## THE IN VITRO IMMUNOGENICITY OF HUMAN PANCREATIC ISLETS AND ACINAR TISSUE

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from

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## ABSTRACT

Islet transplantation is a relatively new treatment for Type I diabetes which offers great potential for normoglycaemia and ultimate avoidance of lifethreatening complications(1). However, the treatment has only been partially successful and poor graft survival rates clinically have contrasted with reversal of diabetes by islet transplantation in rodent models. One major reason for graft loss is rejection of the islets and to study this an *in vitro* model for human allogeneic islet transplantation was developed.

The aim of this study was to develop the mixed lymphocyte islet coculture (MLIC) and so investigate the *in vitro* human allogeneic lymphoproliferative response to untreated human islets. The possibility of developing a short duration MLIC as a pretransplant model for clinical use was initially investigated, and then the parameters and conditions of the MLIC response determined. Titration and kinetic studies of the MLIC (islet immunogenicity) and the MLAC (acinar tissue immunogenicity) showed that ten human islets or acinar tissue pieces (average 150  $\mu$ m diameter) cocultured with 1 x 10<sup>5</sup> HLA mismatched responder PBLs for a duration of nine days, gave an optimal response. Splenocyte, islet and acinar cell stimulator populations from the same donor source showed that the MLR was greater than the MLIC and the response of both were greater than the MLAC.

It was shown that soluble products of untreated acinar cells inhibited lymphocyte proliferation in the MLR and may have led to a reduced MLAC response compared with that in the MLIC. Immunocytochemical investigations showed that upregulation of MHC class II antigen expression on acinar cells and induction on islets by cytokine treatment, did not enhance the stimulatory capacity in this model. The development of the MLIC as a model for *in vitro* islet immunogenicity showed that human islets can stimulate an allogeneic response which is not enhanced by the presence of freshly isolated acinar tissue.

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The material on which this thesis is based is my own independent work except where stated.

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S.M.Swift 1st March 1995

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### **ABBREVIATIONS**

ABS = human serum from AB blood group donor AEC = 3-amino-9-ethylcarbazole ALS = anti lymphocyte serum AP = alkaline phosphatase APC = antigen presenting cell ATCC = American Tissue Culture Collection BCIP = 5-bromo-4-chloro-3-indolyl phosphate BSA = bovine serum albumin cpm = counts per minute Con A = concanavalin ADAB - 3', 3' diaminobenzidine ∆cpm = test cpm minus sum of negative control cpm DMSO = dimethyl sulphoxide DTZ = dithizone (diphenylthiocarbazone) DTZ-DMSO = dithizone dissolved in dimethyl sulphoxide DTZ-ETH = dithizone dissolved in ethanol FACS = fluorescence activated cell sorter FCS = foetal calf serum FDA = fluorescein diacetate Fig = figure FITC = fluorescein isothiocyanate GVHD = graft versus host disease HLA = human leucocyte antigen HP = human pancreas HRPO = horseradish peroxidase Hsp 65 = heat shock protein 65 ICAM-1 = intercellular adhesion molecule 1 IFN-γ = interferon gamma Ig = immunoglobulin IL-2 = Interleukin-2 (IL- followed by number for other interleukins) LDA = limiting dilution assay LFA3 = lymphocyte functional antigen 3 LN<sub>2</sub> = liquid nitrogen LRA = lymphocyte response assay LSM = lymphocyte separating medium Kd = kilodalton

MEM = minimum essential medium MHC = major histocompatibility complex MLAC = mixed lymphocyte acinar tissue coculture MLDC = mixed lymphocyte digest coculture MLIC = mixed lymphocyte islet coculture MLR = mixed lymphocyte response NaCl = sodium chloride NBT = nitroblue tetrazolium NK = natural killer (cell) NOD = non obese diabetic (mouse) PE = phycoerythrin PI = propidium iodide PBLs = Peripheral blood leucocytes PCR = polymerase chain reaction pCTL = precursor cytotoxic T cell PHA = phytohaemagglutinin PLT = primed lymphocyte test PRA = panel reactivity pTHL = precursor T helper cell PWM = pokeweed mitogen RIA = radioimmunoassay SCID = severe combined immunodeficiency SCID-hu = mouse with severe combined immunodeficiency reconstituted with human lymphoid cells S.I. = stimulation index (for coculture results) test cpm divided by sum of negative control cpm S.I. = stimulation index (for insulin assay results) ratio of glucose stimulated insulin release to basal insulin release from islets TBS = tris buffered saline TCR = T cell receptor TNF- $\alpha$  = tumour necrosis factor alpha Tris = trizma base

WHO = World Health Organisation

## INTRODUCTION TO THE THESIS

The prevalence of Type I diabetes, in which the insulin producing beta cells of the pancreas have been destroyed, is currently about 200,000 in the U.K. alone. Although normoglycaemia in these patients can be treated using injected insulin, the absence of fine control leads to complications including retinopathy, microangiopathy and nephropathy(1).

Transplantation of whole pancreas has become increasingly used as an alternative therapy to exogenous insulin administration, but has inherent problems associated with major surgery in which a large amount of unwanted tissue (exocrine) is also transplanted. Transplantation of isolated islets offers many potential advantages including a relatively minor surgical operation to put them in place and, theoretically at least, a simple replacement of healthy islets. As human islets can be maintained in culture, there is an opportunity to transport them either in culture or as cryopreserved tissue to different centres. In addition there is potential for immunomodulation to lesson the chance of rejection.

Since 1974, some 159 human islet transplants have been carried out with varying degrees of success but generally with a low success rate and duration of graft survival. Fourteen Type I diabetic patients have been rendered free of exogenous insulin for a week or more following islet transplantation and in only one case has this period exceeded one year (Edmonton)(2). Although there could be several contributing factors to this observed graft failure, current opinion favours rejection as the major cause of graft functional loss(3).

These studies investigate the apparent rejection of transplanted islets by determining the immunogenicity of human islets in an *in vitro* system. The mixed lymphocyte response assay (MLR) has been used to predict outcome in renal transplant patients and has been found to correlate with accurate tissue typing using DNA-based techniques. An adaptation of this assay, the mixed lymphocyte islet coculture (MLIC), which utilises islets instead of splenocytes as the stimulator population, has been used in rodents to measure the specific immunogenicity and immunomodulation of islets but has not been applied to human islet transplantation. The advantages of

developing a consistent MLIC are that it could allow investigation of the role of islet specific antigens and MHC antigen expression on islet immunogenicity, as well as potentially providing a measure of the effect of *in vitro* immunomodulation of the islets prior to transplantation.

Chapter One briefly reviews the incidence and pathogenesis of Type I diabetes then discusses the results of human pancreas and islet transplantation. Chapter Two describes the current knowledge of the effects of MHC matching on kidney transplantation and the correlation between the MHC, MLR and graft outcome. Chapter Three reviews the published studies using the MLIC and associated studies and the corresponding *in vivo* findings.

Chapter Four outlines the methods used for the experimental studies. The following three chapters contain the experimental details concerning the development of the human MLIC, starting with Chapter Five, which describes the preliminary experiments on the MLIC, Chapter Six, which looks at the parameters of the MLIC, MLDC and background proliferative responses of PBLs, and Chapter Seven, which covers the optimisation and standardisation of the MLIC and MLAC. Chapter Eight describes the studies investigating the limited proliferation of lymphocytes to acinar tissue, and Chapter Nine contains the details of the immunocytochemical staining of islets and acinar tissue for MHC class I and II antigens.

An overall discussion is given in Chapter Ten, conclusions and suggestions for future work are given in Chapter Eleven. The bibliography can be found at the end.

# CHAPTER ONE

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## TYPE I DIABETES, PANCREAS AND ISLET TRANSPLANTATION

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## CHAPTER ONE

## TYPE I DIABETES, PANCREAS AND ISLET TRANSPLANTATION

### 1.1 Type I diabetes

### 1.1.1 Introduction

Diabetes of the young (insulin dependent diabetes mellitus or Type I diabetes) has an incidence ranging from 4.6 individuals per 100,000 population (aged 0 - 14 years) in Northern Greece to 42.9 (aged 0 - 14 years) in Finland(313) and has been found to be a disease with an autoimmune pathogenesis(5) (6), in which the insulin-producing beta islet cells of the pancreas are selectively destroyed(7). This leaves the patient unable to control the level of glucose in the blood leading to hyperglycaemia, potential coma and death(8). Administration of exogenous insulin and careful attention to diet enables the diabetic patient to lead a relatively normal life.

Although exogenous treatment and dietary control have reduced mortality from diabetes, the total rates are higher in diabetic patients than in the normal population, particularly in the younger (<40 years) age group(4). This is partly due to coma caused by diabetic ketoacidosis (blood ketone levels >5 mmol/l), in about 9% overall(9). However, a more common cause of increased mortality in Type I diabetic patients has been found to be renal disease which develops in 45% of cases with a peak incidence 16 years after diagnosis, and leading to renal failure or death from associated vascular disease in half of those cases(10). Microvascular disease has been found to be common and can lead to dermal lesions, loss of peripheral circulation, particularly of the foot, which can require amputation(11). Diabetic retinopathy, also microvascular in origin, has been found in 97.5% of insulin treated patients after 15 years of diabetes, 40% of these suffer from proliferative lesions after 20 years(12). Diabetic neuropathy complications have been found to include sensory abnormalities, muscle weakness/wasting, foot ulceration and impotence(13).

A recent study on glucose control by more frequent insulin injection has clearly shown that better glucose control (3 or more injections a day) is related to a lower incidence of long-term microvascular complications including a 76% reduction of risk for retinopathy, a 35 - 56% reduction of risk for renal disease and a 60% reduction of risk for neuropathy(1). This has been supported by the results of pancreas transplantation which show a lack of deterioration of retinopathy after 3 years successful graft function(14).

Thus in summary, although the use of exogenous insulin has been found to allow relative normoglycaemia in patients with Type I diabetes, the incidence of complications remains high. Evidence has shown that these complications, which include renal failure, vascular disease and retinopathy, can be avoided by tighter control of blood glucose levels.

#### 1.1.2 The normal pancreas

Between 300,000 and 1.5 million islets of Langerhans have been found in the adult human pancreas(3) and they form on average 1.2% (range 0.5% - 3%) of the whole organ(15). Each islet has been found to contain 4 types of endocrine cell arranged around a capillary network -  $\beta$  cells,  $\alpha$  cells,  $\delta$  cells and PP cells. Beta  $(\beta)$  cells have been found to release insulin in response to high blood glucose levels and this has a range of effects on cells, which include increasing glucose, amino acid and fatty acid transport across most cell membranes as well as increasing DNA, RNA and protein synthesis and other related activities. Low blood glucose levels and the activity of the sympathetic nervous system have been found to stimulate alpha ( $\alpha$ ) cells to produce glucagon which in turn stimulates glycogenolysis in the liver resulting in the release of glucose. Glucagon release has also been found to stimulate hepatic gluconeogenesis, ketogenesis, lipolysis, protein catabolism and insulin secretion, many of these effects appear to be mediated by enhancing cyclic AMP activity. Somatostatin which has been found to be released by delta ( $\delta$ ) cells in islets, and by the hypothalamus, inhibits both insulin, glucagon and pancreatic polypeptide release as well as inhibiting release of growth hormone, thyroid stimulating hormone and pancreatic acinar cell enzymes. Pancreatic polypeptide, released by the PP cells and related to gastrointestinal and neuro-peptides, has been shown to respond to

glucose, protein, fat, exercise and stress. Its actions are largely unknown but appear to include inhibition of exocrine pancreatic secretion by modulation of insulin action(7) (8) (16) (17) (18).

In addition, islets contain non endocrine cells which include endothelial cells, neuronal cells, leucocytes, ductal epithelium and stromal cells(7) (19) (20). The non endocrine areas of the pancreas have been found to mostly comprise exocrine tissue made up of acini which secrete lytic enzymes such as amylase, lipase and proteolytic enzymes(16). In addition there are ducts, small blood vessels and stromal cells. Small lymph nodes that have been found to contaminate isolated islet preparations in mice(21) are located in humans in the fat between the splenic artery and vein at the head of the pancreas.

Immunocytochemical studies have shown that human(22) (23) and rat (24) (25) (26) islet endocrine cells weakly express the major histocompatability (MHC) class I antigens, but that rat and human acinar cells do not (25) (26) (24). MHC class I antigen has also been found to be expressed on rat(25) and human(22) (26) vascular endothelium, rat and human macrophages/ dendritic cells(22) (25), duct epithelium(22) (24) (26) and nerve fibres in human pancreas(19).

Most authors have not detected MHC class II antigen expression on rat islets in the pancreas(24) (27) and all authors have found human islet endocrine cells to be MHC class II negative(22) (23) (28) (29). Human acinar cells have also been found to be MHC class II negative(22). MHC class II antigen has not been found to be expressed on rat(24) (27) (30) or canine(31) vascular endothelium but has been found on human(22) (27) and pig(31) vascular endothelium in islets and in exocrine tissue(19).

However, one group using batches of high avidity antibody to MHC class II determinants in the rat has found expression of MHC class II antigen on 20% of rat islets which have shown expression on 20 - 40% of their beta cells, and this has been found to be increased during pregnancy. The same group have found MHC class II antigen to be expressed on 10 - 30% of rat vascular endothelium(25) (32). Scattered duct epithelia in human exocrine tissue have been found to express MHC class II in some cases(19).

Macrophages/dendritic cells and lymphocytes have been described as MHC class II positive in mouse(33), rat(25) (26) (32) (34) and human pancreas(27). In addition, MHC class II positive cells have been found in rat isolated whole islets(25) (32), human monolayer islet cells(28) and sections of rat islets using light microscopy(26). Electron microscopy has detected MHC class II positive cells in rat(25) and dog islets(27).

Using freshly isolated whole islets, zero to fifteen MHC class II positive cells have been counted in individual rat and dog islets with less in human islets(27). Stock has counted five to ten MHC class II positive cells per mouse islet and a total of 2% MHC class II positive cells in dispersed islet cells as measured by FACS(35). Markmann has observed one to two MHC class II positive cells in rat islet sections(36) whereas Flesch has found that 38% of whole fresh rat islets contain ten or more MHC class II positive cells(37). The difference in spread of numbers partly reflects whether the leucocytes are counted in a single plane as in an immunohistology section(36), or by immunofluoresence in a whole islet(27) (35) (37). After two days culture, low numbers of MHC class II positive cells have been found to persist in rat islets(37) and at five to ten days of culture, >85% of canine islets have been found to be free of MHC class II positive cells(31) (38) but MHC class II positive cells have not been detected in rat islets 13 - 14 days post isolation(39).

In the rat pancreas, it has been possible to morphologically distinguish two types of MHC class II positive cells, resembling stellate dendritic cells or macrophages which are morphologically separate from small round lymphocytes(27). In three different strains of rats, 19.6, 6.9, 20.1 macrophages or dendritic cells and 2.22, 0.56, 0.63 lymphocytes have been counted per intact whole islet respectively. These cells have been found to be mostly scattered throughout the islets but the lymphocytes have occasionally been in groups of two to seven cells(32). Using the confocal microscope, Setum has described the two distinct populations as dendritic cells and smaller populations of monocytes or macrophages and B lymphocytes(34).

The adhesion molecule ICAM-1, has been detected on endothelial cells and some ductal cells in normal human pancreas and in islet cell smears immediately after isolation and disassociation. However spontaneous

expression of ICAM-1 has been seen on  $\beta$ , as well as  $\alpha$  and  $\delta$  islet endocrine cells within 72 hours of culture(40).

Thus in summary, the normal pancreas has been found to be a vascularised organ comprising mainly of exocrine acini which secrete lytic enzymes into an extensive ductal system. Approximately 2% of the pancreas consists of islets which contain four endocrine cell types secreting insulin, glucagon, somatostatin and pancreatic polypeptide. In addition, the pancreas has been found to contain blood vessels, a nerve supply, stromal matrix and leucocytes which are interspersed throughout the pancreatic tissue. MHC class I antigens have been found to be expressed weakly on islets, not expressed on acinar cells but expressed on other cell types in the pancreas. MHC class II antigens have been found to be expressed only on leucocytes and on human and pig vascular endothelium in the pancreas. Counts of the number of passenger leucocytes per islet have varied but number approximately between one and fifteen in whole islets.

#### 1.1.3 Aetiology and pathology of the pancreas in Type I diabetes

The initiation and development of Type I diabetes, although still not completely understood, is thought to arise from a combination of genetic predisposition in humans(41) and mice(42) (43) and environmental factors such as viral infection in humans(44) and transgenic mice(45). The genetic factors for predisposition have been found to include DR3 and DR4 genes, particularly in combination, and DQ $\beta$  in which a substitution for aspartic acid at position 57 can confer susceptibility to Type I diabetes in both humans(46) and NOD mice(47).

In humans, Type I diabetes has been found to develop over a long period of time, as shown by the presence of islet cell antibodies (ICA) in up to 80% of patients(48) (49) (50), although the disease does not produce clinical symptoms until about 90% of the beta cells have been destroyed(7). Evidence for the sequence of cellular events leading to Type I diabetes has been studied using post-mortem pathology of pancreata from diabetic patients(7) (15) (20) (51) in which the hyperexpression of major histocompatability (MHC) class I antigen, possibly caused by viral infection(52), may be followed by aberrant expression of MHC class II antigen before the onset of insulitis(51).

Much of the additional information has come from animal models, particularly the non-obese diabetic (NOD) mouse and bio-breeding (BB) rat. Diabetes in the NOD mouse has been observed to start at three to four weeks when an infiltrate of macrophages followed by T lymphocytes, mainly CD4+ve cells, begins to develop around the islets, known as insulitis. Diabetes has been seen clinically at about ten weeks of age, corresponding to the appearance of CD8+ve T cells which penetrate and destroy the islets(53) (54). In NOD mice, insulitis and diabetes have been found to be prevented in the absence of functional MHC class I antigen and CD8+ve T cells ( $\beta$ 2 microglobulin deficient mice) which has suggested that the presence of CD4+ve MHC class II restricted T cells alone are insufficient for progression to diabetes(55).

B lymphocytes may also be involved and a number of islet autoantigens have been identified(56) which are described later and include GAD, although its role in diabetes is still not clear(57). Depletion of the natural killer (NK) cell and T helper cell populations using monoclonal antibodies has been found to completely inhibit the development of insulitis and associated changes in MHC antigen expression(58). Depletion of macrophages using silica has been found to reduce the incidence of diabetes in NOD mice(59) and blocking of an adhesion promoting receptor on macrophages has been found sufficient to prevent the transfer of diabetes from NOD mice via splenic cells(60). In addition, macrophages have been found to be directly cytotoxic to islet cells cultured at high concentrations of glucose *in vitro*(61).

In the BB rat, macrophage infiltration followed by a lymphocytic infiltrate has been observed, and is associated with the specific destruction of beta cells in the islet, leaving the other endocrine cells intact(62). There has been some evidence from immunocytochemical staining that the first cells around the islets are dendritic cells, followed by lymphocytes before scavenger macrophages appear(63). Enhancement of MHC class I antigen expression by the islets and surrounding exocrine cells has been clearly seen but there has been some controversy concerning the observation of MHC class II antigen on islet beta cells(64). MHC class II antigen positive beta cells have been identified in the late stages of insulitis(63) although this has not been confirmed with electron microscopy(65). Using human

pancreas from Type I diabetic patients, the presence of MHC class II antigen has been observed on islet beta cells(20) (51) and on cells simultaneously expressing insulin and MHC class II antigen(66). In cases of recurrence of diabetes in HLA identical pancreas transplants, MHC class II antigen expression has not been detected on islet cells but beta cells have been observed to be engulfed by macrophages giving rise to insulin and MHC class II antigen expression on the same cells(5). In the BB rat, MHC antigen expression has also been found to be confined to lymphoid cells, particularly macrophages, whose ingestion of beta cells could in part also account for simultaneous insulin and MHC class II antigen expression (58) (67).

The islet infiltrate has been found to be comprised predominantly of MHC class II positive cells (macrophages or dendritic cells), CD4+ve cells (T helper cells), and smaller numbers of CD8+ve cells (natural killer and T cytotoxic cells)(58) (64). Using T cell clones generated from a newly diagnosed Type I diabetic patient, CD4+ve T cells have been found that are cytotoxic to islet cells with or without MHC class II antigen restriction(68).

Once the beta cells have been destroyed in the islet, insulitis is no longer observed but a small residual population of cells expressing MHC class II antigen have been seen to remain, which are thought to be residual inflammatory cells, the expression of MHC class I antigen having returned to normal levels. The destruction of the beta cells has been found to have an effect on adjacent acinar tissue which atrophies, possibly due to the drastic reduction in local insulin concentration, which would normally stimulate protein synthesis and cell division(69).

Thus in summary, the pattern of progression for Type I diabetes has been found to be similar in humans, BB rats and NOD mice in that there is an initial lymphocyte infiltrate comprising macrophages, dendritic cells, T helper cells and NK cells, then additionally cytotoxic T cells. The beta cells in islets are specifically destroyed leaving the remaining  $\alpha$ ,  $\delta$  and PP islet endocrine cells unharmed. Following the disappearance of the lymphoid infiltrate from the residual islets, the MHC antigen levels of expression return to normal.

### 1.1.4 The role of cytokines in diabetes

Several cytokines have been shown to affect islet cells and could play a role in their destruction. The expression of interferon-alpha (IFN- $\alpha$ ) which is secreted by T and B lymphocytes, macrophages and fibroblasts, has been found in association with raised levels of MHC class I antigen in pancreata of Type I diabetic patients. In the normal pancreas IFN- $\alpha$  is expressed by spindle shaped cells, as well as cells looking morphologically like tissue macrophages, which have been found in between the acinar cells and occasionally in islets(52). Cytokines interferon-gamma (IFN-y) secreted by T and B lymphocytes and NK cells, and tumour necrosis factor-alpha (TNF- $\alpha$ ) secreted by macrophages, T and B lymphocytes and endothelial cells, in combination, have been found to cause the hyperexpression of MHC class I antigen and to induce expression of human MHC class II antigen on isolated rat(39) and human islets(70). In addition, IFN- $\gamma$  and TNF- $\alpha$  have been found to induce expression of the accessory intracellular adhesion molecule 1 (ICAM-1) but not lymphocyte function associated antigen 3 (LFA-3) on human islet beta cells(40) (71). TNF- $\alpha$  has also been found to inhibit glucose stimulated insulin release and lipid synthesis in isolated human islets (72). When combined, IFN- $\gamma$  and TNF- $\alpha$ , have been found to cause DNA fragmentation and beta cell destruction in rat and mouse islet cell lines (73).

ICAM-1 has been found to be expressed in the normal pancreas on endothelial cells and weakly on a few ductal cells(40). Raised circulating levels of ICAM-1 antigen which aid leucocyte adhesion and migration through vascular endothelium as shown in thyroid of Graves disease patients (314), have been observed in Type I diabetic patients(74). Surprisingly, administered TNF- $\alpha$  has been shown to inhibit insulitis and suppress the development of diabetes in NOD mice and BB rats suggesting that these animals may have a defect in TNF mediated immunoregulation (75). Interleukin 1 (II-1), which is released by macrophages, has been found to modulate insulin secretion in rat(76) (77) and human islets(72) (78) and to exert its cytotoxic effects on islets via the action of nitric oxide(79). The sensitivity of human islets to II-1 $\beta$  has been found to be up to 10 times less than the sensitivity of rat islets (315).

Thus in summary, the principal cytokines implicated in the development of diabetes have been found to be IFN- $\gamma$  and TNF- $\alpha$ , both involved in the

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expression of MHC class I and II antigen. These two cytokines, in addition to IFN- $\alpha$  and IL-1, are produced by leucocytes (including macrophages) in the diabetic pancreas. Expression of the adhesion molecule ICAM-1 has also been found to be raised in the diabetic pancreas.

### 1.1.5 Transgenic mouse models

The development of transgenic mice has produced some intriguing studies which may be particularly relevant to the study of diabetes. Transgenic mice can be produced by linking a specific gene to the insulin promoter gene and inserting the pair into a mouse embryo. In the transgenic adult, transcription and expression of the specific gene is theoretically limited to the beta or insulin producing cells of the pancreatic islets. Studies following the outcome of pancreatic islets in transgenic mice containing genes for cytokines or allogeneic MHC molecules, have helped define the molecular mechanisms in diabetes.

Allogeneic or syngeneic MHC class I hyperexpression by islets using transgenic techniques has been found to be cytotoxic to the beta cells, resulting in diabetes but with no evidence of a lymphocytic infiltrate suggesting that the islet destruction was by a non immune mechanism. Exocrine tissue local to damaged islets was atrophied(80).

The expression of low levels of MHC class II antigen, similar to those of splenic B lymphocytes, in transgenic mice has not necessarily led to islet cell destruction(81) (82). However, islet destruction without lymphocytic infiltrate has been found when the levels of MHC class II antigen expression are much higher than normal class II positive cells(83). Transgenic expression of single MHC class II antigen chains has been shown to result in either a decrease in mRNA insulin levels or a reduction of insulin secretion to background levels without affecting the islet cell morphology or numbers(84).

The expression of TNF- $\alpha$  in islets of transgenic mice has been found to lead to an overwhelming insulitis including penetration of islets, but not adjacent exocrine tissue, and elevated levels of adhesion molecules (ICAM-1 and VCAM-1) but not to reduced insulin content of the islets or diabetes(85). However, transgenic mice, in which the islets produced IFN- $\gamma$ , became

diabetic with an observed lymphocytic infiltrate around the islets. The infiltrate was found to include cells cytotoxic to islets, when tested *in vitro*, and diffuse MHC class II expression on the islets was observed(86).

Transgenic expression of IFN- $\alpha$  in islets has been studied as IFN- $\alpha$ expression has been found to correlate with hyperexpression of MHC class I antigen in human Type I diabetes(52) and on human islets *in vitro*(28). IFN- $\alpha$  can be expressed following viral infection leading to recruitment of NK cells and macrophages, immune cells to which islets are sensitive(61) (87). In IFN- $\alpha$  transgenic mice, islets have been found to develop  $\beta$  cell necrosis, loss of insulin production and an infiltrate comprising a substantial number of T cells(45).

IL-2, which has been shown to cause T cell proliferation *in vitro*, can to cause diabetes in triple transgenic mice. The double transgenic mice (MHC class I and TCR) have been found to delete high avidity T cells to MHC class I expressed by the transgenic islets, but retain peripheral MHC class I self reactive T cells of low avidity which can reject a skin graft without reacting to the islets. Triple transgenic mice in which the islets additionally produce IL-2, have been shown to be associated with rapid onset of diabetes(88).

Thus in summary, experiments with transgenic mice have suggested that hyperexpression of isogeneic or allogeneic MHC molecules is sufficient to disrupt insulin production but that the expression of the allogeneic MHC transgene products is tolerated by the host. However, the transgenic expression of IFN- $\gamma$ , IFN- $\alpha$ , or IL-2 in the presence of normally nonreactive low avidity T cells, can cause a breakdown of self tolerance leading to a cytotoxic lymphocytic infiltrate similar to that seen in Type I diabetes.

1.1.6 T cell, B cell interactions and autoantigens in diabetes

MHC class I and II molecules have been shown to contain a cleft formed by the tertiary protein structure where two spiral helices protrude upwards each side of a striated bed such that the groove so formed is like a mould and this has been found to be complimentary in shape and electrostatic charge to specific peptides(89) (90). The combined MHC-peptide structure has been found to be complimentary to specific T cells via the T cell receptor (TCR). The interaction of the TCR and the MHC + peptide (CD8+ve cells generally

interact with MHC class I molecules containing peptides and CD4+ve T cells with MHC class II molecules containing peptides) stimulates the T cells to proliferate and produce cytokines. The TCR comprises  $\alpha$  and  $\beta$  chains which also form 3-dimensional structures so allowing specificity for the MHCpeptide structure. The molecule is formed using a combination of variable (V), joining (J) and diversity (D) gene sequences to give V $\alpha$ J $\alpha$  and V $\beta$ J $\beta$ D $\beta$  T cell receptors in any combination. The same VJ/VDJ  $\alpha\beta$  combination can be used to detect a range of peptides of different specificities and generally MHC molecules with their associated peptides can interact with a range of TCR molecules. However, it appears that some antigens, particularly autoantigens in association with MHC molecules, may have restricted V $\beta$ usage(91) and although V $\beta$ 1 may have some predominance(92), a wide range of V $\beta$  usage has been found to be possible in the case of autoimmune diabetes in the mouse(93) and man(94). T cell clones expressing TCR V $\beta$ 7 may be associated with superantigen involvement in diabetes (316).

The search for an autoantigen which could trigger the destruction of beta cells both by T cells and autoantibodies, has been pursued vigorously for many years and has been reviewed by Atkinson(56). Several antigens have now been implicated in the disease process in addition to the target antigen of ICA which has been found to be islet but not beta cell specific(95). The identification of an islet antigen with a molecular weight of 64Kd as glutamic acid decarboxylase (GAD) has aroused much interest, this enzyme has been found to be widely distributed and catalyses the conversion of glutamic acid to GABA (gamma-aminobutyric acid). GAD has been found to be the major inhibitory neurotransmitter of the nervous system(96) (97) and exists as a developmentally earlier (67Kd) and a later (65Kd) form(98) (99). Natural antibodies to GAD have not been found in the untreated NOD mouse(100) although in contrast, induced GAD antibody and T cell (Th1) responses in the NOD mouse have been found to correlate with the onset of insulitis and it has been suggested that reactivity to GAD can trigger epitope spreading including an epitope with a region of similar sequence to Coxsackie B virus(101) (102). However, the finding of GAD positive cells in residual islets of diabetic patients and the normal distribution of GAD in the human pancreas which does not include the surface of beta cells but does include the cytoplasm of the  $\beta$ ,  $\alpha$  and  $\delta$  cells, has meant that the distribution of GAD alone cannot explain the beta cell specificity of diabetes(57) (92). In addition, polyendocrine patients with anti GAD antibodies have not been

found to necessarily develop diabetes(103) whereas the presence of ICA antibodies in polyendocrine patients has been found to identify a high risk of progression to diabetes(103).

It has been also been shown that antibodies exist in up to 80% of Type I diabetic patients to a 37Kd/40Kd antigen, to insulin in up to 50% of patients and to a 52Kd (carboxypeptidase-H) and 38Kd antigen in 30% to 25% of patients(95). The human islet cell autoantigen ICA69 has also recently been identified by molecular techniques(104).

Thus in summary, T cells are stimulated to proliferate following the interaction of the TCR with MHC + peptides, restricted V $\beta$  TCR appears unlikely in Type I diabetes. A number of putative autoantigens which could act as targets for T cell or antibody mediated islet destruction have been identified but without apparent beta cell specificity. Despite the interest in anti GAD and other antibodies such as anti carboxypeptidase-H and anti 38Kd, the presence and titre of ICA has been found to correlate most closely with risk to progression of Type I diabetes.

### 1.1.7 Treatment of Type I diabetes

Type I diabetes is mostly commonly treated by the exogenous administration, of either extracted bovine, or porcine or, more recently, human recombinant insulin as one or two injections per day timed to cope with raised blood sugar levels after meals. Careful attention to diet and full awareness of the condition has been found to allow a relatively normal life.

