induced a significantly greater inflammatory response than the transplanted tissue in the row.

<table>
<thead>
<tr>
<th></th>
<th>Acinar</th>
<th>Islets &amp; Acinar</th>
<th>Acinar and Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islets</td>
<td>p = 0.9</td>
<td>p = 0.02*</td>
<td>p = 0.1</td>
</tr>
<tr>
<td>95% Cl -2, -1</td>
<td>95% Cl -2, -1</td>
<td>95% Cl -2, 0</td>
<td></td>
</tr>
<tr>
<td>w = 37.5</td>
<td>w = 54.5</td>
<td>w = 29</td>
<td></td>
</tr>
<tr>
<td>Acinar</td>
<td></td>
<td>p = 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% Cl -3, 1</td>
<td>95% Cl -1, 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>w = 31</td>
<td>w = 43.5</td>
<td></td>
</tr>
<tr>
<td>Islets and acinar</td>
<td></td>
<td>p = 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% Cl -0, 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>w = 49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis of the overall medians for each group using the Mann Whitney U test showed that there was a significant difference between groups 1 (islets alone) and 3 (islets and acinar tissue). The inflammatory response observed was greater when islets were transplanted together with acinar than when islets are transplanted alone. There was no statistical significant difference between any of the other groups.
Figure 4.2 Shows an islet with minimal inflammatory response.

Figure 4.3 Shows an islet with an area of necrosis.
Figure 4.4 Shows an acinar fragment with surrounding necrotic tissue.

Figure 4.5 Necrosis of the liver observed with the transplantation of acinar tissue alone.
4.4 Conclusions.

The results of this study are very important, demonstrating that even if the problem of rejection is overcome, the transplantation of impure islets, engenders an inflammatory response which, even when modulated by immunosuppression, is likely to impair islet implantation and function.

Studies examining the islets transplanted intraportally have shown that in the islets scattered throughout the liver, there is an ingrowth of capillaries. These capillaries connect to both the portal vein and the hepatic artery [293]. From the time of transplantation, islets are bathed in blood, however centres of large islets will become vulnerable to anoxia due to the limitation of gas diffusion when their capillary bed collapses [294]. The central $\beta$ cells have been shown to be the most responsive to glucose [295] and this effect may increase the numbers of islets required to reverse diabetes and factors affecting engraftment. On the H&E sections for the transplantation of islets and acinar, islets were seen to be clumped together with acinar tissue. The necrosis seen in these central islets could therefore be explained by the possible decrease in oxygen supply.
The number of islets required to reverse diabetes in the Wag/Leicester rat was 1000 and, it is for this reason that 1000 islets were used and compared with the effect of 1000 acinar fragments. The study then examined what happens when the number of islets required to reverse diabetes are transplanted with 50% contamination, a realistic scenario in the human. The aim of the study was to investigate the effect of acinar contamination on islet transplantation.

Any affect of the increased volume of islets and acinar tissue when combined was not assessed and would need further investigation. However, the occurrence of thrombosis in the portal vein in this group may be contributed to by the mechanical obstruction caused by the increased volume of tissue being transplanted.

In order to carry out a comparative study a further group of animals would have been required, involving the transplantation of 500 islets and 500 acinar fragments. This was not done in the initial group of experiments since it would have required the use of further animals to create a scenario with no relevance to the clinical situation in which acinar contamination inevitably results in an increase in volume.

Pancreatitis may be triggered by a variety of different mechanisms, although the result is ultimately the same; intraparenchymal enzyme activation, tissue destruction and ischaemic necrosis. Acinar tissue fragment when transplanted into the liver may be expected to produce similar effects. The pancreas is protected from autodigestion by several mechanisms. Pancreatic enzymes are synthesized, transported and secreted in the form of inactive precursors. From the time of their synthesis on the rough endoplasmic reticulum until their secretion by exocytosis, the potentially harmful digestive enzymes are sequestered in membrane bound organelles that contain potent inhibitors capable of inactivating prematurely activated trypsin. The mechanisms underlying the premature enzyme activation seen in acute pancreatitis are poorly understood.

Trypsinogen may be present in necrotic acinar lobules and trypsin could be activated by leucocyte derived cathepsin in a severe acute inflammatory response with parenchymal necrosis. The activation of trypsin may further activate elastase, phospholipase and the kinin system causing additional pancreatitis and impair pancreatic perfusion [296]. The enzymes released by acinar tissue are proteases, amylases and lipases and therefore in the liver these may be secreted thus causing deleterious effects, similar to those seen in pancreatitis with an acute inflammatory response.
In a recent case, the potential hazard of using partially purified pancreatic tissue was highlighted. Embolization via an intraportal site of a combined liver and islet transplant resulted in portal vein thrombosis with loss of the liver allograft [137]. Fortunately another liver became available within 24 hours and the patient survived. This case, together with the results obtained from this study and from previous studies, can only emphasize the need to transplant purified islets and justifies the work described in the rest of this thesis.
CHAPTER 5

THE PRODUCTION OF A PANEL OF MONOCLONAL ANTIBODIES TO THE NON-ISLET COMPONENTS OF THE PANCREAS.

5.1 Production Of A Panel Of Monoclonal antibodies.
5.2 Cross Reactivity Of The Monoclonal Antibodies.
5.3 Conclusions.
5.1. Production of panel of monoclonal antibodies.

Monoclonal antibodies were raised against the non-islet components of human, pig and rat pancreas. The techniques used are described in Chapter 3 Section 3.4. The specificities of the monoclonal antibodies were assessed using the immunohistological staining technique described in Section 3.4iii on frozen sections of the appropriate species pancreas. The fusions that generated positive clones are given in Table 5.1, the antigen used to generate the monoclonal antibodies, the number of times the monoclonal had to be recloned and the specificity are also given. The monoclonal antibodies were designated an LDS (Leicester Department of Surgery) number, also shown in the table.

Table 5.1. Monoclonal antibodies raised against the anti-islet components of the pancreas.

<table>
<thead>
<tr>
<th>FUSION</th>
<th>IMMUNISATION</th>
<th>CLONED</th>
<th>POSITIVE CLONES</th>
<th>SPECIFICITY</th>
<th>LDS NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion 45</td>
<td>Rat</td>
<td>once</td>
<td>F45.4.A3.D9</td>
<td>Rat acinar</td>
<td>LDS 10</td>
</tr>
<tr>
<td>Fusion 68</td>
<td>Human</td>
<td>once</td>
<td>F68.4.D2.19</td>
<td>Human ductal</td>
<td>LDS 5</td>
</tr>
<tr>
<td>Fusion 68</td>
<td>Human</td>
<td>once</td>
<td>F68.4D2.27</td>
<td>Human acinar</td>
<td>LDS 8</td>
</tr>
<tr>
<td>Fusion 92</td>
<td>Rat</td>
<td>twice</td>
<td>F92.19.86.27</td>
<td>Rat acinar &amp; ductal</td>
<td>LDS 35</td>
</tr>
<tr>
<td>Fusion 93</td>
<td>Human</td>
<td>twice</td>
<td>F93.1.51.2p</td>
<td>Human &amp; rat vessels</td>
<td>LDS 44</td>
</tr>
<tr>
<td>Fusion 93</td>
<td>Human</td>
<td>twice</td>
<td>F93.1.51.2p.14</td>
<td>Rat vessel</td>
<td>LDS 41</td>
</tr>
<tr>
<td>Fusion 93</td>
<td>Human</td>
<td>twice</td>
<td>F93.32.85.89</td>
<td>Human acinar</td>
<td>LDS 34</td>
</tr>
<tr>
<td>Fusion 94</td>
<td>Pig</td>
<td>twice</td>
<td>F94.53.2.89</td>
<td>Human acinar</td>
<td>LDS 40</td>
</tr>
<tr>
<td>Fusion 94</td>
<td>Pig</td>
<td>twice</td>
<td>F94.38.16.40</td>
<td>Human vessel</td>
<td>LDS 38</td>
</tr>
<tr>
<td>Fusion 94</td>
<td>Pig</td>
<td>twice</td>
<td>F94.66.21.9</td>
<td>Pig collagen &amp; human scattered cells</td>
<td>LDS 39</td>
</tr>
<tr>
<td>Fusion 95</td>
<td>Pig</td>
<td>twice</td>
<td>F95.8.2.2</td>
<td>Rat scattered cells</td>
<td>LDS 37</td>
</tr>
<tr>
<td>Fusion 95</td>
<td>Pig</td>
<td>twice</td>
<td>F95.8.12.92</td>
<td>Human ducts</td>
<td>LDS 45</td>
</tr>
<tr>
<td>Fusion 95</td>
<td>Pig</td>
<td>twice</td>
<td>F95.20.9p.18</td>
<td>Rat and human vessels &amp; acinar</td>
<td>LDS 42</td>
</tr>
<tr>
<td>Fusion 95</td>
<td>Pig</td>
<td>twice</td>
<td>F95.39.21.13</td>
<td>Human vessels</td>
<td>LDS 46</td>
</tr>
<tr>
<td>Fusion 96</td>
<td>Pig</td>
<td>once</td>
<td>F96.16.54</td>
<td>Human occasional cells</td>
<td>LDS 49</td>
</tr>
<tr>
<td>Fusion 97</td>
<td>Rat</td>
<td>once</td>
<td>F97.38.8</td>
<td>Rat acinar</td>
<td>LDS 43</td>
</tr>
</tbody>
</table>

Figures 5.1 - 5.4 show examples of immunohistological staining (described in Section 3.4iii) of rat and human pancreas sections with the monoclonal antibodies.
Figure 5.1 Immunostaining of rat pancreas with LDS 10, an IgM antibody which reacts specifically with acinar cells (stained red). The islet in the centre is not stained.

Figure 5.2 Immunostaining of human pancreas with LDS 8 an IgM antibody which reacts specifically with human acinar cells (stained red).
Figure 5.3 Immunostaining of human pancreas with LDS 5 an IgM antibody which reacts specifically with human ductal tissue (stained red).

Figure 5.4 Immunostaining of rat pancreas LDS 43 an IgM monoclonal antibody. The antibody reacts with acinar tissue (stained red) leaving the islet unstained.
Ascitic fluid containing high concentrations of specific monoclonal antibodies, was produced by intraperitoneal injection of cell lines into mice. The ascitic fluid was centrifuged to remove cellular debris and then loaded onto the gel chromatography column in 2ml aliquots for purification (Section 3.4via). This process produced 150 2ml fractions of which 110 were in the eluted volume, Vₑ, containing fractionated protein from the ascites, with MW between $6\times10^4$ and $20\times10^6$KD. UV spectrophotometry was used to estimate the total protein content of each fraction. An ELISA was performed to determine the IgM concentration (Section 3.4vib). Fractions containing IgM were then pooled and concentrated by ultrafiltration using an Amicon 8200 unit (Section 3.4vic) To assess for impurities, the fractions were then loaded onto an SDS page gel (Section 3.4vid). All the pure fractions were then pooled and concentrated again using the Amicon to a known concentration, filter sterilised and stored in 1ml aliquots at 4°C for coating Dynabeads®.

The monoclonals LDS 10, LDS 8 and LDS 40 were purified using the above system. Figure 5.5 shows the IgM concentration of the fractions from LDS 8 ascites determined from the ELISA reading at 120 minutes. The fractions were then pooled and loaded onto an SDS page gel shown in Figure 5.6. The gel showed that the native ascites contained a lot of impurities in the molecular weight (MW) range of albumin-65,000. The IgM as a result of denaturation appeared as three bands, one at 65,000 MW, the heavy chain, one at 130,000 MW probably a dimer of the heavy chains and one unresolved band of 20,000MW which appeared at the bottom of the gel. Fractions 80-95 contained all the visible IgM with no impurities and were therefore pooled. Immunohistology and the results from the ELISA had shown that fractions below 80 contained some LDS 8. Therefore, fractions 70-80 were pooled as a lower concentration batch, as there were no visible impurities. Table 5.2 shows the UV adsorption before and after ultrafiltration for the fractions before they were pooled, the final concentration of LDS 8 is also shown.
Figure 5.5 Shows the concentration of the IgM monoclonal antibody LDS 8, in fractions 40-160.

Table 5.2 shows the UV adsorption for fractions of LDS 8 before and after ultrafiltration.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>UV adsorption before ultrafiltration</th>
<th>UV adsorption after ultrafiltration</th>
<th>Protein concentration (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70-74</td>
<td>&lt; std</td>
<td>0.11</td>
<td>0.65</td>
</tr>
<tr>
<td>74-79</td>
<td>&lt; std</td>
<td>0.325</td>
<td>1.93</td>
</tr>
<tr>
<td>80-84</td>
<td>&lt; std</td>
<td>1.695</td>
<td>7.54</td>
</tr>
<tr>
<td>85-89</td>
<td>0.316</td>
<td>2.456</td>
<td>17.29</td>
</tr>
<tr>
<td>90-95</td>
<td>0.260</td>
<td>&gt;2.5</td>
<td>&gt;12.97</td>
</tr>
</tbody>
</table>
5.2. Cross reactivity of monoclonal antibodies.

Immunohistological staining (ABC staining, see Appendix 2) with the monoclonal antibodies, LDS 10 and LDS 8 was carried out on pancreas sections from different species and strains to identify if there was any cross reactivity. Table 5.3 summarises the results. Staining was carried out on formalin fixed and frozen sections due to the possible loss of immunoreactivity of cells caused by cross linking of macromolecules during the fixation process. However, the cell morphology of the frozen sections is not as well defined as the formalin fixed sections.

Table 5.3. Results of immunohistological staining with LDS 10 and LDS 8 on different species.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ANTIBODY</th>
<th>REACTIVITY (formalin fixed sections)</th>
<th>REACTIVITY (frozen sections)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat - WAG</td>
<td>LDS 10</td>
<td>Strong +ve staining of acinar, islets -ve.</td>
<td>Strong +ve staining of acinar, islets -ve.</td>
</tr>
<tr>
<td></td>
<td>LDS 8</td>
<td>-ve staining on all structures.</td>
<td>Strong +ve staining of acinar, islets -ve.</td>
</tr>
</tbody>
</table>
Table 5.3 continued.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>ANTIBODY</th>
<th>REACTIVITY (formalin fixed sections)</th>
<th>REACTIVITY (frozen sections)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat - PVG</td>
<td>LDS 10</td>
<td>Strong +ve staining of acinar tissue only.</td>
<td>+ ve staining of acinar only.</td>
</tr>
<tr>
<td>Rat - PVG</td>
<td>LDS 8</td>
<td>-ve staining but patchy weak +ve staining on internal lining of acinar cells.</td>
<td>Strong +ve staining of acinar only.</td>
</tr>
<tr>
<td>Rat - August</td>
<td>LDS 10</td>
<td>Strong +ve of acinar only.</td>
<td>Strong +ve staining of acinar only.</td>
</tr>
<tr>
<td>Rat - August</td>
<td>LDS 8</td>
<td>Weak +ve staining of acinar tissue only.</td>
<td>Weakly +ve on acinar only.</td>
</tr>
<tr>
<td>Mice - MF1Balb/C</td>
<td>LDS 10</td>
<td>Patchy staining of islets and acinar tissue.</td>
<td>Weak +ve of cells within acinar tissue, islets weak +ve staining.</td>
</tr>
<tr>
<td>Mice - SCIDs</td>
<td>LDS 8</td>
<td>Patchy staining of islets and acinar tissue.</td>
<td>Weak +ve of acinar tissue only.</td>
</tr>
<tr>
<td>Mice - SCIDs</td>
<td>LDS 10</td>
<td>Weak +ve staining on islets, patchy staining of acinar.</td>
<td>Strong +ve staining of acinar tissue and islets +ve.</td>
</tr>
<tr>
<td>Mice - SCIDs</td>
<td>LDS 8</td>
<td>Weak +ve patchy staining of acinar tissue.</td>
<td>Strong +ve staining of acinar, islets +ve.</td>
</tr>
<tr>
<td>Pig</td>
<td>LDS 10</td>
<td>+ve on acinar and islet tissue, and also columnar epithelial cells lining ducts.</td>
<td>Not done.</td>
</tr>
<tr>
<td>Pig</td>
<td>LDS 8</td>
<td>-ve staining.</td>
<td>Not done.</td>
</tr>
</tbody>
</table>

The immunostaining on pancreas sections from the different species using LDS 10 and LDS 8 are shown in Figures 5.7 - 5.10.
Figure 5.7 Immunostaining of frozen sections of August pancreas section with LDS 10 showing positive staining of acinar cells only.