The increasing likelihood of complications with age, such as retinopathy, nephropathy and microvascular disease, incurring a poorer quality of life and substantial expense for the health service schemes in the U.K. and abroad, has encouraged investigations of other treatments. The most important recent study in this area has been the Diabetes Control and Complications Trial, which has compared conventional injected insulin therapy with intensive insulin therapy consisting either of treatment using an external insulin pump or using three or more daily insulin injections. The results, which come from patients with a mean follow-up time of 6.5 years, have clearly shown that the risk of development of complications is significantly reduced in the intensive insulin treatment

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groups. An increase in the number of hypoglycaemic episodes, associated with the intensive therapy regimes, is considered to have been the major adverse event(1).

Studies to develop more intensive forms of insulin therapy include the bioartificial pancreas(105) including microencapsulation of isolated islets, the latter using a variety of alginates which has been hampered by their bioincompatibility leading to fibrosis and effective blockage of the semi-permeable membrane(106). Although some success has been reported in insulin dependent diabetic patients, this should be viewed with caution(107). In addition, genetic engineering techniques to produce a glucose responsive cell line, although potentially useful, are still a long way from clinical use(108). On the other hand, transplantation of whole pancreas, which developed from the pioneering studies in dogs by Minkowski in 1892 (317), or isolated islets offers real hope of good blood glucose control and these procedures are of more immediate application as is discussed in the following sections.

Thus in summary, it has been shown that there is a need for good glucose control in diabetes to lower the long term risk of complications and a variety of methods to improve exogenous insulin treatment or provide endogenous insulin therapy have been investigated. Currently, pancreas or islet transplantation potentially offers the best mode of treatment for control of normoglycaemia in the diabetic patient.

### 1.2 Human pancreas transplantation

Transplantation of the pancreas has had a measure of popularity in the U.S.A. since 1978. The procedure requires a major operation with a large abdominal incision. Complications associated with the safe removal of exocrine secretions produced by the transplanted pancreas from the body, have been recently reduced by attaching the pancreatic duct to a hole in the wall of the bladder. The patients transplanted have been mostly Type I diabetic patients with renal failure who can have a simultaneous kidney and pancreas transplant and are maintained on long-term immunosuppression. Between October 1987 and November 1992, 2870 pancreas transplants were carried out world-wide (2061 in USA, 759 in

Europe, 50 elsewhere) with 66 - 71% graft survival (insulin independence) at one year post transplantation(109).

Although pancreas grafts have been shown to halt the development of some complications in long-term diabetic patients, it is unlikely that they will be used for young newly diagnosed Type I diabetic patients for whom the sideeffects of indefinite immunosuppression pose a greater risk than the possibility of developing secondary complications.

One problem with transplantation of pancreatic tissue into diabetic recipients has been potential recurrence of the autoimmune disease which can destroy the islets of the transplanted pancreas in the same way as the original islets. This has been demonstrated very clearly using isogeneic pancreas transplants in which part of the pancreas has been transplanted from a non diabetic into a diabetic twin (3 cases) and from an HLA identical sibling. Without immunosuppressive treatment, diabetes has been found to recur within six to twelve weeks, although recurrence was halted in one of the four recipients by the use of immunosuppressive therapy. No evidence of a humoral mediated reaction was found, as indicated by the absence of Ig and complement components, in any of the biopsies. The islet infiltrates included numerous CD8+ve cells and MHC class II+ve activated T cells, indicating specific  $\beta$  islet cell destruction by T cells, and there was evidence of macrophage engulfment of  $\beta$  islet cells(5). In contrast, Bosi observed the presence of islet cell antibodies (ICA) which was interpreted as evidence of recurrent autoimmunity in HLA mismatched pancreas transplantation(110).

Thus in summary, pancreas transplantation in specialist centres in the USA and Europe has shown moderate success with 60% graft survival at 30 months. The technical difficulties of the operation and the need for constant immunosuppression has meant that its use has been limited. Living related twin and sibling transplants have illustrated the added potential problems of recurrent autoimmunity in HLA matched combinations.

### 1.3 Human islet transplantation

The first islet transplant into a human was carried in 1894, using a sheep pancreas, some twenty-eight years before the discovery of insulin by

Banting and Best. However, the procedure has only made progress as a treatment for Type I diabetes since 1974 when a team in Minneapolis started to carry out human islet transplantation and the technique has now been tried in 25 centres world-wide(111). The first fully documented account of a Type I diabetic patient made insulin-free was published in 1989(112). Subsequently there has been gradual improvement in the techniques for isolating islets (113) (114) (115) (116).

Compared to whole pancreas transplantation, islet implants have been found to offer a number of potential advantages. Firstly islet transplantation (the majority of grafts have been into the liver) has involved a relatively minor operative procedure which utilises the umbilical vein or, in Leicester, requires a catheter to be guided into the portal vein using ultrasonography through which the small mass of islet tissue is fed in about 150 mls of medium(117). Although islet transplantation overcomes the difficulties associated with drainage of unwanted exocrine secretions from whole pancreas transplants, in cases where the islet preparation has contained considerable exocrine tissue there has been a risk of an unacceptable rise in portal pressure(118). Secondly, human islets have been found to survive well in culture(119) (120), offering the opportunity for immunomodulation(121) and pooling of islets to increase the number of islets per graft(2). Maintenance of islets in culture or by cryopreservation(122) could potentially allow MHC matching and transport between centres.

The Department of Surgery at Leicester University has carried out research into a transplantable replacement for the islets destroyed in Type I Diabetes over the last twenty years. Studies have concerned the relative immunogenicity of pancreatic islet allografts in rats(123) compared with skin and heart allografts and this work suggested that allografts of isolated islet preparations were at risk of rapid rejection following allotransplantation.

The work has continued to the present day. As a department of surgery, a considerable clinical effort has been directed towards helping patients suffering from the complications of diabetes which have included kidney failure resulting in a need for renal transplantation, and microvasculature damage which can result in the need for amputation. The potential

avoidance of these complications by developing techniques of islet transplantation to improve blood glucose control has been the impetus for the work.

Although the advantages of islet transplantation have been clear, it has become apparent that purification of islets poses some difficult problems and considerable effort has been made to overcome these problems of islet yield and purity. Bovine serum albumin (BSA) gradients have been successfully introduced for isolation and purification of rat and human islets(124), as have ficoll based gradients(115). Work on pancreatic islet isolation at Leicester and elsewhere has progressed for human cadaver pancreata with the introduction of the automated method of digestion(113), the COBE 2991 cell separator(114) and the use of large-scale continuous density gradients(116).

Two human islet transplants were carried out in diabetic patients in Leicester during 1991(117). One patient was given a total of 4,700 IEQ/Kg body weight from two fresh islet preparations which subsequently were considered to be insufficient to render the patient normoglycaemic. Another patient was given 7,600 IEQ/Kg body weight pooled from one fresh and two cryopreserved islet preparations. Despite the technical success of the islet isolation procedure and the introduction of islets into the recipient liver, both grafts functionally failed at three and five weeks respectively, corresponding with a rise in T cell levels after initial immunosuppression. During the post transplantation period the peaks of C-peptide (a measure of insulin production by the transplanted islets as opposed to exogenously administered insulin) found on day 9 and 18 respectively in the recipient's serum, indicated insulin production from the graft but neither patient attained insulin independence (maximum C-peptide production of 0.9 ng/ml in the transplanted patients) (normal range = 0.4 - 4ng/ml in subjects with normal oral GTT, C-peptide kit, C-PEP-CT2, CS Biointernational, Gif-fur-Yvette, France)(117). Islet transplantation in a third patient has been more successful (1992) after 3,100 IEQ/Kg from one fresh islet preparation and a donor and recipient HLA match at 4 HLA loci (2x DR, 1xA, 1xB)(125). This patient has continued to produce C-peptide levels (approximately 1 ng/ml) post transplantation for over two years, although insulin independence has not been possible (London, N.J.M. - personal communication)

Islet transplantation programmes elsewhere have reported similar findings. A review of the results of all human islet transplants into Type I diabetic patients up to the end of 1993 has shown encouraging results, particularly over the last few years (126). From the beginning of 1990 to the end of 1993, 94 patients received human islet allografts. Of these, fourteen Type I diabetic patients, all transplanted via the portal vein, were found to be insulin independent for one week or more (ranging from eleven days to just over three years). In respect of the production of C-peptide of  $\geq 1$  ng/ml by transplanted diabetic patients, there has been a one year islet graft survival rate of over 48% since 1992. Guide-lines for treatment of the pancreas prior to isolation, better isolation techniques and improvements to the protocols for immunosuppression have all contributed to increased islet graft survival. The recommendations for human islet transplantation currently include:

a) not less than 6,000 IEQ/Kg (IEQ is equivalent to the number of islets if they were all 150  $\mu$ m diameter), although current evidence suggests that 3,000 - 7,000 IEQ/Kg should be sufficient to produce normoglycaemia(3). b) a pancreas preservation time of eight hours or less,

c) implantation into the liver,

d) carefully controlled immunosuppression with ATG, ALG or OKT3 (111) (127).

Other criteria such as continual monitoring and infusion of insulin using a Biostator with total parental nutrition, intensified insulin therapy, nicotinamide treatment to stimulate islet cell replication, prospective HLA matching, and low temperature culture of highly purified islets have also been described to improve human islet allograft survival(128).

On a cellular level, several events are likely to contribute towards human islet allograft failure. These include islet death, due to poor islet viability at time of transplantation, insufficient islet numbers, lack of vascularisation, failure of reinnervation, inflammation, the initiation of a rejection response or failure due to the harmful effects of immunosuppressive drugs(3). Improvement in islet isolation procedures, tissue culture media and temperature control have been found to increase the quality of islets and reduce potential immunogenicity(129) (130). Islets have been found to lose vascular endothelium post isolation and require up to four days to develop a new vascular system *in vivo* (131) (132). Although, this might be less important for islets implanted into the liver where they have been

found to migrate to the small capillaries and would be expected to have opportunity for nutrient exchange in the immediate post transplantation period. Inflammation, resulting in non specific tissue damage, may destroy the islets in addition to allospecific rejection, and these mechanisms of destruction can result from islet graft immunogenicity and insufficient purification of islets(133) (134). Because of the small size of individual islets which have been found to be dispersed throughout the liver after transplantation, there has been no easy means of detecting them either by biopsy, laparotomy or other visualisation systems.

As with pancreas transplantation, there is evidence of autoimmune recurrence following transplantation clinically(5) as well as in animal models. Following transplantation of islet iso and allografts into BB rats, recurrence of autoimmunity has been seen in the isografts but not in the allografts(64) (135). An interesting study has shown that MHC class I deficient islet allografts have evidence of autoimmune recurrence after transplantation into NOD mice, suggesting that recurrence may not be MHC class I restricted in that model(136).

Thus in summary, human islet transplantation is a technically safe and straightforward procedure but the isolation of islets involves more complex techniques. In addition, the ability to culture and transport islets offers great potential for the future. Protocols designed to improve graft survival have led to a 15% success rate of patients attaining insulin independence for one week or more over the 1990 - 1992 period. Reasons for graft failure include both the quality and quantity of islets as well as revascularisation and immune damage by the recipient.

1.3.1 Non endocrine cell contamination - acinar, ductal vascular, lymphoid

Type I diabetes has been found to cause the selective destruction of beta islet cells in the pancreas, leaving the remaining 98% of tissue intact and functional. The majority of cells comprise the exocrine tissue which is arranged as acini and secretes enzymes into an extensive ductal system leading from the pancreas into the gut. The enzymes produced have been found to include: amylase, which breaks down starch and other carbohydrates; lipase which breaks down neutral fats into glycerol and

fatty acids; proteolytic enzymes such as trypsin and chymotrypsin; carboxypeptidase which cleaves peptides as well as ribonuclease and deoxyribonuclease which break down the ribo- and deoxyribo-nuclear proteins respectively(16). In addition, there are many blood vessels, particularly microvessels, as well as leucocytes and neuronal cells interspersed throughout the exocrine tissue(16) (20) (51).

Reversal of diabetes has been shown following intraperitoneal injection of purified, but not unpurified, islets in the rat(137). In the mouse, Gotoh found that hand-picked islets survive for longer as an allograft than crude islets. In order to determine which of the contaminating elements of the crude preparation reduces survival time, exocrine tissue, ducts and vascular tissue and/or lymph nodes, have been transplanted under the kidney capsule. The addition of lymph nodes to hand picked islets, transplanted either under the same or the colateral kidney capsule, was found to cause a reduction in graft survival time comparable to that of crude islet preparations in mice. In addition, there was some reduction in survival time when exocrine tissue was transplanted together with pure islets, although not when the exocrine was transplanted under the colateral kidney capsule, suggesting that exocrine tissue may induce local inflammation leading to functional loss of the adjacent islets(21). In a similar type of experiment, Gores demonstrated a slight increase in incidence of rejection between purified and unpurified islets but this was found to increase to 100% rejection when splenocytes were added to the unpurified islets(138). The contamination of isolated islet preparations with lymph nodes has been observed in mice(139) and presumably has arisen from outside the pancreas. In the human pancreas small lymph nodes have been found to be located in the fatty tissue between the splenic artery and vein close to the head of the pancreas, tissue which is normally removed during preparation of the human pancreas for islet digestion (personal observation) although the numbers of individual leucocytes or small lymphoid nodules present in the isolated islet preparations has yet to be determined.

Gray has also studied the role of exocrine tissue in rat islet isograft implantation by adding varying proportions of exocrine tissue to 420 pure islets before transplanting under the kidney capsule(134). When exocrine tissue was added to a standard number of islets such that 50% or 90% of the graft comprises exocrine tissue, almost a 50% reduction was found in the

insulin content of the grafts remaining two to three weeks post transplantation. The histological appearance of the grafts was found to comprise scar tissue and giant cells, similar to a foreign body reaction, in the presence of exocrine tissue but not in the presence of pure islets alone. The role of exocrine tissue in graft rejection has thus been attributed to an impairment of islet graft implantation(134). Increasing exocrine contamination of transplanted isogeneic rat islets has also been found to reduce the normal response to glucose three months post transplantation(140).

Hegre compared the renal subcapsular transplantation of 200  $\mu$ g of purified rat allogeneic islets (approx 250 islets) after ten days in culture with nonislet pancreatic components. At 10 - 20 days post transplant, the histological evidence of rejection was found to be significantly increased in the pancreatic non-islet tissue group, from grade 0 to 4. Rejection, in this case, was measured on a scale of 0 - 4, (0 = viable tissue and non-invasive mononuclear cell (MNC) infiltrate, 4 = little or no remaining graft tissue, so mostly scar tissue, and some evidence of MNC infiltrate)(141).

The main complication of exocrine contamination during islet transplantation, has been found to be a rise in portal pressure caused by the large amount of cellular material infused into the liver, leading in one case to splenic rupture(142). However, in monkeys it has been possible to perform autotransplantation with 5 - 10% pure islet preparations without causing systemic complications and to reverse diabetes successfully with an 80% pure islet preparation(143). Gores has shown that although there has been a risk of complications, unpurified pancreatic digest can be successfully transplanted into the liver as a human allotransplant and reverse diabetes(142).

It has been suggested(133) that the major complications arising from the transplantation of exocrine tissue can include: a) systemic/portal hypertension, including shock with systemic hypotension possibly by the release of vasoactive substances eg Kallikrein. Protease inhibitors such as aprotinin have been tried for dog, pig and monkey islet autotransplants without success(144), b) impaired implantation of islets,

c) alteration of the graft immunogenicity,
d) thrombogenic impairment of the graft - there have been attempts to address this problem in autotransplants using heparin without much success(144).

Exocrine enzyme products have been found to be depleted from cultured human islets, prepared by a mincing technique, within four to six days(145). The specific inhibition of acinar tissue products by minced human, dog and rat pancreatic fragments, was investigated after culturing in media containing a combination of aprotinin, which inhibits proteolytic enzymes, as well as cobalt chloride and pilocarpine, which stimulate release of exocrine enzymes (CAP). Cobalt chloride additionally was found to be selectively toxic to exocrine cells. The addition of CAP was found to maintain the insulin but reduce the amylase levels of the tissue over a 24 hour culture period(146).

Finally, it has been shown that contaminating autologous exocrine tissue cannot subsequently be found histologically following intraportal transplantation in dog, pig and rat models, although following transplantation into the spleen or liver parenchyma, the exocrine tissue does persist(3).

Thus in summary, contamination of isolated islets with exocrine tissue does not necessarily increase the relative immunogenicity of islet grafts but may impair implantation, cause local inflammation and lead to complications associated with increased portal pressure due to the large amount of tissue.

#### 1.3.2 Immunomodulation of islets

The results of clinical human islet transplantation have been encouraging but there is a need to improve the functional quality and quantity of islets surviving transplantation. As isolated islets can be maintained in culture, they therefore offer the opportunity for immunomodulatory procedures before transplantation. These techniques have particularly concentrated on the removal of immunogenic MHC class II positive passenger cells from islets.

Successful reversal of diabetes using transplanted allogeneic isolated islets has proved to be substantially easier in rodent models than humans such that it has been found to be progressively more difficult to reverse diabetes moving from mice to rats, to dogs and humans. For example, spontaneous tolerance to mouse islet allografts has been observed(147). Even so, the effectiveness of immunomodulatory techniques, such as culturing mouse islets for seven days and an observed reduction of individual cells expressing MHC class II antigen in rat islets, has been found sufficient to allow indefinite islet allograft survival(148) (149).

The passenger leucocyte concept has been reviewed by Lafferty(150) and evidence indicates that the lymphoid content of the graft is related to the intensity of the allograft reaction. Most importantly, in a model of 2-signal activation of T cells, leucocytes, particularly dendritic cells, carry both the MHC and costimulatory determinants and are likely to be the principal stimulator population for the alloresponse(150).

In addition to purification and culture, there have been a number of treatments designed to remove these MHC class II positive cells. Low temperature (24°C) culture in CMRI. medium was found sufficient to prolong islet allograft survival(119) (129). In support of these findings, the number of MHC class II positive cells in canine islets cultured at 24°C, was found to reduce faster and to reach a lower number than in islets cultured at 37°C, although 0.2 cells per islet were still detectable after eight days of low temperature culture(151). In addition, after culture at 24°C, expression of MHC class II antigen in rat islets was found to be diminished, although some lymphocytes and a greater number of macrophage-dendritic cells were still detectable with antibody at day twelve. Endothelial cell MHC class II positive expression was not detectable after four days in culture(32).

Treatment of rat islets with UV irradiation was found to prolong islet allograft survival without the need for immunosuppression(152). In addition, it was shown that rat islets treated with a low dose of  $\gamma$ -irradiation (250 rads) prior to transplantation led to indefinite allograft survival in recipients treated with cyclosporin(121). UV irradiation and 48 hours culture of canine islets plus pretreatment of the recipients with Cyclosporin A was found necessary for a prolongation of graft survival, possibly due to

the relatively lower purity of dog islets compared to rat and mouse islets(153).

The purity of islet preparations appears to have affected immunogenicity as a direct consequence of the presence of MHC class II positive cells. In studies comparing rejection of MHC class II antigen free rat islets with MHC class II positive non endocrine tissue, evidence of histological rejection was confined to the unpurified tissue(141). Following collagenase digestion of the human pancreas, Ulrichs also found that the vast majority of MHC class II positive cells were isolated with the exocrine component of the pancreas and expressed HLA-DR and occasionally HLA-DQ(29).

Cryopreservation potentially can be of considerable value to human islet transplantation enabling storage and distribution of islets. One additional advantage of cryopreservation may be that dendritic cells have been shown to be more sensitive to cryopreservation than islets(154) and the reduction of MHC class II positive cells can prolong islet allograft survival following transplantation(33).

Removal of MHC class II positive passenger leucocytes by disruption of islets followed by reaggregation, has been found to prolong survival of rat islet allografts (from two to over twenty weeks) and it has been interesting to note that reaggregation of all the endocrine cells of the islet results in a higher number of surviving grafts than reaggregation of the beta cells alone(155). Faustman obtained indefinite allograft survival in non immunosuppressed mice after treating the islets with anti MHC class II antibody and complement prior to transplantation(156). Subsequent injection of splenocytes of donor origin was found to cause rejection of the graft(157) indicating that removal of dendritic cells from the islets can avoid immune recognition but does not induce anergy towards the islets. Rat pancreas allografts perfused with antibody to MHC class II antigen and complement before transplantation, have been found to stimulate a reduced response *in vitro* (MLIC) but this corresponded to only a slight prolongation of survival time(158).

Published reports have concentrated on the MHC class II antigen expression of individual passenger cells in islets but some attention has been paid to parenchymal or endocrine cell MHC antigen expression. Parenchymal islet

MHC class II antigen expression was not found to be prominent in graft rejection such that induced expression of MHC class II antigen, by IFN- $\gamma$  and TNF- $\alpha$  on islet cells, was found insufficient to initiate rejection of allogeneic rat islets(159). In addition, one study found no difference between the levels of parenchymal MHC class I and II antigen expression on rejecting or non rejecting rat renal allografts(160).

The immunomodulation of MHC class I antigen in islets has been less extensively studied but may have a role in allograft rejection via the indirect pathway of presentation as described in Chapter Two. Kneteman did not find an increased rate of rejection after culture of mouse islet allografts despite the presence of enhanced parenchymal MHC class I antigen expression(161). In contrast, two reports have shown that addition of IFN- $\gamma$ to culture medium causing enhancement of MHC class I expression, reduced mouse and rat islet allograft survival time(162) (163) and MHC class I deficient islet allografts survived indefinitely in mice(136). Prolongation of mouse islet allograft survival has also been shown by modulation of MHC class I antigen using  $\beta$ -2 microglobulin gene disruption in transplanted transgenic islets(164) and using anti MHC class I antibody(165). The lack of MHC class I antigen expression, while prolonging allograft survival in the NOD mouse, was not able to prevent subsequent autoimmune recurrence(166).

Hillier has compared allograft survival of rat heart, kidney, pancreas and islets to find that although survival times of allografts have been similar in MHC-incompatible strains (9.5 - 10 days), non MHC incompatibility allows indefinite survival of kidney and heart grafts (over 100 days) but not of pancreas and islet grafts (14 and 12 days)(167). Gotoh found in mice that multiple H-2 incompatible allografts survive better than grafts from a single donor(168).

Thus in summary, immunomodulation of islets prior to transplantation has been principally aimed at the removal of passenger leucocytes from islets. This has been carried out using purified islets with culture at  $37^{\circ}$ C and 24°C, UV and  $\gamma$ -irradiation, antibodies to MHC class II antigens with complement and cryopreservation, and has been successful in prolonging islet allograft survival without substantial impairment of islet beta cell

function. Reduction of islet parenchymal MHC class I antigen expression has been shown to prolong allograft survival.

#### 1.3.3 Rejection of and tolerance to islet allografts

In human clinical intraportal islet transplantation, the identification of rejection has been difficult as the only markers available are the loss of islet cell function indicated by the levels of C-peptide, a by-product of endogenous insulin production, and a concomitant rise in blood glucose levels. In animal models, rejection of islet grafts has been additionally measured by immunocytochemical identification of the cellular infiltrate combined with apparent morphological destruction of the graft.

1.3.3a The pattern of islet allograft rejection in experimental models In the rat, the pattern of acute rejection seen in allogeneic islets has been found to be very similar to that of pancreas allografts. Using the Lew RTU<sup>1</sup> to RTU<sup>1</sup> combination, initiation of rejection was observed between days three and five with lymphocytic destruction of the islets starting on day 8 and was complete by day 10 post transplantation. The recurrence of hyperglycaemia in allogeneic transplanted diabetic rats was recorded on day 9 for both islet and pancreas grafts(132).

Immunohistological analysis of rejection as regards MHC class II antigen expression, has shown that the MHC class II positive infiltrating cells around the islet are dendritic in shape or macrophage-like and principally originate from the recipient. Only a small proportion of these cells have been found to be of donor origin - almost none inside the islets and only a few outside the islets - although the number of donor MHC class II positive cells has been seen to increase outside the islet from day six. Additionally, donor MHC class II positive cells have been found in the peri-islet infiltrate, which resemble ductal and endothelial cells, but without forming any network structures(132). Islet endocrine cells have been found to remain MHC class II negative until destroyed about day ten, but acinar cells have been found to express MHC class I and class II antigen during rejection(24). Evidence of acute rejection in the human pancreas has been described as being characterised by mononuclear cells infiltrating and destroying acinar epithelium, blood vessels and nerves of the graft(5).

Ulrichs was able to detect MHC class II antigen expression on a low percentage of rat beta islet cells and found significantly increased levels of expression 24 hours after transplantation into both syngeneic and allogeneic, nondiabetic and diabetic recipients indicating post operative inflammation rather than a clear alloresponse(32).

The main difference observed between rat islet and pancreas grafts has been the lack of vascularisation of islets grafts until day three, with a corresponding reduction in insulin production. Development of some islet necrosis during the first 48 hours post transplant has also been observed, in the rat this has been found to be compensated for by an increase in mitotic activity in the islets from day four. Similarities between iso and allografted islets up to day five have been found to include the presence of fibrin strands, oedematous fluid and haemorrhage as well as moderate numbers of granulocytes and single macrophages around the islets. Because of their occurrence in both iso and allografts, these features are thought to be associated with the transplantation procedure rather than the alloresponse. It has been suggested that the observed rearrangement of glucagon cells to a polar cluster on day one of islet grafts and day six of duct ligated pancreas grafts may be a response to the lack of adjacent cells(132).

1.3.3b Manipulation of the recipient immune system in islet transplantation Kaufman has selectively depleted three cell types in recipients of islet allografts - macrophages, CD4+ve and CD8+ve T cells, and has shown that primary non-function of the islet allograft is attributable to macrophage activity and that both types of T cell are associated with classic rejection(169). Similarly, Gotoh has shown using antibodies in mice that removal of both CD4+ve T helper (Th) and CD8+ve cytotoxic T (Tc) lymphocytes is necessary to avoid graft rejection(170).

Transplantation of islets into immunologically privileged sites such as the cerebrum(171) have been attempted to protect allografts from immune attack. The most promising has been intrathymic implantation of islet grafts which, in streptozotocin treated rats given anti lymphocyte serum (ALS) as an immunosuppressive treatment, showed indefinite graft survival and unresponsiveness to a second donor strain islet allograft challenge(172).

This induction of tolerance or anergy has received considerable attention because this potentially could allow allograft transplantation without longterm immunosuppression and so would make the procedure more appropriate for young people. Tolerance can avoid the recognition of 'self' antigen and protect against the development of autoimmunity, so theoretically could be utilised to permit specific non recognition of allografted tissues.

The initiation of tolerance has been found to be dependent on contact between T cells and antigen presenting cells. Studies of the role of donor APCs in the induction of anergy as opposed to graft acceptance, have raised some important questions about the limited use of immunomodulation techniques that remove passenger leucocytes from islet grafts. A rat transplantation model has been used to illustrate this point in which two types of allograft maintained with cyclosporin A therapy (14 days post transplantation) were required - a) kidney allografts which were found to contain MHC class II positive passenger leucocytes and b) nonenzymatically isolated perinatal islet allografts which were found to be free of MHC class II positive cells. The results showed that APC-free islet allografts can survive transplantation but not a subsequent challenge with donor dendritic cells. However, the APC-free islets transplanted under the kidney capsule 14 days following kidney transplantation, were found to be protected by the anergic T cell response to the renal graft and to resist donor dendritic cell challenge(173). These studies suggest that the presence of donor APCs may be necessary for the induction of true allograft specific anergy.

The mechanisms for activation of T cells with different cytokine profiles is poorly understood, however it is probable that a dynamic interplay of costimulatory molecules may contribute to this phenomenon. MHC antigen stimulation of T cells via the TCR in the absence of co-stimulation of CD28 on the T cell, has been found to lead to anergy of both CD4+ve and CD8+ve T cells. More than one ligand has been found to exist for CD28, having been identified as the B7 (CD80) family of molecules found on antigen presenting cells and inducible on T cells(174). Anergy has been thought to result from an inability to make active IL-2 and a low capacity for response to IL-2(175). Different populations of T cells have been found to exist, defined by their cytokine profiles, such that Th1 cells secrete IL-2, IFN- $\gamma$  and TNF- $\beta$ , and Th2 cells secrete IL-4, IL-5, IL-6 and IL-10. The specialised activities of these two cell types has been found to be principally related to T cytotoxic cell help (Th1) and B cell help (Th2) to give high and specific induction of antibody production including IgE(176).

Evidence has accumulated to show that blockage of the IL-2 pathway is associated with tolerance of transplanted allografts(177). Support for these findings has come from Rabinovitch, who has analysed the cytokine profile of syngeneic islet grafts in NOD mice and found that production of IL-2 and IFN- $\gamma$  (Th1 cell cytokine profile) correlated with graft rejection. Furthermore, prevention of diabetes development in the NOD mouse following administration of Freud's adjuvant, was shown to correlate with the production of IL-10 (part of the Th2 cell cytokine profile)(178). In addition, T cell clones isolated from rejecting kidney grafts, of CD4+ve and CD8+ve phenotype, were both shown to express IFN- $\gamma$ (179). There has been some evidence that production of IL-2 and IFN- $\gamma$  (produced by Th1 cells) is associated with graft rejection(180) and IL-4 and IL-10 (produced by Th2 cells), with tolerance(176) (181) although the cytokine interactions in transplantation tolerance may be more complex than those seen in other systems(177).

1.3.3c MHC haplotype and islet allograft survival in animal models Animal models have provided some clues to the importance of MHC disparity in islet allograft survival. In the rat, it has been shown that mismatches at both MHC class I (RTI.A) and II (RTI.B) sites are necessary for rejection of renal subcapsular islet grafts and that mismatches at either site alone result in prolonged graft survival(182). In the mouse, MHC class I incompatibility was found to lead to rejection and MHC class II matching was insufficient to ensure graft survival(183). This data has given limited insight into the potential importance of MHC matching in human islet transplantation, the subject is examined further in Chapter Two.

1.3.3d A new in vivo model to study human islet transplantation The opportunities to study the progress of rejection of human islets in a human immune system have been infrequent so the development of a model for this would be welcome. The severe combined immunodeficient (SCID)

mouse can be reconstituted with human lymphocytes (SCID-hu) and it has been shown that an infiltrate of lymphocytes of human origin can be detected around human allogeneic but not human isogeneic islet grafts(184). Currently, the model suffers from variable levels of reconstitution but still offers great potential for the future(185).

Thus in summary, dendritic cells and macrophages have both been implicated in the early stages of the initial immune response against transplanted islet allografts between days three and five post transplantation. This has been found to lead to destruction of the graft by day eight to ten, involving the presence of both CD4+ve and CD8+ve T cells. The progression of rejection in islet and pancreas transplants has been found to differ in the delay caused by revascularisation of islets by the host cells, following the loss of the donor vascular system. Tolerance to allografts is considered to offer great potential for transplantation and has appeared to rely on the presence of donor antigen presenting cells for active induction of the 'anergised' immune state. While MHC class II antigen has been found to play a prominent part in stimulation of the allogeneic response, absence of co-stimulation of CD28 on the T cell can lead to anergy. Cytokines have been shown to affect which subsets of T cells become dominant following stimulation and in addition, cytokine profiles of T cell responses have been shown to relate to graft outcome such that IL-2 has been linked to graft rejection and IL-10 to graft survival. Limited studies on MHC matching in rodent models have indicated that mismatches at both MHC class I and II sites can affect islet allograft survival and studies of islet allograft rejection and tolerance in vivo could come from future use of the SCID-hu model.

## 1.4 Overall summary of Type I diabetes, pancreas and islet transplantation

Type I diabetes has been found to be a disease in which the insulin producing cells of the pancreas are destroyed, principally by an autoimmune process leading to hyperglycaemia. Poor control of blood glucose has been found to lead to a high incidence of complications, particularly renal and vascular disease. The normal pancreas has been found to contain a variety of cell types of which islets containing the

endocrine cells comprise 2%. Expression of MHC antigen, which is important in initiating immune responses, has been found to be limited to a few of these cell types, particularly individual leucocytes scattered throughout the pancreas.

The development of Type I diabetes has been found to result from a combination of genetic and environmental factors, leading to an initial infiltration of dendritic cells, T helper cells, macrophages and cytotoxic T cells, the latter being particularly associated with the destruction of the islet cells. The expression of MHC class I and II antigen, IFN- $\gamma$ , TNF- $\alpha$ , ICAM-1 and IL-1 as well as autoantibodies such as ICA and antibodies to autoantigens such as GAD and carboxypeptidase-H have all been implicated in progression of the disease. Of these, ICA has remained the principal marker of islet cell destruction in pre-clinical disease. Treatments for Type I diabetes have principally been by insulin administration and although whole pancreas transplantation given at the same time as a renal transplant has shown reasonable success, it has not been an option for treatment of the young newly diagnosed patients. Islet transplantation, although a relatively new procedure, offers great potential for manipulation of the islets before transplantation and for successful treatment of Type I diabetes. Evidence from immunomodulation studies has suggested that successful human islet allograft transplantation could be improved by increased islet purity and removal of immunogenic passenger cells from the islets. Alternatively, the recipient immune system potentially could be anergised towards the graft to avoid immune attack, this phenomenon has been shown to occur in the absence of MHC class II costimulatory molecules such as B7.

### The Next Chapter...

The methods and *in vitro* assays that have been used to indicate beneficial donor recipient matches and to predict graft rejection in clinical transplantation are introduced in Chapter Two.

# CHAPTER TWO

### CHAPTER TWO CONTENTS

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### CHAPTER TWO

# MHC AND MLR IN DETERMINATION OF OUTCOME OF CLINICAL TRANSPLANTATION

#### 2.1 Beneficial donor recipient matching

Beneficial matching between donor organs and the immune system of potential recipients requires considerable skill and improves the survival of organ transplants. Prospective matching is carried out in two phases, and first involves testing the serum of potential recipients against a panel of cells of known haplotype, to determine the specificity of cytotoxic antibodies present and the percentage panel reactivity. A high panel reactivity narrows the range of donor haplotypes suitable for transplantation in that potential recipient. The second phase of tests, in cadaver organ transplantation, are carried out when the donor organ becomes available. These tests include:

- a) blood group matching,
- b) lymphocytotoxicity assay (cross matching) and
- c) HLA matching.

In addition, analysis of the frequency of alloreactive T cell precursors for cytotoxic T lymphocytes (pCTL) or T helper lymphocytes (pTHL) may be used as a first phase test to identify potential recipients with low T cell responses to specific haplotypes. The selection of very closely matched donor recipient combinations is slightly different with living related renal and bone marrow transplantation, as there is not normally the same time constraint for selecting a suitable match and this allows the use of the mixed lymphocyte response which takes five to six days (MLR).

#### 2.1.1 Blood group matching

Blood group incompatibility has been found, in the majority of cases, to lead to an immediate hyperacute response post transplantation due to preformed naturally occurring antibodies reacting against blood group antigens on the graft(186). Although blood group antigens were not found to be expressed on islets of Langerhans *in situ* in the pancreas, expression has been detected on islets after isolation, showing that compatible blood groups must be used for these allografts(187).

Thus in summary, compatibility of blood group antigens has been found necessary to avoid immediate rejection of transplanted renal allografts. The same criteria has been applied to islet allografts as human islets also express blood group antigens.

#### 2.1.2 The lymphocytotoxicity assay

The lymphocytotoxicity assay (ICA)(188), also called the cross match, is used to identify recipient cytotoxic alloantibodies that bind to specific HLA antigens. Transplantation following a positive cross match, leads to rapid antibody binding, particularly to the endothelium, causing complement mediated cytotoxicity and early graft loss(189) (190). As indicated above, this test is first carried out using serum from potential recipients, added to a panel of splenocytes from individuals of known HLA specificity, in the presence of complement. Lysis shows the presence of cytotoxic antibodies, and the percentage panel reactivity (PRA) is determined, so that suitable, non reactive combinations of donor and recipient can be identified. For the cytotoxic crossmatch method, splenocytes can be separated as T and non T cells by rosetting, or magnetic bead separation, and incubated together with patient serum and rabbit complement. Cells can be stained with trypan blue, or some other vital dye which is a marker for cell death, and counted to determine percentage lysis. Positive sera have been titrated and the antibody subclass determined. A positive cross match has been described as more than 10% lysis above background of the T and non T cell populations(191).

The lymphocytotoxicity test is repeated when the donor organ becomes available and has been tissue typed. For this, serum from selected potential recipients, previously found to be nonreactive with the donor haplotype, is added to splenocytes from the donor. The difficulty of obtaining good samples of complement fixing antibodies to all determinants has led to the development of alternative methodology. In addition, although IgG antibodies, particularly to MHC class I determinants on the donor cells, are likely to cause hyperacute rejection, IgM antibodies have not been

associated with hyperacute rejection and so could lead to false positive results.

FACS analysis of cytotoxic antibodies in the serum of potential transplant recipients has become widely used, in which phycoerythrin (PE) conjugated monoclonal antibodies to leucocyte antigens and florescein isothiocyanate (FITC) conjugated isotype specific antisera determine a) which haplotypes are targets of the alloantibodies and b) the serum antibody subclass and c) the titre of antibody. The method has been found to improve the identification of weak cytotoxic alloantibodies to B cells. Mouse monoclonal antibodies to HLA antigens have been used to block class I and II antigen positive cells and in some cases determine locus specificity(191).

A positive crossmatch has been defined as an increase in fluorescence of either all leucocytes, B cells and monocytes, or monocytes alone. Karuppan has compared the outcome of grafts with the levels of preformed alloantibodies in the recipient and has found that patients without acute rejection episodes do not have preformed HLA antibodies as determined by FACS(191).

Thus in summary, the cytotoxic crossmatch assay has been routinely used to determine the titre, subclass and specificity of preformed HLA antibodies in potential transplant recipients. The additional use of FACS analysis can identify weak reactivity to B cell antigens and in combination with the conventional crossmatch, improves donor recipient matching. A positive crossmatch has been linked to an increase in the number of rejection episodes and to early graft loss.

#### 2.1.3 HLA matching

The benefits of matching the donor and recipient haplotypes for improved survival rates of renal allografts, has been recognised for many years(192). Currently Eurotransplant recommends matching as far as possible at HLA-DR then -B then A alleles(193). Tissue typing for identification of HLA antigen polymorphisms, has been carried out serologically(194) for many years, more successfully for MHC class I antigen expression than class II. However more recently, DNA based molecular methods of typing, which were introduced in 1981 for HLA-DR (class II) sites(195), have become the

method of choice for determination of both MHC class I (HLA-A, -B, -C) and II gene polymorphisms (HLA-DR, -DQ, DP)(196). Before transplantation, potential recipients of allografts are tissue typed to determine which of the HLA polymorphic alleles they carry. The donor haplotype is determined as soon as organ donation becomes a possibility.

#### 2.1.3a Serological typing

Serological HLA antigen determination, introduced in 1964, has involved microdroplet testing for HLA-A, -B and -C antigens using T cells from peripheral blood and for HLA-DR using B cells from peripheral blood or spleen. Small aliquots of lymphocytes from the donor are mixed with polyclonal antisera or monoclonal antibody of known specificity, then incubated with rabbit complement. Cell lysis, indicating positive identification, has been determined after fixation using formaldehyde with the addition of eosin(194), or more commonly propidium iodide or ethidium bromide and acridine orange(193) and counted using an inverted phase contrast microscope or fluoresence microscope as appropriate. Results are scored as five categories: 0 - 10% = negative, 11 - 20% = probably negative, 21 - 50% = weak positive, 51 - 80% = positive, 81 - 100% = strongly positive(193).

#### 2.1.3b DNA based typing

DNA based methods, including restriction fragment length polymorphism (RFLP) and sequence specific primers(194), in particular oligonucleotide probes, have all been shown to be more specific and reliable than serological typing. This has particularly been the case for MHC class II polymorphisms, where B cells are required and identification of some alleles such as DRw52 (DRB3 domain) and DRw13 (DRB1.13), has not been possible serologically(197). Sequence specific oligonucleotide identification of HLA-D genes involves the amplification of genomic DNA from granulocyte pellets or blood by the polymerase chain reaction (PCR), denaturation and neutralisation followed by blotting and hybridisation. The reaction can be detected using <sup>32</sup>P labelled oligonucleotide probes, then autoradiography(197) or more recently, digoxygenin-11-2', 3'-dideoxy-uridine-5'-triphosphate (DIG-11-ddUTP) labelled single strand oligonucleotide probes followed by anti digoxygenin alkaline phosphatase, AMPPD solution and exposure to X-ray film(198).

#### 2.1.3c HLA matching and graft outcome

Most of the data comparing graft outcome and HLA matching has related to renal cadaver transplantation, although data from non renal transplantation such as heart and pancreas has shown early signs of similar findings. The introduction of cyclosporin as a renal transplant immunosuppressant (in 1981) has significantly improved graft survival from a half life expectancy of 9.7 years to 11.6 years, with the five year survival rate increasing from 49.2% to 66%(195). The benefits of sharing organs between centres has been highlighted by results from UNOS which show an 88% survival rate at one year for six antigen matched renal allografts, as opposed to 79% for non HLA mismatched grafts in the same centres(199). An analysis of the costs of treating rejection episodes post transplantation, in which there was a correlation between the number of rejection episodes and DR matching (such that 63% of the 0 DR mismatches, 33% of the 1 DR mismatches and 9% of the 2 DR mismatches in unsensitised males were found to be rejection free), showed that HLA matching gives benefit in financial as well as patient welfare terms(200).

Non renal transplantation has had less years of experience to draw on, but there has been evidence to show that HLA-DR matching might also be beneficial in heart allografts(201). Liver transplantation has been successfully performed across major HLA differences for reasons that are not clearly understood(202), however the acute rejection rates have been found to be high, up to 80%. Recent improvements in availability of livers and their storage, as well as the condition of patients on referral have meant that some prospective HLA matching has been possible(203). Results from the European Pancreas Transplant Registry, reported at the AIDSPIT meeting in Igls in January 1994, have shown that although no improvement in pancreatic allograft survival has been seen following simultaneous pancreas kidney transplantation, an improvement has been shown in pancreatic graft survival rates of pancreas after kidney transplantation. Survival rates of these pancreas grafts which are normally lower than simultaneous grafts, show that zero to one total HLA mismatches (HLA-DR, -B, -A, -C) have almost twice the survival rate of two to six mismatches (80% compared to 44% at one year)(204). These findings have supported the recommendation for HLA matching in pancreas transplantation(205). Results of the Islet Transplant Registry from the same meeting have shown that insufficient data exists to determine any effect of HLA matching, this

has been particularly due to the use of multiple organ donors in order to obtain sufficient numbers of islets for a single transplant(111).

HLA matching for bone marrow transplantation has posed a different problem because donors are living and harvested cells can be stored in liquid nitrogen banks until required, such that time is available for pretransplant testing. Grafts have generally been very well matched between donor and recipient, often from family members, but a degree of mismatching by using matched unrelated donors has allowed similar levels of graft survival but some increase in GVHD occurrence(206).

Thus in summary, improvements in techniques of tissue typing, particularly DNA based methods of HLA-D analysis, have led to more accurate identification of beneficial donor-recipient renal transplant matches. Comparison of HLA donor recipient matches with renal graft outcome has shown the benefits of optimal HLA antigen matching in addition to the improvements in modern immunosuppressive treatment and despite the delay caused by transport of organs between centres. Data from non renal transplantation is gradually accumulating and has also shown some correlation between HLA matching and graft outcome.

#### 2.1.4 Frequency of pCTL and pTHL lymphocytes

The variable frequency of HLA specific T cells in individuals has been considered a possible means of selecting potential recipients for allografts, such that those with low frequencies of T cells reactive with specific HLA loci would be expected to accept an allograft of that specificity(207). Limiting dilution analysis (LDA) can detect both the frequency of precursor T helper (Th) lymphocytes, with a range of 1:2,000 to 1:500(208), and precursor cytotoxic T lymphocytes (Tc), with a range of 1:100,000 to 1:5,000. Some cases of non responsiveness to specific HLA antigens in individuals were observed(209). These precursor T cell frequencies were shown to be consistent over a period of months between one responder individual and a particular stimulator individual and were found to relate to specific HLA disparity(209) (210). The variable frequency of memory T lymphocytes responding to tuberculin and tetanus toxoid between five humans was found to range from 1:1,600 to 1:300 for tuberculin and from 1:11,500 to 1:750 for tetanus toxoid(210).

Using a mouse model and a mixed lymphocyte islet coculture to generate pCTLs, a low frequency of pCTLs has been shown to correlate with a reduced rejection rate of pancreatic islet and heart-lung allografts. In these studies using the H-2<sup>k</sup> (graft) to H-2<sup>b</sup> (recipient) combination, and a frequency of pCTLs at 1 in 3477, the rejection rate of islet allografts has been found to be 86%, and that of heart-lung allografts to be 100%. However, using the reverse combination, the H-2<sup>b</sup> (graft) to H-2<sup>k</sup> (recipient), the frequency of pCTLs has been found to be 1 in 50,129 and the rejection rate of the islet allografts to be 28%, and of that of heart-lung allografts 47%(211).

A study in rats using two MHC class I mismatched combinations, in which heart allografts normally either reject within eight days or survive indefinitely, showed no differences in the frequencies of pCTL and the authors suggested that the frequencies of both cytotoxic and T helper cells may be important in determining the outcome of MHC class I mismatched allografts(212). Frequencies of pTHLs in individuals to MHC class II mismatched allogeneic peripheral blood mononuclear cells (PBMC), was found to vary between 1:500 and 1:2,000(208). Frequencies of pTHLs to syngeneic Epstein-Barr (EBV) virally transformed B cells (B-LCL cells) was shown to be higher in EBV immune individuals (approx. 1:1,500) compared to non immune individuals (<1:100,000)(213).

The LDA method for pCTL has been described using serial dilutions of human PBLs (5 x 10<sup>4</sup> - 0.25 x 10<sup>4</sup>) in the presence of  $\gamma$ -irradiated allogeneic human PBLs (5 x 10<sup>4</sup>) with T cell growth factors and sufficient irradiated autologous filler cells per well to give a total of 1 x 10<sup>5</sup> cells per well and 30 -60 wells per responder concentration. To measure the pCTL frequency, the proliferating T cell population was incubated with phytohaemagglutinin (PHA) stimulated <sup>51</sup>Cr labelled blast cells, generated from human PBLs and used as targets for the cytotoxic T cells. Frequency was determined by maximum likelihood estimation(209).

The LDA method for pTHL has been described using serial dilution of responder human PBLs ( $2 \times 10^4 - 0.03125 \times 10^4$  per well) with 24 wells per dilution in the presence of irradiated syngeneic or allogeneic human PBMC ( $1 \times 10^5$ ) or EBV transformed B lymphoblastoid cell lines (B-LCL cells) ( $5 \times 10^4$ ). These two cell types were incubated for 18 hours and then further

irradiated before adding CTLL cells  $(1 \times 10^3)$  which proliferate in the presence of IL-2. The amount of IL-2 is proportional to the proliferation of Th cells and so used as an estimate of the frequency of pTHL. The proportion of negative wells for each sample size of responder cells was found to be linearly related to the frequency of responder cells according to the Poisson distribution(208) (213).

Thus in summary, the frequency of T cytotoxic lymphocytes has been found to correlate with the outcome of transplantation in rodents. The methods for analysing pCTLs and pTHLs involve limiting dilution analysis to determine the frequency and this has been found to be lower for cytotoxic T cells than T helper cells. Low precursor T cell frequency has been shown to correlate with graft survival in animal models.

#### 2.2 The mixed lymphocyte response (MLR)

As this thesis focuses on a technique adapted from the MLR, the method and relevance of the MLR to transplantation will be discussed here in some detail.

#### 2.2.1 Historical aspects of the MLR

The correlation between the proliferation of allogeneic lymphocyte culture and the outcome of a skin graft on pre-sensitised individuals has been known since 1964(214).

Initial studies with the mixed lymphocyte culture used  $7.5 \times 10^5$  cells/ml in medium containing 20% FCS and found a peak of mitoses on day seven in addition to a peak of "large cells" on day 9, during a total culture period of eleven days. The response was measured by counting cells and mitoses using light microscopy or incorporation of tritiated [<sup>3</sup>H]thymidine by the proliferating cells. The kinetics of the MLR have been described as an increase in proliferation commencing about 40 hours after the start of the mixed culture and increasing up until 130 hours (5.4 days) as a peak, with very few new cells produced after 170 hours (day seven). These proliferating lymphocytes were shown to peak at 6 - 8 hours when 80 - 100% of mitoses are labelled with [<sup>3</sup>H]thymidine, then to remain relatively

stable at 18 - 24 hours when about 60% of the mitoses are labelled(215). The exogenous [3H]thymidine has since been shown to be incorporated into DNA during replication via the salvage pathway in which purine bases are formed by hydrolytic degradation of nucleic acids and nucleotides. The period of isotope incorporation has been found to be longer than the S phase of the cell cycle but shorter than the cell cycle itself, usually 18 hours(216).

By 1971, the combined use of 5-bromodeoxyuridine (BUdR) and [<sup>3</sup>H]thymidine to measure lymphocyte proliferation, had shown the specificity of allogeneic antigen recognition by the induction of proliferation to a secondary alloantigen (measured using [<sup>3</sup>H]thymidine) after killing the cells responding to a first alloantigen (measured using BUdR then killed by UV light which causes the BUdR to inactivate those cells)(217). After the introduction of smaller quantities of lymphocytes used in the assay(218), the present day MLR methodology has become widespread and the details described here are as used by the Trent Regional Transfusion Centre(219).

#### 2.2.2 Methodology of the modern clinical MLR

The current MLR used at the Trent Regional Transfusion Centre(219) involves using patient and donor samples of unclotted blood, two control samples and a pool of eight random samples as control stimulator populations. Sedimentation of the erythrocytes occurs after the addition of dextran and iron powder, and following centrifugation of the supernatant over lymphoprep, the PBLs can be aspirated from the interface, before washing and resuspension in RPMI medium + 10% fetal calf serum (FCS). The cell suspensions are divided such that one set (non irradiated) are used as the responder population and the other used as three populations - donor, potential recipient and controls (y-irradiated) as stimulators. The mixed culture can be set up as a chequer board test in 96 microwell plates with 50  $\mu$ l of the appropriate cell suspension(s) and 50  $\mu$ l media as necessary, in triplicate. On the fifth day of coculture, [<sup>3</sup>H]thymidine is added to each well then harvested five to six hours later and, after overnight drying, the  $\beta$ emission counted in scintillation fluid on a beta counter. A diagrammatic representation of the method can be seen in Figure 2.1.

### Method for the one way MLR



Beta emission as cpm

Fig 2.1 Method for the one way MLR

The results at Trent Regional Transfusion Centre are expressed as the relative response using the formula:

$$RR = \frac{ABx - AAx}{AYx - AAx}$$

where A = responder, B = stimulator, Y= pool of control cells,  $Ax_Bx_Yx =$  corresponding irradiated cells(219).

Thus in summary, the MLR has been used since 1964, and improvements in the technique including the use of  $[^{3}H]$ thymidine to measure proliferation and the use of microwell plates, have led to a standard methodology since 1971. The responder and  $\gamma$ -irradiated stimulator lymphocyte populations are normally incubated together for five days and the  $[^{3}H]$ thymidine uptake counted as beta emission (cpm) then expressed as a relative response compared to a control response.

#### 2.2.3 Correlation between MLR and graft survival

Because the MLR takes six days to complete, the assay has not been used for prospective prediction of outcome of cadaveric donor grafts, but it has been used as a pretransplant assay for potential living related donors and recipients, and as a retrospective indication of cadaveric graft outcome.

Currently the results of MLR assays are expressed as raw counts per minute and clinically, a negative response is used to predict beneficial graft survival. The results of MLR assays can be expressed in different ways including the relative response, as given above, and the stimulation index (S.I.). Additional computations have included a normalised, double normalised and stabilised relative response which show an association between the MLR and graft survival(216) (220). An S.I. of less than five has been found to correlate with a lower acute rejection rate among living related donor kidney transplants(221) whereas an S.I. of less than two has been found to correlate with increased survival of living related donor renal grafts(222).

So-called minor non MHC antigens have also been found to be important in transplantation. These antigens which are mostly non tissue specific, have been found to be detectable by MLR only after *in vivo* immunisation, in contrast to MHC antigens which can be detected in a primary MLR. Disparity between minor histocompatibility antigens can lead to GVHD in bone marrow grafts between HLA identical siblings and, like MHC antigens, can lead to rejection of living related renal grafts(223).

Thus in summary, the MLR has been used for prediction of living related transplant outcome, and low proliferation in the MLR has been found to correlate with increased graft survival and a lower acute rejection rate of

renal allografts. Renal and bone marrow non MHC transplantation antigen disparities may not be detected by the MLR without prior *in vivo* stimulation.

#### 2.2.4 Relationship between PCR HLA-D typing and MLR

Several factors have led to development of new DNA based methods of tissue typing, which have included the difficulty of obtaining reagents and B cells for DR identification but principally that the new methods are easier and reproducible. Disparity has been found between serologically typed HLA-DR matched donor recipient combinations and MLR reactivity and has generated a number of reports of comparisons between accurate typing and the MLR(220) (224). Among serologically DR matched unrelated donor recipient combinations, oligonucleotide typing has been found to identify 53% inaccuracy and all of these identified mismatches have been found to generate a response in the MLR(225). Good correlation between MLR reactivity and mismatches of HLA-DR2, DR4 and DRw52 alleles has been found, although HLA-DRB1\*11 (DR5w11) has been associated with MLR reactivity in oligonucleotide matched combinations, the reasons for which are not yet clear. Mismatches of the DRB3 domain (HLA-DRw52) which have been serologically typed as DR3 and/or DRw6 have been found to stimulate a significantly lower response than other combinations(225). RFLP and oligonucleotide typing of bone marrow transplant related and unrelated donor recipient combinations, has shown that using accurately DNA typed HLA-DR and DQ matching, with an absence of HLA-DP mismatches, gives a lower stabilised relative response (SRR) than one or two HLA-DP mismatches, such that the matched related and unrelated responses have been similar(226). RFLP typing in combination with MLR has been used for matching donors and recipients and for analysing the importance of HLA-DP alleles. Mismatches at HLA-DP sites have been found to correlate with high MLR reactivity (one way and two way MLR) and an increase in occurrence of severe acute graft versus host disease (GVHD) although no correlation has been found with other parameters such as rejection(227). Using human monocyte hybridomas selectively expressing HLA-DR, -DQ, -DP antigens, an investigation of their separate stimulatory capacities in the MLR has been possible and has shown that all three can stimulate lymphoproliferation. In particular, the stimulatory capacity of HLA-DR+ve hybrids has been shown to be markedly reduced in the absence of the HLA-

DQ and HLA-DP determinants indicating the importance of all HLA-D antigens in the alloresponse(228) (229).

Thus in summary, the MLR has been found to correlate particularly with HLA-D disparity and many of the differences between the two in the past have been traced to inaccuracies in the serological determination of HLA-D polymorphisms. The relevance of all MHC class II polymorphisms using PCR based methods such as oligonucleotide typing, to MLR reactivity and graft outcome has yet to be determined.

#### 2.2.5 The MLR in animal models

Animal models of the MLR vary only slightly from the clinical assay. In PVG allogeneic rats, four days incubation of quadruplicate samples of 2 x 10<sup>5</sup> irradiated stimulator lymphocytes and 2 x 10<sup>4</sup> responder lymphocytes followed by 18 hours incubation with [<sup>3</sup>H]thymidine has been found to stimulate T helper cell proliferation. An additional six to eight days incubation has been found necessary to generate resting memory Th cells(230). An investigation of the relative roles of MHC class I and II antigen in the autologous and allogeneic human MLR has involved seven day triplicate culture followed by eight hours incubation with [<sup>3</sup>H]thymidine(231). The results have been expressed as the Stimulation Index (S.I.), which is the response of the recipient to own cells (responders) divided by the response of the recipient cells to the stimulator (donor) cells(222), or expressed more directly as uptake of [<sup>3</sup>H]thymidine counts per minute (cpm)(230) (232) (233). A detailed discussion of the different means of expressing results is given in Chapter Ten.

Thus in summary, the MLR used in animal models is essentially the same as that used clinically but with greater flexibility, for instance in duration of culture. The results of the MLR can be expressed both by the S.I. and direct cpm.

#### 2.2.6 The in vitro alloresponse of the MLR

Details of allostimulation in the MLR have been described by Steinman(234) and have been found to be primarily due to the presence of dendritic cells which are potent antigen stimulators, up to 100 times more potent than T or

B cells. Human dendritic cells have not been easy to identify except morphologically when activated in culture with their large "veil-like" projections giving them an enormous surface area. Their surfaces express high levels of MHC class I and II antigens, leucocyte common antigen and complement receptors but the cells are negative for Fc receptors, surface Ig, macrophage specific antigens, natural killer cell antigens, T lymphocyte antigens (CD1, CD3, CD4, CD5, CD8) and B lymphocyte antigens. Dendritic cells can be positively identified only in mice using the 33D1 monoclonal antibody(235) (236). The capacity of spleen cells to stimulate lymphoproliferation in the MLR has been found to be a function of the number of dendritic cells present rather than any of the other cell types (T, B cells or macrophages). Dendritic cell function has been found not to be inhibited by treatment with  $\gamma$ -irradiation but has been found to be inactivated by heat treatment (45°C for 30 minutes) or fixation (0.15% glutaraldehyde/ formaldehyde for 10 minutes)(234) (237).

Following the incubation of allogeneic rat lymphocytes in an MLR, clusters have been found to form, comprising dendritic cells and T blast cells, followed by the production of IL-2(230). Dendritic cells can proliferate in the cluster but not once they have left it(238). The role of dendritic cells in the alloresponse has been found to be essential to the passenger leucocyte concept(150) in which these cells act as the prime stimulators of an alloresponse against islets or other tissues which carry them. Although it has been found that Th cells are principally stimulated in the MLR, the proliferating cells also include cytotoxic T cells which can be directly stimulated in the apparent absence of T helper cells (CD4+ve)(237) and this includes stimulation by the HLA-DQ and DP loci(228). After a peak of proliferation at four to six days, the T cells have been found to return to a resting stage as T memory cells which can be restimulated in a secondary MLR(230). The naive cells have been found to express the CD45RA isoform of leucocyte common antigen and the memory cells to express the CD45RO isoform.

MHC class II molecules, HLA-DR, -DQ and to a lesser extent HLA-DP, have been considered as the primary allostimulatory molecules in the MLR(216). However, the role of MHC class I has also been investigated using monoclonal antibodies against specific MHC class I antigens (HLA-A2). Blocking the MHC class I antigens has been found to reduce the allogeneic

MLR by 25% (60% reduction using antibodies against both MHC class I and II determinants) and to inhibit, completely, the autologous MLR(231).

Studies by Asano in mice have shown that CD4+ve T cells in the MLR predominantly proliferate to disparate MHC class II stimulators and that CD8+ve T cells proliferate to disparate MHC class I stimulators. However, MHC antigen recognition alone has been found insufficient for T cell proliferation and additional costimulatory signals are required(233). Production of IL-2 by Th cells, although sufficient to produce Tc activation *in vitro*, has been considered to be insufficient for Th-Tc cell collaboration *in vivo*(239). One important costimulatory molecule has been shown to be B7, its receptor on T cells, the CD28 molecule(240) was described earlier in Chapter One.

Thus in summary, the MLR has been shown to be a model for the early stages of the alloresponse in which stimulator antigen is presented, principally by dendritic cells expressing high levels of MHC class II antigen. Clusters of dendritic cells and T cells occur, initiating the proliferation of T cells, principally T helper lymphocytes, together with the production of the cytokine IL-2. A peak of proliferation has been found after about 4 - 6 days followed by maintenance of memory T cells.

2.2.7 Direct and indirect pathways of antigen presentation in the primary MLR

Presentation of peptides by MHC class I and II antigens to the T cell receptors (TCR) of allogeneic Tc (CD8+ve) and Th (CD4+ve) cells respectively, has been called the direct pathway of alloantigen presentation and is considered to be the major effector in early graft rejection(241). In addition, there has been evidence that an indirect pathway of alloantigen presentation exists in which donor or graft MHC molecules may be processed by recipient antigen presenting cells, and presented as 'foreign' peptides to the recipient T cells in combination with recipient or 'self' MHC class II antigen(242) (243). This could explain why CD4+ve Th cells, in the absence of CD8+ve Tc cells, have been found to initiate rejection of MHC class I disparate grafts, involving the presentation of allogeneic MHC class I peptides by recipient MHC class II molecules(244). Generally MHC class I molecules have been found to present endogenous peptides and MHC class II

molecules to present exogenously derived peptides, the TCR has been found to recognise the combined MHC-peptide site although there is some evidence that limited recognition of empty MHC binding sites may also occur(241).

Thus in summary, both direct and indirect pathways of alloantigen presentation have been demonstrated using the MLR and this involves presentation of the antigenic peptides of the stimulator population, both by the stimulator (direct) and responder (indirect) antigen presenting cells.

#### 2.2.8 Cytokines in the primary MLR

Proliferation of T helper cells in the MLR has been found to be associated with IL-2 production(208) which correlates with stimulation of the Th1 subset(176). A lack of IL-2 production has been associated with the generation of 'suppressor' T cells, rather than Th and Tc cells in the MLR and can be demonstrated following heat inactivation of the stimulator population. The production of IL-1 and IL-3 was found in the MLR following heat inactivation of the stimulator population as well as following  $\gamma$ -irradiation inhibition of the stimulator cell population(245). Interleukins IL-1 and IL-6 were found to be produced following mitomycin C and  $\gamma$ -irradiation treatment of stimulator PBMCs in the MLR but to only have a marginal effect on responder T cell proliferation(246).

Because IL-2 production has been associated with activated and proliferating T lymphocytes, its detection (using CTLL IL-2 sensitive cells) has been used as an early indicator of T cell proliferation, thus shortening the length of time for the MLR (mouse and human) from six days to two or three days(247).

Differences have been found to exist in the ability of human endothelial cells to stimulate a proliferative lymphocytic response compared with epidermal cells, fibroblasts and smooth muscle cells which do not have this ability, despite the presence of MHC class II antigen and ICAM-1 on all cells (induced by IFN- $\gamma$  treatment) and the addition of cytokines II-1, II-2 or TNF- $\alpha$ . This may have been due to a lack of costimulatory molecules involved in the early stages of activation or to the generation of inhibitory molecules(248).