Figure 5.8 Immunostaining of formalin fixed PVG pancreas with LDS 10, showing positive staining of the acinar tissue only.
Figure 5.9 Immunostaining of formalin fixed SCID pancreas with LDS 8 IgM antibody raised against acinar tissue, showing weak patchy staining of the acinar tissue.

Figure 5.10 Immunostaining of formalin fixed Pig pancreas with LDS 10 IgM antibody raised against acinar tissue. The antibody stains both islets and acinar tissue.
5.3 Conclusions.

A panel of 15 IgM monoclonal antibodies to the non-islet components of the human and rat pancreas were produced. The monoclonal antibodies were purified from ascitic fluid by the use of gel filtration chromatography and then concentrated by ultrafiltration. The purity of the antibodies was confirmed by gel electrophoresis, IgM ELISA, UV absorption and immunohistology. LDS 10 and LDS 8 were found to be strong antibodies against rat (Wag, PVG and August strains) and human acinar tissue respectively.

Preliminary studies investigating the use of immunomagnetic techniques for islet purification, were carried out in the rat model using the monoclonal antibody LDS 10 and Dynabeads®. This work is detailed in the following chapters. The panel of monoclonal antibodies produced against various non-islet components in the human pancreas, could be utilised for the purification of the human islets once the system has been optimised.
CHAPTER 6.

DEVELOPMENT AND OPTIMISATION OF AN IMMUNOMAGNETIC DEVICE FOR PURIFICATION OF ISLETS USING DYNABEADS.

6.1. Introduction.

6.2. Determination of the diameter and the optimal magnetic field used for immunomagnetic purification.
   i. Eppendorf vs test-tube.
   ii. A comparison of unipolar vs bipolar magnetic fields.
   iii. Test tube vs universal.
   iv. Assessment of the effect of polarity of the magnetic field.
   v. Mode of entry of digest into the magnetic field.
   vi. A comparison of bipolar vs quadripolar.
   vii. Optimisation of the magnetic field.
   viii. Concentration of the magnetic field.
   ix. The use of a continuous flow through system.

6.3. Conclusions.
6.1 Introduction.

Due to the inconsistent availability of human pancreata, and the problem of unreliable collagenase digestion, the experimental work of designing and developing a system using immunomagnetic separation techniques for islet purification, was carried out using pancreatic digest from WAG/Leicester rats. Using this model, collagenase digestion routinely produced pancreatic digest identical in tissue fragment size to that obtained in the human pancreas. Therefore, once a system has been developed and optimised, transferring it to human islet purification should not incur any problems.

Chapter 2 discusses the development of the Dynabead® and its application to islet purification. Initial work carried out in the Department of Surgery determined the conditions for the binding of the antibody to Dynabeads® (Chapter 3 section 3.5) and the bead to target the cell ratio (500 beads to 1 acinar fragment).

In this chapter, the developmental work carried out to produce a system for the secondary purification of islets using immunomagnetic separation techniques is described. This chapter is written as an evolving sequence of experiments with the direction of each investigation determined by the results of the previous experiment.

6.2 Determination of the diameter and the optimal magnetic field used for immunomagnetic purification.

6.2i Eppendorf vs Test-tube.

Introduction.

Most of the work described using Dynabeads® for cell separation utilised the magnetic particle concentrators produced by Dynal, either the MPC®-E, designed to hold eppendorfs or the MPC®-6, designed to hold test tubes (Chapter 2, Section 2.2iiib). The majority of the work described using these systems, has involved segregation of single cell suspensions. In this situation Dynabeads® are incubated with the cells in either the eppendorf or the test tube, which is then placed against the magnet and left for a short period of time. The cells that are not immunomagnetically labelled, remain suspended in the medium and are removed as the non-magnetic fraction (NMF). The immunomagnetically labelled cells which are attracted to the magnet, are collected following resuspension and are known as the magnetic fraction (MF).

One major contrast with the work described in this thesis and previous work described using Dynabeads® is that the pancreatic digest is not a single cell suspension. This problem is highlighted by the bead to target cell ratio. For single cell separation by negative selection, Dynal recommend a ratio beads to target cell ranging from 10:1-
The optimal ratio for Dynabeads® to acinar tissue fragments has been found to be 500:1. The actual separation of the immunomagnetically labelled tissue (acinar) from the unlabelled tissue (islets) cannot occur simply by incubating the cells in an eppendorf (or test tube) and then placing this into a magnetic field, since, due to the tissue fragment size the gravitational force would be greater than the magnetic force, and the majority of the digest would settle to the bottom of the tube. Therefore, the immunomagnetically labelled digest had to be released into the magnetic field and allowed to drift through it under gravity. The most effective device for releasing the digest into the magnetic field was found to be a pasteur pipette.

The first investigation therefore compared the use of the eppendorf with the test tube as a vessel for the purification of islets.

**Method**

Following density gradient purification, aliquots of 100 handpicked rat islets and an estimated 2000 acinar particles (the number of particles in 50µl of resuspended acinar pellet were counted by 2 observers and extrapolated to determine the volume containing 2000 particles) were incubated with Dynabeads coated with LDS 10 (at a concentration of 8µg/ml) in 0.5ml RPMI in eppendorfs. The ratio of beads to acinar particles used was 500:1. The aliquots of digest were incubated with the beads at room temperature on a rock 'n' roller for 30 minutes to allow binding.

The immunomagnetically labelled digest was drawn up into a hand-held pasteur pipette which was used to slowly release the immunomagnetically labelled digest into either an eppendorf containing 1ml RPMI+10% FCS or a test-tube containing 9.5ml RPMI+10% FCS. The magnetic field was created by the MPC®-E for the eppendorf or the MPC®-6 for the test-tube (Figure 2.8, Chapter 2).

The immunomagnetically labelled digest was allowed to drift vertically through the side pull magnetic field and left to settle for 10 minutes. One ml of the non-magnetic fraction (NMF) was then aspirated off using a pasteur pipette, care being taken to avoid disrupting the magnetic fraction, by the creation of shear forces. The magnetic fraction (MF) was then resuspended in 1ml RPMI. Each experiment was performed in duplicate. Following centrifugation, the fractions were finally resuspended in 500µl MEM and stained with dithizone. The number of islets and the percentage of acinar tissue in each of the fractions were estimated by two observers.
The percentages of islets and acinar from the two observers scores are shown in Table 6.1, the bold line shows the mean. The results showed that the eppendorf enabled efficient separation of islets and acinar tissue, producing very pure islets (only 7% contamination) and only 21% of the islets were retained in the MF, due to trapping. The test tube however produced higher yields of islets but of much lower purity than with the eppendorf.

Table 6.1. The percentages of islets and acinar(Ac) in the NMF and the MF using an eppendorf and a test tube. The results for each experiment were assessed by 2 observers, a and b.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>NMF</th>
<th>MF</th>
<th>NMF</th>
<th>MF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>78</td>
<td>5</td>
<td>22</td>
<td>95</td>
</tr>
<tr>
<td>1b</td>
<td>83</td>
<td>7</td>
<td>17</td>
<td>93</td>
</tr>
<tr>
<td>2a</td>
<td>84</td>
<td>5</td>
<td>16</td>
<td>95</td>
</tr>
<tr>
<td>2b</td>
<td>69</td>
<td>10</td>
<td>31</td>
<td>90</td>
</tr>
<tr>
<td>Mean</td>
<td>78.5</td>
<td>7</td>
<td>21.5</td>
<td>94</td>
</tr>
</tbody>
</table>

Examination of the correlation and British Standards Institution repeatability coefficient [297] of the 64 measurements carried out initially between the 2 observers, showed that for islet counts there was a significant correlation of r=0.91 p<0.001 with a mean difference of 1.9 islets (+/- 10%) between observers. For the estimation of acinar tissue the correlation was even better r=0.91 p<0.001, with a mean difference in the percentage of acinar of 0.05% (+/- 9%).

The result of increasing the number of islets and the volume of digest using the eppendorf was then investigated. Table 6.2 shows the results obtained using different numbers of islets and acinar fragments (maintaining a 500 beads to 1 acinar particle ratio). The control experiments use the same experimental conditions however, the beads are blank (they have not been incubated with LDS 10, only borate buffer). Each experiment was repeated in duplicate and again assessed by two observers.

In the previous experiments the number of islets and acinar fragments post purification, had been counted. As the number of islets and acinar fragments increased, it became increasingly difficult to visually assess their relative distributions in the magnetic and non-magnetic fractions accurately. Work from this Department [155] had
already shown that assaying the relative distribution of insulin and amylase contained in different fractions, correlated highly with visual assessment of islet and acinar tissue distribution (p<0.001 for acinar tissue and p=0.022 for islet tissue), with the assay being regarded therefore as the more objective measurement. For the rest of this work therefore, the relative distribution of islet and acinar tissue was estimated by assay of the distribution of insulin and amylase content of the magnetic and non-magnetic fractions after sonication (see Appendix 4). Table 6.2 shows the mean percentages of insulin and amylase containing tissue in the magnetic only are shown.

Table 6.2. Results obtained using the eppendorf with increasing numbers of islets and acinar fragments. The percentages of insulin and amylase containing tissue retained in the magnetic fraction and the number of islets and acinar fragments in the non-magnetic fraction post purification, are shown.

<table>
<thead>
<tr>
<th>Experiment No</th>
<th>Pre purification</th>
<th>Post purification</th>
<th>Post purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N° Islets</td>
<td>N° Acinar</td>
<td>% Insulin</td>
</tr>
<tr>
<td>1 control</td>
<td>100</td>
<td>2,000</td>
<td>24</td>
</tr>
<tr>
<td>a</td>
<td>100</td>
<td>2,000</td>
<td>35</td>
</tr>
<tr>
<td>2 control</td>
<td>100</td>
<td>10,000</td>
<td>9</td>
</tr>
<tr>
<td>a</td>
<td>100</td>
<td>10,000</td>
<td>9</td>
</tr>
<tr>
<td>3 control</td>
<td>100</td>
<td>20,000</td>
<td>60</td>
</tr>
<tr>
<td>a</td>
<td>100</td>
<td>20,000</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>10,000</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>20,000</td>
<td>65</td>
</tr>
<tr>
<td>6</td>
<td>5000</td>
<td>100,000</td>
<td>60</td>
</tr>
</tbody>
</table>

Although the eppendorf purifies islets the final degree of purity is variable and dependent on the volume of tissue. This is highlighted when the number of islets is increased to 500 with an initial purity of 5% the system becomes overloaded and no effective purification occurs. In addition, with the increase in the volume of tissue the number of beads bound to the acinar tissue also increases. This causes trapping of islets in the magnetic fraction (due to cross linking of the beads) as highlighted in the control experiments. This suggests that the volume of the eppendorf is becoming a limiting factor in the separation process as the volume of tissue increases.
The eppendorf clearly limited the volume of tissue that could be efficiently separated with the percentage of acinar tissue being removed and islets trapped becoming identical. The experiments were therefore repeated in a test tube to allow the effect on final purity of the volume of the separation vessel to be determined. The results are shown in Table 6.3, again the experiments were repeated twice and assessed by two observers. The mean results are shown.

Table 6.3. Results obtained using the test tube with increasing volumes of tissue. The mean percentages of insulin and amylase containing tissue retained in the magnetic fraction and the number of islets and acinar fragments in the non-magnetic fraction are shown.

<table>
<thead>
<tr>
<th>Experiment No</th>
<th>N° Islets</th>
<th>N° Acinar</th>
<th>% Insulin</th>
<th>% Amylase</th>
<th>N° Islets</th>
<th>N° Acinar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 control</td>
<td>100</td>
<td>2,000</td>
<td>2</td>
<td>2</td>
<td>98</td>
<td>1,960</td>
</tr>
<tr>
<td>a</td>
<td>100</td>
<td>2,000</td>
<td>1</td>
<td>87</td>
<td>99</td>
<td>260</td>
</tr>
<tr>
<td>2 control</td>
<td>100</td>
<td>10,000</td>
<td>0</td>
<td>12</td>
<td>100</td>
<td>8,800</td>
</tr>
<tr>
<td>a</td>
<td>100</td>
<td>10,000</td>
<td>10</td>
<td>94</td>
<td>90</td>
<td>600</td>
</tr>
<tr>
<td>3 control</td>
<td>100</td>
<td>20,000</td>
<td>39</td>
<td>50</td>
<td>61</td>
<td>10,000</td>
</tr>
<tr>
<td>a</td>
<td>100</td>
<td>20,000</td>
<td>26</td>
<td>72</td>
<td>74</td>
<td>5,600</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>10,000</td>
<td>42</td>
<td>92</td>
<td>290</td>
<td>800</td>
</tr>
<tr>
<td>5</td>
<td>1000</td>
<td>20,000</td>
<td>41</td>
<td>93</td>
<td>590</td>
<td>1,400</td>
</tr>
<tr>
<td>6</td>
<td>5000</td>
<td>100,000</td>
<td>61</td>
<td>91</td>
<td>1,950</td>
<td>8000</td>
</tr>
</tbody>
</table>

Discussion.

Although the eppendorf efficiently separated up to 100 islets from 2000 acinar particles, it is clearly a small volume container and releasing greater quantities of digest resulted in a dramatic deterioration in purification, until with 1000 islets and 20,000 acinar fragments no purification at all occurred.

The test tube on the other hand is able to cope with the larger volumes of tissue and produces reasonable purification throughout the range of tissue mixtures examined, although the problem of islet trapping increased with the size of the attempted separations. The problem of non-specific trapping is highlighted by the control.
experiments. As the volume of tissue increases, the mesh work formed by the cross-linking of the beads traps tissue. This is illustrated in Figure 6.1. The results in this second set of experiments using the test tube were much better than the first making the test tube quite clearly the better separation vessel.

**Figure 6.1.** The mesh work formed by cross linkage of beads traps tissue non-specifically.
6.2ii. A comparison of a unipolar vs bipolar magnetic field.

Introduction.

In order to reduce the mesh work effect created by the beads on exposure to the magnetic field, the effect of a magnetic field created by two repelling magnets, on the purification process, was compared to that created by a single side pull magnet. Figures 6.2 and 6.3 illustrate the magnetic field created by a single side pull magnet and two repelling magnets, using iron filings. Neodymium-iron-boron permanent magnets (BLNI 00680) supplied by Magnet developments (Swindon U.K.) were used, Figure 6.4. 96

Figure 6.2 The magnetic field shown by the use of iron fillings, created using a side pull magnet.
Figure 6.3 The magnetic field created using two repelling magnets.

Figure 6.4 Neodymium-iron-boron magnets.
Dynabeads® coated with LDS 10 (at a concentration of 8μg/ml) were incubated with 12 aliquots of 300 islets and 10,000 acinar particles at a ratio of 500:1. The incubation and release steps are detailed in Section 6.2. Six aliquots were released through each type of field. The system was left to settle for 10 minutes, the NMF was collected in 2 fractions NMF1 and NMF2. NMF 1 was the bottom 1ml of RPMI and NMF 2 was the remaining volume of RPMI in the test tube. NMF1 and 2 were aspirated off using a pasteur pipette, and the MF resuspended. Following centrifugation the fractions were resuspended in 2mls of MEM, sonicated and assayed for insulin and amylase content (Appendix 4).