The production of TNF- $\alpha$ , and also IFN- $\gamma$ , from T cells activated in the autologous MLR, has been found to suppress T cell proliferative responses in a non MHC restricted manner(249). In the allogeneic MLR, the effect of IFN- $\gamma$  induced downregulation of HLA-DQ and DP in a HLA-DR positive monocyte hybridoma cell line used as the stimulator cell population, was to reduce lymphoproliferation. In contrast, IFN- $\gamma$  was found to upregulate the expression of HLA-DQ and HLA-DP on HLA-DR negative stimulator cells, leading to an increased proliferation in the MLR(229).

Thus in summary, cytokines have been shown to play an important role in the *in vitro* alloresponse. IL-2 has been associated with the proliferation of T helper cells (Th1) and can be used as an early indicator of the T cell response. In addition, IL-1, IL-3 and IL-6 have been detected in the MLR but have not been found to substantially affect T cell proliferation. The combined expression of MHC class II antigen, IL-2 and ICAM-1 in the absence of costimulatory molecules, have been found insufficient to generate a T cell response. The use of cytokine IFN- $\gamma$  has shown that all HLA-D loci have a role in stimulation of the MLR.

#### 2.2.9 The secondary MLR or primed lymphocyte test

Following 48 hours of primary stimulation in the MLR, T cell clusters around dendritic cells can be isolated and have been shown to contain two types of T cell, alloantigen primed T memory cells which are IL-2 insensitive small lymphocytes, and sensitised IL-2 responsive lymphoblasts. These latter cells have been found to respond to alloantigen presentation by dendritic cells, macrophages, small B lymphocytes and pre-stimulated B lymphoblasts according to the expression of cell surface MHC class II molecules. In contrast to the potency of dendritic cells in the primary MLR, at this stage, dendritic cells were found to be only one or two times more potent than the macrophages and B cell blasts and 5 - 20 times more potent than the B lymphocytes(235). The small rounded T memory cells were found to be restimulated in a secondary MLR by dendritic cells as in the primary MLR but more rapidly(230), the addition of IL-2 alone was shown to have no effect on these cells(235).

Thus in summary, the secondary MLR has been found to constitute a rapid restimulation of memory T cells and IL-2 sensitive T cell blasts

produced in the primary MLR and represents a model for the primed *in vivo* response to a specific antigen.

#### 2.2.10 The autologous MLR

Proliferation of lymphocytes to "self" antigens has been observed in the autologous MLR in which responder and  $\gamma$ -irradiated stimulator cell populations are from the same source. Although evidence has been found to link the use of serum in culture to the autologous response(235), the use of cells from specific mouse strains and F1 hybrids have shown that the response is specific(250) (251). Studies with the autologous MLR have shown that dendritic cells, but not macrophages, B cells or T cells, can stimulate a primary T cell autoresponse. This response has been found to be 10 - 30 times less active than the allogeneic response and no subsequent development of the CTL response has been seen. Mouse dendritic cells have been found to form clusters with the T cells, as has been seen in the allogeneic MLR, but clustering has not been observed in the autologous human MLR(235) (237).

The importance of MHC class II antigen in the autologous MLR has been stressed. However, the involvement of MHC class I antigens has additionally been shown by complete inhibition of the human autologous MLR in the presence of anti MHC class I antibody and it has been suggested that autoreactive T cells may respond to MHC class I peptides presented in the context of MHC class II antigens(231).

Thus in summary, the autologous MLR has been found to be mainly driven by MHC class II expression rather than by serum components. Autologous proliferation can be stimulated by dendritic cells, and has been found to be a low response that does not lead to cytotoxic T cell production although MHC class I antigen has been found necessary for the human autologous MLR.

#### 2.2.11 The cytotoxic T cell assay

Cytotoxic T cells have been found to be generated in the primary MLR. To measure the relative proliferation of cytotoxic T cells, responder lymphocytes from a five day MLR were stimulated and used as effector cells

(cytotoxic T cells) in the MLR-CTL assay. Concanavalin A stimulated autologous or allogeneic lymphoid cell blasts, were labelled with  $^{51}$ Cr for one hour at 37°C and used as the target cell population (2 x 10<sup>3</sup> per well) with effector:target ratios of 50:1 to 100:1, in triplicate(252). The amount of  $^{51}$ Cr released into the supernatant by the lysed target cells over a four hour incubation with effector cells, was found to correlate with the number of  $\gamma$ -irradiation counts in the supernatant, and were recorded as percentage lysis using the following formula(211):

### experimental cpm - spontaneous cpm x 100 total cpm - spontaneous cpm

There has been some evidence to show that autologous dendritic cells in the responder population of the MLR can contribute towards the generation of CTLs, because the addition of responder dendritic cells to the coculture of dendritic cell free stimulator spleen cells was found to increase the proliferation of alloreactive CTLs(235).

Thus in summary, the cytotoxic T cell assay (MLR-CTL) has been used to show the generation of Tc cells in the MLR. Firstly, effector cells have been obtained from a primary MLR, then radioactively labelled target cells have been added. The percentage lysis has been used as a measure of the presence of cytotoxic T cells.

# 2.3 The effect of using non-lymphoid tissue to stimulate the MLR

This thesis has aimed to use an adaptation of the MLR in which a non lymphoid cell population is used to investigate the relative contribution of tissue specific antigens to the alloresponse. Several investigators have described a modified MLR assay, particularly using endothelial cells or kidney cells as the primary stimulator population. The use of pancreatic islets in this context is described more fully in the next chapter.

#### 2.3.1 Mixed lymphocyte endothelial coculture (MLEC)

The number of reports of cocultures investigating the response of lymphocytes to non-lymphoid tissues *in vitro* has been relatively few. One report used the MLEC with triplicate cocultures, using  $1 \times 10^6$  responder lymphocytes and  $1 \times 10^5$  mitomycin C treated stimulator lymphocytes per well in Medium TC199 with 20% human AB serum, to investigate the stimulatory properties of human umbilical vein endothelial cells (UVECs). Freshly explanted and cultured UVECs have both stimulated allogeneic lymphocytes to proliferate but to a lesser degree than allogeneic lymphocytes(253).

The ability of human endothelial cells to function as accessory cells in the MLR has been investigated using purified T lymphocytes stimulated by mitogens. No proliferation has been observed in the absence of monocytes from the responder population of pure T cells but the addition of monocytes or allogeneic endothelial cells was shown to lead to a dose dependent mitogen induced proliferation. The optimum number of monocytes required to supplement the purified T cell cocultures in the presence of mitogens was found to be in the range  $5 \times 10^4 - 1 \times 10^5$ . Endothelial cells have been shown to retain this capacity to stimulate purified T lymphocyte proliferation in the presence of mitogens, even following the loss of MHC class II (but not class I) antigen expression after prolonged culture. Activation of the coculture has been found to depend on cell to cell contact between the monocytes or endothelial cells and T cells(254). Unlike endothelial cells, fibroblasts stimulate little proliferation when cocultured with lymphocytes(253) (254).

The kinetic activity of the human MLEC has been found to show a peak proliferation at 7 days with a maximum of just over 50,000 cpm. The results were expressed as cpm with a range of 1,641 - 7,238 cpm for the MLEC and 2,140 - 14,408 cpm and 2,877 - 23,648 cpm for the MLR (n = 8 in each range), cocultures have been incubated in Medium 199 containing 20% human AB serum(253).

Results of mitogen stimulated T cell + monocyte cocultures (triplicate wells) have been expressed as  $\Delta$ cpm (test cpm minus control cpm) for  $10^5$  monocytes per well and were in the range 34,000 - 80,000 cpm (PHA) 23,000 -

30,000 (Con A), 9,000 - 20,000 cpm(PWM) after four days incubation (n = 2)(254).

Thus in summary, the MLEC has been shown to give a lower response than the MLR, relying on cell to cell contact for responder cell proliferation. Endothelial cells can act as accessory cells for mitogen stimulation of purified responder T lymphocytes. In the MLEC there was a peak of responder cell proliferation at seven days and a maximum cpm reported of 50,000 cpm.

#### 2.3.2 Mixed lymphocyte kidney cell coculture (MLKC)

The mixed lymphocyte coculture has been carried out using mouse, canine and human allogeneic kidney cells as stimulators from normal and rejecting transplanted kidneys(255) (256). One aim was to investigate the relative immunogenic effects of putative tissue specific antigens such as a 90Kd antigen found with enhanced expression in rejecting mouse kidneys(251). In a series of experiments, the canine autologous MLKC (3,944 cpm, n = 8) was found to be higher than the autologous MLR (570 cpm, n =14) suggesting stimulation by tissue specific antigens, and the autologous MLKC response was found to be higher using rejecting kidney cells as the stimulator population (8,232 cpm n = 2) compared with normal kidney cells (3,221 cpm, n = 6). In addition, the normal allogeneic MLKC (2,359 cpm, n = 6)10) was less than the MLR (3,326 cpm, n = 11)(256), as found using human cells(255), however, allogeneic canine cocultures containing rejecting kidney cells showed a greater proliferative response (6,823 cpm, n = 2) than those containing normal kidney cells (2,548 cpm, n = 6). The kinetics of the MLR and normal MLKC have shown the canine allogeneic proliferative response to continue to increase at day 9 (MLR = 10,000 - 12,000 cpm, MLKC = 3,000 - 5,000 cpm maximum mean,  $n = 8 \times 2$ ) using triplicate wells and incubating the cocultures in Click's medium containing 15% canine serum(256).

Some correlation was found between the increased level of MHC class II antigen expression and an increased stimulatory capacity in the autologous and allogeneic canine MLKC but this was found to be incomplete, suggesting the importance of co-stimulatory factors as well as tissue specific antigens(256). Similar incomplete correlations were also found between the

expression of MHC class II antigen on rat nephron components and their alloimmunogenicity *in vivo*(257).

Thus in summary, the MLKC response has been used to determine the relative immunogenicity of tissue specific antigens, especially in rejecting kidneys. The autologous and allogeneic MLKC were both found to be higher using rejected kidney cells as the stimulator population. The normal allogeneic MLKC was shown to be lower than the allogeneic MLR with an increasing proliferation at day nine to a maximum of 5,000 cpm. It has been suggested that costimulatory factors, in addition to the expression of MHC class II antigen, are necessary for the allogeneic MLKC response and that tissue specific antigens have a role in the autologous MLKC response.

## 2.4 Overall summary of MHC and MLR in determination of outcome of clinical transplantation

A number of different methods have been used to assess the potential outcome of transplanted allografts. Blood group matching has been the first consideration in choice of recipient as a mismatch has been found to lead to rapid graft failure due to the presence of naturally occurring antibodies against blood group antigens. Another important assay has been the cytotoxic crossmatch, which can be carried out as a conventional cell lysis assay and, more recently, by FACS analysis. The crossmatch is a measure of specific HLA cytotoxic antibodies present in the serum and has been found to correlate with rejection episodes and early graft loss. Transplantation in the presence of a positive cross match has been found to lead to hyperacute rejection and graft loss.

HLA matching has been carried out serologically and more recently using PCR based methods, particularly for HLA-D typing. For renal, and to a certain extent non renal, transplantation, it is now generally recognised that HLA-DR, -B and -A matching is beneficial and prolongs long-term graft survival, so policies are in operation to maximise well matched donor recipient pairs. The frequency of precursor cytotoxic T cells (pCTL) or T helper cells (pTHL), in a potential recipient's blood, measured by LDA analysis varies considerably between individuals and in rodents has been shown to correlate with allograft outcome.

The MLR, which was introduced in 1964 and standardised in 1971, has been used to measure the proliferation of responder (potential recipient) lymphocytes to stimulator (potential donor) lymphocytes and shows a correlation with graft survival as well as accurately typed HLA-D antigen disparity. Unfortunately in the case of cadaver transplantation, there is insufficient time for the 5-day MLR assay to be carried out so use of the MLR has been restricted to living related donors. The *in vitro* response has been shown to involve dendritic cell clustering prior to an induction of T cell proliferation, mainly T helper cells, and production of IL-2. Both the direct and indirect pathways of allopresentation have been demonstrated in the MLR. In addition to IL-2 production in the primary MLR, IL-1, IL-3 and IL-6 have been detected and although they appear to have little or no effect on the proliferating T helper cells. Cytokine induced upregulation of MHC class II antigen has shown that all HLA-D loci have some role in the MLR.

The secondary MLR or primed lymphocyte test, which has been described as a rapid restimulation of T cells which have responded in the primary MLR, can be used to model the responses of primed lymphocytes to antigen. The autologous MLR which has been shown to be stimulated by dendritic cells, as has the allogeneic MLR, has been used to identify tissue specific antigens and potential self reactive T cells.

Two assays which have not become part of the testing for beneficial donor recipient matches include the MLR-CTL and tissue specific MLKC but they are included here for completeness. The MLR-CTL can be used to determine the relative numbers of cytotoxic T cells in the primary MLR. Effector cells are used from a five day MLR and the percentage lysis of isotope labelled allogeneic or tissue specific target cells recorded. The mixed lymphocyte culture can also been adapted to use non lymphoid cells as stimulators, particularly endothelial cells (MLEC) and kidney cells (MLKC). In each case the coculture response has been less than the MLR and with extended kinetics, the MLEC showing a peak at seven days and the MLKC continuing at nine days.

### The Next Chapter...

In addition to the above assays, studies have been carried out based on the MLIC, an adaptation of the MLR using pancreatic islets as the stimulator cell population. As these published studies are fundamental to the thesis, they are described in detail in Chapter Three.
### CHAPTER THREE

### CHAPTER THREE CONTENTS

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### CHAPTER THREE

### MIXED LYMPHOCYTE ISLET COCULTURE (MLIC)

### 3.1 Assessing the potential immunogenicity of human islets

Of all the tissues used for transplantation, isolated human islets of Langerhans have presented an almost unique opportunity for investigations of diabetes and transplantation studies. Isolated pancreatic islets can be maintained in culture medium before transplantation which potentially allows sufficient time for estimates of culture sterility, islet viability and function, time for assays to measure islet immunogenicity in specific donor recipient combinations and for immunomodulation procedures. Transport between centres either in culture medium or as cryopreserved islets potentially allows beneficial donor recipient matching

Although animal models of islet transplantation have been relatively successful and have provided valuable insights into the immunogenicity of islet grafts, human islet transplantation has had limited success and the specific nature of human islet immunogenicity has had to be addressed(3). The purpose of this thesis has been to develop the MLIC - an adaptation of the MLR using islets as the stimulator population instead of lymphocytes, and to use this as an *in vitro* model of human islet immunogenicity. Published studies using the MLIC and similar *in vitro* models have formed the basis for this thesis and are described below.

3.1.1 Introduction to the published MLIC and related studies

Summaries of the published MLIC studies are briefly described below, in turn, by author and broad aims. In addition, data from the studies can be found at the end of Chapter Three, in tabulated form for easy reference, as Tables 3.1 - 3.18. Of these, Tables 3.1 - 3.9 describe the detailed methodology and parameters and Tables 3.10 - 3.18 describe the results.

The aims, methods and results of published studies are described for the rodent MLIC, canine MLIC, fetal porcine MLIC, human MLIC, xenogeneic MLIC and secondary MLIC. For completeness, MLIC-CTL studies and measurements of insulin levels to indicate loss of function and direct islet cell killing by leucocytes have been included.

Published studies have used the MLIC and associated methodology to look at a number of immunological problems relating to the immunogenicity of islets as well as more general aspects of the immune response. A small number of investigations have used the MLIC to focus on the nature and progress of the lymphocytic response in Type I diabetes but the major efforts have been directed towards reducing the immune response to transplanted allogeneic islets.

3.1.2 Use of the MLIC and related assays to investigate Type I diabetes

The nature of the lymphocytic response in diabetes has been shown using the MLIC loss of function assay in which the ability of lymphocytes from Type I diabetic patients to suppress insulin release, has been measured. This phenomenon was first described by Boitard in 1981(258).

Using the MLIC-CTL assay, Charles (in 1983) investigated the ability of human lymphocytes from diabetic patients and normal individuals, to kill rat islet cells(259) McEvoy (in 1981)(260) described the appearance of insulitis around islets in the multi-dose, compared to the single dose, streptozotocin mouse model and has also described an *in vitro* MLIC-CTL which has shown that lymphocytes from the multi-dose model only, are capable of killing islet (RIN) cells. More recently, Harrison used the MLIC to investigate the effect of human lymphocytes from patients with preclinical and clinical Type I diabetes as well as normal patients(261) on MHC matched human islets and fetal pig proislets.

Investigations combining the diabetic syngeneic and alloresponses using the MLIC-CTL, include the lysis of islets by lymphocytes from diabetic and diabetes-prone BB rats, as described by Mackay in 1985(262).

Thus in summary, a range of studies starting in 1981, used islet and lymphocyte coculture in several forms, as a model for investigating the nature of Type I diabetes. These studies included the streptozotocin treated mouse and BB rat models as well as the use of lymphocytes from human diabetic patients in combination with autologous human islets and xenogeneic mouse or fetal pig islets.

3.1.3 Use of the MLIC to investigate potential responses to transplanted islets

The earliest studies which have described the use of the MLIC as a model for islet transplantation were reported in 1981, by Rabinovitch, using a canine MLIC and involving multiple combinations of responder cell, stimulator cell and primed cell populations to investigate allogeneic and islet specific antigens on islets(263). Roth, using human islet enriched pancreatic fractions(255), investigated the relative role of MHC class II in the allogeneic and autologous response to islets.

Schwizer (1984) studied the role of macrophages in islet destruction using a MLIC loss of function assay to measure direct killing(61), which has had implications both for the development of diabetes and for the potential immunomodulation of islets prior to transplantation. The MLIC has been used to measure the immunomodulation of islets, and the first studies of this type were carried out by Shizuru (in 1986), using an immunotoxin coupled to MHC class II antibody(264).

Starting in 1987 and working with a mouse model, Stock reported a considerable number of studies using the MLIC-CTL in which the generation of cytotoxic T cells, rather than T helper cells (as in the MLIC), were measured. These studies aimed to investigate unprimed and primed lymphocytic killing of islets and the relative role of MHC class I and II antigen expression in the generation of these cytotoxic T cells(35) (242) (252) (265) (266).

Demidem (in 1988), working with human insulinoma cells, investigated the effect of long term culture (30 days) on HLA-DR antigen expression(267). This was followed in 1989, by a large number of studies using the MLIC and related systems to study the immunomodulation of islets. Yoneda(268) used

fetal pig islet cells to show the relationship between low temperature culture and the MLIC response, and the additional effect of major and minor transplantation antigen mismatches on the response of lymphocytes to islets. The effect of low dose irradiation on islets and the relationship between transplantation in cyclosporin-treated allogeneic rats and proliferation in the MLIC, was reported by James(121). Ulrichs(29) (269) immunomodulated isolated human islets using monoclonal antibodies against MHC class II antigens to remove the passenger leucocytes. Lloyd(158) investigated MHC class II antigen expression of isolated islets after incubating islets with anti MHC class II antibody while still in the pancreas, prior to isolation, and compared the in vitro response with pancreas graft immunogenicity. Also, as part of the immunomodulation studies, Markmann investigated the reduction of MHC class II antigen expression after culture(39), and then studied the effect of cytokine upregulation of MHC antigen expression on islet immunogenicity(162). Additional studies of IFN-y regulation of lymphoproliferative responses were reported in 1993 for the MLIC and MLKC(270).

Kenyon used canine islets to show the fine balance between effective immunomodulation of islets by UV irradiation and minimal damage to islet function(153).

A number of studies using the MLIC were reported at the Fourth International Congress on Pancreatic and Islet Transplantation held in Amsterdam 1993 all of which have aimed to functionally remove passenger leucocytes from pancreatic islets. Following on from Kenyon's work, Behamou investigated the effects of UV-B irradiation on human(271) and pig(272) islet immunomodulation and function. Another study from the same group(130) looked at the effect of culture temperature on human islets, similarly, Zeevi(273) also looked at the effect of low-temperature culture of islets as well as the effect of purity on *in vitro* immunogenicity. Finally, Gramberg(274) studied the relative immunogenicity of isolated pig islets after isokinetic centrifugation to increase purity.

Thus in summary, since 1981 there have been a number of studies describing a whole range of investigations which have used the MLIC to identify aspects of immune responses to autologous and allogeneic islets. The majority of these have been directed towards immunomodulation and

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reducing the immunogenicity of islets by removing MHC class II positive passenger cells, with the ultimate aim of improving the survival of human islets after allogeneic transplantation.

### 3.2 Methodology of the MLIC used in published studies

The following paragraphs describe the published MLIC and related studies in some detail, by the methodology and then the results. Eighteen tables have been prepared as an easy reference and are included as separate pages at the end of Chapter Three. Tables 3.1 - 3.9 summarise the methodology and Tables 3.10 - 3.18 summarise the results of published MLIC studies. Each group is divided according to the species and model used so that Tables 3.1 and 3.10 concentrate on the primary MLIC in rodents, Tables 3.2 and 3.11 on canine primary MLIC, Tables 3.3 and 3.12 on fetal porcine primary MLIC and Tables 3.4 and 3.13 on the human primary MLIC. In addition, information on studies using the secondary allogeneic MLIC and different species, are shown in Tables 3.5 and 3.14 and studies on the xenogenic MLIC are summarised in Tables 3.6 and 3.15. Two pairs of tables describe studies in which the killing of target cells has been measured by radioactive chromium release as in the MLIC-CTL assay (Tables 3.7 and 3.16) or by loss of insulin secretion (Tables 3.8 and 3.17). Lastly, details are given of the mixed lymphocyte coculture experiments using stimulator cell populations from a source other than lymphoid tissue or islets (Table 3.9 and 3.18).

### 3.2.1 The primary MLIC

The primary MLIC has been adapted from the MLR for which lymphocytes, as responders, are mixed with islets, islet cells or other cells as stimulators, from the same source (autologous) or an MHC disparate (allogeneic) source, and incubated together to allow lymphocyte stimulation and proliferation. This proliferation has been found to consist mainly of T helper cells stimulated by MHC class II antigen presentation associated with the stimulator cell population. The level of proliferation can be measured by the uptake of [<sup>3</sup>H]thymidine by the dividing cells which is recorded as counts per minute (cpm). See Figure 3.1 which describes the primary MLIC and MLR method.

### Method for MLIC and MLR.



Fig 3.1 Method of the primary MLIC and MLR.

The information given in Tables 3.1 - 3.9 can be referred to and have been summarised below. The variations in the basic MLIC methodology used by each author, can be identified from the tables so have not been separately referenced.

Stimulator cell populations have included rat, dog, fetal pig and human islets, insulinoma cells or RIN cells with single cells at  $1 - 5 \times 10^5$  cells per well or 25 - 200 whole islets per well, the cells or islets have been used fresh, cultured, cryopreserved or treated with  $\gamma$ -irradiation or mitomycin C.

Responder cell populations have included between  $1 \ge 10^5$  and  $1 \ge 10^6$  lymphoid cells per well from peripheral blood, the spleen, lymph nodes or as purified T cells. For comparison, the MLR has been used with  $\gamma$ -irradiation or mitomycin C treated PBLs, lymph node cells, splenocytes or dendritic cells.

The plates used have included both round and flat bottomed microtitre plates (96 wells) but reports have rarely specified type of microwell plate and it is probable that tissue culture treated plates have generally been used. Microwells have been prepared as triplicate or quadruplicate samples, bulk culture has only been used to generate cells for the secondary MLIC or the MLIC-CTL.

RPMI has been shown to support lymphocyte growth(275) and has been the preferred medium for MLR and MLIC studies described here, except the work by Kenyon with dog tissues which used Waymouth's medium. The addition of serum has varied according to the species and the study series and has included 2% syngeneic rat or 10% foetal calf or bovine serum for rodent cell cocultures, 15% normal dog serum for dog cell cocultures and 5% autologous or 10 - 15% human AB serum for human cell cocultures.

Incubation of the coculture for the MLIC has varied from three to nine days, with the majority incubated for six days. Two studies on the kinetics of the MLIC have been for 3, 5, 7 and 9 days. The isotope marker used to measure proliferation has been [<sup>3</sup>H]thymidine in every case, used at 5 - 20  $\mu$ Ci/ml and incubated with the proliferating cells for 6 hours or 18 - 24 hours before harvesting.

### 3.2.2 The secondary MLIC or primed lymphocyte test

In this type of assay, the responder lymphoid cells have been 'primed' using a variety of stimulator cell populations in what is effectively a primary MLR or MLIC which has been found to generate T helper cells which progress to

a resting stage. Restimulation of the lymphoid cells with the same or related MHC type as the stimulator cells leads to a more rapid proliferation of the primed T cells, which is measured by the uptake of [<sup>3</sup>H]thymidine and is recorded as counts per minute (cpm) as for the primary MLIC. See Figure 3.2 which describes the secondary MLIC method.

### Method for Secondary MLIC and MLR.



Fig 3.2 Method of the secondary MLIC, MLAC and MLR.

To summarise the information in the tables, the following conditions have been used for the secondary MLIC:

Priming has been carried out using PBLs from primary MLR or MLIC allogeneic or autologous bulk or pooled microcultures after ten days. Stimulators used for this have been  $\gamma$ -irradiated (1,500 rads) cells in the form of islets (100) or islet cells (1 - 2 x 10<sup>5</sup>), hepatocytes or lymphocytes (1 - 2 x 10<sup>5</sup>)(263). Another method used purified CD4+ve T cells in bulk cultures with  $\gamma$ -irradiated allogeneic stimulator lymph node cells added at two week intervals together with 10% supernatant from Con A activated splenocytes(39). These sensitized T cell responders from the bulk cultures or pooled microcultures were used as 5 x 10<sup>4</sup> or 1 x 10<sup>5</sup> cells per coculture well.

Stimulation of primed lymphocytes has been carried out using allogeneic or autologous islet cells ( $5 \times 10^3$ ,  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $5 \times 10^4$ ) (fresh, culture treated, IFN- $\gamma$  treated) or 1 - 2  $\times 10^5$  fresh islets (100) or 150 IEQ/ml, 300 IEQ/ml, 600 IEQ/ml (islet suspensions), or for the secondary MLR, lymph node cells ( $5 \times 10^3$ ,  $1 \times 10^4$ ,  $2 \times 10^4$ ,  $5 \times 10^4$ ) or dendritic cells (80,  $1.5 \times 10^2$ ,  $3 \times 10^2$ ,  $6 \times 10^2$ ).

Incubation of primed lymphocytes and stimulator cells has been for three days(39) (273) or as a kinetic study for 2, 3, 5, 7, or 9 days(263).

The isotope used to measure proliferation was  $[^{3}H]$ thymidine in each case, used at 20  $\mu$ Ci/ml or 1  $\mu$ Ci/well and incubated with the proliferating cells for 18 hours before harvesting.

### 3.2.3 Stimulation of purified T cells by Con A

These experiments have investigated the stimulation of  $2 \times 10^5$  purified T cells in the presence of Con A with or without APC (which are necessary for Con A stimulation) and this has been shown to be independent of endocrine parenchymal MHC class II expression. Stimulator cell populations used have included syngeneic splenocytes, fresh islets, cultured islets or culture + IFN- $\gamma$  treated islets, also RINm5F cells (which lack APC). All of these have been used at a concentration of  $10^5$  cells per well and with triplicate samples. In each case, [<sup>3</sup>H]thymidine has been used at 20 µCi/ml for ten hours before harvesting on day three(39).

### 3.2.4 The MLIC-CTL assay

The method used for the MLIC-CTL has similarities with the secondary MLIC or primed lymphocyte assay, in that responder lymphocytes are normally primed and proliferate in a primary MLR or MLIC. These primed cells are called effector cells. The MLIC-CTL assay aims to measure the relative number of cytotoxic T cells produced by the primary culture and does this with the addition of labelled 'target' cells which are lysed by the MHC class I restricted cytotoxic T cells.

To summarise the information in the tables, the following have been used for the MLIC-CTL:

Effector lymphoid cells from a variety of sources and the different methods used to prime them, distinguish each group of studies. One series of studies used splenocytes from BB rats(262), another used lymph node cells from alloimmunised rats(39), another used splenocytes from streptozotocin multiple dose or single dose treated mice(260) and another used human PBLs which were unprimed *in vitro* but originated from Type I diabetic patients or normal controls(259). Splenocytes have also been used as effector lymphoid cells after selective depletion of CD8+ve T cells, CD4+ T cells or APCs(242).

Primary incubations to generate the effector cells have used lymphocytes for two days duration treated with Con A, for six days using  $\gamma$ -irradiated allogeneic lymph node cells, for five days using mitomycin C treated RIN cells (25 µg/ml for 10 minutes), or for five days using whole islets (50 islets) or purified/ unpurified beta cells (5,000 cells).

Most incubations between primed effector cells and target cells have been carried out in microwells with three or four repeats per sample, with the exception of McEvoy who used  $11 \times 50$  mm plastic tubes(260), and six repeats per sample, and Charles(259) who also used tubes but three repeats per sample.

Effector cell number has ranged from  $1.25 \times 10^5$  to  $4 \times 10^6$ , with  $5 \times 10^4$  -

5 x  $10^5$  for the larger tubes and 3 - 5 x  $10^5$  cells per microwell in other studies.

The target cells have been  ${}^{51}$ Cr labelled at a concentration of 200 - 330  $\mu$ Ci/ml for 60 - 90 minutes. The  ${}^{51}$ Cr labelled target cells for the CTL part of the assay have included allogeneic RIN cells or GH3 pituitary cells, islet endocrine cells, syngeneic or allogeneic Con A blasts, autologous Con A blasts or the allogeneic P815 (mouse H-2<sup>d</sup>) cell line.

Coculture of primed effector and labelled target cells has been in the target: effector ratio of 1:2 - 1:20 or 1:30 or 1:6.25 - 1:200 or 1:50 - 1:100 with an incubation time of four, five or eight hours in RPMI + 10%FCS, or in one case(39) 2% syngeneic rat serum.

### 3.2.6 MLIC direct killing measured by insulin depletion

Three studies have measured lymphocyte mediated damage and lysis of islets by the depletion of insulin rather than  $^{51}$ Cr release. They have included the incubation, between 18 hours and six days in microwells, of human PBLs(258), rat splenocytes(262) and mouse macrophages(61) with xenogeneic mouse islets, syngeneic islet cells/allogeneic RIN cells or syngeneic/allogeneic islet cells respectively. Estimations of insulin levels in the remaining target cells or in the supernatant have been carried out using static incubation and radioimmunoassay (RIA) where appropriate.

3.2.6 Primary MLIC using a variety of stimulator cell populations

Studies using an adaptation of the primary MLR with stimulator cell populations other than lymphocytes or islets, have investigated the *in vitro* relationship between MHC class II antigen expression and immunogenicity of kidney, liver cells and fibroblasts compared with leucocytes(276). Esquenazi investigated the MLKC in dog using lymphocytes from normal and rejecting kidneys(256).

In addition, two authors compared the MLIC with the MLKC and MLR, Roth studying the different peak human allogeneic and autologous proliferative responses(255) and Fernandez studying the effects of the presence of IFN- $\gamma$  and anti IFN- $\gamma$  antibody on compatible and incompatible canine

cocultures(270). Lastly, cocultures were studied using cells with minimal immunogenicity as potential 'neutral allografts' including fibroblasts, smooth muscle cells and epidermal cells. The response to these cell types was observed with or without IFN- $\gamma$  stimulation and compared to that of endothelial cells(248).

Thus in summary, studies of islet immunogenicity have used a variety of methodologies including the primary and secondary MLIC, the MLIC-CTL and a direct killing assay using insulin loss as the marker of cell death. In addition, tissues used as the stimulator cell population have included, in addition to islets and lymphocytes, kidney cells, liver cells, fibroblasts, smooth muscle cells, epidermal cells and endothelial cells. Rodent models including mouse and rat have been used, as well as larger animal models such as pig and dog, and the immune responses studied have been autologous, allogeneic and xenogeneic. Most studies have been carried out in microwells with approximately 10<sup>5</sup> responder lymphocytes and up to 100 stimulator islets. RPMI with 10% serum added has been favoured as the incubation medium and the duration of the MLIC has predominantly been five to six days. Cell proliferation has been measured using [<sup>3</sup>H]thymidine incorporation and the majority of results expressed as counts per minute (cpm).

### 3.3 Results of the MLIC in published studies

3.3.1 The primary MLIC - titration, kinetics and levels of proliferation

Titration studies have been reported using different numbers of islets per microwell including 2, 20, 50, 100 rat islets(264), 25, 50, 100, 200 dog islets(263), 5, 10, 20, 30, 40, 50 crude islet preparations(269) and 150 IEQ/ml, 300 IEQ/ml and 600 IEQ/ml human islets from preparations of variable purity(273). These results showed the peak islet number to be variously 100 islets per well(263) (264), 40 islets per well(269), and 600 IEQ/ml(273).

Only two kinetic studies have been carried out which investigated MLIC allogeneic proliferation on days 3, 5, 7 and 9 in the dog(263) and human(255). These studies showed that the time course is important, such

that both authors record an increasing proliferation up to day nine in the allogeneic MLIC without the establishment of a true peak response. The MLIC using dog 'MLC non reactor' combinations showed a true peak response at day seven for allogeneic and autologous islets(263) and an autologous peak response at day seven for human autologous islet enriched pancreas fractions(255).

The results of nine of sixteen studies on the primary MLIC have been expressed as cpm and seven of sixteen expressed as stimulation index. Peak results for the primary allogeneic MLIC have ranged from 6,000 to 62,000 cpm and the stimulation index from 1.5 to 52. Results of the autologous MLIC have been found to range from 1,300 to 10,000 cpm and the stimulation index from 1.26 to 2.1. The equivalent allogeneic MLR results have ranged from 2,453 to 34,600 cpm, and this can be compared with an autologous MLR carried out as part of the MLIC studies which has been recorded as 300 cpm(39).

The question of 'back proliferation', that is the proliferative response of cells in the stimulator population in response to the responder population, has been addressed by Rabinovitch(263). In these studies 100 islets were used as the stimulator population and the passenger cells were found to constitute approximately 2% of the islet cell population. This was calculated to represent the stimulatory capacity of 4,000 lymphocytes per well which Rabinovitch found to be insufficient to stimulate responder cell proliferation. However, this finding has to be considered in the light of Steinman's work with dendritic cells, which show that as few as 1,000 mouse dendritic cells can stimulate allogeneic proliferation of approximately 10,000 cpm(235).

The results of immunomodulation studies are described later.

Thus in summary, studies on the titration of islets in the primary MLIC and the kinetics of the response have reported a wide variation in optimal islet number and relative proliferation with a peak duration of seven days or more which is longer than the five to six day incubation used for much of the MLIC work. More authors have expressed their MLIC results as cpm than as stimulation indices.

### 3.3.2 The secondary MLIC or primed lymphocyte test

The secondary lymphocyte response has been used to measure the proliferation of primed T lymphocytes to stimulator cell populations of islets(263) (273), lymph node cells or dendritic cells(39). The responder cell populations have been primed using islets, islet cells(263) (273) or irradiated lymph node cells supplemented with Con A supernatant(39).

The secondary allogeneic MLIC has been shown to generate about half the level of proliferation of the secondary MLR similar to the results seen in the primary MLIC. The peak responses have been recorded as 6,000 cpm for the secondary MLIC and 12,000 cpm for the secondary MLR in the dog(263), and 8,000 cpm for the secondary MLIC and 18,000 cpm for the secondary MLR in the rat(39). One study reported a stimulation index of 22 (70 - 90% pure islets) for the human secondary MLIC(273).

The preprimed proliferative response has been shown to be related to the number of stimulator cells used(39) and to follow a curve over time, increasing from two days with a peak at three to five days and finishing at about nine days(263). Studies with the allogeneic and autologous secondary MLIC and MLR using islet or lymphocyte primed T cells, showed that priming T cells with allogeneic islets abrogated their subsequent response to allogeneic lymphocytes but proliferation was subsequently recorded against allogeneic islets. Priming the responder lymphocytes with allogeneic islets, which indicated that both islets and lymphocytes can be used to prime T cells for the secondary MLIC, but that islets cannot be used to prime T cells for the secondary MLR(39).

Thus in summary, the secondary MLIC shows the same rapid proliferation of primed responder lymphocytes as seen in the secondary MLR but the MLIC was found to be lower than the secondary MLR as would be predicted from the primary response. However, lymphocytes primed with islets were found not to respond to subsequent stimulation with lymphocytes, but in contrast, both islets and lymphocytes were found to have the capacity to prime responder lymphocytes to islets.

### 3.3.3 Immunomodulation studies using the MLIC

Most of the work using the MLIC has been directed towards reducing islet immunogenicity. Although disassociation of islets to single cells has been found not to alter the MLIC response, neither has the treatment of different cell types (lymphocytes and hepatocytes) with collagenase used during the digestion of the pancreas(263). Immunogenic MHC class II positive passenger cells in islets have been found to survive digestion of the pancreas and islet separation so that they are found in isolated islets and islet preparations in culture(26) (27). Immunomodulatory methods designed to inactivate or remove these immunostimulatory cells have included increasing purity of the islet preparation, low temperature culture, low dose  $\gamma$ - or UV irradiation and treatment with antibodies against MHC class II antigen bearing cells, in addition the effect of cytokine treatment of islets on proliferation in the MLIC has been studied. These different approaches are summarised below.

3.3.3a Effect of purity of isolated islets on immunogenicity

Increasing the purity of islet preparations has been shown to decrease the immunogenicity and capacity to stimulate allogeneic lymphocyte proliferation in the MLIC. Increasing the purity of human islet preparations from 30 - 50% to 70 - 90% was shown to reduce the MLIC response by approximately half (from a stimulation index of 52 to 29 for 600 IEQ/ml)(273) and from 6,000 cpm to 2,000 cpm for crude islet preparations containing approximately 40 islets compared to 40 dithizone handpicked islets(269). Isokinetic gradients have been used to separate out the small fragments (<100 µm diameter) remaining in islet preparations after an initial density gradient centrifugation to separate the majority of exocrine tissue from the islets. This secondary purification has resulted in a reduction in the xenogeneic (human PBLs vs 100 pig islets) MLIC response of 58.4 - 67.4% dependent on the volume of the gradient(274).

Thus in summary, it has been shown that the increased purity of islet preparations correlates with decreased immunogenicity in the MLIC both after density gradient centrifugation, which produces islet preparations of variable purity, and after secondary isokinetic centrifugation.

3.3.3b Effect of culture on the immunogenicity of islets in the MLIC As can be seen in Table 3.19 below (included in the cytokine section), culture for 14 days reduced the allogeneic rat MLIC response from 7.4 to 1.6 (stimulation index) but did not affect the already low autologous response(39). Human insulinoma cells used as stimulators in the MLIC, either fresh, or after culture for one month at 37°C, were found to have lost the 2% of cells expressing MHC class II antigen in the crude insulinoma preparations(267). Stimulation indices in the allogeneic MLIC have been shown to fall from 7 and 3 to 1.6 after culture. Little difference was shown in the stimulation indices of the pre (1.26) and post (1.04) culture autologous response to insulinoma cells(267). Similarly low temperature culture (24°C) for one week was found sufficient to decrease the secondary human MLIC (pPLT) response approximately five-fold to background levels when stimulated by islet suspensions containing 600, 300 or 150 IEQ/ml(273). Using single cell suspensions of human islet cells and three to four days low temperature (24°C) culture, a reduction of stimulation index in the MLIC from 18.3 to 6.8 (n = 10) was found comparable with the stimulation index of islet cells cultured for eight to nine days at 37°C(130). Earlier the same group showed a reduction in MLIC response following low temperature of fetal pig islet cells for one week although culture for more than eight days at 24°C was found to have a deleterious effect on islet cells(268).

Thus in summary, these studies have all shown a reduction in the primary and secondary MLIC following culture of the stimulator islet population, and showed that incubation at low temperature (24°C) can produce the same effect over a shorter time scale.

3.3.3c Effect of  $\gamma$  and UV irradiation of islets on immunogenicity in the MLIC Using a rat model, treatment of islets with low doses of  $\gamma$ -irradiation was shown to correspond to a dose-related reduction in proliferation of T cells against the islets in the MLIC. This was found to correlate with increased graft survival *in vivo* provided that the rats were immunosuppressed with cyclosporin(121). Immunomodulation was also demonstrated using UV irradiation of whole islets or dispersed islet cells in the canine MLIC(277) and this showed some degree of correlation with prolonged islet graft survival in immunosuppressed unrelated dogs(153). A similar reduction in MLIC immunogenicity induced by UV-B irradiation was shown using allogeneic fetal pig islets (67 - 91% reduction at 300 - 500 J/m<sup>2</sup>)(271) or

human islets  $(43 - 81\% \text{ at } 300 \text{ J/m}^2 \text{ and } 51-70\% \text{ at } 500 \text{ J/m}^2)(272)$  without substantial impairment of islet function as measured by static incubation, perifusion and histology after implantation into athymic mice.

Thus in summary, both  $\gamma$ - and UV irradiation have been shown to reduce the immunogenicity of islets in the MLIC with minimal impairment of islet function and some degree of correlation with increased graft survival *in vivo*.

### 3.3.3d Effect of anti MHC class II antibody treatment on islet immunogenicity in the MLIC

Immunomodulation techniques using antibodies have included treatment of the whole pancreas with anti MHC class II antibody and complement to destroy the MHC class II +ve antigen presenting cells in the islets prior to isolation. This led to a reduced MLIC response although the reduction in immunogenicity was less clear *in vivo*(158).

Other techniques have involved treating the islets after isolation, for instance, using 30 - 40 islets as the stimulator population in the MLIC, pretreatment with anti HLA-DR/DP antibody showed a reduction of the lymphocyte proliferative response from 22,000 cpm to 10,000 cpm(269). Similarly, the incubation of human islet enriched fractions with anti MHC class II antibody showed a reduction in the proliferative response of the MLIC (9,000 cpm vs 2,000 cpm) and this was further reduced by passing the islet enriched fractions through an anti-DR sepharose column (<1,000 cpm) to remove the MHC class II positive cells from the fractions before use(255). A more moderate reduction of the proliferative response in the autologous MLIC was seen after anti MHC class II antibody treatment of the islet fractions (10,000 cpm to 4,000 cpm and 2,000 cpm)(255). Studies in vitro using rat islets showed that treatment of islets with an anti MHC class II immunotoxin (anti MHC class II antibody coupled to ricin toxin) can reduce the MLIC response in a dose dependent manner which is thought to be due to removal of the immunogenic cells from islets (the antibody-toxin conjugate has been shown not to impair insulin release from islets). The abrogation of the MLIC response by concentrations of  $10^{-7}$  M and  $10^{-8}$  M MHC class II immunotoxin, indicate the important role for MHC class II in the stimulation of the MLIC(264).

Thus in summary, MHC class II antibody treatment with complement, either before or after isolation, or coupled to ricin toxin, which are all treatments designed to remove antigen presenting cells from islets, have shown some correlation with a reduced MLIC response although with no clear *in vivo* correlation.

3.3.3e Effect of cytokine treatment on islet immunogenicity in the MLIC The cytokine IFN- $\gamma$ , was used to increase MHC expression on islets in order to study the effect of altered MHC antigen expression on islet immunogenicity as measured by the proliferation of purified CD4+ve T helper cells in the MLIC compared to the MLR(39) (162). The results are shown in some detail in Table 3.19.

Table 3.19. Comparison of MLIC response using fresh, cultured and IFN- $\gamma$  treated islets.

Stimulator- responder combination	Splenocytes	fresh islets	cultured islets	IFN-y treated islets
Allogeneic	34.6 <u>+</u> 3.2	7.4 <u>+</u> 1.5	1.6 <u>+</u> 0.7	0.9 <u>+</u> 0.2
Syngeneic	0.3 +0.1	1.3 +0.3	1.8 + 0.1	1.4 + 0.2

Allogeneic and syngeneic MLIC results mean cpm  $\pm$  S.D. x 10<sup>-3.</sup> (39).

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From the results shown in Table 3.19, it can be seen that although freshly isolated allogeneic islets were found to cause less proliferation than stimulator lymphocytes, cultured islets (14 days), with or without the addition of IFN- $\gamma$ , invoked only a minimal proliferative response. Therefore no correlation was seen between MHC class II expression on the islet endocrine or parenchymal cells and the proliferation of T helper cells in the MLIC. However, other studies using the MLIC-CTL and *in vivo* models have shown that a positive correlation exists between MHC class I expression and islet allograft immunogenicity(39).

The secondary MLR or MLIC utilising presensitized alloreactive lymphocytes has given similar results to those obtained using the primary MLR or MLIC. For this the stimulator population has included dendritic cells, lymph node cells, fresh islets and cultured islets with or without IFN- $\gamma$ treatment. It has also been shown that purified T helper cells do not proliferate in response to Con A stimulation in the absence of antigen presenting cells but the addition of  $10^3$  syngeneic macrophages,  $10^2$ purified dendritic cells or fresh islets can restore the Con A response. However, this response has not been found to be restored by the addition of cultured (dendritic cell free) islets before or after IFN- $\gamma$  treatment(39).

Thus in summary, it has been shown using the MLIC, that treatment of islets with the cytokine IFN- $\gamma$ , cannot restore the effects of depleting passenger MHC class II positive cells from islets. However, enhanced MHC class I expression caused by IFN- $\gamma$  has been found to correlate with increased immunogenicity in the MLIC-CTL and increased rejection rates *in vivo*.

### 3.3.4 The role of islet specific antigens in the MLIC

The role of tissue specific antigens has been studied using the differences between the allogeneic and autologous MLIC and MLR. The allogeneic MLIC response was shown to be less than the allogeneic MLR (6,000 cpm vs 12,000 cpm) and the autologous MLIC response to be lower (3,000 cpm vs 6,000 cpm) than the allogeneic MLIC. However the autologous MLIC was shown to be considerably higher than the autologous MLR (3,000 cpm vs <1,000 cpm) suggesting that islet specific antigens as well as alloantigens have a role in stimulating lymphocyte proliferation. Stimulatory tissue specific antigens have not been found on all cell types as has been demonstrated using dog liver cells as the stimulator population cocultured with responder autologous lymphocytes which showed a low autologous response (1,200 cpm) comparable to the autologous MLR(263).

A comparison of the T lymphocyte response to kidney cells (MLKC) and non T lymphocytes (MLR) as well as islets (MLIC) has been used to further support the role of tissue specific antigens in the immune response. The autologous MLKC (peak = 9,148 cpm at day five) showed considerable proliferation although less than the allogeneic MLKC (peak = 28,738 cpm at day seven). In addition, the autologous MLIC (peak day seven) was found to be similar to the allogeneic MLIC (peak day nine) (12,000 cpm) and the removal of MHC class II positive cells, using an anti-DR sepharose column, lowered the proliferative response to allogeneic islets (2,000 cpm) more than the response to autologous islets (4,000 cpm)(255).

Thus in summary, it has been shown that islet specific antigens are involved in the autologous response to islets *in vitro*.

### 3.3.5 The effect of insulin levels on the MLIC

One group has studied the effect of insulin on lymphocyte proliferation in the autologous and allogeneic MLR. Exogenous porcine insulin was added to the coculture wells at three concentrations - 0.1  $\mu$ g/ml (about the level found to be in microwells containing 100 islets and 10<sup>5</sup> lymphocytes) which has been found to be stimulatory (20,000 cpm vs 12,000 cpm at day nine), 1  $\mu$ g/ml, which has been found to have no effect, and 10  $\mu$ g/ml which has been found to be inhibitory (<1,000 cpm vs 12,000 cpm at day nine). The effect of different levels of insulin secreted by the islets in the culture media (72 - 127 ng/ml) for up to seven days, was found to be the same for reactive and non reactive MLIC combinations. The addition of insulin to the allogeneic MLR was found to increase proliferation but this was not the case with the autologous MLR for which the addition of insulin had no effect. Therefore, insulin levels alone cannot explain the differences seen between proliferative responses to lymphocytes and islets(263).

Thus in summary, insulin has been found to be stimulatory to lymphocytes in allogeneic combinations at the concentration detected in the MLIC and so the presence of insulin cannot explain the low MLIC response compared to that of the MLR. Similarly, the lack of effect of insulin on the autologous MLR has shown that insulin cannot account for the increased proliferation in the autologous MLIC compared to the MLR.

### 3.3.6 The use of the MLIC to investigate the diabetic response to islets

Only one MLIC study has been carried out using human lymphocytes from patients with overt and preclinical Type I diabetes compared to non diabetes related controls. The difficulty of obtaining sufficient samples to HLA-DR match the responder lymphocytes with the stimulator islets was reflected in the relatively few tests in this study that used human islets (four tests using PBMCs from clinically diabetic patients, and one test using PBMCs from a patient with preclinical diabetes). The purpose of this study of the MHC matched MLIC was to determine the potential T cell autoreactivity of diabetic patients with a view to monitoring preclinical diabetes and potential subsequent immunotherapy. The results(261) are shown in Table 3.20.

Table 3.20. MHC class II matched or xenogeneic MLIC using lymphocytes from preclinical and overt diabetic patients.

Stimulator islets	Control	No. in group	Preclinical diabetes	No. in group	Diabetes	No. in group
human	1.9	3	4.3	1	2.9	4
total inc. pig proislets	2.7	12	8.7	6	5.2	11

Results are expressed as stimulation index.

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The results in Table 3.20 indicate that preclinical diabetic and diabetic individuals showed an increased lymphocyte reactivity to islets and although the number of experiments using human islets was small, the inclusion of results using fetal pig proislets as stimulators led to a significant difference between the groups (p < 0.02)(261).

Thus in summary, islet specificity of proliferative lymphocytes from Type I diabetic patients has been demonstrated against human and fetal pig proislets.

3.3.7 The use of the MLIC-CTL to investigate the diabetic response to islets

A similar study to that described above, was carried out using diabetic, diabetes prone and diabetes resistant BB rats, but using the MLIC-CTL assay instead of the MLIC(262). The results are evidence for the existence of lymphoid cells in diabetic and diabetes-prone BB rats capable of lysing, and decreasing insulin content of, MHC compatible and incompatible islet cells *in vitro*, as can be seen in Table 3.21.

Table 3.21. MLIC-CTL using lymphocytes from diabetes resistant, diabetes prone and diabetic BB rats expressed as percentage specific cytotoxicity

Target cells	Diabetes	No. in	Diabetes	No. in	Diabetic	No. in
	resistant	group	prone	group		group
RIN cells MHC incompatible*	10.0	8	15.1	7	18.9	8
rat islet cells MHC compatible**	164	17	124	17	106	16

\* expressed as cell lysis by  $^{51}$ Cr release as % specific cytotoxicity

\*\* expressed as insulin content ng/well

In addition to the results shown in Table 3.21, the islet specificity rather than allospecific nature of this killing was supported by the observation that GH3 allogenic pituitary cells were not lysed by effector cells from diabetic and diabetes-prone BB rats(262). This lysis was shown to be due to NK cell activity(87).

Another model for investigating the role of lymphocytes in diabetes has been to use low multiple dose streptozotocin treatment (multi-dose) in mice, in which diabetes with associated insulitis is induced(260). Single dose treatment with streptozotocin, although inducing diabetes via a toxic effect, was not found to be accompanied by insulitis. It has been shown that the timing of a cytotoxic response against islets, as identified using the MLIC-CTL, coincides with the timing of the immunohistological appearance of insulitis in the mouse model. Although the RIN cells used as primary stimulators in these MLIC-CTL studies meant that this was a xenogeneic coculture, no lysis was seen against a variety of other cells including syngeneic, allogeneic or xenogeneic lymphocytes, or was detected using cells from the single dose mouse model. The authors concluded that the appearance of cytotoxic splenocytes, which may be cytotoxic T cells or NK cells with specificity for insulin producing RIN cells, correlated with the appearance of insulitis in the streptozotocin multi-dose mouse model(260). Taken together these studies have shown the importance of cytotoxic lymphocytes specific for islets in diabetic models, however the exact nature of the cells in these models of diabetes has yet to be fully defined.

Thus in summary, studies have shown the presence of cytotoxic lymphocytes from Type I diabetic patients or from animal models of diabetes, capable of functional inhibition or lysis of islets or islet derived cells. This lysis has been shown, in one model at least, to be islet specific without MHC restriction and to include killing by NK cells.

3.3.8 The use of the MLIC-CTL as a model for the role of cytotoxic T cells in islet graft rejection

### 3.3.8a Depletion of MHC antigens

A series of studies based on the mouse MLIC-CTL have investigated the relative effects of MHC class I and class II depletion by removing passenger leucocytes or antigen presenting cells, on the immunogenicity of islets *in vitro*. Depleting the islets of antigen presenting cells by treatment with anti MHC class II antibody and complement was found to lower the CTL response but not to abolish it(265).

An extension of this work has used whole mouse islets, dispersed beta cells or FACS purified beta cells as primary stimulators, with responder allogeneic splenocytes to produce effector cells for P815 tumor cell line targets (haploidentical with the stimulators). The results showed that an allospecific CTL response can be generated both by using whole islets (I+ve, II+ve) and purified beta cells (I+ve, II-ve), but that the response is abolished by anti MHC class I antibody even in the presence of MHC class II antigen(35).

In addition, when the responder splenocyte population was separately depleted of T helper cells or cytotoxic T cells, each was found to separately abolish the CTL response when stimulated by whole islets. Coculturing a responder cell population depleted of APCs with a stimulator cell population comprising either whole islets or purified beta cells, showed that depletion of APCs from either the responder population or the stimulator islets (purified beta cells), reduced but did not abrogate the MLIC-CTL. However, depletion of APCs from both responder and stimulator populations did abolish the response. The MLIC-CTL activity was partially restored by adding APCs back to the responder population but complete restoration of the cytotoxic activity required the APCs also to be integral with the stimulator population. These results have shown that both the indirect and direct

pathways are involved in the alloimmune response to pancreatic islets in which responder APCs potentially can present peptides of stimulator MHC class I molecules to responder T helper cells. Removal of APCs from the stimulator islet population does not remove the ability to generate a cytotoxic T cell response against islets, therefore, the authors have suggested that immunomodulation regimes should additionally include the down regulation of MHC class I antigen(242) (266).

### 3.3.8b Upregulation of MHC antigens

As already described, Markmann showed that MHC class II antigen, induced on the islet endocrine cells using IFN- $\gamma$ , did not lead to an increase in the MLIC response and that APCs have to be present to induce proliferation. In addition, the effect of upregulating MHC class I antigen expression using IFN- $\gamma$  was studied using the MLIC-CTL assay, with the result that lysis of islets was shown to increase after MHC class I enhancement and that blocking the MHC class I expression significantly decreased lysis of islet cells. Together these studies have shown that, in the absence of APCs, the induction of MHC class II antigen expression on the islet endocrine cells is, in itself, not enough to cause rejection but that MHC class I antigen expression is and so may play an important role in the immunogenicity of islets(39).

**Thus in summary,** antigen presenting cells from either the responder or stimulator cell population can contribute to the generation of cytotoxic T cells in the MLIC-CTL and the expression of MHC class I antigen has been found to be necessary for CTL stimulation.

3.3.9 Predictive value of the MLIC-CTL for islet allograft survival

The prediction of islet graft survival is not possible using the MLR and a series of studies has investigated the relationship between the MLIC-CTL and islet allograft rejection. For this, stimulator islets were cocultured with responder splenocytes to produce effector cells, followed by addition of Con A blasts as target cells. The MLIC-CTL was found to show more correlation with the rate of survival of islet allografts than the MLR-CTL when splenocytes were used as the stimulator cell population as shown in Table 3.22(278).

Table 3.22. A comparison between the rejection rate of islet allografts and percentage lysis in the MLIC-CTL and MLR-CTL(278)

Donor	Recipient	Percentage rejection	Stimulators	Responders lysis	Percentage
H-2 <sup>b</sup> islets	H-2 <sup>k</sup> mouse	26%	H-2 <sup>b</sup> islets	H-2 <sup>k</sup> splenocytes	20%
			H-2 <sup>b</sup> splenocytes	H-2 <sup>k</sup> splenocytes	68%
H-2 <sup>k</sup> islets	H-2 <sup>b</sup> mouse	74%	H-2 <sup>k</sup> islets	H-2 <sup>b</sup> splenocytes	54%
			H-2k	H-2b	63%
			splenocytes	splenocytes	

The same group also showed the value of the MLR-CTL (lymphocytes as responders, stimulators and targets) in generating pCTL for measuring their frequency in the responder population. Results in the mouse showed that a lower frequency of pCTLs corresponded to higher survival rates of pancreatic islet and heart-lung allografts(211).

There has been no work published on MLIC-CTL assays using human islets and lymphocytes.

Thus in summary, it has been shown that the frequency of precursor cytotoxic T cells as well as the percentage lysis in the MLIC-CTL, but not in the MLR-CTL, correlates with islet graft survival in mouse models.

### 3.3.10 MLIC, direct killing as measured by loss of insulin secretion

The three studies using this method have aimed to investigate different aspects of the immune response to islets.

Two studies have concentrated on the effect of PBLs from normal and diabetic individuals on the insulin release of islet cells. For this, human

PBLs from normal and diabetic patients (between two days and several years post diagnosis), were incubated with mouse islet cells as a xenogeneic coculture(258). In addition, PBLs from diabetes resistant, diabetes prone and diabetic BB rats, were incubated with Wistar rat islet cells (syngeneic coculture) or RIN cells (allogeneic coculture)(262) as part of the studies described earlier in Table 3.21. Both studies showed that lymphocytes from diabetic individuals can functionally damage islet cells and the latter studies showed these to include NK cells(87).

Boitard measured the inhibition of insulin release by islet cells after incubation with lymphocytes using static incubation and perifusion methods and found an inhibition of insulin release after islet cells were cocultured with lymphocytes from diabetic but not non diabetic individuals(258).

The special role of macrophages in islet destruction has also been studied. Schwizer investigated allogeneic and syngeneic mouse macrophage activity in mixed islet cell and macrophage coculture, in which cytotoxicity was measured morphologically and by estimates of insulin loss in both culture medium and cells. The islet cell monolayers were cultured for seven days, macrophages added for a further four days, then the cocultures refed daily for two days. Cytotoxicity was found to be associated with direct contact between either allogeneic or syngeneic macrophages and the islet cells, and this cytotoxicity could not be reproduced using macrophage conditioned media. Controls for non-specific lysis included fibroblasts and sarcoma cells, which showed few or no cytotoxic interactions with macrophages(61).

Thus in summary, it has been shown that cytotoxic leucocytes, particularly NK cells, from diabetic and diabetes prone disease sources, are primed to kill islets. In addition, macrophages have been found to kill islets in the mouse.

3.3.11 Primary MLIC using a variety of stimulator cell populations

Coculture studies comparing the immunogenicity of porcine kidney cells, liver cells and fibroblasts have shown that kidney cells express MHC class II antigen and stimulate the MLKC, fibroblasts do not express MHC class II

antigen and do not stimulate lymphocytes in the MLFC but that liver cells, which not express MHC class II antigen, do stimulate lymphocytes in the MLLC(276). The canine MLKC showed that kidney cells taken from a rejecting renal transplant, strongly stimulate autologous lymphocytes, more so than kidney cells from a non rejecting kidney or an allogeneic MLKC. In addition, T cells from the rejecting kidney responded more strongly to kidney cells from the rejecting kidney than did autologous PBLs. Enhanced stimulation by the rejecting renal distal tubular cells, correlated with MHC class II expression. However, the expression of MHC class II antigen, induced by coculture on cells normally negative for this antigen, was not found to correlate with an increased MLKC, and the authors have suggested that two costimulatory molecules are required(256). Comparing the MLKC with the MLIC, Roth(255) and Fernandez(270) have shown that the allogeneic MLKC has a proliferative peak at day seven and is approximately four times greater than the autologous MLKC but that the allogenic and autologous MLIC have similar proliferative responses, continuing at day nine and at day seven respectively(255). Fernandez also showed that IFN-y presence can moderately enhance the allogeneic MLR, the autologous and allogeneic MLKC and MLIC but not the autologous MLR and has suggested that local IFN-y production might play a crucial role in antigen presentation by specific tissues(270). Coculture studies using other human cell types have focussed on endothelial cells, which have been found to express MHC class II following IFN-y treatment and to show enhanced stimulation of the lymphoproliferative response. Other cell types (including fibroblasts, smooth muscle cells and epidermal cells), were found not to be modulated in this manner and so are thought to lack costimulatory factors necessary for T cell activation(248).

### 3.4 Overall summary of the mixed lymphocyte islet coculture (MLIC)

Considerable efforts have been made to investigate the immunogenicity of pancreatic islets of Langerhans in rodent as well as larger animal models. The principal *in vitro* model used has been the primary MLIC, but other models such as the secondary MLIC, the MLIC-CTL and an MLIC measuring direct killing by insulin loss, have given further insights in the immune reactions stimulated by islets. In most cases the studies have focussed on the

problems of rejection following transplantation but a small number of studies have investigated the immune reactions in the progression of disease in autoimmune diabetes.

Despite the use of different methods, various species and a range of parameters for responder-stimulator combinations in the MLIC, some continuity can be seen in the methodology and the results obtained. A variation in the optimal number of islets used in the MLIC for optimal stimulation has been found to range from 40 - 100, and a peak duration of coculture to be of seven days or more.

Secondary MLIC studies have shown that islets can be used to prime lymphocytes prior to a secondary islet, but not a lymphocyte challenge, and that stimulator lymphocytes can be used to prime allogeneic effector lymphocytes for the secondary MLR and MLIC.

The immunomodulation of islets has been studied by increasing the purity of islet preparations, culturing the islets at  $37^{\circ}$ C or  $24^{\circ}$ C, treating the islets with  $\gamma$ - or UV irradiation, or treating islets with antibody against MHC antigens. All these techniques have shown some degree of reduction of the immunogenicity of islets. Treatment of islets with the cytokine IFN- $\gamma$ , has been found to upregulate MHC antigen expression but not to restore MHC class II antigen related immunogenicity to APC depleted islets. Enhanced MHC class I antigen expression has been found to correlate with increased MLIC-CTL activity and *in vivo* rejection. Specific islet antigens have been found to contribute to autologous islet immunogenicity, and insulin, although stimulating lymphocyte proliferation, cannot account for the comparatively low MLIC vs MLR response and the high autologous MLIC vs MLR response. These results have been supported by studies with the MLKC.