Results.

The results for the six release experiments in each group are shown in Table 6.4 (the insulin and amylase content is assumed to equate to the percentage of islet and acinar tissue present respectively). The relative percentages of insulin and amylase in each fraction, using the side pull and the facing magnets, were compared and analysed for statistical significance using the Mann Whitney U test (Minitab Statistical Software; Minitab, State College, Pennsylvania, US.). Experiments found that combining NMF 1 and 2 increased the yield of islets and only slightly decreased the purity. The percentages removed in the magnetic fraction only are therefore used for the comparisons of the effectiveness, ideally this should contain 100% amylase containing tissue and 0% of the insulin containing tissue. There was no significant difference in either the percentage of amylase containing tissue retained in the magnetic fraction (p=0.6, 95% confidence interval -6, 5, n=43) or the percentage of insulin containing tissue trapped (p=0.5, 95% CI -7, 17, n=44), using either the side pull or the facing magnets. Although the median percentage of islets trapped in the MF is only slightly higher, the range of values is wider encompassing higher values.

Discussion.

The results obtained using the side pull magnet and the facing magnets are remarkably similar, suggesting that the magnetic field had little effect using this set up. Although the two facing magnets (a bipolar magnetic field) were not significantly better than the side pull magnet (a unipolar magnetic field), further investigations were carried out using the bipolar magnetic field. The bipolar field was felt to be theoretically better with increasing magnetic field intensity making it potentially more reliable (range of islet trapping 30-51% compared with 32-63%). The use of a slightly larger diameter vessel, with the two repelling magnets, for the separation procedure was then investigated.
Table 6.4. The percentages of insulin and amylase containing tissue retained in the magnetic fraction when a single side pull magnet or two facing magnets are used. The mean is highlighted in bold.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Single side pull magnet</th>
<th>Facing magnets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Insulin</td>
<td>% Amylase</td>
</tr>
<tr>
<td>1</td>
<td>44</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>63</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>94</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>47</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>53</td>
<td>88</td>
</tr>
<tr>
<td>Median</td>
<td>45.5</td>
<td>95.5</td>
</tr>
</tbody>
</table>

6.2iii. Test tube vs universal.

Introduction

The bipolar magnetic field appeared to trap a smaller range of values of islets in Section 6.2ii. The use of a slightly larger diameter vessel, the universal with a 2.3cm diameter was therefore compared with the test tube, with a 1.5cm diameter using the facing magnets. The hypothesis was that the slightly larger diameter would prevent the digest from becoming attracted to the magnet immediately on entry into the field (trapping islets in the process), allowing it instead to disperse under gravity.

Method.

Six aliquots of 300 islets and 10,000 acinar particles were incubated with Dynabeads® coated with LDS 10 (8µg/ml) at a ratio of 500 beads per acinar particle. The digest was released into a universal through a bipolar magnetic field, allowed to settle for 10 minutes, then using a pasteur pipette, NMF1 and 2 were aspirated off as one fraction NMF, and the MF resuspended. The insulin and amylase content were assessed following centrifugation and sonication as in Section 6.2ii. The results for the bipolar field using the test tube, obtained in Section 6.2ii (unipolar vs bipolar) were used for this comparison.

Results.

The percentages of insulin and amylase containing tissue retained in the MF for each of the 6 release experiments are shown in Table 6.5. Again the mean is shown in bold. Statistical significance was assessed using the Mann Whitney U test. The test tube, although there was no statistical significant difference, tended to retain more acinar tissue in the MF (p=0.2, 95% CI -2, 7, w=48) and trapped fewer islets (p=0.5, 95% CI -15, 11, w=34).
Table 6.5. The percentages of insulin and amylase containing tissue retained in the MF using the test tube or universal and a bipolar magnetic field.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Test tube</th>
<th>Universal</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Insulin</td>
<td>% Amylase</td>
<td>% Insulin</td>
</tr>
<tr>
<td>1</td>
<td>44</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>97</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>93.5</td>
</tr>
<tr>
<td>5</td>
<td>38</td>
<td>95</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>87</td>
</tr>
<tr>
<td>Median</td>
<td>45.5</td>
<td>95.5</td>
</tr>
</tbody>
</table>

A Comparison Of The Use Of The Test Tube And The Universal

Figure 6.5. The results obtained for 6 release experiments using:
I. Test tube with a unipolar magnetic field
II. Test tube with a bipolar magnetic field
III. Universal with a bipolar magnetic field.
The median is represented by a horizontal line and the interquartile ranges are highlighted as a column.
Discussion.

The increase in diameter of the vessel did not improve on the results initially obtained using the bipolar magnetic field and the test tube. The increased diameter reduced the magnetic field strength and slightly reduced the acinar removal. Figure 6.5 summarizes the results obtained using the test tube with a unipolar and bipolar magnetic field and the universal with a bipolar magnetic field.

Dynal recommend the use of neodymium-iron-boron magnets (for Dynabead purification systems). This type of magnet was chosen for use in this study because the represent the strongest permanent magnets that are commercially available. The effect of field strength, while not examined in terms of differences in the permanent magnets used to create it, were analysed in terms of the diameter of the tube across which the magnetic field was created. The narrow eppendorf resulted in unacceptable trapping while the wider universal resulted in a small decrease in the acinar tissue retained in the MF. It was concluded, therefore, that magnets acting across a tube of 1cm diameter created a MF which optimised the balance between islet trapping and acinar removal.

On the basis of the first 3 experiments (6.2i-iii) the test tube with a bipolar magnetic field was felt to be marginally the best system for the purification of islets, though there was no statistically significant difference between most of the systems examined.

6.2iv. Assessment of the effect of polarity of the magnetic field.

Introduction.

With a quadripole of magnets, four different magnetic fields can be created. All the magnets can be repelling (i), opposite magnets can repel, but neighbouring ones attract (ii), opposite magnets attracting (iii) and, two repelling, two attracting (iv). Figure 6.6 diagrammatically represents these different combinations. Figure 6.7 shows the magnetic field created using 4 repelling magnets.

The four different combinations were compared with each other to see if there was any statistical significance between them for the purification of islets.
Method.

24 aliquots of 300 islets and 10,000 acinar particles were incubated with Dynabeads coated with LDS 10 as in Section 6.2iii. Using the test tube, six aliquots were released through each type of field shown in Figure 6.6. The system was allowed to settle for 10 minutes, NMF1 & 2 were aspirated off, the MF resuspended. Following centrifugation and sonication the insulin and amylase content were assessed.

![Diagrammatic representation of the different combinations of magnetic field created by a quadripole of magnets.](image)
Figure 6.7. The magnetic field created by a quadripole of repelling magnets.

Results.

The percentages of islets and acinar retained in the MF for each of the six separations, using the 4 different magnetic fields are shown in Table 6.6, with the mean in bold. Statistical analysis was carried out as before using the Mann Whitney U test, Table 6.7 shows the p values and Figure 6.8 summarizes the results graphically.

Table 6.6 shows the relative percentages of insulin and amylase in the magnetic fraction with the mean in bold.

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>NNNN % insulin</th>
<th>NNNN % amylase</th>
<th>NSNS % insulin</th>
<th>NSNS % amylase</th>
<th>NSSN % insulin</th>
<th>NSSN % amylase</th>
<th>NNNS % insulin</th>
<th>NNNS % amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>90</td>
<td>85</td>
<td>97</td>
<td>68</td>
<td>91</td>
<td>63</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>90</td>
<td>48</td>
<td>88</td>
<td>51</td>
<td>80</td>
<td>63</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>85</td>
<td>82</td>
<td>96</td>
<td>38</td>
<td>63</td>
<td>55</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>89</td>
<td>76.5</td>
<td>90.5</td>
<td>49</td>
<td>88</td>
<td>37</td>
<td>91</td>
</tr>
<tr>
<td>5</td>
<td>52</td>
<td>88</td>
<td>71</td>
<td>94</td>
<td>45</td>
<td>99</td>
<td>54</td>
<td>93</td>
</tr>
<tr>
<td>6</td>
<td>71</td>
<td>84.5</td>
<td>77</td>
<td>91</td>
<td>46.5</td>
<td>87</td>
<td>36</td>
<td>88</td>
</tr>
<tr>
<td>Median</td>
<td>57.5</td>
<td>87.5</td>
<td>77</td>
<td>92.5</td>
<td>48</td>
<td>87.5</td>
<td>54.5</td>
<td>91.5</td>
</tr>
</tbody>
</table>
Table 6.7. P (am = amylase; in= insulin) values, 95% confidence intervals and w score, obtained from the Mann-Whitney U test for the comparison of the use of the four different magnetic fields for the dispersal of digest.

Where the p value is asterisked, the ‘column’ method is significantly “better” than the ‘row’ method eg. the first comparison, the NSNS is significantly better at retaining acinar tissue than NNNN.

<table>
<thead>
<tr>
<th></th>
<th>NSNS*</th>
<th>NSSN*</th>
<th>NNNS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNNN</td>
<td>Pam = 0.02*</td>
<td>Pam = 0.9</td>
<td>Pam = 0.02*</td>
</tr>
<tr>
<td></td>
<td>95% -9,-1</td>
<td>95% -9,-22</td>
<td>95% -9,-1</td>
</tr>
<tr>
<td></td>
<td>w = 24</td>
<td>w = 40</td>
<td>w = 24</td>
</tr>
<tr>
<td></td>
<td>Pin = 0.07</td>
<td>Pin = 0.07</td>
<td>Pin = 0.4</td>
</tr>
<tr>
<td></td>
<td>95% -30, 0.3</td>
<td>95% -2, 22</td>
<td>95% -7, 2</td>
</tr>
<tr>
<td></td>
<td>w = 27</td>
<td>w = 51</td>
<td>w = 45</td>
</tr>
<tr>
<td>NSNS</td>
<td>Pam = 0.2</td>
<td>Pam = 0.9</td>
<td>Pam = 0.03*</td>
</tr>
<tr>
<td></td>
<td>95% -26, 3</td>
<td>95% -5, 4</td>
<td>95% -40.5, -8</td>
</tr>
<tr>
<td></td>
<td>w = 29</td>
<td>w = 38</td>
<td>w = 25</td>
</tr>
<tr>
<td>NSSN</td>
<td>Pam = 0.1</td>
<td>Pin = 0.02*</td>
<td>Pin = 0.8</td>
</tr>
<tr>
<td></td>
<td>95% -26, 2</td>
<td>95% -30, 0.3</td>
<td>95% -17, 13</td>
</tr>
<tr>
<td></td>
<td>w = 29</td>
<td>w = 51</td>
<td>w = 37</td>
</tr>
</tbody>
</table>

Previous results obtained using a bipolar field NN were then compared with the quadripolar magnetic fields created by the four combinations NNNN, NSNS, NSSN or NNNS. The results from the Mann Whitney U test are shown in Table 6.8.
Table 6.8. shows the p values, the 95% confidence intervals and the \( w \) score obtained using the Mann Whitney U test. As in Table 6.7 the asterisked p value indicates that the column method is significantly better than the row method.

<table>
<thead>
<tr>
<th></th>
<th>NNNN*</th>
<th>NSSN</th>
<th>NSNS*</th>
<th>NNNS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN</td>
<td>Pam = 0.03</td>
<td>Pam = 0.1</td>
<td>Pam = 0.8</td>
<td>Pam = 0.3</td>
</tr>
<tr>
<td>95% 2, 10</td>
<td>95% -3, 24</td>
<td>95% -3, 5</td>
<td>95% -4, 5</td>
<td></td>
</tr>
<tr>
<td>w = 53</td>
<td>w = 49</td>
<td>w = 41</td>
<td>w = 46</td>
<td></td>
</tr>
<tr>
<td>Pins = 0.008</td>
<td>Pins = 0.2</td>
<td>Pins = 0.008</td>
<td>Pins = 0.2</td>
<td></td>
</tr>
<tr>
<td>95% -27, -5</td>
<td>95% -21, 4</td>
<td>95% -44, -18</td>
<td>95% -24, 8</td>
<td></td>
</tr>
<tr>
<td>w = 22</td>
<td>w = 31</td>
<td>w = 22</td>
<td>w = 31</td>
<td></td>
</tr>
</tbody>
</table>

Figure 6.8. Shows the results obtained using a quadripole of magnets in various configurations
I. NNNN all magnets repelling each other.
II. NSNS opposing magnets repelling but neighbouring magnets attracting.
III. NSSN opposite magnets attracting.
IV. NNNS one pair of opposing magnets attracting, one pair repelling.
The median is represented by a bar and the interquartile ranges are highlighted.
Discussion.

The different combinations of the quadripolar magnetic field all remove a comparable percentage of acinar, approximately 90%, although NSNS and NNNS are significantly better than NNNN. Also there is with NSNS a difference in the percentage of islets retained in the MF, with significantly more islets trapped than with NSSN and NNNS.

Thus the use of either NNNN or NNNS appears to be better on balance. In theory the strongest magnetic field using a quadripole of magnets is created by 4 repelling magnets (NNNN) (personal communication from Mr Baden Fuller, Department of Engineering, University of Leicester). The magnetic field lines for the different combinations of a quadripole of magnets are shown in Figure 6.9.

When the results using the quadripolar magnetic field created by NNNN or NNNS were compared with the bipolar magnetic field, neither were found to be
significantly better at acinar removal but the bipolar field trapped significantly fewer islets than 2 of the 4 quadripole fields. The magnetic field with a quadripole is complex and in theory intense, this may explain the increase in islet trapping.

It was therefore felt that the most favourable combination was that of two repelling magnets using a hand held pasteur pipette.

6.2v. The mode of entry of digests into the magnetic field.

Introduction.

One observation that had been noted during the experimental procedure, was that before entry into the magnetic field, the digest could be seen to form clumps in the pasteur pipette. These clumps failed to disperse on entry into the field, and the immunomagnetically labelled digest when attracted to the magnet was therefore unable to release the islets trapped within it. Methods of dispersing the digest before entry into the magnetic field, were therefore investigated.

Methods.

Two methods of dispersing the digest and maintaining it in suspension during release were investigated. The first one consisted of vibrating the pasteur pipette containing the digest before release into the field. A fish tank pump which cause vibration, (Second Nature, Whisper 200, Interpet Ltd., Dorking, UK.) was adapted to allow the attachment of a pasteur pipette. An air valve was attached to the top of the pasteur pipette to allow the synchronous slow release of the digest (Figure 6.10).

The second method was the addition of BSA to the digest prior to release, increasing the density of the RPMI to maintain the digest in suspension and to reduce nonspecific cell binding to the pipette and tissue aggregation. The release of digest using a hand-held pasteur pipette was used as the control for this comparison.

Six release experiments using the standard 300 islet, 10,000 acinar aliquots, were performed for each of the following methods;

i. Hand held pipette with no additions.
ii. The vibration of the pipette during release.
iii. The addition of BSA to the digest prior to release.
iv. The vibration of the pipette and the addition of BSA prior to release.

The digest was released through a magnetic field created by 2 repelling magnets. As with previous experiments the digest was allowed to settle for 10 minutes and then
the NMF1 & 2 aspirated off, the MF resuspended, then the insulin and amylase content
determined following centrifugation and sonication.

Table 6.9 shows the results of the four methods. The results were compared
using a Mann-Whitney U test and the P values, the 95% confidence intervals and the w
score, are given in Table 6.10.

Figure 6.10. The system designed to vibrate the pipette during the release of the
digest into a 10ml conical tube, which is placed inside a magnetic field.
Table 6.9 shows the percentages of insulin and amylase in the MF fraction, the mean for each series of six release experiments is highlighted in bold at the bottom.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Control</th>
<th>Vibration</th>
<th>BSA</th>
<th>Vibration &amp; BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% insulin</td>
<td>% amylase</td>
<td>% insulin</td>
<td>% amylase</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>87</td>
<td>61</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>87</td>
<td>56</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>91</td>
<td>33.5</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>96</td>
<td>54</td>
<td>57</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>88</td>
<td>43</td>
<td>69</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>96</td>
<td>51</td>
<td>36</td>
</tr>
<tr>
<td>Median</td>
<td>43.5</td>
<td>89.5</td>
<td>52.5</td>
<td>50</td>
</tr>
</tbody>
</table>

Discussion.

The analysis of the results showed that the control, the hand held pasteur pipette system without vibration and BSA, remained significantly better at retaining acinar tissue in the magnetic fraction than any other system (median 89.5% range 87-96%). However, the use of vibration and BSA together trapped less islets compared to all the other systems (median 22% range 11-52%).

The other 2 systems of either vibration or BSA alone appeared to offer no advantages resulting in no effective purification with similar percentages of acinar and islet tissue ending up in both the NMF and the MF.

The use of vibration and BSA together appeared to be effective at decreasing clumping and maintaining the digest in suspension over the 5 minutes required to complete the release, resulting in decreased islet trapping. However, the use of vibration and BSA either separately or together, reduced the ability of the bipolar field to remove the acinar tissue. The possible explanations for this include the fact that the tip of the pasteur pipette vibrating at the top of the test tube creates shear forces which may disrupt the immunomagnetic purification process. Secondly, the increase in the density of the RPMI once BSA is added carries the digest more quickly through the field not allowing time for acinar removal by the magnetic field.
Table 6.10. *P* values obtained from Mann-Whitney U test for the comparison of the use of vibration, BSA, and vibration together with BSA for the dispersal of digest. Where *p* value is asterisked, the 'column' method is significantly “better” than the ‘row’ method, eg. in the first comparison, the control is significantly better at retaining acinar tissue in the MF (*P* is the *p* value for the amylase and *P*ins was the *p* value for the insulin). The 95% confidence intervals and the *w* score are also given.

<table>
<thead>
<tr>
<th></th>
<th>Vibration*</th>
<th>BSA*</th>
<th>Both*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P</em> = 0.005</td>
<td><em>P</em> = 0.005</td>
<td><em>P</em> = 0.005</td>
</tr>
<tr>
<td></td>
<td>95% -57,-27</td>
<td>95% -66,-35</td>
<td>95% -40,-30</td>
</tr>
<tr>
<td>Control</td>
<td><em>w</em> = 21</td>
<td><em>w</em> = 21</td>
<td><em>w</em> = 21</td>
</tr>
<tr>
<td></td>
<td><em>P</em>ins = 0.6</td>
<td><em>P</em>ins = 0.4</td>
<td><em>P</em>ins = 0.065*</td>
</tr>
<tr>
<td></td>
<td>95% -11.5, 24</td>
<td>95% -22, 34</td>
<td>95% -40, 3</td>
</tr>
<tr>
<td></td>
<td><em>w</em> = 43</td>
<td><em>w</em> = 45</td>
<td><em>w</em> = 27</td>
</tr>
<tr>
<td>Vibration</td>
<td><em>P</em> = 0.4</td>
<td><em>P</em> = 0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% -27, 8</td>
<td>95% -12, 40</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>w</em> = 33</td>
<td><em>w</em> = 44</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P</em>ins = 0.9</td>
<td><em>P</em>ins = 0.03*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% -23, 22</td>
<td>95% 4, 42</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>w</em> = 40</td>
<td><em>w</em> = 53</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td><em>P</em> = 0.9</td>
<td><em>P</em> = 0.04*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% -17, 32</td>
<td>95% -51, 19</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>w</em> = 40</td>
<td><em>w</em> = 26</td>
<td></td>
</tr>
</tbody>
</table>

6.2vi. A comparison of bipolar vs Quadripolar.

Introduction.

The use of vibrating the pipette and the addition of BSA to the digest prior to release was significantly better at preventing the trapping of islets in the MF. However, only 40% of the acinar tissue was being retained in the MF. Possibly, a stronger field might improve this retention. Therefore, the effect of using a quadripolar field together with vibrating the pipette and the addition of BSA to the digest was investigated.
Method.

A series of six release experiments were performed each using one of the following methods:

i. A bipolar magnetic field with a hand held pipette
ii. A bipolar magnetic field with vibration and BSA
iii. A quadripolar magnetic field with a hand held pipette
iv. A quadripolar magnetic field with vibration and BSA

The bipolar magnetic field was created using two repelling magnets and the quadripolar magnetic field was created using four repelling magnets. The configuration NNNN was chosen because, as mentioned before, in theory this should create the strongest magnetic field. The 3 fractions NMF1, 2 and MF for each separation were assessed for insulin and amylase content as in previous experiments.

Results.

The results obtained using methods i - iv for each series of six release experiments are shown in Table 6.11. Statistical analysis was carried out using the Mann Whitney U test and the results are presented in Table 6.12.

Table 6.11. The percentages of insulin and amylase retained in the magnetic fraction are shown, the mean is given in bold. The results of the bipolar magnetic field previously presented in Table 6.9 are represented for ease of comparison.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Bipolar magnetic field</th>
<th>Quadripolar magnetic field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hand held %</td>
<td>Vibration &amp; BSA %</td>
</tr>
<tr>
<td></td>
<td>insulin</td>
<td>amylase</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>87</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>91</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>38</td>
<td>96</td>
</tr>
<tr>
<td>Median</td>
<td>43.5</td>
<td>89.5</td>
</tr>
</tbody>
</table>

The use of vibration and BSA with a quadripole of magnets significantly reduced the trapping of islets compared with the hand held pipette (median 22%, interquartile range 15) and retained significantly more acinar tissue than the bipolar field
with vibration and BSA (median 81%, interquartile range 28). In order to verify these results a further series of six release experiments were performed using vibration and BSA, with a quadripolar magnetic field. The median percentage of acinar retained in the MF was 90%, there was no significant difference between the first and second series of release experiments (p=0.5, 95% CI -33, 5.5). The median percentage of islets trapped in the MF was 32%. Again there was no significant difference between the the two experiments (p=0.2, 95% CI -33, 8). The results are graphically illustrated in Figure 6.11.

Table 6.12. shows the p values, 95% confidence intervals and the w score, obtained using the Mann Whitney U test. As in Table 6.7 the asterisked p values indicate that the column method is significantly better than the row method.

<table>
<thead>
<tr>
<th>Bipolar Vibration &amp; BSA*</th>
<th>Quadripolar Hand held*</th>
<th>Quadripolar Vibration &amp; BSA*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bipolar Hand held</strong></td>
<td><strong>Quadripolar Hand held</strong></td>
<td><strong>Quadripolar Vibration &amp; BSA</strong></td>
</tr>
<tr>
<td>Pam = 0.005</td>
<td>Pam = 0.3</td>
<td>Pam = 0.2</td>
</tr>
<tr>
<td>95% -80, -30</td>
<td>95% -1, 8</td>
<td>95% -4, 38</td>
</tr>
<tr>
<td>w = 21.0</td>
<td>w = 46.0</td>
<td>w = 47</td>
</tr>
<tr>
<td>Pins = 0.07</td>
<td>Pins = 0.2</td>
<td>Pins = 0.02*</td>
</tr>
<tr>
<td>95% -40, 3</td>
<td>95% -30, 4</td>
<td>95% 6, 40</td>
</tr>
<tr>
<td>w = 27.0</td>
<td>w = 30</td>
<td>w = 54</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bipolar Vibration &amp; BSA*</th>
<th>Quadripolar Hand held*</th>
<th>Quadripolar Vibration &amp; BSA*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bipolar Vibration &amp; BSA</strong></td>
<td><strong>Quadripolar Hand held</strong></td>
<td><strong>Quadripolar Vibration &amp; BSA</strong></td>
</tr>
<tr>
<td>Pam = 0.005</td>
<td>Pam = 0.02*</td>
<td>Pam = 0.02*</td>
</tr>
<tr>
<td>95% -71, -27</td>
<td>95% -66, -13</td>
<td>95% -66, -13</td>
</tr>
<tr>
<td>w = 21</td>
<td>w = 24</td>
<td>w = 24</td>
</tr>
<tr>
<td>Pins = 0.01</td>
<td>Pins = 0.8</td>
<td>Pins = 0.8</td>
</tr>
<tr>
<td>95% -50, -16</td>
<td>95% -13, 23</td>
<td>95% -13, 23</td>
</tr>
<tr>
<td>w = 23</td>
<td>w = 41</td>
<td>w = 41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Quadripolar Hand held*</th>
<th>Quadripolar Vibration &amp; BSA*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quadripolar Hand held</strong></td>
<td><strong>Quadripolar Vibration &amp; BSA</strong></td>
</tr>
<tr>
<td>Pam = 0.7</td>
<td>Pam = 0.008*</td>
</tr>
<tr>
<td>95% -6, 35</td>
<td>95% 22, 49</td>
</tr>
<tr>
<td>w = 56</td>
<td>w = 41</td>
</tr>
</tbody>
</table>

**Discussion.**

The results demonstrate considerable variation between each run of experiments. Given that the animals were of the same strain and the same batch of collagenase was used throughout, the only variable that occurs during the process of
separation is the incubation time of the pancreatic digest with the beads. The separations were carried out by one observer sequentially over 1 hour, resulting in gradually increasing the incubation times of beads with pancreatic digest, for the consecutive runs. However the effect of incubation time on the final purification results was not one of the variable assessed. It is possible that this may be one of the causes of the variation noted. There may have been other unidentified factors such as different sex and weights of rats, which contribute to the variation between the two groups using quadripolar magnets with vibration and BSA (groups 4 and 5, Fig. 6.11).

Initially the results obtained using the quadripolar magnetic field with vibration and BSA (group 4) were significantly better in terms of preventing islet trapping. However, when the experiments were repeated to verify these results, although there was no significant difference between the original and repeated results (groups 4 and 5) there was no longer a significant difference between groups 1 and 5. This highlighted the problem of using the minimal number of 6 for statistical significance and also the variation in the different runs of experiments as discussed above. If however, the results of groups 4 and 5 (n=12) were combined, then the results for islets trapped in the MF were significantly less from group 1 p=0.04.

The system using the quadripolar magnets with vibration and BSA, despite variation within and between the 2 groups, represented a significant improvement on the previous optimal system, the bipolar magnetic field.

Although these results demonstrate a significant degree of islet purification there are, nevertheless, still significant islet losses (median 22% for optimal system). The removal of only 88% of acinar tissue together with these losses of islet tissue demonstrates that there remains room for improvement.

The results reiterate those obtained in section 6.2v: the use of vibration and BSA decreased islet trapping and the quadripolar field significantly reduced trapping. However, with the bipolar magnetic field, the use of vibration and BSA prevents acinar removal. The use of a quadripole field has demonstrated improved removal of acinar tissue and is thought to reflect the benefit of a more intense magnetic field. This combination of vibration and BSA to maintain the digest in suspension, together with an intense quadripole magnetic field to remove the immunomagnetically labelled acinar tissue, produced an optimal purification system.
A Comparison Of A Bipolar And Quadripolar Magnetic Field And The Mode Of Digest Release

Figure 6.11 comparison of method of digest release into;
I. A bipolar magnetic field using a hand held pasteur pipette.
II. A bipolar magnetic field using vibration and BSA.
III. A quadripolar magnetic field using a hand held pasteur pipette.
IV. A quadripolar magnetic field using vibration and BSA.
V. A repeat of IV to check that the results were reproducible.
The median is represented by a bar and the interquartile range is highlighted.

6.2vii. Optimisation of the magnetic field.

Earlier experiments (section 6.2iv) investigating the effect of the different configurations of a quadripolar field showed that the NNNS combination gave the better separation results. To ensure that the most effective magnetic field was utilised, a comparison of the two combinations NNNN and NNNS using vibration and BSA was carried out. At the same time the use of two quadripolar magnetic fields one above the other was investigated (using only the NNNS combination). The bottom quadripole of magnets had the same combination, NNNS, but turned through 90° to the above one, with the hypothesis that this would create a longitudinally spiralling field.
Method.

Six release experiments were carried out using the magnetic field created by two repelling and two attracting magnets NNNS, or 2 quadripolar magnetic fields, combination NNNS at 90° to each other with vibration and BSA. The percentages of insulin and amylase in each fraction were then assessed.

Results.

The results for each of the six separations are presented in Table 6.13, the data for the results of separations using the combination NNNN were previously presented in Table 6.11, results from statistical analysis are presented in Table 6.14.

Table 6.13. The percentage of insulin and amylase retained in the magnetic fraction, the mean for each series of six release experiments is highlighted in bold.

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>NNNN % insulin</th>
<th>NNNN % amylase</th>
<th>NNNS % insulin</th>
<th>NNNS % amylase</th>
<th>2 Quadripolar magnetic fields % insulin</th>
<th>2 Quadripolar magnetic fields % amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>92</td>
<td>35</td>
<td>88</td>
<td>45</td>
<td>82</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>89</td>
<td>46</td>
<td>92</td>
<td>37</td>
<td>91</td>
</tr>
<tr>
<td>3</td>
<td>51.5</td>
<td>82</td>
<td>37</td>
<td>72</td>
<td>49</td>
<td>86</td>
</tr>
<tr>
<td>4</td>
<td>26.5</td>
<td>90</td>
<td>77</td>
<td>79</td>
<td>47</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>83</td>
<td>43</td>
<td>91</td>
<td>29</td>
<td>86</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>93</td>
<td>39</td>
<td>85</td>
<td>53</td>
<td>83.5</td>
</tr>
<tr>
<td>Median</td>
<td>32</td>
<td>89.5</td>
<td>41</td>
<td>86.5</td>
<td>46</td>
<td>86</td>
</tr>
</tbody>
</table>

Statistical analysis using the Mann Whitney U test showed that there was no significant difference between use of the two fields, either in the retention of amylase or for the percentage of islets trapped in the MF. The use of the 2 quadripolar magnetic fields was not significantly better than the either of the single quadripolar fields. The median (86%) for the retention of acinar in the MF was slightly lower than the NNNN combination (89.5%) and slightly higher for the islets trapped in the MF (median 46%, NNNN median 32%). It was therefore felt that the optimal magnetic field combination should be NNNN with the use of vibration and BSA, even though the results were not statistically significant. Figure 6.12 summarizes these results.
Table 6.14 Statistical analysis using the Mann Whitney U test. P values, 95% CI and w score are given. Where p value is asterisked, the 'column' method is significantly "better" than the 'row' method.

<table>
<thead>
<tr>
<th></th>
<th>NNNS*</th>
<th>NNNN*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NNNS</td>
<td>P_{nm} = 0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% -13.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>w = 33</td>
<td></td>
</tr>
<tr>
<td>2 x NNNS</td>
<td>P_{nm} = 0.8</td>
<td>P_{nm} = 0.6</td>
</tr>
<tr>
<td></td>
<td>95% -6, 12</td>
<td>95% -7, 4</td>
</tr>
<tr>
<td></td>
<td>w = 41</td>
<td>w = 35</td>
</tr>
<tr>
<td></td>
<td>P_{nm} = 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95% -12, 24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>w = 37</td>
<td></td>
</tr>
</tbody>
</table>

Discussion.

Overall, including Chapter 7, 24 release experiments have been performed during the course of the work described here using the configuration NNNS with vibration and BSA. The overall percentage of acinar tissue removed was 83%, with only 25% of the islets retained in the MF. Compared with any other mode of purification this is highly reproducible and effective purifying 300 islets with 10,000 acinar fragments to approximately 225 islets and 1700 acinar fragments.