Immunomodulation studies have also shown that both responder and stimulator APCs can be used to facilitate a T cell proliferative response and that the expression of MHC class I antigen is necessary for CTL stimulation. The frequency of precursor T cells and the MLIC-CTL have both been shown to correlate with islet graft survival in mice.

Lymphocytes taken from diabetic patients have shown specificity for islet functional inhibition and destruction, which has implications for islet

transplantation into Type I diabetic patients. In addition to cytotoxic T cells, cytotoxicity for islets has been shown by NK cells from diabetic sources and macrophages.

In combination, these reports have shown that useful information about the immunogenicity of islets can be gained from *in vitro* studies. However, the variability of methods, parameters and species, combined with the records of poor human islet allograft survival *in vivo*, have meant that there is a need to extend the *in vitro* studies on human islets and this includes standardisation of the human allogenic MLIC.

### 3.5 Aim of the thesis

The aim of this thesis was to develop a reproducible *in vitro* model for determining the potential immunogenicity of human isolated pancreatic islets. The objectives were to:

a) reproduce basic parameters of the MLIC to obtain a measurable responseb) determine whether a short duration MLIC was feasible, for clinical useprior to transplantation

c) identify the optimal conditions and parameters of the MLIC

d) identify the relative immunogenicity of acinar tissue in vitro

e) determine whether cytokines upregulate MHC antigens on human isolated islets and acinar tissue

e) observe the effect of cytokine treatment of stimulator cells on the lymphoproliferative response.

This model, once clearly defined, could be of great benefit in clinical islet transplantation with potential for prediction of human islet graft survival.

### The Next Chapter...

The methods used during the development of the human allogeneic MLIC model and the experimental design are described in Chapter Four.

# 3.6 CHAPTER THREE - TABLES 3.1 - 3.18

These tables summarise the methods (3.1-3.9) and results (3.10-3.18) of published studies using the MLC and related *in vitro* assays which measure the potential response of hymbocynes to allogenesic or spreeted practices. For the MLC, the tables describe the rodent, canine, teal pig and human primary MLC, the secondary MLC that the tables describe the rodent, canine teal primary MLC funct stilling experiment in which ymphocynes damage is measured by insulin loss and the MLC card, and to a variety of different stimulator populations. The contents of these tables are further described in the text of Chapter Three.

### METHODOLOGY

### Table 3.1. Summary of rodent MLIC, published methodology.

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25 untreated talets or lo trradiation (250R-10	spienocytes 6 days 25 untreated talets or 1c introduction (250R-1C	4 3x10 <sup>5</sup> splenocytes 6 days 25 untreated talets or 1c 250%-1C	t - 4 3x10 <sup>5</sup> splenocytes 6 days 25 untreated talets or 16 appendent (250R-16
100 handpicked, ytradia (2000R) from normal MHC class II + comple treated pancreas	purtified T cells from 5 days 100 Ibandpicked, yarada splenocytet ± 10 <sup>5</sup> Adys 100 Down Sormation splenic APCs ADPCs 100 Down Sormation Society APCs 100 Down Sormation splenic APCs 100 Down Sormation Society Society APCs 100 Down Society	3         Zz 10 <sup>4</sup> putter T cells from 100         5 days         100         1000         7000000 (20000) from zormal paternotyres 10 <sup>4</sup> 3x 10 <sup>4</sup> 3x 10 <sup>4</sup> 100         200000         200000         200000         200000           3x 10 <sup>4</sup> 3therady of the tells         3therady of tells         3therady of tells         200000         200000         200000	t fact bottom 3 22 10 <sup>4</sup> purdiad. Tel from 1 5 days 100 (2000) from stand Rectom protective 210 <sup>4</sup> pilomoyres 10 <sup>5</sup> (2000) from romania Rectom pilomoyres 10 <sup>2</sup> (2000) from romania pilomoyres 10 <sup>2</sup> (2000) from romania p
3x10 <sup>5</sup> 7 irradiated RivinSF islet endocrine cells	purfied T cells 5 days 3x10 <sup>5</sup> 7 traditied Rthm5F kide endocrine cells	5 3x10 <sup>5</sup> purified T cells 5 days 3x10 <sup>5</sup> 1 predieted T cells 5 days 1 and 1 a	t Liebro U bottom 3 3x10 <sup>5</sup> purified T cells 5 days 3x10 <sup>5</sup> 1 modated T cells 5 days 1 modated 1 moda
s 2,5,10,20, y irradiated islets 50,100	lymph node cells 3,5,7,9 days 2,5,10,20, y irradiated islets 50,100	3 2.5X10 <sup>5</sup> Itymph node cells 3,5,7,9 days 2,5,10,20, 1 traditied talets 30,100	Faicon tissue 3 2.5x10 <sup>2</sup> lymph node cells 3.5,7,9 days 2.5,10.20, 1 tradiated isless     culture     for therem     for the second secon

### Table 3.2. Summary of canine MLIC, published methodology. Authors

Author	Stimulator Isl et species	Plates	Repeats	Responder lymphocyte number	Source of lymphocytes	Duration - from set up to harvest	Stimulator Islet number	Stimulator islets	Number of non islet stimulator cells	Treatment of non islet tissue	Medium	( <sup>2</sup> H) Thymidine
Fernandez 1993	<b>3</b> 9		3		18d	9 days		r trradiated (4500R)	-	y trradiated kidney cells, lymph node cells		
Kenyon 1990	ŧ,			1x10 <sup>5</sup>	PBL	7 days	50 50	r trradiated + UV ittradiated islet cells islets	4	4	Vaymouths +	
Rabinovitch 1981	ġ	flat-bottom	E.	1x10 <sup>5</sup>	PBLs	3,5,7,9 days	25,50, 100,200	Islets	1x10 <sup>5</sup>	y irradiated PBI, hepatocytes, lymph	RPMI + 1596 dog serum	1uCl/well for 18 hrs

## Table 3.3. Summary of fetal porcine MLIC, published methodology.

Author	Stimulator	Plates	Repeats	Responder	Source of lymphocytes	Duration - from	Stimulator	Stimulator Islets	Number of non	Treatment of non islet	Medium	tu <sub>6</sub> ]
	isl et species			lymphocyte number		set up to harvest	islet number		islet stimulator cells	tissue		Thymidine
Benhamou 1994	pig (fetal)							control and UV irradiated				
								islet cell clusters cultured				
								for 1-29 days prior to use				
Yoneda 1989	pig (fetal)			5x 10 <sup>5</sup> or 5x 10 <sup>6</sup>	PBLs (various MHC	5 days	5x 10 <sup>5</sup> or	r irradiated for MLIC	,			9 HRS
					major or minor		5x 10 <sup>6</sup> fetal	(3,000R), islet cells				labelling
					mismatches)		islet cells	previously cultured at 37°C				
								or 24°C for 7 days				

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### Table 3.4. Summary of human MLIC, published methodology.

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Author	Stimulator Islet species	Plates	Repeats	Responder Lymphocyte number	Source of lymphocytes	Duration - from set up to harvest	Stimulator Islet number	Stimulator islets	Number of non islet stimulator cells	Treatment and type of non talet tissue	Medium	[ <sup>3</sup> H] Thymidine
Benhamou 1994	human			-				r irradiated + UV-B irradiated isiets				
Demidem 1988	buman	U bottom	8	1×10 <sup>5</sup>	PBLs	6 days	1X10 <sup>5</sup>	allogenetc insulmoma cells fresh or cultured (30 days)	1×10 <sup>5</sup>	y irradiated PBL, hepatocytes, lymph node cells	RPMI + 15% human AB serum	
Harrison 1991	human	Limbro round bottom	4	4x 10 <sup>5</sup>	DR matched PBMC from diabetic patients, preclinical relatives and controls	6 days	3-10 tilets	DR matched	-	fetal liver, kidney, thyroid cells, also used instalin and anti T cell receptor antibody as controls	RPMI 1640 + 5% human autologous or AB serum	1 uCl/well for 14-18 hrs
Liu 1994	terman		9	1x 10 <sup>5</sup>	PBLs	32,5,7,9 days	23-150 IBQ	islets 70-90% purity	-	1	RPMI 1640 (supplemented)	luCt/well for 18 hrs
Roth 1984	buman	flat bottom	3	1×10 <sup>5</sup>	PBIA, autologous spienocytes	3,5,7,9 days	100 2x10 <sup>5</sup>	talet enriched pancreas fractions, tingle cells of above	1×10 <sup>5</sup>	y trradiated Iddney cells, spienocytes	RPMI + 1596 human serum	
Stein 1994	human				PBLs	5 days	-	single cells or small clusters				
Ulrichs 1990	namul	fiat bottom	3	1×106	PBLs	6 days	s, 10, 20, 30, 40, 50, peak - 40	cryopreserved, mitomycin C pure and crude tilets			RPMI + 10% AB serum	
Zeevi 1994	uneuunuj					6 days	600/ml, 300/ml, 150/ml		30-50% vs 70-90% purity			

### Table 3.5. Summary of secondary MLIC, published methodology.

Author	Primer islet species	Plates	Repeats	Responder lymphocyte number	Source of lymphocytes	Duration of priming incubation	Priming cells	Duration of secondary incubation	Stimulator islet cells number	Stimulator islet treatments	Number and type of non tslet primer cells	Treatment and type of non islet tissue	Medhum	[ <sup>2</sup> H] Thymidine
Markmann 1969	191	Linbro U bottom	m	sx104	purified CD4+ve T cells	primed at 2 week intervals + 10% Con A supn	irradiated allogeneic LN celis	3 days with islet cells	SX10 <sup>4</sup> cells ZX10 <sup>4</sup> cells IX10 <sup>4</sup> cells SX10 <sup>4</sup> cells	allogenet: fresh, culture or yIPN-treated + culture islet cells (all yirradiated)	IN cells = 5x10 <sup>3</sup> , 1x10 <sup>4</sup> , 2x10 <sup>4</sup> and 5x10 <sup>4</sup> Dendritic cells = 80, 1.5x10 <sup>2</sup> , 3x10 <sup>2</sup> , 6x10 <sup>2</sup>	yltrachation	RPMI + 2% syngenets rat serum	20 uCVml for 18 hrs
Rabinovitch 1961	8	flat bottom	m	1X 102	5184	10 days altogeneic and autologous pooled microcultures	PBLs or islets	2, 3, 5, 7, 9 days allogeneic and autologous culture	100 or 1-2x10 <sup>5</sup> cells	4	1-2x 10 <sup>5</sup> allogeneic and autidogous hepatocytes or LN cells	virradiation 1,500R	RPMI +15% dog serum	IuCl/well for 18 hrs
Zeevi 1994*	naman	-		-	-	6 days	islet preps	3 days	600/ml, 300/ml, 150/ml	culture for 1 week at 24°C also islet purity 30-50% vs 70-90%	-		,	

N.R. Marituman 1999 has also described the stimulation of 2x 10<sup>5</sup> purified T cells in the presence of Can A with and without APC (which are necessary for Can A simulation) in the form of syngeneic spiencoytes, fresh, cultured or JFN treated + cultured states also RNMs in the APC of the start and curred out in triplicate, 5H thymidine was used at 20uC/m [for 10 his before harvesting on day 3.

### Table 3.6. Summary of xenogeneic MLIC, published methodology.

AUTOOL	Sumuator	Plates	Kepears	Kesponoer	Source of lymphocytes	Duration - from	Stimulator	Stimulator islet treatments	Number of non	Treatment and type of	Medium	H
	islet species			lymphocyte number		set up to harvest	Islet number		islet stimulator cells	non islet tissue		Thymidine
Gramberg 1994	pig	-		1x 10 <sup>5</sup>	human PBMC		100 IBQ	separated on normal or tsokinetic gradients				
Harrison 1991	pig (fetal)	Limbro round bottom	4	4x 10 <sup>5</sup>	human PENC	6 days	5-15 proisiets		1x 10 <sup>4</sup> , 1x 10 <sup>4</sup> , 2.5x 10 <sup>4</sup>	fetal liver, kidney, thyroid cells, also used insulin and anti T cell receptor antibody as controls	RPMI 1640 + 5% human autologous or AB serum	1 uCL/well for 14-18 hrs
Helser 1992	pig		-	5x 10 <sup>4</sup>	human PBLs	1	40 or optimal dilution of crude islets	DTZ selected islets	5x 10 <sup>4</sup>	allo human MLR, xeno PBLs, splenocytes LN cells with <sub>Y</sub> irradiated stimulators		

### Table 3.7. Summary of MLIC-CTL, published methodology.

Author	Stimulator	Plates	Repeats	Effector cell	Source and treatment	stimulator cells	Duration of	Number of target cells	Target cells	Duration of cytotoxic	Medium	<sup>51</sup> Cr label
	isl et species			number	of effector lymphocytes	or stimulation method	primary culture			assay		
Charles 1983	rat	tubes	£	1x 10 <sup>4</sup>	PBLs from human IDDM and control patients			5x 10 <sup>4</sup> , 1x 10 <sup>5</sup> , 2x 10 <sup>5</sup> to give target effector ration of 1:5, 1:10 and 1:20	rat islet cells	8 hrs	DMEM + 10% FCS	100ul for 10-20x 10 <sup>6</sup> cells
Mackay 1985	rat	Costar 96 microwell	4	<b>5</b> K 10 <sup>5</sup>	spienocytes from 4,dp or dr BB rats*	± culture in Con A (5x10 <sup>6</sup> cells in 5ug/ml Con A)	2 days	2.5x 10 <sup>4</sup> target effector ratio of 1:20, also used 1:8 and 1:2	RIN (islet) or GH3 (pituitary) cells (allogeneic)	8 hrs	RPME + 1096PCS	330 uCL/ml 90 mins
Markmann 1989	Tat	Linbro U bottom	m	3×105	LN cells from spienocyte allo- immunised (7-10 days) rats	y irradiated (2000R) allogeneic LN cells	6 days	1x 10 <sup>4</sup> /well target:effector ratio of 1:30	syn- and allogenet. Con A blasts (3 days at 5ug/ml) or islet endocrine cells	4 hrs	RPMI + 2% syngenetc rat serum	200-250 uCU/페 1 hr
McBroy 1984	mouse	11mm x50mm plastic tubes	9	4x106, 2x106, 1x106, 5x105, 2.5x105 1.25x105	spienocytes from control, multiple low- dose streptozotocin treated mice	Kin cells (xenogeneic) treated with Mittomycin C 25ug/mi for 20 mina	5 days	2x10 <sup>4</sup> /well to give target:effector ratios of 1:200, 1:100, 1:50, 1:25, 1:12.5, 1:6.25	Con A biasts (3 days at Sug/mi) or RIN cells	S las	RPMI + 10% FBS	20 cpm per cell for 90 mins
Stock 1988 - 1991	. norte	U bottom	m	Sx10 <sup>5</sup> or to give effector:target ratio between 5:1 and 100:1	mouse spienocytes selectively depieted of APCs or CD8+ve or CD4+ve T cells	50 whole islets or 5000 FACS- purified or unpurified beta	5 days	2x10 <sup>3</sup> /well to give target:effector ratios of 1:100 to 1:50	autologous Con A blasts (1x10 <sup>7</sup> splenocytes for 48hrs at 5ug/ml) or P815	4 brs	RPMI + 10% FCS	۲.

' d = diabetic, dp = diabetes prone, dr = diabetes resistant

N.B. RINmSF cells do not contain any APC so act as a negative control (Markman 1989).

NR Sock used the MLC to stimulate cytotock T cells with 30-100 lakets or SAIO<sup>4</sup> laket cells or FAG purified bera cells. The presence of MHC class II +ve cells in the stimulator population was estimated by coculture with MHC class II antigen sensitive T cell clones as an MLC The results have shown that whole lakets and laket cells on the that purified bera cells do not (Sock 1991). NR Charles also carried out saxys for antibody dependent reludint cytotocking (ADCC), complement augmented ADCC (CADCC),

# Table 3.8. Summary of MLIC-direct killing as measured by loss of insulin secretion, published methodology.

Author	Stimulator islet species	Plates	Repeats	Responder lymphocyte number	Source of lymphocytes	Duration - from set up to harvest	Stimulator Islet number	Stimulator islets	Medium	Insulta detection
Boitard 1981	mouse	Falcon microtest	9	4x 10 <sup>5</sup>	human PBLs (xenogeneic)	18 hrs	5x10 <sup>3</sup> islet cells	cell suspension made from handpicked islets of DBA/2 mice	supplemented MEM+ 10% FCS (basal = 5.5mmol/1 glucose or stimulatory = with 16.5mmol/1 glucose and 5mmol/1theophylithe	static incubation and RIA
Mackay 1985 Mackay 1986	rat	Costar 96 well	9	5x10 <sup>5</sup>	spienocytes (syngenekc and allogenekc) (d.dr.dp)*	2 days	2.5×10 <sup>4</sup>	tatet cells (syngenetc) RIN (tatet) cell monolayer (allogenetc)	RPMI + 10% FCS	cell extract - RIA
Schwizer 1984	mouse			2.5×10 <sup>4</sup>	macrophages (allogeneic and syngeneic)	4 days + reled coculture for 2 days	20 İtilets	islets grown as monolay ers	DMEM +10% FBS (basel - 5.5mmol/1 glucose or stimulatory - with 16.5mmol/1 glucose and 5mmol/itheophylitre	static incubations of islet cells, supn and cell extracts RIA
Stock 1988a	tinouse			5x10 <sup>5</sup> in MIIC then 2x10 <sup>5</sup> as effectors for cytotoxic determination	splenocytes (allogenek) in MLIC so primed by islets as effectors	5 days + 8-24 hrs	10 islets in MIJC	fresh	RPMI (11mMol glucose) then DMEM (25 mMol glucose)	static incubation of islets and RIA
<ul> <li>splenocytes were from</li> </ul>	n diabetic (d	l), diabetes-prone (	(dp), diabete	es resistant (dr) sou	rres.					

Table 3.9. Comparison of mixed lymphocyte cocultures using a variety of cell types as the stimulator population.

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Author	Stimular	Plates	Repeats	Responder	Source of	Duration - from	No. and treatment of	Stimulator	Medhum	(H)
	species			lymphocyte number	lymphocytes	set up to harvest	stimulators	population		Thymidine
Fernandez 1993 (MLIC on Tables 3.2 and 3.11)	30g		3		PBLs	9 days	r irradiated 3,000R- lymphocytes, 9,000R kidney cells	lymph node cells, kidney cortical cells	,	
Pawelec 1979	porcine	microtiter	8	Zr 10 <sup>5</sup> lymphocytes		MLR peak = 120 hrs all others = 144 hrs	x-irradiated 3,000R, all allogeneic, compatible at 0.6x 10 <sup>4</sup> - 2x 10 <sup>5</sup>	leucocytes liver kidney fibroblasts		
Roth 1984 (MLIC on Tables 3.4 and 3.13)	buman	flat- bottomed microtiter	£	1X 10 <sup>5</sup> PBLs	PBLs emriched T cells	3, 5, 7, 9 days	1x 10 <sup>5</sup> y trradiated 1,500R allogeneic or autologous	kidney cortical cells	RPMI + 15% human serum	đ
Esquenazi 1987	ar Se	Limbro flat or round- bottomed microtiter	£	1x 10 <sup>5</sup>	lymphocytes or T cell clones from rejecting kidneys	3, 5, 7, 9 days	IX 10 <sup>5</sup> allogenetc <sup>1</sup> irradiated (1,500R lymphocytes, 4,500R for kidney cells),	kidmey cortical cells lymphocytes	Clicks medium + 15% dog serum	1 µCl for 18 hus
Theobald 1993	umud	microtiter	£	1x 10 <sup>5</sup>	PB13	6 વેશપ્ર	2x 10 <sup>4</sup> mitomycin C treated, allogeneic	leucocytes dermal fibroblasts smooth muscle cells endothelial cells cells	RPMI + 9% autologus serum	1µCJ/ well
## RESULTS Table 3.10. Summary of rodent MLC, published results.

Author	Stimulator species	Peak islet number	MLIC qpm*	MLIC Stimulation Index	MIR	immunomodulation	non islet component	Additional information	Overall comment
Bretzei 1993	rat	used 100 islets	3751Acpm 3113Acpm	4.7 1.6		anti MHC class II antibody + complement, depleted MHC class II-ve cells in 86% of islets (control =6,5%)		No increase in MHC class II depietion with 5 days additional culture	Concludes that MILC response is not related to rejection
Flesch 1991	rat	used 100 islets	5,500cpm (n=7)(allo) 2,300cpm(n=7)(syn)	4.7 (alio) 1.6 (syn)	-	anti MHC class II antibody + complement		incomplete MHC class II+ve cell removal using antibody + complement	Reduced MHC dass lit-ve cells = reduced MLIC but no prolongation of graft survival
James 1989	Tat	no titration, used 25 islets	20,000 cpm	1	30,000 cpm	r kradiation 250R = 12,500 cpm without impairing id et function in vivo	,	Also carried out in vivo approvention (lipid soluble) soluble) to show the effect immunosuppression was mecessary to show the effect and 250k in prolonging graft survival	Low dose irradiation functionally removes dendritic cells from islets
Поуд 1989	Ħ	no titration used 100 islets	18,000 cpm (in vitro treatment cournel) 62,000 cpm (in vivo treatment control) (NB no added APCs)		40,000 cpm (+10 <sup>4</sup> APCs), 120,000 cpm (+3x 10 <sup>6</sup> APCs), 140,000 cpm (+10 <sup>5</sup> APCs)	In vitro anti MHC class Il treatment of islets = 10,000 cpm In vivo anti MHC class II treatment of islets = 5,000 cpm	purified T cells from splenocyces	addition of APCs of stimulation of APCs of efficient than adding APCs of responder strain no prolongation of rejection even post transplantation of seen post transplantation of dass if	removal of MHC class II cells from lakets reduces prodiferation in MLIC but no correlation with latet survival in vivo
Markmann 1989	Iat	no titration, used 3x 10 <sup>5</sup> isiet cells	7,400 cpm fresh (allogeneic) 1,600 qm cuitured (allogeneic) 1,300 fresh (autologous)		34,600 cpm (allogenetc), 300 cpm (autologous)	IPN, treated cultured allogeneic talets = 900 gym		Used purified T cells as responders Also carried our CTL expts and in vivo expts	IPM, can enhance MHC class I and Induce MHC IL yIPN treated islets are rejected farter in vivo than non treated, do proliferation in MLIC proliferation in MLIC
Shizuru 1986	rat	titration of 2, 20, 50, 100 islest, peak - 100 isless (day 5), less islets - longer duration to peak	33,000 cpm peak day 5 (100 talets) 5 (000 talets) 6 (30 talets), peak day 7 (2,20 talets) day 7 (2,20 talets)			Pre-incubation of islets with immunotoxin coupled to anti class II antibody, reduced proliferative response eg. highest doses 10 <sup>-6</sup> and 10 <sup>-7</sup> were 1,000 cpm vs 18,000 cpm untreated		carried out gluccee stimulation of maultin studies at well as gross morphology under the Hight microscope	Suggest that the allostimulatory MHC class II cells in the falet can be removed without damage to the islet using firmunotoxin coupled to anti class II anti class II
* results cpm - back	ground cpr	a							

## hadichad MLIC - interest 2 Table 3.11. Sumn

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Author	Stimulator species	Peak islet number	MLIC cpm*	MLIC Stimulation Index	MLR	Immunomodulation	non islet component	Additional information	Overall comment
Fernandez 1993	ġ		15,256 (allo) 2,471 (auto)		35,509	IFN, 0.008-2U/ml x 10 <sup>4</sup>	MIKC + auribody (see Table3.18)	No effect of IFN, on alloMLIC <0.4U/ml but at 2U/ml = 31,500cpm (allo), 6.559cpm (auto)	IFN, enhanced alto and auto MIIC and alto MIR but not auto MIR (see MIKC Table 3.18)
Kenyen 1990	dog	no titration, used \$x10 <sup>5</sup> balet cells	14,274 26,271 26,690 74,00 32,402	123 107 36 11	5,807 2,453 31,393 26,380 -	UV irradiation at 600 1) m <sup>2</sup> was necessary to reduce MLIC response (19,813 cpm to 5,860 epm) but even 200 J/m <sup>2</sup> reduced glucose atimulated tursulin (400-250 %basal	pure	lates stimulators in ML/C also virtuated Also carried out in two expts, it was necessary to pretrast dops with CAA and to culture islets for 48 hrs post UV tradiation (600 post UV tradiation (600 surviva	Immunomodulatory results in robents are not mitrored by those in larger animals as shown here in dog and by results of human isle transplants perhaps due to impurity of isle preparations.
Rahmovitch 1981	ă,	tttration of 25,50,100 and 200 islets, peak = 100 islets or 2x10 <sup>5</sup> islet cells	6,000 cpm peak day 9 (allogendes) 3,000 cpm peak day 5 (autologous)		12,000 cpm peak day 9	Culturing isless for 7 days at 37° and 24° had no effect on MLIC response	Porcine insulin was stimulatory at 0. lug/mid (approx level in wells) and inhibitory at 10ug/mi	Also carried out Zudary MLIC which augested priming to islet specific autologous antisen also presence of talets inhibited lymphocyte proliferation to PHA	Suggest that both suggest that both aplogense and islet apecific antigens exist on taket cells and that the former might inhibit the response to the latter
<ul> <li>results cpm - back</li> </ul>	ground cpn								

Table 3.12. Summary of fetal porcine MLIC, published results.

Author	Stimulator	Peak islet	MLIC cpm	MLIC Stimulation	MLR	immunomodulation	non islet component	Additional information	Overall comment
	species	number		Index					
Benhamou 1994	pig (fetal)					67-91% reduction of		see also human islet data	300J/m2 reduces islet
						MIJC at 300J/m <sup>2</sup> and			immunogenicity without
						600J/m <sup>2</sup> , no effect on			impairing function
						insults secretion at			
						3001/m <sup>2</sup> .			
Yoneda 1989	pig (fetal)	no titration 5x	(responder alone	1.5 - 17.8		culture at 24°C		measured percentage MHC	reduction in MLIC after
		10 <sup>5</sup> or 5x 10 <sup>6</sup>	com - 205 + 56 and			SI = 0.2 - 3.2 (1 wk),		class II+ve cells in islet	culture at 24°C but more
		islet cells used	1693 + 316)			culture at 37°C		cells did not correlate with	than 8 days culture at
			4			SI = 0.9 - 10.3 (1 wk)		SI In MUC,	24°C was deleterious,
								also transplanted 24°C	some survival of islet
								cultured cells and islet cell	cell grafts in major MHC
									and the second s

## Table 3.13. Summary of Human MLJC, published results.

				All to Color de la delas	400	And a	non telse company	Additional information	Customer Comment
Author	summator	reak suer number	MLIC OF	Index	MLK		TION IN ALCOURDONALI		
Benhamou 1994	namut	-		1		MLIC at 300J/m <sup>2</sup> = 43-81% of controls and at \$00J/m <sup>2</sup> = \$1-70% of controls	1	need to irradiate immediately post isolation	UV-B can reduce islet immunogenicity and despite some alteration in insulin release, in vivo function was normal
Demidem 1938	human	no titration - used 10 <sup>5</sup> insulinoma cells	14,768cpm (allogeneic) 4,232cpm (allogeneic) 2,224cpm (autologous)	7 (aliogeneic) 3 (aliogeneic) 1.26 (autologous)	9,303cpm (allogeneic)	Long-term culture (insultnoma cells) - 4,303qm, SI = 1.00 (allogentc) 3,326qm, SI = 1.04 (autologous)	1	(zto islets)	Long term culture is necessary to reduce immunogendity in MLIC of insultionna cells corresponding to a loss of MHC class II+ve cells
Harrison 1991	human	no titration - used 5		1.9, 2.1, 1.7 (c)* 4.3 (pd)* 7.5, 1.7, 0.9, 1.5 (d)* (all DR matched )				GM-CSP concentration in MLIC correlated with <sup>3</sup> H thymidue uptake, <sub>1</sub> HN was inconsistent and TNFu was inconsistent and TNFu was xenogeneic MLIC.	increased SI using lymphocytes from predimical and diabetic patients but very few humnan-human results
lin 1994	Thuman .	50 IEQ (peak day 7)		1		low temperature culture 22°C	1	2 MLIC using low temp. cultured talets and 5 MLIC control idets with HA-DR mismatch PBLs, also 2 MLIC mismatch PBLs, also 2 MLIC with 1 HLA-DR match, all - no significant effect	no significant effect of HLA-DR matching on SI or of low temp. immunomodulation.
Roth 1984		no thration - used 100 islet emriched pancress fractions or 2x10 <sup>5</sup> islet cells	peak 9,000cpm on day 9 (allogenetc) peak 10,000cpm on day 7 (autologous)			2000cpm peak day 9 2000cpm peak day 7 (attologous) peak day 7 (attologous) after anti class II treatment		MLKC (kidney cells) anogeneic peak - 25,738 cpm on day 7, and antologue peak - 9,148 cpm on day 5)	Suggest that islet immunogenic and that class II is involved in allogeneic but not attologous reaction
Stein 1994	unuuu			Culture at 37°C 6.1 ± 2.2 (1day) 18.3 ± 6.0 (3-4 days) 6.1 ±1.3 (8-9 days) in culture ()-days in culture	4	Culture at 24°C 2.4 ± 0.4 (1 days) 5.6 ± 1.3 (3-4 days) 5.0 ±1.3 (3-4 days) days at 3.7°C) ()-days in culture			3-4 days culture at 240C reduces the timmunogenicity of talets in the MIJC
Uinchs 1989, 1990	thuman	uttration of 5, 10, 20, 30, 40, 50, peak = 40 crude talets	6,000 -22,000 on day 6 (no kinetics)	peak stimulation index=7.1		58% inhibition of proliferation with anti class II mcab	68% immunogenicity due to exocrine contamination, 2,000cpm (pure), 2,000cpm (pure DTZ handpicked)		Immunogenicity of islers decreases with increased purity and preincubation with anti class II mcabs
Zeevi 1994	human	titration of 150/ml, 300/ml or 600/ml, peak = 600/ml		52 (30-50% purity) 22 (70-90% purity)	1	culture for one week at 24°C	< half the timmunogenicity by 40% increase in purity	Also carried out a 2ndary MLIC using primed responders	Increasing the purity and culture at low temperature decreases the immunogenicity of islets in the MLIC

Stimulator Index = mean com expl/mean com background \* (c) = non diabetic control PBMC, (pd) = preclinical diabetes PBMC, (d) = overt diabetes PBMC

Table 3.14. Summary of secondary MLIC or pFII, published results.