However, there is clearly room for improvement. In the situation whereby this system would be used as a secondary purification process, following density dependent purification, the initial purity would be approximately 50%. The experimental work in this chapter developing the secondary purification system has been carried out using islets of an initial purity of 3%. As there is no experimental support for extrapolating the data described here it can only be speculated that, using this system, it may be possible to purify 7500 islets contaminated with only 1700 acinar fragments from a starting point of 10,000 islets plus acinar fragments. This level of purity would be clearly acceptable for transplantation, and the work in Chapter 4 suggests that there would be substantial benefits from improved purity in terms of a decrease in inflammatory reaction.
Figure 6.12. Summary of results obtained using vibration and BSA with:
I. A quadripole of magnets combination NNNN.
II. A quadripole of magnets combination NNNS.
III. Two quadripolar magnetic fields combination NNNS at 90° to each other.
The median is represented by a bar and the interquartile range is highlighted.

6.2viii. Concentration of the magnetic field.

Introduction.

The best separation results produced so far have been by releasing the digest using vibration and BSA into a quadripolar magnetic field. The next series of experiments investigated the effect of concentrating the magnetic field using a mesh. The effect of placing a mesh within the magnetic field would, in theory, concentrate the lines of flux of the magnetic field.

Method.

The mesh was a ferromagnetic stainless steel torroidal ring, (0.5mm spacing) supplied by Knitmesh LTD (Holywell, Clwyd, Wales). This was placed inside the test tube adjacent to the magnets. The magnetic field was created once again by four repelling magnets. The digest was released with vibration and BSA into a test tube with or without the mesh.
Figure 6.13. The torroidal mesh placed inside the magnetic field to concentrate the lines of flux.

Results.

Table 6.15 shows the percentages of amylase and insulin retained in the MF and Table 6.16 shows the data from statistical analysis using the Mann Whitney U test.

The statistical analysis showed that the use of the mesh did not improve the purification process. The use of vibration and BSA was significantly “better” in terms of acinar retention with a median 89.5% retained in the MF and the prevention of islet trapping (median 32%).
The percentage of amylase and insulin retained in the magnetic fraction, the mean for each series of six release experiments is highlighted in bold.

<table>
<thead>
<tr>
<th>Exp N°</th>
<th>Vibration &amp; BSA</th>
<th>Vibration &amp; BSA With Mesh</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% insulin % amylase</td>
<td>% insulin % amylase</td>
</tr>
<tr>
<td>1</td>
<td>38 92</td>
<td>75 79</td>
</tr>
<tr>
<td>2</td>
<td>22 89</td>
<td>53 70</td>
</tr>
<tr>
<td>3</td>
<td>51.5 82</td>
<td>61 74</td>
</tr>
<tr>
<td>4</td>
<td>26.5 90</td>
<td>66 86</td>
</tr>
<tr>
<td>5</td>
<td>15 83</td>
<td>53 78</td>
</tr>
<tr>
<td>6</td>
<td>55 93</td>
<td>35 84</td>
</tr>
<tr>
<td>Median</td>
<td>32 89.5</td>
<td>57 78.5</td>
</tr>
</tbody>
</table>

Table 6.16 The p value, 95% confidence interval and the w score obtained using the Mann Whitney U test for the data in Table 6.15. As before, the asterisked p values indicate that the column method is significantly better than the row method.

<table>
<thead>
<tr>
<th>Vibration &amp; BSA With Mesh*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pam = 0.03</td>
</tr>
<tr>
<td>95% -18.3</td>
</tr>
<tr>
<td>w = 25</td>
</tr>
<tr>
<td>Pin = 0.004</td>
</tr>
<tr>
<td>95% 1.44</td>
</tr>
<tr>
<td>w = 52</td>
</tr>
</tbody>
</table>

Discussion.

The internal diameter of the test tube was reduced considerably by the presence of the mesh, which possibly did not enable dispersion of the digest causing a higher degree of trapping to occur. Another reason could be that, as in the initial quadrupole experiments without vibration and BSA where islet trapping increased, there is an optimal field strength which allows the islets to pass through whilst retaining the acinar tissue. The mesh may produce a too intense magnetic field. Increasing the diameter of the separation vessel might produce better results, but has not so far been investigated.

6.2ix. The use of a continuous flow through system.
One problem that had been noted during the experimental process was that during collection of the non-magnetic fractions (containing the purified islets), care had to be taken not to dislodge the acinar tissue from the magnets. The beads being considerably smaller than the acinar particles but highly magnetic, were easily dislodged from the acinar fragment but not the magnet (see Figure 6.14).

**Figure 6.14.** Diagrammatic representation of the large 500μm diameter acinar fragment magnetically held onto the magnet by 4.5μm Dynabeads.
Figure 6.15 A diagram showing the flow through system for the purification of islets.
It was therefore felt a flow through system that would enable continuous collection of the non-magnetic fraction, would reduce the risk of dislodgement of the acinar from the magnet during collection of the separated fractions in the 'static system'. Such a system would enable islets to be collected in RPMI and cultured without any centrifugation. It would also potentially increase the amount of digest able to be released by allowing a virtually continuous infusion through the magnetic field, without the suspending medium overflowing the top of the test tube.

**Method.**

A test tube had a small hole bored through the bottom of it and an air tight rubber bung was placed in the top with a hole large enough to allow the insertion of the end of pipette through it. The system could therefore be sealed, apart from the small hole at the bottom (Figure 6.15). A series of six release experiments were carried out using 4 repelling magnets with vibration and BSA. The digest was released into the test tube, the air valve allowed entry of the digest at a controlled rate and the small hole in the bottom of the test tube allowed medium to escape at the same rate. The NMF (all the RPMI in the tube) was collected from the bottom of the test tube into a petri dish. The MF was then resuspended. Both fractions were assayed for insulin and amylase content.

**Results.**

Table 6.17 presents the results of the separation using the 'static' and the flow through system. Figure 6.16 graphically shows these results.

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>% insulin (static system)</th>
<th>% amylase</th>
<th>% insulin</th>
<th>% amylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>92</td>
<td>34</td>
<td>93</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>89</td>
<td>42</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>51.5</td>
<td>82</td>
<td>47</td>
<td>93</td>
</tr>
<tr>
<td>4</td>
<td>26.5</td>
<td>90</td>
<td>63</td>
<td>92</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>83</td>
<td>53</td>
<td>91</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>93</td>
<td>59</td>
<td>87</td>
</tr>
<tr>
<td>Median</td>
<td>32</td>
<td>89.5</td>
<td>50</td>
<td>92</td>
</tr>
</tbody>
</table>
These results were not significantly different from those using the “static system”, for either acinar retention p=0.1 (median 92%, 95% CI -1, 10) or for islets retained in the MF p=0.1 (median 50, 95% CI -5, 36.5). The median percentage of islets retained in the MF appears higher however in the static system.

Discussion.

With the flow system the volume of RPMI released is no longer a problem so the system has enormous potential for purification of large amounts of tissue, perhaps releasing it slowly in at the top from a syringe driver containing 50mls of RPMI. The amount of medium used and the rate of flow could clearly be important variables and in this experiment were controlled by the size of the hole in the test tube and the rate of air bled into the pasteur pipette. Further experiments altering the flow rate may therefore improve the results.

A Comparison Of The Use Of The 'Static' System With That Of The Flow Through System.

Figure 6.16 Shows the percentages of insulin and amylase retained in the MF using a “static” and flow through system
I. The use of the static system with vibration and BSA.
II. The use of the flow system with vibration and BSA.
The median is represented by a bar and the interquartile range is highlighted.
6.3. Conclusions.

The work detailed in this chapter demonstrated that it was possible to purify islets effectively by the use of immunomagnetic techniques. After incubating the digest with M-450 Dynabeads® coated with LDS 10, addition of BSA prior to releasing the digest from a vibrating pipette (section 6.2v) into a magnetic field created by 4 repelling magnets (Section 6.2vi) enabled the separation of islets from acinar tissue. Islets, from which on average 88% of the acinar contamination has been removed, could be obtained using this method. Trapping of islets in the magnetic fraction due to cross linking of beads and non specific cell aggregation and adherence caused a mean loss of 25%. Attempts to concentrate the magnetic field by the introduction of a mesh and the use of a series of two sequential magnetic quadripoles failed to reduce the islet loss (Section 6.2vii and 6.2viii). The purification may be further improved by optimisation of the continuous flow through system, however further work is required to reduce the loss of islets into the magnetic fraction. This system, if it could be optimised, would enable the purification of islets which could be cultured immediately following collection, minimising mechanical trauma to the islets and consequently improving the islet viability.
CHAPTER 7.

A COMPARISON OF IMMUNOMAGNETIC BEADS FOR ISLET PURIFICATION IN THE RAT.

7.1 Introduction.
i. Magnetic Inducible Microspheres, their production and use in islet purification.
ii. Dynabeads, their production and use in islet purification.

7.2 Methods.

7.3 Results.

7.4 Conclusions.
7.1 Introduction.

Two types of beads, Magnetic Inducible Microspheres (MIMS) and Dynabeads®, of different composition using different ligands have been reported for their application to islet purification. This study compares the two.

i. Magnetic Inducible Microspheres, their production and use in islet purification.

Secondary purification of islets using immunomagnetic separation techniques has been investigated using both MIMS and M-450 Dynabeads®.

<table>
<thead>
<tr>
<th>0.5ml suspension made from</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 66% human serum</td>
</tr>
<tr>
<td>2. 15% SpA</td>
</tr>
<tr>
<td>3. 19% Fe₃O₄ (15-20nm)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>60ml cotton seed oil</th>
</tr>
</thead>
</table>

Homogenized by sonication

Homogenate added drop wise to cotton seed oil

200ml cotton seed oil constantly stirred at 120-125°C for 10 mins

Washed 4 times in diethyl ether by centrifugation for 15 mins at 2000g

Stored at 4°C

Before use washed in 1ml diethyl ether containing 1 drop of Tween-80 then resuspended in 0.2ml saline containing 0.1% Tween-80

Figure 7.1 The method developed by Widder et al., for preparation of MIMS.

Kandzia et al. first used magnetic microspheres in 1981 for the purification of lymphocytes [242]. They adapted the technique of Widder et al. [298] for bead production (Figure 7.1), by altering the relative proportions of the reagents and the sonication to produce an homogeneous suspension of 400μm diameter microspheres. Lymphocytes labelled with these were then separated by passage through a glass
column adjacent to a permanent magnet. The recovery of cells was 96% of which 97% were viable.

Three years later the same group, Kandzia et al. [280] described the preparative procedure for MIMS of 200, 300 and 500\(\mu\)m diameters. The smaller MIMS were prepared by further modification of Widder’s method. The Fe\(3\)O\(4\) and Staphylococcus

protein A (SpA) were dissolved in a small amount of water and mixed with cotton seed oil at 26°C and then cooled to 4°C. Instead of sonicating just once as in Widder’s method, the homogenate was sonicated 3 times for 1 minute each time. The homogenate was then squirted (not added drop by drop) into 100ml of hot oil, hardened physically for 10 minutes and then washed 4 times in diethyl ether, centrifuged at 2600g and stored at 4°C. This method produced MIMS which were stable, with strong antibody binding and magnetic responsiveness.

The optimum MIMS to target cell ratio was then determined for the separation of lymphocytes (HLA BW6\(^+\) and HLA BW4\(^+\)). Following the synthesis of MIMS they were coupled to the monoclonal antibodies, 2BC4 (anti-HLA BW6) and T648 (anti-HLA BW4) by incubation in PBS azide at pH 8 for 30 minutes at room temperature. Using a ratio of 2mg MIMS:10\(^6\) cells the yield was 96% with 98% purity and only 5% of non-target cells were trapped in the magnetic fraction [299].

In 1987, the use of MIMS for islet purification was first reported[279]. The MIMS were activated with 2.5% glutaraldehyde and then incubated with a lectin at a concentration of 100\(\mu\)g/mg MIMS.

Lectins are proteins or glycoproteins with two or more binding sites that recognise a specific sequence of sugar residues. They were originally isolated from plants but are found in all types of organisms. Two examples of lectins are Wheat-germ agglutinin and Phytohemagglutinin (from red kidney bean). Lectins bind to cell surface glycoproteins or glycolipids and are widely used to locate and isolate sugar containing plasma membrane molecules.

Several lectins were used to purify approximately 1500 impure rat islets. The crude digest was incubated with 5-6mg lectin bound MIMS at room temperature for 25 minutes. The digest was transferred to a culture flask and passed four times into a powerful magnetic field generated by an electromagnet. The magnetic fraction was retained on the flask wall and the unbound fraction containing the islets was collected from the bottom of the flask (Figure 7.2). This procedure was repeated using different lectins. Immunofluorescent staining was carried out using fluorescent activated lectins.
and the purified islets were transplanted intraportally into diabetic Lewis rats. The lectin *Ulex europaeus agglutin-1* (UEA) showed strong binding to rat acinar tissue, confirmed by immunofluorescence staining. Also reported was the use of the lectin *Maclura pomifera agglutinin* (MPA) that apparently bound to the acinar component of the human pancreas but no further data were presented.

![Diagram](image)

**Figure 7.2** Diagrammatic representation of the system for islet isolation using MIMS and an electromagnetic field.

The study also demonstrated that the yield and degree of purity achieved was determined by the pancreatic fragment size produced by the collagenase digestion and by the magnetic field. The transplantation of MIMS purified islets reversed diabetes for a mean of 100 days, data from control experiments was however not presented. Interestingly the immunofluorescence studies using MPA showed binding to islets as well as acinar, however after culturing the islets for 10 hours the staining of the islets was reduced to minute amounts, apparently due to acinar tissue being attached to the periphery of the islets. Viability studies showed that the technique did not alter islet function. They concluded that the lower yield of islets (75%) might be due to magnetic labelling of islets carrying undectable acinar cells present on their surface and hypothesised that the yield might be improved if the magnetic field strength were to be reduced, although this might be at the expense of a decrease in the islet purity.
Further studies of human rather than rat islet purification using MIMS were reported 2 years later [300]. The lectin UEA-1 as reported earlier did not show any binding specificity to human acinar or endocrine tissue. Therefore, 19 other lectins were tested for their binding capacity to human acinar tissue. Two previously frozen preparations of pancreatic digest were used in the study, one contained well digested islets and the other islets which were underdigested. Fluorescence studies were carried out as in the previous studies with some alterations to the binding method. The incubation time was 40 minutes instead of 25 minutes, it was carried out at 4°C rather than 22°C and 2-3mg of lectin coupled MIMS were incubated with 200-300 crude islets (in the previous study 1500 crude islets were incubated with 5-6mg lectin coupled MIMS). No reason for the alterations was given. Out of the 19 lectins only two showed strong specific binding to human acinar tissue. These were Wisteria floribunda (WFA) and as previously noted MPA. The lectin MPA was reported in this study to show only weak specific reactivity in fluorescence and no binding with acinar tissue after coupling to MIMS, which was not consistent with the previous study. They concluded that immunofluorescence studies alone do not predict the efficacy of a lectin for immunomagnetic labelling, and binding experiments need to be carried out to confirm this.

For the magnetic separation of human digest, therefore, the lectin WFA was used. Of the two digest preparations (previously frozen following digestion), the first one, which contained cleaved islets, gave a yield of 70% which was 90-95% pure. The second one which contained islets with a rim of acinar tissue, gave yields of only 20% although they remained 90-95% pure. Islets were thought to be retained in the magnetic fraction due to under digestion, non-specific trapping and the design of the flow system.

Shortly after this, a further paper [301] was published on the functional integrity of the β cells after magnetic separation, both rat and human, using a new ELISA. This supported the results from the previous study [300] that the lectin WFA was the only usable one out of the 19 tested. It also showed that insulin secretion by β cells was unaltered by magnetic separation.