Author	Stimulator species	Peak tslet number	MLIC cpm*	MLIC Stimulation Index	MLR	immunomodulation	Additional information	Overall comment
Martmann 1989	ž	5x 10 <sup>4</sup>	8,000cpm (fresh) <1,000cpm (culture) <1,000cpm (gIFN + culture)		18,000cpm on day 3 (LN cells)	culture, <sub>Y</sub> IFN + culture	1	Loss of stimulation by cultured islets was not restored by freating them with yIFN
Rabinovich 1981	<b>2</b>	100 talets or 2x10 <sup>5</sup> talet cells	6,000cpm peak day 3 (responders primed and allo lymphod 4,000cpm peak day 3 (responders primed with allo islets)		12,500cpm peak day 5 (responders (responders allo hymphocytes) -4,000cpm (responders plained with allo isites)		Actoragence > MLC 4,000cym pesk day 3 (arto 4,000cym pesk day 3 (arto Hile primed lymphocytes + 4,000cym pesk day 5.7 4,000cym pesk day 5.7 (arto tate primed lymphocytes or hepauocytes lymphocytes or hepauocytes	secondary NLIC is only half the response of secondary MLR comparison of allogeneic and autologous autologous thimmarigeneic himmarigeneic determinants on isles.
Zeevi 1994	human	150/mi, 300/mi, 600/mi		40, 110, 100 (30- 50% pure) 7, 9, 22 (70-90% pure)		culture for one week at 24°C = SI of 5 fold less than culture at 37°C	4	Increasing purity of talets and culture at 2.4°C decreases proliferation in the MIJC
<ul> <li>results cpm - back</li> </ul>	ground cpi	8						

# Table 3.15. Summary of Xenogeneic MLIC, published results.

Granters 1944         Pit         Institution         2364: 100?         2364: 100?         Removed for and 100         Removed for and 100 <th< th=""><th>Author</th><th>Stimulator species</th><th>Peak islet number</th><th>MLIC cpm</th><th>MLIC Stimulation Index</th><th>MLR</th><th>Immunomodulation</th><th>non islet component</th><th>Additional information</th><th>Overall comment</th></th<>	Author	Stimulator species	Peak islet number	MLIC cpm	MLIC Stimulation Index	MLR	Immunomodulation	non islet component	Additional information	Overall comment
Burnton 1991         Def (real) motify used ()         Description (mail) motify used ()         Constantion (mail) motify used ()         Constantion ()         Constantion ()         Constantion ()         Constantion ()         Constantion	Gramberg 1994	3jd	no titration, used 100 IEQ	3256 ± 1303 * 11903 ± 7311**			Isokinetic gradient centrifugation to decrease non islet contamination			removal of small <100um non talet tissue reduces immunogenicity in MIJC
Diedeer 1992         Dig         dot one optime         Concentration         25.56,000         Increases in purity by concentration         Personance not with all of the concentration         Sector         Sector         Sector         Sector         Concentration         Concontentration         Concentration	Harrison 1991	pig (fetal)	1.5 proteters but mostly used 10 proteters	41,000 (optimum from patient ao 6 preclimical) background = 8,000	11.0, 4.7, 9.5, 14.0, 8.6 (prectimical) 5.4, 11.0, 5.6, 6.2, 10.0, 2.1, 4.9 (diabetic) 7.4, 2.5, 0.8, 2.0, 1.7, 2.2, 3.0, 4.3, 3.0, (control)			no stimulation using liver or ixdray, using ibyroid (2.5x 10 <sup>4</sup> cells) 2 climical and 1 corriol cased and a response but lower than to protidets	CM-CSF concentration in MLLC correlated with <sup>3</sup> H thymdiate upstake, HTN was inconsistent and TNFe was not detected. See also human MLLC.	Correlation between increased or over diabetes (also with 64K positivity) so MLIC could help detect preclinical diabetes
	Hetser 1992	\$jd	40 or an optimal crude taket preparations	42,000cpm crude sist preparation gicked islets picked islets		25-56,000 alton fhuman alton 24.62,000 cpm (xento PBLs) PBLs) PBLs) PBLs) PBLs) alton cpm (xento cpm (xento cp	hacrease in purity by handpicking	response to Pillso spiencoytes lymph node cells		zero responze not very different to alloresponze and condrude hat directions hat eccognition is a major stimulatory path in zeno combination

Table 3.16. Summary of MLK-CTT, published results.

Author	Stimulator species	Peak islet number	Cyroxidity <sup>51</sup> Cr release in MUIC-CTL	<sup>51</sup> Cr release in MLR-CTL	farmunomodulation	non islet companent	Additional information	Overall comment
Charles 1983	1.at	no titration 1x10 <sup>4</sup> and T:E ratio 1:10	1 3+2% (diabetic lymphocytes) 7+1% (controls)		,	spleen and macrophages as targets		monomuclear cell- mediated islet cell killing is associated with diabetes, antibody mechanisms seem less active
Mackay 1985	88 rat	no titration, used 2.5x10 <sup>4</sup> cells	Bon primed effectors 18.9 ±3.3 (d)° 15.1 ± 2.5 (dp)° 10.0 ± 2.3 (dp)° 37.7 ± 7.3 (dp)° 30.4 ± 4.5 (dp)°		,		GH3 pitutiary cells were nor lysed by the effector cells cytotoxicity range 3.6-7.6 overall	diabetic and diabeties groups Bis ans have lymphoid cells capable of lysing kiet cells in vitro
Markman 1969	181	peak target:effector ratio 1:100	49% (allo lympho against fafet cell targes) <2% (yn lympho <2% (yn lympho gainst fafet cell gainst fafet cell targes)	6.9% (allo lympho primed affectors against islet cell argens) <2% (yn lympho primed affectors against islet cell against islet cell	65% (allo lympho primed effectors against yIFN treated talet cell targets)		The addition for factor against MHC class I antigen suppressed taket lysts but those against class II or rat Ig did not.	Cytoroxic T cells can lyse sister cells, this effect is enhanced after yFN resument to increase MHC antigen expression
McEvoy 1984	asnou	no thration, used 2x10 <sup>4</sup> cells/well effector ratio 1:100, peak cytojash 10 dytojash 10 multi-strep	Non primed sylenocytes 9.0+1.1% = multi-strep 0.8+0.5% = straje dose strep RUN cell + TCGP primed sylenocytes 10.14.0.9% = controls 53.34.7.9% = multidose strep		4		Detection of cyrotoxic T cells connoted with insultive seen using histology.	Cytotoxic T cells specific to IUN cells appear in mouse 10 days after multicos strep but not after single dose strep. The specificity may not blic restricted at the targets were set the targets were
Stock 1987cm	House	no titration, used 50 islets	6596	80%			Cyrotoxic T cells can be sensitised to falets	Cytotoxic T cells can destroy islets so may play a role in talet graft destruction
Stock 1988c	mouse	no titration, used 50 falets	MLIC = 20% and 34% respectively see MLR	MLR - 68% and 63%	-			MLIC-CTL but not MLR- CTL correlates with graft outcome
Stock 1989d	moute	no titration, used 50 islets:class II depeleted islets	cytotoxicity of isleticidass II depleted islets = 34:22, 42:33, 49:40					isiet MHC class II depietion doesn't significantly affect CTL response
Stack 1989f	·	so titration, used 1x10 <sup>4</sup> ister cells:MBC depleted beta cells	cytotookitty of talet cellsciaas 11 depleted talet cells = 82:80, 87:78, 86:84, 76:76		Cynoroxicity of later cellaciass II depleted later cells (both treated with anti class I mcab) = 3:5, 10:4		In following state meab specific to donor strain class I was found necessary in the poise of the sponse of it may be possible to treat rejecting grafts with alloppedific anti class I alloppedific anti class I	Presence of class I but not class II is necessary to stimulate CTL response
Stack 1990) Stack 1991k	mouse	no titration, used 50 kilets (dass ive or dass live) and \$5000 kilet cells (dass ive or dass live or dass live or			when responder lymphocyte were depleted of either T helper of T cytotook cells, this abbrogated CTL response			Class II cells have to teneved both from isless and responder lymphocytes to abbrogate CTL response
cytotoxicity = 100 x ex	pt cpm-backg n diabetic (d	pround cpm /total (), diabetes-prone (	cpm (2000 cells) - cpm ba (dp), diabetes resistant (d	ckground tr) sources.				

allografts (Stock 1989e) eatic islet and heart-lung ŧ N.B. One study has determined the pCTL frequency in cells generated by the MIC-CTL and found the frequency to

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AULDOT	SUBULIER	Peak Incl	Insum reese	AGGINODAL INFORMATION	OVER'S LOOP CONTRACTOR
	isler species	number	ng/ml or uU/well*		
Boltard 1981	mouse	no titration, used 5x10 <sup>3</sup>	xenogeneic control PBLs + Islets*	No cytotoxicity against fibroblasts was detected,	PBLs from IDDM patients suppress
		cells	prestim = 11.7±0.8	also carried out ICA on	insulin release from
			basal = 8.8±0.9	human and mouse pancreas	mouse islets, half of the
			stim = 18.5±1.8	sections	IDDM patients had no detectable and insults
			PRIs 4 felanet		antibodies, but most had
			prestim = 10.5+0.9		ICA. However no exact
			basai - 10.7±1.1		correlation between ICA
			stim = 12.3±1.6		and insults inhibition
MacKay 1985	rat	no titration,	Syngeneic	Used NK cell senstive cell	allogeneic and syngeneic
MacKay 1986		used 2.5x10 <sup>4</sup>	155 ± 18 control	line to identify cytotoxic	splenocytes from
		islet or RIN	106 ± 13 (d)	cells as NK cells	diabetic and diabetes
		cells	124 ± 12 (dp)		prone rats show
			164 ± 18 (dr)		decreased insulin
			Allogeneic		content and so survival
			6.5 ± 2.8 control		OF DEER CELLS,
			1.5±0.7 (d)		spienocytes from
			$2.4 \pm 0.8$ (db)		diabetes relistant rats
			5.4+1.8(dr)		caused background
			(m) on The		levels of tslet lysis only
Schwizer 1984	moute	no titration	basal = 125	no effect of me conditioned	reduction of insulin
		used 20 islets	islets alone = 210	medium alone (4 days	release and lysis of
		grown as a	islets + syn mp = 70	culture), morphological	tslets in presence of allo
		monolayer	islets + allo mp = 60	evidence shows need for	or syn macrophages so
				cell-cell contact for lysis	beneficial to remove
					macrophages from islets
					prior to transplantation
Stock 1988a	mouse	50 allogeneic	islets + allo naive	morphological examination	inhibition of
		islets	spien = 576-1529	showed destruction of islet	allospecific beta cell
			islets + primed allo	cells within 24 hrs of	function with primed
			spien = > 12,800	coculture	lymphocytes within 8
			islets + islet primed		has of coculture
			1 009'71<- Unids	and the second	and the second se

\* spienocytes were from diabetic (d), diabetes-prone (dp), diabetes resistant (dr) sources.

Table 3.18. Results of mixed lymphocyte cocultures using a variety of cell types as the stimulator population.

Author	Species	Peak	Stimulator	Peak response	Stimulation Index	Comments
		stimulator	population	,		
		number				
Fernandez 1993	ğ		kidmey contical cells	4,405cpm (allo)		IFNg treatment + antibodies to IFNg
				3,304cpm (auto)		and to kidney specific ag showed
			lymph node cells	35.509cpm (allo)		IPNe enhancement of response to
				176cpm (auto)		MLKC rather than MLR
Pawelec 1979	porcine	1x 10 <sup>5</sup>	leucocytes	38,000dpm		Kidney is MHC la-like+ve and
		6x 10 <sup>3</sup>	liver	30,0004pm	10.55 (allo)	stimulates in MLKC, liver is MHC
					2.7 (compatible)	Ia-like-ve but also stimulates MLIC
		1.25x 10 <sup>4</sup>	tidney	45,000dpm	17.7 (allo)	but fibroblasts (also la-like -ve)
					6.0 (compatible)	do not
		,	fibroblasts	no proliferation		
Roth 1984	human	1x 10 <sup>5</sup>	kidmey cortical cells	28,738cpm (allo)		earlier auto than allo peak, MHC
(islets on Table				9,148cpm (auto)		class II minimally involved in
3.4 and 3.12)						autologous response
Esquenazi 1987	ş	used 1x 10 <sup>5</sup>	kidmey contical cells	\$,000mm		MHC class II expression alone is not
			lymphocytes	10,000cpm		enough to increase stimulatory
					,	capacity in MLKC, a tissue response
						could stimulate an activated system
Theobald 1993	human	used 2x 10 <sup>3</sup>	leucocytes	100,000cpm		5 days 500U/ml IFM; optimal for
		· 5x 104	dermal fibroblasts	15,000cpm		MHC I and ICAM-1 enhancement,
			smooth muscle cells	10,000cpm		MHC II induction correlated with
			epidermal cells	5,000cpm		increased response to endothelial
			endothelial cells	20,000cpm		cells only.

## CHAPTER FOUR

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## CHAPTER FOUR

#### MATERIALS AND METHODS

#### 4.1 Isolation and storage of human islets

4.1.1 Automated method and COBE centrifugation for isolating human islets

Human islets were isolated from cadaver pancreata retrieved with appropriate consent from brain-dead heart beating kidney or multi-organ donors. The pancreata were retrieved after perfusion *in situ* with hyperosmolar citrate (Baxter Healthcare, Thetford, UK) or University of Winconsin solution (UW) (Du Pont, Delaware, USA) and transported to the laboratory (normally within two - three hours) for processing.

The pancreas (designated an HP number) was cleaned under sterile conditions in a Class II Microbiological safety Cabinet (Medical Air Technology Ltd, Manchester) and then cut in two (head and tail) before distention, via the common bile duct, with 3 mg/ml collagenase (Serva, Heidelberg, Germany, cat no 17449). The pancreas was placed in a sterile stainless steel chamber, then digested using the automated system described by Ricordi(113). The pancreatic digest was collected and washed, then stored for up to one hour in UW solution before being loaded onto a COBE 2991 cell processor (COBE, Lakewood, Colorado, USA) for density gradient centrifugation(114). Digest was either bottom loaded onto a discontinuous gradient of Euro-Ficoll(279) or bovine serum albumin (BSA) (Advanced Protein Products, Dudley, UK)(114) or by top loading onto a continuous gradient of BSA or Giessen Ficoll-sodium diatrizoate based density medium (Hering - personal communication). The gradient was collected in aliquots from the centrifuge and fractions containing islets or exocrine identified by dithizone staining and microscopical examination. After washing the fractions twice including centrifugation (800g for two minutes) (Jouan, St Nazaire, France) and supernatant replacement, the islet and exocrine tissue was placed into sterile non tissue culture 180 mm petri dishes (Sterilin, Stone, Staffs., UK), 150 µl tissue per dish in complete RPMI + 10% FCS as described below. Purity was estimated by two independent observers after

staining with dithizone-DMSO (1.44 mM DTZ in 10% DMSO and MEM) (Sigma, Poole, UK, cat no D5130 and Fisons, Loughborough UK, cat no D4121).

Human islets, exocrine and digest were maintained in culture in a Queue incubator (Parkersburg, WV26102, USA) for up to 48 hours at 37°C in 95% humidified air/5%CO<sub>2</sub> in RPMI tissue culture medium (Gibco-BRL, Paisley, Scotland, cat no 31870-025) containing 10% foetal calf serum (FCS) (Gibco-BRL, cat no 01106290) and supplemented with 20 mmol Hepes (Gibco-BRL cat no 15630056), 100 units/ml each of penicillin and streptomycin (Gibco-BRL cat no 15070-022), 2 mmol/L L-glutamine (Gibco-BRL 25030024), 1 mmol sodium pyruvate (Sigma cat no P2256) and 0.1 mmol 2-mercapto-ethanol (Sigma, cat no M6250).

Handpicking was carried out using a Wild Stereo microscope (Heerbrugg, Switzerland) with incident lighting. Islets and equivalent sized pieces of acinar or digest tissue were selected with an average size of  $150 \mu m$ diameter either from an unstained sample so that the handpicked tissue was representative of the purity and range of tissue in the sample (the preliminary experiments), or (for the standardisation experiments) were handpicked from a dithizone-ethanol (DTZ-ETH) stained sample(280) so that pure islets or pure acinar tissue pieces were used.

To stain the islets and acinar tissue for handpicking, the tissues were suspended in a 0.026 mM solution of DTZ-ETH in minimum essential medium (MEM) for 10 minutes after which the islets were stained pink/red as shown in Figure 4.1.

This preparation and concentration of dithizone has been shown not to alter the function of rat islets *in vitro* or *in vivo* (280). The tissue was then washed in MEM containing 5% FCS and resuspended in complete RPMI containing 10% human serum from a donor of blood group type AB (ABS) (Regional Blood Transfusion Centre, Sheffield). The stain was found to persist for 60 - 90 minutes during which the islets were picked as a positive selection and acinar tissue pieces as a negative selection.



**Figure 4.1.** This photograph shows the effect of staining a human isolated islet preparation with 0.026 mM dithizone in MEM. Islets are pink, acinar tissue is white and a mixture of complete and incomplete disassociation between the islets and acinar tissue can be seen. Rounded well cleaved islets  $(100 - 200 \ \mu\text{m}$  diameter) without acinar tissue attached were used and intact acinar tissue pieces of similar size. The photograph was taken using incident light, magnification x 40.

#### 4.1.2 Method for cryopreservation of human islets

The majority of the human islets used for this work were fresh and kept in culture for up to 48 hours before use. However, for the preliminary experiments, cryopreserved human islets were used which had been frozen and thawed using Rajotte's method(281). Briefly 10,000 human islets were placed in 1 ml of RPMI in a 5 ml freezing vial at room temperature, then DMSO was added in increasing amounts until a total of 3 ml DMSO had been added over a 45 minute period. The tubes containing islets and the RPMI/DMSO mixture were cooled on ice, left at -7.5°C for 30 minutes, nucleated after 15 minutes, then cooled on a programmable temperature controller (Planar Products Ltd, Sunbury on Thames, UK cat no PTC300) at 0.25°/min to - 196°C. The 5 ml vials of islets were stored in an XLC LN<sub>2</sub> bank. To use the islets for the MLIC, the vials were thawed rapidly at 37°C then transferred to ice before adding increasing volumes of 0.75 M sucrose solution to a total of 8 ml over a 50 minute period.

## 4.2 Collection and preparation of lymphocytes for the MLR and cocultures

#### 4.2.1 Responder PBLs

Peripheral blood was taken from tissue typed normal volunteers from the Department of Surgery using 5 x 10 ml monovette syringes containing heparin (Sarstedt, Germany), diluted 50:50 with MEM, layered over lymphocyte separating medium (LSM) (Techngen UK, cat no 9221397) then centrifuged at 2000 rpm for 20 minutes without braking. Peripheral blood lymphocytes were aspirated from the interface, washed twice in MEM containing 5% FCS and counted after staining with trypan blue using a haemocytometer. Between 1 - 2 million cells per ml were obtained from each volunteer. After the final washing, cells were resuspended in freezing medium (50% RPMI, 40% FCS, 10% DMSO (Fisons, Loughborough) at 7 x  $10^6$  cells per vial and frozen by incubation at  $4^{\circ}$ C for 1 hour, 20°C for 1 hour, overnight at -70°C and then put into liquid a nitrogen (LN<sub>2</sub>) bank.

Before use, PBLs cells were quickly thawed at  $37^{\circ}$ C, washed twice in MEM containing 5% FCS, live cells counted using a haemocytometer with trypan blue to detect dead cells. PBLs were resuspended in RPMI containing 10% FCS or ABS, normally at 1 x 10<sup>6</sup> cells per ml. The cell suspension (100 µl) was added to the coculture plates to give 1 x 10<sup>5</sup> cells per well. Concentrations of PBLs other than 1 x 10<sup>5</sup> per well are indicated in the text.

#### 4.2.2 Stimulator splenocytes

An amount of human spleen (3 cm cube minimum) was obtained from the same cadaver donor source as each pancreas, transported to the laboratory in new born calf serum (NBCS) (Advanced Protein Products Ltd, Brierley Hill, cat no AS-202-50) and processed within 12 hours of resection. Using sterile techniques, the spleen was first cut into small pieces then pushed through a 250  $\mu$ m nylon mesh. The mesh was washed with MEM, the cell suspension collected and overlaid onto LSM before centrifugation at 2000 rpm for 20 minutes without braking. The splenocytes were removed from the interface using a pasteur pipette, washed twice in MEM + 5% FCS, counted in a haemocytometer using trypan blue to detect the dead cells then resuspended at 1 x 10<sup>6</sup> cells/ml in complete RPMI containing 10% FCS

or ABS. Before adding to the MLR, the splenocytes to be used as the stimulator cell population were irradiated (3000 rads) using a Gammacell (Vinten Instruments, Atomic Energy of Canada Ltd Radiochemical Co.) and then 100  $\mu$ l of the splenocyte suspension used in the microwell plates to give 1 x 10<sup>5</sup> splenocytes per well. On occasions where the splenocytes were not used within 24 hours, the splenocytes were stored frozen and thawed, when required, by the method described above for PBLs.

## 4.2.3 Tissue typing and mismatched responder-stimulator populations

Splenocytes from cadaver donors (representing stimulator splenocytes, digest, islets, acinar tissue) and responder PBLs from normal volunteers were tissue typed by the Regional Blood Transfusion Centre, Sheffield. The techniques used included serological testing for MHC class I loci A,B and C and DNA oligotyping for the DR and DQ loci. Blood groups were identified and all cadaver donors were additionally tested for CMV and HIV. Stimulator and responder cell populations were HLA mismatched at the DR and A, B, and C loci except where indicated otherwise.

#### 4.3 MLIC, MLAC, MLR and Con A assays

#### 4.3.1 Setting up the assays

Microtitre plates (96 microwells) were used for the MLIC, MLAC, MLR and Con A assays. For the preliminary studies, three different types were used -Nunc tissue flat bottomed culture plates (Gibco-BRL, cat no 1-63320), Primaria coated flat bottomed tissue culture plates (Becton Dickinson, Oxford, UK, cat no Falcon 3872) and Linbro/Titertek round bottomed non tissue culture plates (Flow-ICN, cat no 76-242-05). Subsequently, only the Linbro 96 microwell round bottomed non tissue culture plates were used.

A plate diagram was used for each experiment to record the location and combination of cells. For the MLIC, MLAC and MLDC, samples of islets, acinar tissue or digest were taken in aliquots from the large petri dishes and the tissue either handpicked immediately or after dithizone staining as described above. The appropriate number of stimulator islets, acinar tissue

or digest pieces, averaging 150  $\mu$ m in size (100 - 200  $\mu$ m), for each experiment, were handpicked from small petri dishes and placed in the appropriate microwells using a finely drawn out pasteur pipette. In general, islets, digest and acinar tissue pieces were handpicked in random order to give a cross section of the tissue present. In most cases the acinar tissue was of similar size to islets (see Figure 4.1), but for a few preparations the tissue was dispersed as loose groups of cells and an estimate was made of the appropriate volume. For occasions where insufficient time was available, the plates containing the handpicked tissue were left overnight in an incubator at 37°C and the remaining cells added the following day.

For the MLR, 100  $\mu$ l of  $\gamma$ -irradiated splenocytes were added to the appropriate wells to give 1 x 10<sup>5</sup> cells per well, or as indicated for individual experiments. Concanavalin A (100  $\mu$ l) (Sigma, cat no C2010, type IV-S) was added to appropriate wells to give 0.4  $\mu$ g per well.

The responder population of HLA mismatched PBLs was added to all wells as appropriate (100  $\mu$ l) to give 1 x 10<sup>5</sup> cells per well. The final volume in the wells was made up to 250  $\mu$ l with RPMI medium containing 10% FCS or ABS. An example of an MLIC well containing 1 x 10<sup>5</sup> responder PBLs and 10 human islets is shown in Figure 4.2.



**Figure 4.2.** This photograph shows the MLIC after 3 days coculture, the white population is the proliferating responder PBL population and the central grey areas the 10 handpicked islets. The photograph was taken using incident light, magnification x 40.

#### 4.3.1a Pretreatment of stimulator populations

There was some difficulty in the preliminary experiments with fibroblast outgrowth from islets and acinar tissue which caused adhesion to the microwells, by collagen deposition, leading to high background counts for the stimulator population controls and adding to the variability of results. Fibroblast outgrowth was avoided in subsequent experiments by using non tissue culture 96 microwell plates.

The stimulator splenocytes for the MLR wells were  $\gamma$ -irradiated (3000 rads) to prevent them proliferating in response to the PBLs. The islet, acinar tissue and digest stimulator populations were not normally pretreated (except where indicated) for the MLIC, MLAC and MLDC. This was because these tissues do not proliferate in culture(282) and so would not be expected to add to the amount of responder PBL proliferation. However, the effect of such pretreatments was investigated on islets and acinar tissue and included  $\gamma$ -irradiation (3,000 rads) (as for the standard MLR) or mitomycin C (12.5 µg/ml) (Sigma, cat no M0503) pretreatment by incubation for 20 minutes then washing twice(269). In addition, Trasylol or aprotinin, a protease inhibitor (Bayer Newbury, UK) (28 µg/ml) was used, by adding to the appropriate wells for the duration of the experiment(146) and glutaraldehyde at 0.05 - 0.1% for 1 minute, which retains antigen epitopes but inhibits cell function (Londei M, personal communication).

#### 4.3.1b Incubation of MLIC, MLDC, MLAC, MLR plates

Microwell plates set up according to the appropriate plate diagram were incubated at  $37^{\circ}$ C and 95% humidified air/5% CO<sub>2</sub> for the appropriate time, normally 3, 5, 7 or 9 days.

#### 4.3.1c Pulsing with $[^{3}H]$ thymidine, harvesting and counting

Plates were pulsed by adding 1  $\mu$ Ci of [<sup>3</sup>H]thymidine using a Hamilton dispenser and 500  $\mu$ l gas tight glass syringe (Hamilton, Nevada USA, cat no PB-600-1) and a 21g needle. After further incubation at 37°C for 18 - 24 hours to allow incorporation of the isotope, the contents of the wells were harvested onto filter paper using a cell harvester (Titertek cell harvester, Flow-ICN), the filter discs put into scintillation vials, 2 ml of scintillation fluid added (Hi-safe Optiscint Fisons, Loughborough, UK, cat no sc/40 20/21)

and the beta emission of the vials read on a Beta counter (1217 Rackbeta liquid scintillation counter, LKB-Wallac, S Croydon, UK).

A summary of the MLR, MLIC and MLAC method is shown in Figure 4.3.



**Figure 4.3.** Diagrammatic summary of the MLR, MLIC and MLAC method in which only the nature of the stimulator cell population changes (splenocytes, islets or acinar tissue pieces). The results were expressed either as raw data (test cpm),  $\Delta$ cpm or S.I.

#### 4.3.1d Analysis of data

as described below.

Data was obtained in the form of counts per minute (cpm), the mean of quadruplicate samples were used as raw data for the calculation of  $\Delta$ cpm ( $\Delta$ cpm = test cpm minus sum of the responder and stimulator control cpm)

or stimulation index (SI = test cpm divided by sum of responder and stimulator control cpm). Results were also calculated as percentage changes and statistically analysed. At one period where the Beta counter was temporarily faulty and irregularly produced single anomalous results, then these, if different from the other three results by an order of magnitude, were omitted. Statistical analyses were carried out using the non parametric Mann Whitney U test together with the median and interquartile range or using Student's t test with the mean and standard error of the mean. The Apple Mac 'Minitab' statistics software was used and graphs were constructed using the 'Cricket Graph' and 'MacDraw' software programs.

## 4.4 Maintenance and use of CTLL cells, (interleukin-2 dependent)

CTLL cells originated from a mouse lymphoma cell line (European Collection of Animal Cell Cultures, Salisbury, UK) and are dependent on exogenous IL-2 and other cytokines for survival. They show proliferation that, at low concentrations, is linearly related to the amount of IL-2 present in the supernatant. The cells were maintained in tissue culture conditions using complete RPMI + 10% FCS with the addition of 10 units/ml recombinant IL-2 (Boehringer-Mannheim, Germany, cat no 799068). The cells were sub-cultured three times per week and the amount of IL-2 in the medium was depleted before using the cells in the CTLL assay. Cells were non adherent and were harvested from the tissue culture flask by aspiration then counted using a haemocytometer with trypan blue to identify the dead cells. Cells were resuspended in RPMI with additives including 10% FCS, but no IL-2, over the previous night (18 hours), at concentrations of 1 x  $10^4$  and/or 1 x  $10^5$  cells/ml for use in the CTLL assay.

### 4.4.1 CTLL cell assay for presence of IL-2 in MLR and coculture supernatants

Non tissue culture 96 well microtitre plates similar to those used for the cocultures were used for the CTLL assay. Supernatant (50  $\mu$ l) from the coculture wells was removed and transferred to the CTLL assay plate as appropriate and recorded on the plate diagram. The appropriate numbers

of IL-2 starved CTLL cells were added to each well as 100  $\mu l$  of cell suspension.

A dose response curve for the CTLL cells against known concentrations of IL-2 (including 10, 5, 2.5, 0.6, 0.3, 0.15, 0.075, 0.0375 units/ml) was constructed. For the test wells, supernatants from the control wells were taken from the coculture plates. After incubation at  $37^{\circ}$ C overnight, or as indicated for each experiment, the wells were pulsed with 1 µCi of [<sup>3</sup>H]thymidine (Amersham, UK, cat no TRK300) in 10 µl of RPMI containing 10% FCS or ABS using a Hamilton dispenser with a 500 µl gas tight glass syringe (Hamilton, Nevada USA, cat no PB-600-1) and a 21g needle. After further incubation at  $37^{\circ}$ C for 6 hours, the wells were harvested onto filter papers using a cell harvester (Titertek cell harvester, Flow-ICN Laboratories, Irvine, Scotland), the filter discs put into scintillation vials, 2 mls of scintillation fluid added (Hi-safe Optiscint, Fisons, Loughborough, UK, cat no sc/40 20/21) and the beta emission of the vials read on a Beta counter (1217 Rackbeta liquid scintillation counter, LKB-Wallac, S Croydon, UK).

4.4.2 Control lymphocyte response assays

These experiments were carried out to investigate the positive (Concanavalin A stimulated) and negative control (PBLs alone) responses of PBLs in medium containing serum of different types (FCS, pooled human serum, AB serum, autologous human serum) and batches (ABS batches 1 - 3). For this 1 x 10<sup>5</sup> PBLs were incubated in RPMI medium in the presence of either 10% FCS or 10% human serum with or without the addition of Con A (0.4  $\mu$ g/well). Microwells containing quadruplicate cocultures of PBL + Con A + medium or PBL + medium were included for responder PBLs from three different volunteers. The duration for these assays was three to five days.

4.4.3 Viability assay for islets and acinar tissue

The purpose of these experiments was to determine whether incubation in medium containing 10% human ABS had any effect on viability of cells in the MLR, MLIC and MLAC. Human islets and acinar tissue were cultured in microwells with and without lymphocytes and viability assays as described below, were carried out by microfluorimetry on islets and acinar tissue and

by trypan blue staining on lymphocytes. For microfluorimetry, a combination of fluorescein diacetate (FDA) and propidium iodide (PI) was used. FDA is a non polar ester which passes through cell membranes and is internally hydrolysed to form free fluorescein (green) which accumulates within the intact membranes of a living cell and PI can only penetrate the membranes of dead or dying cells where it binds to nucleic acids and fluoresces (red)(283).

#### 4.4.3a Microfluorimetric determination of viability

The contents of each microwell after coculture were placed into a shallow well on a glass slide containing 50  $\mu$ l of a solution of propidium iodide (PI) (Sigma, cat no P4170) (50  $\mu$ g/ml) and fluorescein diacetate (FDA) (Sigma, cat no F7378) (1 mg/ml in acetone - 75 mM) (188  $\mu$ l PI + 125  $\mu$ l FDA + 4190  $\mu$ l PBS) and incubated for 30 minutes. Measurements of fluorescence were made on an Olympus BH-2 inverted microscope using a blue filter (B460) green light (FDA) and separately a red filter (R610) (PI). Measurements of the background fluorescence as well as the tissue with each filter, were made. The measurements were used to calculate percentage viability in the following equation.