Not only was the specificity of the lectin found to be important for MIMS binding but many other variables also had a profound influence. The pH and composition of the incubation media was shown to be important for the purification of porcine islets [302].
Serum free media with a range of pH values 7.0-7.8 were used to evaluate the influence of medium pH on the binding effect and subsequent islet viability; the effect of binding was determined microscopically and islet viability by the use of FDA/PI staining[303]. It was found that binding specificity increased with increasing pH, and at pH 7.8 MIMS complexes were binding to islets as well. The islet viability however was found to reach a peak of 98% at pH 7.4. The optimal balance of pH for WFA lectin was shown to be pH 7.4 for cell viability and binding to acinar. However, they proposed that the optimal pH for each lectin should be determined. Data on the effect of temperature during incubation was not presented, although the authors reported that there was no difference between 4°C and 24°C.

The effect of different incubation media on the MIMS-WFA acinar complexes was also investigated. Incubation with RPMI-1640 and 5% FCS, CMRL 1066 and 5% FCS were compared to serum free medium. The use of serum was shown to decrease the binding of MIMS complexes to acinar tissue and also result in non-specific binding. Optimal purification was achieved using the serum free medium. The authors felt that FCS components may be deposited on endocrine tissue, causing non-specific binding.

MIMS-WFA complexes were incubated with D-glucose (at a concentration of 1, 2 and 10%) for 24 hours at 4°C to investigate the influence of D-glucose on the specificity and strength of binding of MIMS-WFA complexes to porcine acinar tissue. Microscopic examination was used to determine the strength and specificity of binding. D-glucose at a concentration of 2%, was found to improve significantly the binding of the MIMS complexes to the acinar tissue. The reason for the increase in binding specificity when D glucose is present was not fully understood.

ii. Dynabeads®, their production and use in islet purification.

M-450 Dynabeads® are commercially available and do not require pre-treatment for antibody binding. This section will however give a brief review of the development of Dynabeads® and their reported application to islet purification.

In the 1980’s, John Ugelstad, a Norwegian scientist, addressed the problem of producing uniform microscopic monosized particles. Small polymer particles of up to 0.5μm could be satisfactorily prepared by a process of emulsion polymerisation, however to prepare particles above 2μm suspension techniques had to be used which resulted in a wide variation in particle size. Attempts using emulsion polymerisation to produce particles with diameters larger than 2μm, produced either clusters of particles or the formation of new smaller particles in large numbers. Either way, size uniformity was destroyed. These problems could be overcome by a polymerisation process.
occurring under gravity free conditions, in space and monospheres of 10μm were produced in this way. However, the financial implications of creating such conditions were a major disadvantage.

Uglestad developed a new process that produced monospheres, or “Dynospheres” of 0.5μm to more than 100μm in diameter. The original method which has since been modified, involved a monomer being added to a dispersion of polymer ‘seed’ particles in water. The monomers diffused through the aqueous phase and the seed particles swelled, polymerisation was then effected by the action of an initiator. One limitation of the technique was that the seed particles were only capable of absorbing monomer in a volume ratio of 0.5 to 5, a process which was dependent on the type of polymer and particle size used. Preparation of a 10μm diameter particle from a 0.5μm particle, requiring an 8000 fold increase in volume, would therefore mean that the swelling process would have to be repeated many times (personal communication with Dynal (UK.) LTD).

The modified method now used for the production of “Dynospheres” involves a two-step swelling process. The particles are first swollen with a small amount of an “activating agent”. The activating agent enables the particles to adsorb more than 10,000 times their own weight of monomer. The monomer is evenly distributed amongst the original particles, thus uniformly sized droplets are formed. Very little of the original polymer and activating agent are contained in each droplet. The problems of the ‘sticky stage’ (where coalescence and conglomeration of the particles as a result of changes in the density initially resulting in ‘creaming’ as the swollen particles become less dense than water and then ‘settling’ as polymerisation increases their density) are eliminated and monosized particles of more than 100μm diameter can be produced. The company Dyno Particles AS. of Lillestrom Norway, acquired production and marketing rights and DYNAL AS was formed as a joint venture with Apothekernes Laboratorium AS.

The only reported use of Dynabeads® for islet purification was by Soon-Shiong et al. in 1989 [282]. They reported on the use of anti-acinar monoclonal antibodies for canine and rat islet purification, two methods were employed. Firstly, a cytotoxic McAb was used for the depletion of acinar tissue. The yield and viability of islets were compared to that obtained using handpicking. Secondly, in the rat model the use of M-450 Dynabeads® was assessed. The monoclonal antibodies used were directed to (ABH and Lewis) blood group antigens expressed on acinar tissue, but not islets. Clayton et al. [304] subsequently demonstrated, however, that although islets in situ do not express (ABO) blood group antigens, following isolation using collagenase they do, making the use of such antibodies for immunomagnetic islet purification uncertain.
In this study, collagenase digestion was performed on pancreata obtained from Lewis rats weighing 300-350g [282]. Twenty-five mg of M-450 Dynabeads® coated in monoclonal antibody suspended in 50ml of RPMI/2% PBS for 15 minutes at 4°C with rotation, and then incubated with the pellet of unpurified digest (approximately 1ml). Microscopic examination ensured adequate binding of Dynabeads® to acinar tissue. Non-specific trapping was prevented by diluting the digest 1:2 with RPMI/2% PBS. The digest was then transferred to a 50ml flat bottomed tissue culture flask. The flask was placed for 1 minute horizontally in a magnetic field created by an electromagnet and for 2 minutes in a vertical position. Purified islets were then removed from the bottom of the flask, and the process was repeated twice.

Insulin secretion studies were carried out on handpicked islets and Dynabeads® purified islets and in vivo studies to assess the viability of the islets were also carried out. Islets were transplanted into diabetic Lewis rats, again comparing islets isolated by handpicking and using Dynabeads®. Handpicking gave a higher yield of islets - 27% more than immunomagnetic techniques. In vitro studies showed no significant difference between the viability of islets prepared by the two methods. In vivo studies showed that all recipients achieved normoglycemia, though the handpicked islet groups attained normoglycemia faster than Dynabead® purified islets. They concluded that the loss of yield of islets was both acceptable and expected, and agreed with Mueller and Kandzia that purification depended on enzymatic digestion. This work was published later in 1990 [281].

The work in this chapter aimed to directly compare MIMS coupled with the lectin UEA which were supplied by Winoto Morbach and Dynabeads® prepared as detailed in Chapter 3.5 for islet purification. To allow direct comparison of beads without the confounding issue of the differing ligands, MIMS were also directly coupled with the same antibody, LDS 10, used for Dynabead® purification. The LDS 10 antibody is an IgM antibody and since MIMS preferentially bind IgG molecules a third group of MIMS were covalently bound with a secondary antibody (goat IgG against mice IgM) with specificity for the primary antibody LDS 10 (see Figure 2, Chapter 2).

Using the static separation device developed in the studies in Chapter 6, purification using the following immunomagnetic bead complexes were then compared in terms of islet yield and purity.
1. MIMS coated with UEA.
2. MIMS coated with LDS 10.
3. MIMS coated with IgG and LDS 10.
The following controls were used;
1. Uncoated Dynabeads®.
2. Uncoated MIMS.
3. No beads.

7.2 Methods.

Following activation with 2.5% glutaraldehyde, MIMS were coated with the
lectin UEA (20µg/mg) or the antibody LDS 10 (8µg/mg) by incubation in PBS for 2
hours. The beads were then washed with PBS then stored in PBS with 0.02% azide,
1% BSA and 0.5% Tween at 4°C. Before use, the MIMS were washed 3 times with
PBS then reuspended in RPMI/10% FCS. Dynabeads® were coated using the method
described in Chapter 3.

Aliquots of 300 Wag/Leicester rat islets and 10,000 acinar particles, prepared by
the use of discontinuous BSA density purification (Chapter 3), were incubated with the
different beads at room temperature on a spiramix for 30 minutes.

The immunomagnetically labelled digest was then released from a pasteur
pipette using vibration and BSA into RPMI in a 10ml test-tube with a magnetic field
created by a quadripole of repelling magnets (Chapter 6). The system was left for 10
minutes to allow the islets to settle to the bottom of the tube under gravity. Using a
pasteur pipette the islets contained in the bottom 2 mls of RPMI, were then aspirated
off, as the non magnetic fraction 1 (NMF1), the remaining 7.5mls RPMI were removed
as non-magnetic fraction 2 (NMF2). The tube was then removed from the magnetic
field and the tissue retained in the magnetic field was resuspended in RPMI, as the
magnetic fraction (MF).

Each fraction was then assayed after sonication for insulin and amylase content
using the RIA assay and the Phadebas amylase assay (see Appendix 4). As in Chapter
6, the percentage of tissue retained in the magnetic fraction was used to compare the
efficiency of purification by the various beads.

A series of 6 release experiments were carried out in each group to allow a
comparison of the relative percentages of insulin and amylase in each fraction. The data
was entered into Minitab (Minitab Statistical Software; Minitab, State College,
Pennsylvania, US.) and the Mann Whitney U test was used to assess any statistical
difference between the purification achieved by the different beads.
7.3 Results.

The percentages of insulin and amylase containing tissue retained in the MF of the controls (uncoated Dynabeads®, uncoated MIMS and no beads) are shown in Figure 7.3. Each of the 6 values is plotted, the median is represented by a horizontal line and the interquartile ranges are highlighted as a column. High percentages of insulin in the magnetic fraction indicate that trapping of tissue is occurring while high percentages of acinar tissue indicate good purification. An optimal balance of these two parameters is required.

**Insulin And Amylase Obtained In The Magnetic Fraction For The Control Experiments.**

![Graph showing results](image)

**Figure 7.3.** shows results obtained using

I. Blank Dynabeads
II. Blank MIMS
III. No beads.

The median is shown by a horizontal line and the interquartile range is highlighted as a column.

When no beads are present in the digest, then minimal amylase containing tissue (acinar tissue) is retained in the magnetic fraction (MF) (median 0%, range 0-15%). There was a similarly small percentage of insulin containing tissue (islets) in the magnetic fraction (median 4%, range 1.5-7%).
When uncoated MIMS were used, there was no significant difference in the percentage of acinar tissue retained in the MF (median 0.7%, range 0.5-1%) compared to the use of no beads at all, (p=0.06). Significantly more of the islets were retained in the magnetic fraction (median 10%, range 7-15%) compared with no beads at all (p=0.005).

The uncoated Dynabeads® trapped significantly more acinar tissue in the MF (median=30%, range 12-44%) compared to when no beads were present (p=0.008), and compared with the MIMS (p=0.005). There was also a significant increase in the percentage of islets retained in the MF with Dynabeads® (median=23%, range 11-37%) compared with when no beads were present (p=0.005) and with MIMS (p=0.02).

Figure 7.4 compares MIMS coated with the lectin UEA, LDS 10 or IgG & LDS 10 and Dynabeads® coated with LDS 10. Again the percentages of insulin and amylase retained in the MF are shown for each type of bead. The statistical comparisons of amylase and insulin in the MF using the Mann Whitney U test are shown in Table 7.1. MIMS coated with the lectin UEA retained significantly more acinar tissue (median 83%, range 76.5-88%) than MIMS coated with LDS 10 (median 42%, range 25-65%). However, the MIMS coated with UEA also trapped significantly more islets (median 33, range 26-45%) than MIMS coated with LDS 10 (median 18%, range 11.5-40%).

MIMS coated with UEA were also significantly better at retaining acinar tissue in the MF compared with MIMS coated with IgG & LDS 10 (median 43%, range 38-61%), but again trapped significantly more islets than MIMS with IgG & LDS 10 (median 15%, range 13-19%).

There was no significant difference between the acinar and islets retained in the MF between MIMS coated with LDS 10 and MIMS coated with IgG & LDS 10, demonstrating the use of a primary antibody (IgG), to enable the attachment of the IgM antibody to the MIMS, did not significantly improve the binding of LDS 10 to the MIMS.
A comparison of the use of MIMS and Dynabeads.

Figure 7.4. shows the results obtained using:
I. MIMS bound with UEA lectin.
II. MIMS bound with LDS 10.
III. MIMS bound with IgG and LDS 10.
IV. Dynabeads® bound with LDS 10.

The median is represented by a horizontal line and the interquartile ranges is highlighted as a column.

Dynabeads® were significantly better at removing acinar tissue (median 77%, range 66-84%) than MIMS coated with LDS 10 directly or indirectly. However, both types of MIMS were significantly better at preventing islet trapping than Dynabeads® coated with LDS 10 (median 35%, range 30-51%). The results demonstrated no significant difference between MIMS coated with UEA and Dynabeads® coated with LDS 10 either for acinar removal or islet trapping in the magnetic fraction.
Table 7.1. Statistical analysis using the Mann Whitney U test. Where the p value is asterisked, the 'column' beads were significantly "better" than the 'row' beads for that comparison e.g. the first comparison, MIMS coated with IgG and LDS 10 with MIMS coated with UEA, the retention of acinar tissue in the MF is significantly better using MIMS with UEA but significantly fewer islets are retained using MIMS with IgG and LDS 10. (Pam = p value for amylase comparison and Pins = p value for insulin).

<table>
<thead>
<tr>
<th></th>
<th>MIMS IgG+LDS 10*</th>
<th>MIMS LDS 10*</th>
<th>Dynabeads® LDS 10*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIMS UEA</td>
<td>Pam = 0.005</td>
<td>Pam = 0.005</td>
<td>Pam = 0.09</td>
</tr>
<tr>
<td></td>
<td>Pins = 0.005*</td>
<td>Pins = 0.045*</td>
<td>Pins = 0.4</td>
</tr>
<tr>
<td>MIMS IgG + LDS 10</td>
<td>Pam = 0.8</td>
<td>Pam = 0.005*</td>
<td>Pam = 0.005*</td>
</tr>
<tr>
<td></td>
<td>Pins = 0.4</td>
<td>Pins = 0.005*</td>
<td>Pins = 0.03*</td>
</tr>
<tr>
<td>MIMS LDS 10</td>
<td>Pam = 0.005*</td>
<td>Pam = 0.005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pins = 0.03*</td>
<td>Pins = 0.03*</td>
<td></td>
</tr>
</tbody>
</table>

Repeating the experiments using a different collagenase (Figure 7.5), showed there was now a significant difference between the 2 types of beads. The amount of acinar tissue retained in the magnetic fraction in this second group of experiments was significantly greater with Dynabeads® (median 87%, range 55-95%), compared with the MIMS with UEA (median 46%, range 13-71%) p=0.01. There was no significant difference in the islets retained in the MF, p=0.5 (Dynabeads® median 14%, range 6-25%, MIMS with UEA median 10%, range 6-18%).

A further 6 release experiments were then performed using the 2 types of beads that gave the best results. The batch of collagenase was changed for the second set of experiments (from Serva 030923C to Serva 13073C), and as a result the acinar tissue fragment size increased slightly.

The results obtained with the two different collagenases but the same beads were analysed. The p values are given in Table 7.2. There was a significant change in the percentage of amylase retained in the MF using MIMS, with the median percentage retained dropping from 83% with Serva I to 46% using Serva II. With MIMS there was also a significant change in the insulin retained in the MF with less islets trapped in the MF using serva II (median of 10%, compared with serva I median of 33%).
When comparing the results using Dynabeads®, the opposite was seen. The use of Serva II did not significantly change the amount of amylase retained in the MF. However, there was a significant decrease in the islet tissue retained in the MF with the median percentage dropping from 35% with Serva I to 14% with Serva II.

![Figure 7.5.](image)

Figure 7.5. The amylase and insulin tissue retained in the magnetic fraction with MIMS and Dynabeads® using Serva II. The median is represented by a horizontal bar and the interquartile range is highlighted as a column.
Table 7.2 P values obtained with two Serva batches of collagenase using MIMS and Dynabeads®. Where the p value is marked by an asterisk, the beads at the top of the table are significantly better for that comparison.

<table>
<thead>
<tr>
<th></th>
<th>MIMS + UEA Serva II*</th>
<th>Dynabeads® Serva II*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIMS + UEA Serva I</td>
<td>$P_{am} = 0.005$</td>
<td>$P_{am} = 0.6$</td>
</tr>
<tr>
<td></td>
<td>$P_{in} = 0.005^*$</td>
<td>$P_{in} = 0.005^*$</td>
</tr>
<tr>
<td>Dynabeads® Serva I</td>
<td>$P_{am} = 0.01$</td>
<td>$P_{am} = 0.3$</td>
</tr>
<tr>
<td></td>
<td>$P_{in} = 0.005^*$</td>
<td>$P_{in} = 0.005^*$</td>
</tr>
<tr>
<td>Dynabeads® Serva II</td>
<td>$P_{am} = 0.01$</td>
<td>$P_{in} = 0.5$</td>
</tr>
</tbody>
</table>

7.4. Conclusions.

These results, as with previous experiments in Chapter 6, show a degree of variation between separations using the same bead. This may be due to the development of clumping during the separations which were performed by a single person using one set of apparatus and therefore took a period of about an hour from start to finish. The variation is, however, dramatically less than that seen using density dependent methods where islets can sometimes not be purified at all. Nevertheless it makes comparison of different beads difficult. These problems were minimised by performing 6 separations with each bead and comparing the results using a non-parametric statistical test. The number of separations performed (6) was specifically chosen to minimise the number of animals involved but at the same time allowing statistical analysis of the results, while this appears as a very small number the statistical test contrives to detect differences which would only have a 5% probability of occurring by chance.

Controls.

When the digest was allowed to settle under gravity through the apparatus without any beads present, virtually all the acinar tissue settled to the bottom of the tube and was removed as the first non-magnetic fraction. There was however a small percentage of islet tissue (1.5-6.5%) retained in the magnetic fraction even in the absence of any magnetic label. This could be due to some of the islets sticking to the test tube or binding non-specifically to it during the separation and becoming resuspended in the magnetic fraction. Even in large scale separations, however, the percentage of islets lost in this way is probably of little relevance and the process by which they are lost may be rapidly saturable reducing losses still further the larger the scale of separation.
When the immunomagnetic beads are added even without a ligand to allow them to bind specifically to the tissue, significant amounts of tissue start to be retained with the beads in the magnetic fraction. Both the Dynabeads® and the MIMS non-specifically retained 10-20% of the islets, with the MIMS retaining significantly fewer. The MIMS also non-specifically trapped significantly less of the acinar tissue. It seems likely that the smaller MIMS with their lower magnetic moment, cross link less within the magnetic field trapping less tissue, and more of them would be required to bind nonspecifically to generate the same magnetic moment. The different surface coating of the two particles may also result in significantly different non-specific binding of the tissue to the particles, with the hydrophobic surface of the polystyrene particles such as the Dynabeads® generally recognised as increasing non-specific interactions with cells. The coating of hydrophobic surface particles such as Dynabeads® with antibody, in theory reduces such interactions which may therefore become less of a problem in 'non-control' conditions.

It is therefore interesting to note that the 23% of islets non-specifically trapped by the coated Dynabeads® in these experiments is almost identical to that trapped overall by beads without antibody coating. It may be that more effective blocking of such sites following antibody coating of the beads might help to reduce the amount of trapping which occurs. However the MIMS, by non-specifically trapping less islets are in this respect at least, potentially more effective than the Dynabeads®.

Comparison of the beads.
The MIMS coated with the antibody LDS 10, directly or indirectly, did not prove effective at separation. There are numerous potential reasons for this. The conditions used for the antibody binding to the bead were those found to be most effective for coating the Dynabeads® and had not specifically been modified for the MIMS, this might have reduced the uptake of primary antibody onto the bead surface. The surface of the MIMS bead may not allow presentation of the antibody, which does become bound in a stearic configuration, which subsequently allows it to bind to tissue. This could be improved by an indirect method in which the antibody is incubated with the tissue first followed by the bead.

There is no doubt that following good digestion, which produces small acinar fragments and cleaved intact islets, both the MIMS coated with UEA and the Dynabeads® coated in LDS 10 are equally capable of producing routine, efficient, islet purification with the removal of about 80% of the contaminating acinar tissue and a loss of only 10-35% of the islets. However, the MIMS were significantly less effective.
when a different batch of collagenase was used and the size of the acinar particles increased.

In this study the Dynabeads® coated with LDS 10 were therefore the most reliable particle studied for immunomagnetic islet purification. However, the minimal non-specific trapping of islets by the MIMS in the control group could give them the greater potential unless Dynabeads® can be improved in this respect.

The effect of different types of collagenase on the pancreatic digest is highlighted by this experiment. Collagenase used for islet isolation is produced by Clostridium histolyticum. It is produced commercially in large fermentation vats, a simple purification step removes the bacteria and the enzyme is lyophilised. This method of production is non-specific and non-reproducible and results in extreme batch variation. Crude collagenase is a mixture of 12 different components. Collagenase is one of the major components but also present are trypsin, neutral proteases and elastase. There are six different types of collagenase present in crude preparations and these are divided into two classes, class I and class II based on their activities towards collagen [305]. Studies are currently being carried out to determine the role of collagenase components on human islet isolation. The role of components both individual and in combination, must be assessed. One of the major problems with this area of research is that in the rodent it is possible to control the inter-pancreatic variation between rat pancreata, however, this is not possible in the human.
CHAPTER 8
CONCLUSIONS AND FUTURE WORK.

8.1 Conclusions.
8.2 Future Work.
8.1 Conclusions.
Islet transplantation, to reverse diabetes and to prevent the development of secondary complications, is now becoming a feasible option. There have been a few successful cases where patients have remained insulin independent for more than 2 years following transplantation. One of the limiting factors in the success of islet transplantation has been the purification of large numbers of islets. There have only been a few studies investigating the effect of exocrine contamination on islet transplantation. In animal models and also in humans, studies have shown that it is preferable to transplant highly purified islets. The transplantation of unpurified islets reduces the implantation rate [82], increases the immunogenicity [80] and has been shown to risk portal hypertension[137]. However, there have been transplants of unpurified islets carried out intraportally without any complications[136].

In this thesis, the effect of transplanting impure islets was addressed (Chapter 4) in the rodent model. The number of islets required to reverse diabetes in the WAG/Leicester rat were transplanted purified, unpurified and also acinar tissue alone was transplanted. The study showed that, even with a small number of animals used in each group, there were significant differences in the inflammatory response observed when unpurified islets were transplanted compared to purified islets or acinar tissue alone. The are two possible explanations for this observation. Firstly, as originally suggested by Gotoh et al., when unpurified islets are transplanted, the acinar tissue causes an inflammatory reaction to occur which contributes to damage of the islets. Other studies, as mentioned above, have shown that the presence of acinar tissue decreases the implantation rate. Secondly, and possibly the most plausible explanation, is that it is the effect of the increase in volume of tissue being transplanted, leading to necrosis of the liver and portal vein thrombosis. In order for the effect of the volume of tissue transplanted to be correctly addressed, further experiments need to be carried out. The effect of transplanting 2000 islets and 2000 acinar fragments alone needs to be assessed. Further experiments that could be of value would be the staining of the liver to ascertain which cells are involved in the inflammatory response. Even though the issue of why there is an inflammatory response to the transplantation of unpurified islets has not been resolved, this work has supported the need to transplant purified islets intraportally.

The purification of islets on the basis of physical differences using density gradients has been extensively researched. Due to the overlapping in densities of much of the islet and acinar tissue, purification on the basis of the physical differences will never allow complete separation. The production of new density gradient media will not improve the purification process, whatever the modification of the biochemical
composition of the solvents in which established gradient media are dissolved [306]. Consequently, an alternative method for purification, or a method of secondary purification is needed. It was, therefore, the overall aim of this thesis to develop a system that would be used after the pancreatic digest had been purified by the use of density gradients with the COBE 2991 allowing retrieval of pure islets from the relatively impure fractions of the gradient. Different methods of cell separation were reviewed in Chapter 2 which concluded that the use of immunomagnetic methods offered a highly attractive alternative to density dependent methods, with the techniques being rapid, gentle and reproducible.

One of the aims of this thesis was to develop a panel of monoclonal antibodies against the various exocrine components of the pancreas, in the hope that these could be bound to Dynabeads® and used as a 'cocktail mix' to remove the non-islet tissue from the impure fractions produced by the COBE 2991. Several monoclonal antibodies were raised against acinar tissue, ductal tissue and vascular tissue and were discussed in Chapter 5. The development of the system for the purification of islets using immunomagnetic separation techniques was carried out in the WAG/Leicester rat, due to the inconsistent supply of human pancreata. Therefore, only one monoclonal antibody, LDS 10, an IgM antibody raised against rat acinar tissue, was used. Preliminary experiments in the human, carried out using LDS 8, an IgM antibody raised against human acinar tissue, showed binding to acinar tissue. However, the human scenario is different from that seen in the rat and this will be discussed later.

As mentioned above, the development of the immunomagnetic purification system was carried out using inbred rats and the monoclonal antibody LDS 10 (Chapter 6). The system was developed as a series of sequential experiments, with the results of one experiment determining the methodology of the next.

There were two significant limiting factors to adapting immunomagnetic separation developed for single cell separations to islet purification. Firstly, due to the large size of the acinar fragments, compared to the beads, any shear forces created during separation causes dislodgement of acinar fragments from the magnetic beads, thus contaminating the purified islets. Secondly, as the beads enter the magnetic field they become magnetic and cross link with each other, forming a mesh work and trapping islets in the process.

The first problem was addressed by investigating the magnetic field used. The use of 4 repelling magnets across a test tube was found to create the strongest magnetic field. Although it was not significantly better than the magnetic field created by 2
repelling magnets, it was theoretically stronger. The second problem of the cross linking of beads on entry into the magnetic field was addressed by investigating the mode of entry of the digest into the magnetic field since it was noted that the digest tended to 'clump' during release. It was found that if BSA was added to the digest prior to release and the pipette was vibrated during the release of the digest the trapping of islets was reduced. Several problems concerned with the design of the experiments were highlighted during this study. Considerable variation between each 'run' of 6 experiments was observed and this may have been due to the length of incubation of the pancreatic digest with the beads. Another problem highlighted by repeating an experiment to test for reproducibility, was the use of the minimal number of 6 for statistical significance. The minimal number of 6 was chosen in order to try and minimise the number of animals required. The use of a quadripole of repelling magnets surrounding a test tube, with the digest being released into the tube from a vibrating pipette and the addition of BSA prior to release, was able to purify islets to a purity of greater than 80% with a loss of 25%. The system is gentle, rapid and simple to use.

The system was developed using an inbred strain of rats and two batches of collagenase and conditions for the preparation of islets, such as the ischaemic time, were constant throughout. The effect of variations such as different batches of collagenase was highlighted in Chapter 7. In the comparison of different types of immunomagnetic beads a second batch of collagenase was used. This produced acinar fragments which differed in size. In the human scenario, the batch of collagenase is constantly changing due to the activity of the enzyme decreasing or the lack of availability. Such variations might have a significant impact on the effectiveness of immunomagnetic separation.

In the rat, collagenase digestion of the pancreas routinely produces well cleaved islets (cleavage index of greater than 80%). In the human, collagenase digestion is extremely variable and the cleavage index can vary from as little as 20% to over 80% with a good collagenase. There are many factors thought to play a role in this for example, the batch of collagenase used, the period of warm ischaemia and even the cause of death.

Although a system has been developed that is capable of purifying islets, it was developed using an initial purity of islets of approximately 3%. The system used as a secondary purification in human islet would be used when the initial islet purity of approximately would be 50%. As discussed in Chapter 6, it can only be speculated that using this system with an initial purity of 50%, similar degrees of purification would be achieved and this obviously needs to be established. The system in the rat was
developed using very rigid conditions which do not apply in the human scenario. Investigations need, therefore, to be carried out using human pancreatic digest to assess the effect of collagenase digestion, cleavage index and ischaemia on immunomagnetic purification of islets.

8.2 Future work.

Further work on optimising the use of the flow system described in Chapter 6 to investigate if islet purification could be improved needs to be carried out. This needs to examine the effects of variation in flow rate and perhaps different lengths of ‘test tube’ with more than one quadripole of magnets. The effect of increasing the diameter of the ‘test tube’ in combination with the quadripole magnet and the possible inclusion of a torroidal ring in such a device should also be re-examined. The system could then be used for the secondary purification of human islets.

It is foreseen that immunomagnetic purification would be used as a secondary process following ‘debulking’ of the digest from the COBE continuous density gradient (described in Chapter 3). The fractions containing pure islets would be kept, the pellet discarded and the fractions between would be ‘cleaned up’ using immunomagnetic techniques resulting in significant increases in islet yield together with a beneficial effect on overall purity.

In order to allow not just acinar fragments but also lymph nodes, vascular and neurological tissue to be removed, a panel of monoclonal antibodies to each of these may be necessary. Immunohistological examination of the COBE fractions would help to predict the optimal ratio of beads coated with such a panel of antibodies to maximise purification.

One of the major limiting factors that has hindered the development of the system for human islet purification has been the lack of an efficient collagenase to produce pancreatic digest. This problem has been experienced world-wide and research is now being carried out to investigate the active components of collagenase. Clearly immunomagnetic separation, like all other methods of purification, relies heavily on a collagenase which produces cleaved intact islets in large numbers. On going research in this area may make an important contribution to the efficacy of our technique.

Current large-scale density-dependent islet isolation techniques using the COBE 2991 cell processor produce, on average, 200,000 x 150 μm islet equivalents (IEq) [507]. The cost of producing this quantity of islets is approximately £300 depending on the density gradient medium used. If immunomagnetic methods could be successfully
transferred to human islet isolation, then to further purify the more dense fractions, which may for example contain an additional 200,000 islets of approximately 20% purity, would (in this example) add a further £41 to the overall cost and double the yield of islets from one pancreas. With the number of islets transplanted being one of the factors crucial to the success of human islet transplantation [99] such an increase could dramatically improve the results of transplantation. In addition, by improving the chances of one to one donor to recipient transplantation, the HLA matching of transplants would improve, further increasing the chances of success.

In conclusion, the work in this thesis has produced a system for the purification of rat islets which could potentially be adapted for the secondary purification of human islets. In the rodent model, prototypes of the system are currently being tested by Dynal A.S. Oslo, Norway, with a view to commercial production. Optimisation of the system for human islet purification would enable an increase in the yield of islets from one donor. Therefore, using density gradient purification followed by secondary purification of the impure fractions normally discarded, would make the important end point of one donor to one recipient islet transplantation a more realistic prospect.
APPENDICES

APPENDIX 1-FORCE ON A PARTICLE IN A MAGNETIC FIELD.

APPENDIX 2-SOLUTIONS

APPENDIX 3-HISTOLOGY AND IMMUNOSTAINING TECHNIQUES

APPENDIX 4-ASSAYS
APPENDIX 1
FORCE ON A PARTICLE IN A MAGNETIC FIELD.

Susceptibility
Susceptibility is defined by equation 1:

\[ \chi = \frac{M}{H} \]

where \( M \) is the magnetisation (the magnetic moment per unit volume of a solid, \( M = \frac{m}{V} \)) and \( H \) is the magnetic field strength.

Force on a particle in a magnetic field
If a particle, \( X \), is moving at a constant velocity it is said to have an amount of momentum, given by equation 2:

\[ \text{momentum} = \text{mass of } X \times \text{velocity} \]

Newton's second law states that a force acts on particle \( X \) which is equal to the change in momentum per second. Therefore, if \( F \) is the magnitude of the force acting on a constant mass \( m \) then equation 3 states that;

\[ F = kma \]

where \( k \) is a constant, \( a \) is the acceleration.

The Newton is defined as the force which gives a mass of 1kg an acceleration of 1ms\(^{-2}\), from this \( k = 1 \), hence equation 4;

\[ F = ma \]

The force on a particle in a magnetic field created by a bar magnet, is defined by equation 5;

\[ F = F_m - F_d \pm \delta F_a \]

where \( F_a \) is the gravitational force, \( F_m \) is the magnetic force and \( F_d \) is the drag force. \( \delta \) takes the value, +1 if the direction of the magnetic field and magnetic force is vertically
down, -1 if the direction is vertically up and 0 if the direction is horizontal. This is for a particle with a density greater than that of the fluid.

Figure 1a shows the magnetic force on a sphere in a magnetic field. The magnetic force $F_m$ on a magnetized sphere is defined below in equation 6:

$$F_m = V M \frac{dH(R)}{dR}$$

where $V$ is the volume of the sphere, $M$ is the magnetic moment of the spheres (Gauss or emu/cm$^3$) and $dH(R)/dR$ is the flux gradient (in units of Gauss/cm).

The drag force $F_d$ is given in equation 7:

$$F_d = 6 \pi \mu r u$$

where $r$ is the radius of the sphere, $\mu$ is the viscosity of the carrier fluid and $u$ is the velocity of the sphere. This only applies if Reynolds number ($Re$) is $\leq 1$. ($Re = \frac{\rho v d}{\mu}$, where $\rho$ is the particle density, $v$ is the particle velocity and $\mu$ is the liquid viscosity).

In the case of steady state motion, the magnetic force is just balanced by the viscous force, so that:

$$F_m = F_d$$
Therefore, the velocity of a single sphere is given by equation 9;

\[ v = \frac{\nu M}{6\pi \eta} \frac{dH(R)}{dR} \]

The magnetic force around a rectangular magnetic bar is complex and therefore it is easier to approximate the magnet to a magnetic sphere (Figure 2) and the field decay straight out from the surface of one pole is given by;

\[ H(R) = H_s \frac{r_{eq}^3}{R} \]

where \( H_s \) is the magnetic field strength on the surface of the magnet, when using an equivalent radius \( r_{eq} \), \( r_{eq} = \frac{6hw}{\pi} \). \( H(R) \) is the field at a distance \( R \) away from the centre of the sphere along the direction of the magnetisation. The flux gradient needed to calculate the velocity is therefore given by;

\[ \frac{dH(R)}{dR} = -\frac{H_s}{r_{eq}} \frac{r_{eq}^4}{R} \]

Figure 2a. A magnetic bar may be approximated by an equivalent magnetic sphere, of radius \( r_{eq} \). \( H(R) \) is the field at a distance \( R \) from the center of the sphere.
MEM for human islet isolation.

10 x MEM 400ml
Penicillin & Streptomycin 100U/ml
Fungizone 40ml
HEPES 40ml
Sterile H2O 3360ml

Dithizone (Dithiophenylcarbazone) for staining of islets.
To make up stock solution of Dithizone;
Dithizone 33.3mg (Sigma D5130)
95% Ethanol 50ml
adjust to pH 7.8 with 28.4% ammonium hydroxide.
Before use add 5ml of stock dithizone to 50ml of MEM.

Final Suspension medium for monoclonal antibody production.
RPMI 500ml
DME 500ml
HEPES 24mls 24mmol
Penicillin & Streptomycin 20mls 100U/ml
Sodium pyruvate 2mls 1mmol
L-glutamine 10mls 2mmol

HA medium
Azaserine Hypoxanthene 50X 1 vial
dissolve in 500ml of serum free 50:50 DME (final suspension medium).

Gelatin Coating of multipot microscope slides.
Gelatin powder 1.5g (BDH Chemicals).
H2O (Warm) 300ml
Chronic potassium sulphate 0.15g (Fisons)
Multipot slides (Hendley Loughton, Essex, UK.) were placed in a slide rack and then immersed in the gelatin solution for 10 minutes and dried before use.
Fluorescence Medium (negative control) for immunohistology.
MEM 100ml
FCS 10ml
Sodium azide (0.02%) 0.02ml

Tris Buffered Saline 10x (TBS) for immunohistology.
Trizma Base 60.55g (Sigma) 50mmol
NaCl 85.2g (Fisons) 150mmol
The above were dissolved in 500ml ddH2O and adjusted to pH 7.6 with HCl 2M then made up to a final volume of 1 litre and stored at 4°C. The TBS was used at 1:10 dilution with H2O.

Chromogenic substrate for immunohistology.
Trizma base 1.2g (Sigma)
H2O 100ml
Using 1M HCl pH adjusted to 8.2, stored at 4°C ready for use.
Before using:
Naphthol As-BI phosphoric acid 1mg/ml (Sigma N-5000)
Fast Red 1mg/ml (Sigma F1500)
vortex well and add just before filtering through a Whatman paper,
Levamisole 24mg/ml (Sigma L-9756)
were added and the substrate used within 15 minutes.

Freezing Mix for freezing monoclonal antibodies.
FCS 20%
DMSO 10%
Hepes 1M 2%
50:50 RPMI/DME 68%

PBS for the ELISA.
KH2PO4 3.402g
K2HPO4.3H2O 5.706g
NaCl 1.461g
Made up to 500ml with ddH2O and pH adjusted to 7.2.

Glycine Buffer.
Glycine 7.507g
MgCl2 0.2033g 0.001mmol
ZnCl2 0.1363g 0.001mmol
Made up to 1 litre with DH₂O and pH adjusted to 10.4.

**Resolving Buffer for PAGE**

Trizma Base 121.1g/l
Dissolve in H₂O and pH to 8.8 with HCl.

10% SDS
Sodium Lauryl sulphate (SDS) 10g/100ml H₂O. Fisons.

Acrylamide 50%
Acrylamide 50g (BDH No. 44349W)
Bisacrylamide 1.3g (Sigma No. M7256)
Made up to 100ml with ddH₂O.

7.5% acrylamide resolving gel for PAGE
H₂O 13.28ml
Resolving Buffer pH8.8 11.25ml
50% Acrylamide 4.5ml
10% SDS 0.3ml
Temed 0.02ml
1.5% Ammonium persulphate 0.68ml

For a 7.7% acrylamide stacking gel for PAGE
H₂O 7.7ml
Resolving Buffer pH8.8 1.25ml
50% Acrylamide 0.6ml
10% SDS 0.1ml
Temed 0.01ml
1.5% Ammonium persulphate 0.35ml

**Running Buffer for PAGE.**

Trisma base 15.14g
Glycine 72.00g
SDS 5.0g
Made up to 1 litre with ddH₂O and adjusted to pH8.3 with HCl.
Coomassie brillant blue G & R for PAGE
Coomassie brillant blue G 75mls
Coomassie brillant blue R 50mls
Methanol 250mls
Acetic acid 25mls
Water 225mls

Destain for PAGE.
Acetic acid 75mls
Methanol 50mls
Water 875mls.

Borate Buffer for coating of Beads.
Citric acid 21.02g (BDH)
Boric acid 6.18g (Fisons)
NaH₂PO₄⋅H₂O 13.8g (Aldrich chemical CO Ltd.)
Each chemical was dissolved in 100ml of H₂O and then mixed. 250ml of this mixture
was then diluted with 400ml H₂O and the pH adjusted to 9.5 with NaOH 10M and
made up to 500ml with H₂O.

Phosphate buffered saline (PBS) 0.01M.
Na₂HPO₄ 1.9g 8.4mmol alkali
NaH₂PO₄ 0.22g 1.6mmol acidic
NaCl 8.76g 150mmol
Dissolved in 100ml H₂O and pH adjusted to 7.4.

0.5% BSA/PBS for washing Dynabeads®.
PBS 500ml
BSA 35% 7.16ml
Filter sterilised with a 0.22mm Falcon 7105 bottle top filter (Becton Dickinson
Labware, New Jersey, USA).

0.1% BSA/PBS storage medium for Dynabeads®.
PBS 500ml
BSA 35% 1.43ml
Make up as with 0.5% BSA/PBS.
Paraffin sections were cut by the Department of Pathology and then the following staining techniques were carried out on them.

**Haemotoxylin and Eosin Staining Technique.**
Sections were dewaxed by placing in an oven at 60°C and then placed in xylene for 10 minutes and then into 2 solutions of xylene containing 99% and 95% ETOH for 3 minutes each time. Finally, the sections were then washed in water (this process is known as taking to water).

Sections were transferred to Mayers Haemotoxylin solution for 5 minutes and then washed under running tap water until the sections turned blue. The sections were then transferred to Eosin stain for 1 minute then rinsed briefly in tap water, dehydrated, cleared and mounted.

**Mayer's Haemotoxylin**

- Haemotoxylin 2g (BDH monohydrate 34037)
- 99% IMS 10ml
- Distilled H₂O 2L
- Potassium alum 100g
- Sodium iodate 0.4g
- Citric acid 0.4g
- Chloral hydrate 100g

**Eosin Stain.**

- Eosin 10g
- Tap water 2L
- Formaldehyde 2ml

**Avidin biotin complex (ABC) HRP technique (monoclonal).**

1. The sections were taken to water (as described above).
2. The sections were then placed in 6% hydrogen peroxide solution for 10 minutes and then for 2 minutes in water.
3. The sections were then washed in PBS and incubated with normal rabbit serum (1:20 in PBS) for 10 minutes. After this period excess liquidated was drained off.
4. Appropriately diluted primary antibody was added, 100μl to each section and incubated for 60 minutes at room temperature. Sections were then washed in PBS for 20 minutes.

5. 100μl rabbit anti mouse immunoglobulin at 1:400 dilution was added and sections left at room temperature for 30 minutes followed by a wash for 20 minutes in PBS.

6. Using preformed ABC complex (from the ABC complex kit, 2 drops of avidin, 2 drops of biotinylated HRP and 5mls of PBS made up 30 minutes before use at room temperature) sections were incubated with 100μl of the complex for 30 minutes then washed with PBS for 20 minutes.

7. To each sections DABs (5mg frozen in 0.5ml PBS, 10mls of PBS and 70μl of 3% hydrogen peroxide filtered and used immediately) was added, then washed in water for 2 minutes.

8. Sections were placed in haematoxylin for 30 seconds and washed in water for 2 minutes.

9. The reverse process of step 1 “taking to water” was carried out, the sections were then mounted.
APPENDIX 4
ASSAYS

PHADEBAS AMYLASE ASSAY.
Phadebas ® Amylase Test (Pharmacia Diagnostics, AB Upsala, Sweden)
0.5M NaOH (S/4845/60, Fisons).

1) Double distilled water (ddH2O) (4ml) was pipetted into appropriately labelled
10ml conical tubes.
2) To each tube, 200µl of sample to be assayed was added, ddH2O was used for a
blank.
3) The tubes were incubated in a waterbath and allowed to equilibrate to 37°C.
4) To each tube, 1 Phadebas tablet was added using forceps and then the tube was
vortexed immediately. For serial assays, the tablets were added at 15 second
intervals.
5) All tubes were incubated for exactly 15 minutes at 37°C.
6) After 15 mins, 1ml of 0.5M NaOH was added to each tube at 15 second intervals,
then vortexed immediately to stop the reaction.
7) The tubes were then spun at 1500g for 5 minutes
8) The absorbance at 620nm was read against the blank, using the LKB Ultrospec
and semi-micro cuvettes with a 1cm light path.
9) High absorbance samples were diluted, (usually 1:10) before reading, with
ddH2O.
10) The amylase values in U/L were then determined from the supplied standard
curve data sheet.

INSULIN ASSAY
Buffer
PBS + 0.5% BSA - Sigma No. A7030

Serum
Guinea pig anti-bovine serum (ICN Biomedicals) aliquotted out in 500µl of 1/1K
dilution + 9.5 ml buffer = 10 ml of 1/20K working concentration. Can be stored and
refrozen once.

Standards;
Rat serum (Novo Biolabs LTD., Cambridge, UK.)
40μl of rat serum in 960μl of RIA buffer was 8ng/ml. A serial dilution of the 8ng standard was carried out, to make 4, 2, 1, 0.5 and 0.25 and 0ng standards.

125I Insulin
IM166 activity 1.85MBq (Amersham International LTD., Amersham, UK.) ; 20 μl aliquots were made up to 5-6mls of RIA buffer to give 20-30K counts per tube.

To each tube, 5-6 ml assay buffer was added to give approximately 20-30 K counts per tube.

Immunoprecipitating agent
Sac Cel (IDS, Washington, UK., A-Sac-3). Donkey anti-guinea pig coated cell suspension. Diluted 1/2 with PBS.

Wash solution.
0.1% Triton X-100 (Sigma T6878) in distilled water.

ASSAY PROCEDURE
1. Appropriately diluted samples were assayed in duplicate, using the following system;
   Tube no.
   1 = Total (50μl of 125I Insulin)
   2 = Blank (50μl assay buffer and no serum)
   3 = Zero (50μl assay buffer and 50μl serum)
   4 = 0.25ng std (50μl std and 50μl serum)
   5 = 0.5ng std ("
   6 = 1.0ng std ("
   7 = 2.0ng std ("
   8 = 4.0ng std ("
   9 = 8.0ng std ("
   10 onwards = samples (50μl sample and 50μl serum)

2). All tubes were vortexed and left covered at room temperature for 30 minutes.
3). To all tubes 50μl 125I insulin was added and mixed.
4). Tubes were then left covered at room temperature for 1.5 hours.
5). 50μl sac cell was added to tubes 3 onwards and mixed.
6). Tubes were then left at room temperature for 30 minutes.
7). 1 ml water was added to tubes 2 onwards.
8). The tubes were then centrifuged in a Jouan centrifuge at 3500g for 6 minutes at 18°C with no brake.

9). The supernatant was decanted off and any remaining liquid aspirated off. The pellet was not very solid so care had to be taken so as not to dislodge it.

10). The tubes were capped, then counted in gamma counter. The standard curve and concentration of insulin was calculated in ng/ml, using the Multi Data Management Package (Pharmacia, Milton Keynes, UK.)
PRESENTATIONS AND PUBLICATIONS ARISING FROM THE WORK IN THIS THESIS.

PRESENTATIONS


Robertson GSM, Davies J, Chamberlain J, Bell, PRF, James RFL, London NJM. Mode of digest release into the magnetic field significantly affects immunomagnetic islet purification. Medical Research Society, London, 5-6th November 1992. I was the presenting author. (Winner of Best Poster Award in the Free Poster Session).

Robertson GSM, Davies J, Chamberlain J, Bell, PRF, James RFL, London NJM. Mode of digest release into the magnetic field significantly affects immunomagnetic islet purification. British Society for Immunology, London, 18-20th November 1992. I was the presenting author.

Robertson GSM, Davies J, Chamberlain J, Bell PRF, James RFL, London NJM. The application of immunomagnetic separation methods to pancreatic islet purification. 12th Workshop of the EASD Study Group AIDSPIT, Igls/Austria, 24-26th January 1993.


Robertson GSM, Davies J, Swift S, Chamberlain J, Bell PRF, James RFL, London NJM. Mode of digest release into the magnetic field significantly affects immunomagnetic islet purification. 4th International Congress on Pancreas and Islet Transplantation, Amsterdam, 27-30th June 1993. I was the presenting author.

comparison of different magnetic fields in the rat. First International Meeting of the Pancreatic Islet Study Group, Alicante, November 25-28 1993.


PUBLICATIONS;

ABSTRACTS


PAPERS.


BOOK CHAPTERS

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