%tage viability = 
$$(FDA, test - bg)$$
 x 100  
(FDA, test-bg) + (PI, test-bg) - (0.04 x [FDA, test-bg])

(test = tissue being tested, bg = background)

#### 4.4.3b Trypan blue dye exclusion test

This was carried out by aspirating the total lymphocyte suspension from the glass slide wells, adding an equal volume of 0.2% trypan blue in 0.9% saline (Sigma, cat no T5526) then counting the number of live (unstained) and dead (blue) cells using a haemocytometer. Results were calculated as the total number of cells and the percentage live cells.

## 4.5 Measurement of the effect of soluble products of acinar cells (amylase) and islets (insulin) on MLR, MLIC and MLAC

For these experiments, the effect of enzymes secreted by the acinar tissue on lymphocyte proliferation was investigated using a system based on

commercially available 'transwells'. MLR assays in which 1 x 10<sup>5</sup> human responder PBLs (after LN<sub>2</sub> storage) were incubated with 1 x 10<sup>5</sup>  $\gamma$ irradiated (3,000 rads) stimulator HLA mismatched PBLs or fresh
splenocytes in 96 microwell plates were used in addition to MLIC and MLAC
assays.

Handpicked acinar tissue or islets (10 acinar cell clumps or 10 islets) were placed into a nucleopore polycarbonate filter cone with a 4  $\mu$ m pore size (Costar, High Wycombe, UK, cat no 110407) and inserted into each microwell such that the medium inside the cone could exchange with the medium in the microwell without the acinar cells or islets being in direct physical contact with the lymphocytes. A diagram showing the nucleopore filter cone inserted into the microwell is shown in Figure 4.4.



**Figure 4.4.** Nucleopore filter cone containing acinar cell clumps, inserted into the top of a microwell containing an MLR or coculture. The soluble products of the tissue in the cone, but not the tissue itself, was in contact with the coculture in the well.

Figure 4.5 shows a microwell plate in which half of the wells (white) contain nucleopore filter cones.

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The cultures were incubated for seven days and pulsed with  $[^{3}H]$ thymidine for the last 18 - 24 hours before harvesting and measuring the cpm in a beta counter as described above. Results were expressed as  $\Delta$ cpm (test cpm minus sum of appropriate negative controls).



**Figure 4.5.** This photograph shows a microwell plate containing wells of MLR, MLIC or MLAC combinations either without (pink) or with (white) inserted nucleopore filter cones.

#### 4.5.1 Amylase measurements

Measurements of amylase in the supernatant of microwell cocultures was made using a Phadedas Amylase kit (Pharmacia Biotechnology, St Albans, cat no 105 38033). Briefly 200  $\mu$ l of the supernatant sample was incubated with tablets containing a starch-colour complex (which releases dye on breakdown by amylase) for 15 minutes then the reaction stopped with 0.5 N sodium hydroxide. A standard curve of known concentrations of humylase (human amylase) was also set up. After centrifugation, absorbance was read at 620 nm wavelength on a LKB Ultrospec 4050. Calculations of amylase content of the supernatant sample were made by subtracting the absorbance of the blank from that of the sample then reading the figure against the standard curve.

#### 4.5.2 Insulin measurements

The insulin concentration in supernatants was measured using radioimmunoassay incorporating a standard curve of 0, 0.25, 0.5, 1, 2, 4, and 8 ng/ml human <sup>125</sup>I insulin (Amersham cat no Im166) and appropriate

positive and negative controls. Samples of 50  $\mu$ l supernatant and 50  $\mu$ l guinea-pig anti bovine insulin antiserum were incubated for 30 minutes at 4°C then 50  $\mu$ ls <sup>125</sup>I insulin in RIA buffer (PBS + 0.5% BSA) added for 90 minutes at room temperature. Sac Cel (50  $\mu$ l) (Donkey anti guinea pig coated cellulose) (IDS Ltd, Bolden, Tyne on Wear, cat no A-SAC-3) was added for 30 minutes incubation at room temperature and after centrifugation at 3500g for 6 minutes the supernatant was decanted and the pellet counted in a LKB Wallac 1282 Compugamma CS gamma emission counter.

The standard curve was calculated as:

mean of each non zero standard - mean of blank controls x 100 = Y

Х

where X = mean of the zero standards - mean of blank controls 100 - Y = % inhibition

The % inhibition vs ng/ml was plotted as a semilog graph for the standard curve.

The sample concentrations were calculated as:

<u>mean of sample - mean of blank controls</u> x 100 = % inhibition x

The insulin concentration of the samples (ng/ml) were read from the standard curve.

## 4.6 Measurement of the effect of cytokine upregulation of MHC antigens on the MLIC and MLAC

These experiments were in two parts and involved the treatment of handpicked islet and acinar stimulator populations with IFN- $\gamma$  (50 - 500 units/ml) and TNF- $\alpha$  (10 - 100 units/ml) (incubation for three to six days) and subsequent determination of a) whether the cytokine treatment altered the levels of MHC antigen expression and b) the effect on their immunogenicity as measured in the MLIC and MLAC. The procedures for b) are covered as part of the MLIC and MLAC descriptions above. The procedures for a) are covered by the descriptions for immunocytochemistry below.

#### 4.7 Immunocytochemistry

Immunocytochemical studies were carried out as a number of studies which included the determination of:

1) the efficacy of the antibodies chosen,

2) the investigation of light microscope double staining techniques,

3) the identification, distribution and quantification of MHC class I and II antigen positive cells in the pancreas as well as islets and acinar tissue immediately after isolation and after seven days culture at  $37^{\circ}$ C, 4) the effect of IFN- $\gamma$  and TNF- $\alpha$  cytokine treatment on MHC class I and II

expression of islets and acinar tissue and

5) the identification of different cell types in pancreatic digest.

4.7.1 Collection and frozen storage of pancreas, islets and acinar tissue

The following tissues were collected for the studies listed above:

1) and 2) = three different pancreata,

3) = 10 different pancreata each with islets and acinar tissue immediately after isolation and after seven days culture,

4) = islets and acinar tissue pieces as used in the relevant MLR, MLIC and MLAC experiments and

5) = samples of pancreatic digest from ten different donor pancreata.

For each pancreas, a small piece of tissue was taken from the head of the pancreas before distension with collagenase, cut into small blocks 3 mm x 10 mm, placed on a cork block, covered in Tissue-Tek OTC cryoprotectant (Miles, Elkhart, USA, cat no 4583) and frozen by being slowly immersed in isopentane (Fisons, cat no P/1030) cooled to approximately -21°C using LN<sub>2</sub>. The block was stored in a 1 ml freezing vial in a LN<sub>2</sub> bank (LR-40, Union Carbide, Darlington) until sectioned. Islets, acinar tissue and digest were frozen either immediately after digestion (digest) or isolation (islets and acinar tissue) (Day 0) or after one week in culture (islets and acinar tissue) (RPMI + 10% FCS), and were aspirated into a soft plastic microwell before filling the well with cryoprotectant. The well was frozen by being slowly immersed in isopentane cooled to approximately -21°C using LN<sub>2</sub>, then the

plastic cover cut away and the block stored in a one ml freezing vial in a  $LN_2$  bank.

#### 4.7.2 Cutting sections, storage, thawing slides

Sections of all tissues were cut at 5  $\mu$ m thickness onto multispot (Hendley-Essex, Loughton, UK, cat no PH-068, ) slides (four sections per slide) using a cryostat (Reichert-Jung 2800 Frigocut). Sections were dried at room temperature then stored at -20°C in dessicated air tight boxes. To thaw for use, the box was kept at 4°C for 30 minutes then at room temperature for 30 minutes before opening. Slides were fixed by immersion in acetone for 10 minutes at room temperature then dried in an airstream before being stained using immunocytochemistry.

#### 4.7.3 Immunocytochemical staining of sections

Two methods were used for this work. The principal method was the biotinavidin 3-layer method that has been previously successfully used in this laboratory. In addition, attempts were made to develop a double staining method using markers detectable with the normal light microscope, this was unsuccessful. Both are described below.

4.7.3a Biotin-avidin alkaline phosphatase, napthol phosphate-fast red For this method the primary antibodies (mouse anti human monoclonal antibody) as listed below, were incubated overnight, at 4°C in an incubation chamber, on sections fixed in acetone. After washing the sections in tris buffered saline (TBS) (6.055 g trizma and 8.52 g sodium chloride per litre at pH7.6), the appropriate secondary antibody, polyclonal antibody (to mouse IgG or IgM or human IgG) conjugated to biotin (Sigma, cat no B4765 - anti mIgG, B9265 - anti mIgM, B1518 - anti hIgG) and diluted 1:1000 in TBS, was incubated with the sections for 45 minutes at room temperature. After washing again with TBS, the sections were incubated, with extravidin conjugated to alkaline phosphatase (Sigma, cat no E2636) and diluted 1:400 in TBS, for 20 minutes at room temperature. After washing, the sections were incubated for 15 minutes at room temperature with napthol AS-BI phosphate sodium salt (Sigma, cat no N2250) and Fast red TR salt (Sigma, cat no F8764) with 0.1 M levamisole (Sigma, cat no L9756) to precipitate excess phosphatase in an acidic chromogenic substrate (1.21g trizma base and

4.8 ml of 1 M hydrochloric acid in 100 mls  $H_2O$ , pH 8.2). Acid haematoxylin (Sigma, 285-2) was used to counterstain the sections and they were mounted using glycerol gelatin (Sigma, GG-1). The resulting positive stain showed as a red localisation on a blue background.

## 4.7.3b Double staining method using horseradish peroxidase (HRPO) and alkaline phosphatase (AP)

This method was developed as a double staining procedure based on primary antibody isotype differences which would be visible under the light microscope and with two colours (red and blue-black) sufficiently different to identify specific antigens in the sections. One stain was used for the primary antibodies of isotype IgG2a and IgM, which identified the insulin-producing beta cells in islets (HB124) and acinar cells (LDS8), and the secondary polyclonal antibodies against mouse IgG2a or IgM were conjugated to alkaline phosphatase (Serotec, Kidlington, Oxon, cat no AACO3A and Sigma cat no A7784) and developed with 5-bromo-4-chloro-3indolyl phosphate (Sigma, cat no B8503-BCIP) and nitroblue tetrazolium (Sigma, cat no A5754-NBT) to give a blue-black stain. The contrasting stain was for primary antibodies of IgG1 isotype, which defined various molecules involved in stimulating an immune reaction, conjugated to HRPO (Serotec cat no SERT103P) and developed using 3-amino-9-ethylcarbazole (AEC) (Sigma, cat no A5754) to give a red stain.

4.7.3*b* i) Alkaline phosphatase colour development with BCIP/NBT For this method of immunocytochemical staining, acetone fixed sections were incubated overnight at 4°C using either IgG2a (HB124) or an IgM (LDS8) antibody. After washing with TBS, the sections were incubated for 45 minutes at room temperature with a secondary antibody specific for mouse IgG2a or mouse IgM conjugated to alkaline phosphatase at a 1:1000 dilution in TBS. After washing in TBS and buffer 3 (1.5 ml of 1 M tris, 1.5 ml of 5 M sodium chloride, 0.75 ml of 1 M magnesium chloride and 11.25 ml distilled water), the sections were developed for approximately 50 minutes using BCIP/NBT (1 ml buffer 3, 3.3  $\mu$ l of 50% BCIP in 70% dimethyl formamide [DMF], 4.4  $\mu$ l of 75% NBT in 70% DMF, with 1  $\mu$ l of 1 M levamisole). After washing in distilled water, the sections were counterstained with Mayer's haematoxylin for 5 minutes, rinsed in tap water, dried and mounted using glycerol gelatin.

#### 4.7.3b ii) HRPO colour development with AEC

For this method of immunocytochemical staining, acetone fixed sections were incubated overnight at 4°C with an IgG1 antibody specific for MHC class I antigens (HB120) or class II antigens (HB145). After washing with TBS, the sections were incubated for 45 minutes at room temperature with a secondary antibody to mouse IgG1 conjugated to HRPO (Serotec, cat no SERT103P) at a 1:200 dilution in TBS. After washing in TBS, sections were washed in acetate buffer pH 5.0 (14.8 ml of 0.2 M acetic acid, 35.2 ml of 0.2 M sodium acetate in 100 ml H<sub>2</sub>O) before incubation for 15 minutes in filtered AEC (0.5 mls of 0.4% AEC in 70% DMF, 9.5 mls acetate buffer pH 5.0, 25  $\mu$ l of 12% hydrogen peroxide). After further washing in distilled water, the sections were counterstained for 5 minutes with Mayer's haematoxylin then dried and mounted in glycerol gelatin.

### 4.7.3b iii) Combination of the two techniques alkaline phosphatase - BCIP/NBT and HRPO-AEC.

For the double stain technique, a combination of the two methods in sequence was used.

First the full method of the BCIP/NBT immunostaining was carried out using IgM or IgG2a primary antibodies, followed by incubation with the appropriate polyclonal antibodies (Sigma cat no A7784, Serotec cat no AACO3A) conjugated to alkaline phosphatase. The colour reaction was developed using BCIP and NBT. For the second stage using AEC immunostaining, the section was incubated with IgG1 primary antibody for 45 minutes, washed off with TBS, then incubated with the appropriate polyclonal antibody conjugated to HRPO (Serotec cat no SERT103P) for 45 minutes. After washing with TBS followed by acetate buffer, the AEC substrate was used for colour development. The sections were counterstained using Mayer's haematoxylin.

#### 4.7.4 Antibodies used in immunocytochemical staining

Antibody	Antigen	Isotype	Source
HB124	insulin	IgG2a	ATCC cell line
LDS8	acinar cells	IgM	Leicester University(284)
W6/32	HLA-A,-B,-C	IgG2a	ATCC cell line
HB120	HLA-A,-B,-C	IgG1	ATCC cell line
HB55	HLA-DR	IgG2a	ATCC cell line
HB145	HLA-DR	IgG1	ATCC cell line
MCA532	ICAM-1	IgG1	Serotec MCA532 anti CD54
Li8	IFN-a	IgG1	(T. Stewart, Genetech)
CB1375	TNF-α	IgG1	ATCC cell line
HB8291	IFN-y	IgG1	ATCC cell line
MC4243	Hsp65	IgG1	W.H.O. antibody

All primary antibodies used were mouse anti human antigen. ATCC = cells were obtained from the American Tissue Culture Collection, Rockville, Maryland USA, and grown in house. The supernatant was used neat as primary antibody.

W.H.O. = World Heath Organisation, Centres for Disease Control, Atlanta, USA.

4.7.5 Scoring slides and analysis

Microscopic sections were examined under the microscope and scored on a scale as follows:

- no evidence of any stain including artefactual stain
- -/+ minimal background stain, negative
- +/borderline between high background stain and weak positive positive +
- strongly positive ++

Comments were recorded for each section including an indication of the quality of morphology and the relative amount of tissue (post isolation). Analysis involved a comparison of positives from ten different pancreata and total numbers of islets or acinar tissue from one or more pancreata. The results are represented using photographs and graphs.

#### EXPERIMENTAL DESIGN

#### 4.8 Preliminary experiments

The first experiments were designed to ascertain:

a) whether an MLIC response could be measured

b) the suitability of the MLIC model as a pretransplant (three day) assay

c) a brief investigation of basic parameters of the MLIC.

#### 4.8.1 A test of the method

Experiment	Duration	Plates	No. Responders	No. Stimulators	Treatment of Stimulators
Expt 1-3	6 days in RPMI	NUNC	1-2 x 10 <sup>5</sup>	40 islets	cryopreserved

#### 4.8.2 Suitability as a pretransplant assay

These experiments reduced the duration to three days, to be within the expected pretransplant culture period of human islets. In addition, IL-2 production was measured, which precedes T helper cell proliferation and so could be used to shorten the assay duration.

Experiment	Duration	Plates	No. Responders	No. Stimulators	Treatment of Stimulators
Expt 4	3 days in RPMI + 10% FCS	NUNC Primaria Linbro	2 x 10 <sup>5</sup>	40 islets <u>+</u> irrad	cryopreserved
Expt 5 CTLL-1A CTLL-1B	6 days 2 days 6 days in RPMI + 10% FCS	NUNC Primaria Linbro	1 x 10 <sup>5</sup>	40 islets 1x 10 <sup>5</sup> irrad splenocytes	cryopreserved
Expt 6 CTLL-2A CTLL-2B	3 days 2 days 3 days in RPMI + 10% FCS	NUNC Linbro	1 x 10 <sup>5</sup>	40 islets $\pm$ irrad 1x 10 <sup>5</sup> irrad splenocytes	fresh (<24 hrs)

Expt 7 CTLL-3A CTLL-3B	3 days 2 days 3 days in RPMI + 10% FCS	Linbro	2 x 10 <sup>5</sup>	40 islets <u>+</u> irrad 40 digest <u>+</u> irrad	fresh (<24 hrs) fresh (<24 hrs)
Expt 8 CTLL-4A CTLL-4B	3 days 2 days 3 days in RPMI + 10% FCS	Linbro	2 x 10 <sup>5</sup>	40 islets islet ag 40 digest digest ag 1 x 10 <sup>5</sup> irrad splenocytes	fresh (<48 hrs) freeze/thaw fresh (<48 hrs) freeze/thaw
			. *	1 x 10 <sup>5</sup> irrad PBLs	
Expt 9	3 days in RPMI + 10% FCS	Linbro	2 x 10 <sup>5</sup>	40 islets <u>+</u> irrad	fresh (<48 hrs)

#### 4.9 Establishing parameters

These experiments looked at the titration and kinetics of the MLIC and MLAC/MLDC and determined the parameters to be used in later studies. (Linbro plates used and all islet, digest and acinar stimulators used fresh, normally within 48 hours of isolation, medium was RPMI + 10% FCS).

#### 4.9.1 Titration and kinetics of digest tissue in the MLDC

Islets were not available during this period and digest, containing all cell types found in the pancreas, was used as a substitute.

Experiment	Duration	No. Responders	No. Stimulators
Expt 10	3,5,7,9	1 x 10 <sup>5</sup>	20,30,40,50 digest 1 x 10 <sup>5</sup> irrad splenocytes
Expt 11	4,5,7,9	2 x 10 <sup>5</sup>	20,30,40,50 digest 1 x 10 <sup>5</sup> irrad splenocytes
Expt 12	3,5,7,9	1 x 10 <sup>5</sup>	20,30,40,50 digest 1 x 10 <sup>5</sup> irrad splenocytes

#### 4.9.2 Titration and kinetics of digest tissue and islets

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Islets were available for these experiments and the same pattern of titration and kinetic experiments to those above was followed.

Experiment	Duration	No. Responders	No. Stimulators
Expt 13	3,5,7,9	1 x 10 <sup>5</sup>	10,20,30,40 islets, digest 1 x 10 <sup>5</sup> irrad splenocytes
Expt 15	3,5,7,9	1 x 10 <sup>5</sup>	10,20,30,40 islets, digest 1 x 10 <sup>5</sup> irrad splenocytes
Expt 17	4,5,7,9	1 x 10 <sup>5</sup>	10,20,30,40 islets, acinar tissue 1 x 10 <sup>5</sup> irrad splenocytes
Expt 18	3,5,7,9	1 x 10 <sup>5</sup>	10,20,30,40 islets, acinar tissue 1 x $10^5$ irrad splenocytes
Expt 19	3,5,7,9	1 x 10 <sup>5</sup>	10,20,30,40 islets, acinar tissue 1 x 10 <sup>5</sup> irrad splenocytes

## 4.9.3 Comparison of different treatments of stimulator population

This experiment was designed to detect any substantial enhancement of immunogenicity caused by treating the islets, digest and acinar cells with irradiation or mitomycin C (treatments used by others to arrest stimulator cell growth in the MLC or MLIC) or Trasylol which is a protease inhibitor and would be expected to reduce the possible detrimental effect of the acinar cell enzymes on the proliferating T cells.

Experiment	Duration	No. Responders	No. Stimulators	Treatment of Stimulators
Expt 16	3,5,7,9	1 x 10 <sup>5</sup>	20 islets	<u>+</u> irrad, mitomycin C,
	in RPMI		20 digest	and trasylol
	+ 10% FCS		20 acinar	
		*+	$1 \ge 10^5$ irrad	
			splenocytes	

#### 4.9.4 Transplant patient donor-recipient combination for MLIC

This was an experiment permitted by the chance acquisition of islets from a batch used for transplantation into a diabetic patient. PBLs from the recipient, another well matched potential recipient as well as a non diabetic HLA mismatched source. The limited number of islets allowed a titration at seven days only.

Experiment	Duration	No. Responders	No. Stimulators
MUCIA	7 4	1 - 105	10 20 20 40 5-1-4-
MLIC14	in RPMI	PBLs from	10,20,30,40 islets
	+ 10% FCS	recipient,	
		HLA match,	
		HLA mismat	ch a

4.9.5 Comparison of effect of different number of responder cells

This was one experiment designed to check the difference in response generated by a range in numbers of lymphocytes. Previously either 1 x or  $2 \times 10^5$  PBLs were used as responders without any detectable difference between the two numbers.

Experiment	Duration	No. Responders	No. Stimulators
Expt 20	3,5,7,9 in RPMI	1 x 10 <sup>3</sup> 1 x 10 <sup>4</sup>	10 islets 1 x 10 <sup>5</sup> irrad
	+ 10% FCS	$1 \ge 10^5$	splenocytes

#### 4.9.6 Effect of using dithizone to handpick islets for the MLIC

The initial concept behind these experiments was to work with the islet preparation in the same condition as used for clinical transplantation. However, the preliminary experiments showed low and variable immunogenicity of islets and digest and a way to identify the separate

contribution of islets and acinar cells became necessary. Latif(280) has reported a concentration of dithizone which stains islets without impairing function and this was used to positively identify islets and subsequently to negatively identify acinar tissue.

Experiment	Duration	No. Responders	No. Stimulators	Treatment of Stimulators
Expt 21-22	3,5,7,9 in RPMI + 10% ABS	1 x 10 <sup>5</sup>	10 islets	± DMSO-DTZ, 0.13 mM DTZ-ETH, 0.026 mM DTZ-ETH

#### 4.9.7 Viability of islets, acinar tissue and lymphocytes

These experiments were carried out to determine whether 10% human AB serum damages islets, acinar cell clumps or lymphocytes.

Experiment	Duration	No. Islets or Acinar tissue
VA1	3,5,7,9 days	10 islets, 10 acinar tissue pieces
(all	+ 1 x 10 <sup>5</sup> PBLs ar	ad all in RPMI + 10% ABS)

VA2 3,5,7,9 days 10 islets, 10 acinar tissue pieces 5 islets + 5 acinar tissue pieces (all + 1 x 10<sup>5</sup> PBLs in either RPMI + 10% ABS or RPMI + 5% ABS)

#### 4.10 Standardisation of the model

#### 4.10.1 Titration and kinetics

These experiments consolidated the information gained from the previous work. As the background cpm of the negative PBL controls had in some cases been high, the use of 10% ABS as growth supplement for the medium was advised. The combination of ABS and DTZ for handpicking formed a basis for further titration and kinetic experiments. The titration followed the range given by Ulrichs(29) which showed 40 islets generating an optimal proliferative response. Higher numbers than 40 per well were not practical with the limited amount of human tissue available but following the results of the first titrations, the numbers per well were reduced to ascertain whether immunogenicity was detectable using low numbers of this scarce resource. The use of islets and equivalent sized pieces of acinar tissue from the same source allowed a direct comparison of the immunogenicity of both tissues.

Titration and Experiment	<b>l kinetics of</b> Duration	<b>10-40 islets</b> No. Responders	and acinar No. Stimulators	tissue pieces MLR	
Expt 23, 24 and 26	3,5,7,9	1 x 10 <sup>5</sup>	10,20,30,40 islets and acinar tissue	1 x 10 <sup>5</sup> splenocytes	
Titration and	d kinetics of	f 2-10 islets			
Experiment	Duration	No. Responders	No. Stimulators	MLR	
Expt 25	5,7,9	1 x 10 <sup>5</sup>	2,5,10 islets		
Titration and Experiment	<b>1 kinetics of</b> Duration	1-10 islets No. Responders	and acinar No. Stimulators	tissue pieces MLR	
Expt 27, 28 and 29	3,5,7,9	1 x 10 <sup>5</sup>	1,2,5,10 islets and acinar tissue	1 x 10 <sup>5</sup> splenocytes	
Extension of Experiment	kinetics of Duration	10 islets and No. Responders	d acinar tiss No. Stimulators	ue pieces MLR	
Expt 30, 32 and 35	7,9,11*,13*	1 x 10 <sup>5</sup>	10 islets and acinar tissue	1 x 10 <sup>5</sup> splenocytes	
* ± fresh media	ım				
<b>Titration of</b> Experiment	5-20 islets a Duration	and acinar C No. Responders	<b>ell Clumps</b> No. Stimulators	MLR	
Expt 33, 34 and 36* * no acinar tis	7,9 days sue used in this	1 x 10 <sup>5</sup> s expt.	10 islets and acinar tissue	1 x 10 <sup>5</sup> splenocytes	
(all experimen DTZ-ETH, med	ts above used f ium RPMI +10%	resh islets or a 6 ABS and HLA	cinar tissue, ha mismatched PB	ndpicked with 0.02 Ls as responders.	6 mM

4.10.2 'Back' proliferation of untreated stimulator islets and

acinar tissue pieces against HLA mismatched PBLs

This experiment was to determine whether the non proliferating islet and acinar stimulator cells substantially contributed to the overall number of proliferating cells recorded by the MLIC and MLAC.

Experiment	Duration	No. Responders Si	No. timulators
Expt 39,41	7,9 days	10 islets 1 x 10 <sup>5</sup> PBLs	1 x 10 <sup>5</sup> PBLs 10 islets

#### 4.11 Immunogenicity of acinar tissue

Some reports have described exocrine tissue as particularly immunogenic in the MLIC(29) (269) (273) and so the similarity of the proliferation to acinar tissue and islets found in these experiments was investigated further. Two approaches were taken:

a) to determine the effect of the soluble products of acinar cells on T cell proliferation, this was carried out using nucleopore filter cones to separate the acinar tissue but not its products from the MLR, and b) to upregulate the MHC antigens on acinar tissue using IFN- $\gamma$  and TNF- $\alpha$ ,

to determine whether this would increase the proliferative response.

#### 4.11.1 Effect of acinar cellular products on the MLIC and MLR

Two types of experiment were undertaken both using acinar cell clumps in nucleopore filter cones suspended in the medium of an MLIC or MLR. The first experiments (Expts 31, 37, 38, 40) included MLIC and MLR, the latter with either 1 x  $10^5$  y-irradiated splenocytes or 1 x  $10^5$  y-irradiated PBLs as the stimulator population combined with HLA mismatched PBLs.

Experiment	Duration	No. Responders	No. MLIC stimulators	MLR stimulators
Expt 31*,37, 38,40	7, 9 days	1 x 10 <sup>5</sup> PBLs	10 islets	1 x 10 <sup>5</sup> splenocytes
Expts MLR1, MLR2	7, 9 days 5, 7 days	1 x 10 <sup>5</sup> PBLs 1 x 10 <sup>5</sup> PBLs		1 x 10 <sup>5</sup> PBLs 1 x 10 <sup>5</sup> PBLs
* day 7 only				

All microwells were  $\pm$  10 handpicked acinar tissue pieces in suspended nucleopore filters so that cell products but not the cells were in contact with the cocultures. Amylase estimations were made for the supernatant in each well as a marker for the presence of protein products from the acinar cells above the filter appearing in the medium under the filter. 4.11.2 Effect of pretreatment of islets and acinar tissue pieces with cytokines on MLIC and MLAC.

The concentration of cytokine and duration of treatment is shown for each experiment.

<b>Treatments</b> Expt No. Pancreas	Post Isolation	Cytokine Incubation	units/ml IFN-γ TNF-α
Expt 42 HP327 Expt 43 HP333	1 day 0 days	3 days 6 days	50 10 500 100
Expt 44 HP340 MLIC and MLAC	2 days	4 days	500 100
Experiment Duration	No. Responders	No. Stimulators	
Expt 42, 43, 7, 9 days 44	1 x 10 <sup>5</sup> PBLs	10 IFN- $\gamma$ /TNF- $\alpha$ treated islets 10 IFN- $\gamma$ /TNF- $\alpha$ treated acinar clumps	

Immunocytochemical staining was carried out on islets and acinar cell clumps at an equivalent time to the cytokine pretreatment. This allowed correlation between the level of MHC expression and the allogeneic T cell proliferation. The relevant pancreas numbers are for Expt 42 - HP327, Expt 43 - HP333 and Expt 44 - HP 340.

4.11.3 Persistence of amylase in supernatant of cultured acinar tissue

Experiment	Duration	No. of Acinar Tissue Pieces	Treatment
Persistence of amylase	9 days total, 1 sample removed every 24 hrs	30 µl packed digest per 10 ml medium	fresh
Expt Aci 1	24 hrs duration for 9 days	10 pieces in 4 ml medium	fresh, γ–irradiation, mitomycin C
Expt Aci 2	48 hrs duration for 8 days	50 pieces in 4 ml medium	fresh, γ–irradiation, mitomycin C, 0.1% glutaraldehyde fix
Expt Aci 3	72 hrs duration for 6 days	30 pieces in 200 μl medium	fresh, <sub>Y</sub> -irradiation, mitomycin C, 0.1% glutaraldehyde fix
Expt Aci 4a 48 hrs duration for 4 days 30 pieces in transwells over 200 µl medium fresh, γ-irradiation, mitomycin C, 0.05% glutaraldehyde fix

Expt Aci 4b 48 hrs duration for 4 days

100 pieces in 4 ml medium fresh, <sub>Y</sub>–irradiation, mitomycin C, 0.05% glutaraldehyde fix

## 4.12 Immunocytochemical histology

4.12.1 Control levels of MHC class I and II antigen on pancreas, islets and acinar tissue

Immunocytochemical staining for insulin, acinar cells, MHC class I and II antigen were carried out on ten different pancreata and subsequent islets and acinar tissue post isolation at day zero and post culture at day seven.

PANCREAS ISLETS DAY O ACINAR TISSUE DAY 0 ISLETS DAY 7 ACINAR TISSUE DAY 7

Human pancreas numbers: HP257, HP258, HP259, HP260, HP261, HP263, HP264, HP266, HP267, HP270

4.12.2 Effect of cytokines IFN- $\gamma$  and TNF- $\alpha$  on MHC expression of islets and acinar tissue

As indicated above the following human pancreas numbers corresponded to the MLIC and MLAC cytokine experiments:

HP327 - Expt 42 HP333 - Expt 43 HP340 - Expt 44

4.12.3 Immunocytochemical staining to determine the presence of different cell types in pancreatic digest

This was carried out on digest from ten different pancreata as follows: HP255, HP257, HP258, HP260, HP261, HP263, HP264, HP266, HP267, HP270

# CHAPTER FIVE

## CHAPTER FIVE CONTENTS

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## CHAPTER FIVE

## PRELIMINARY STUDIES USING THE MIXED LYMPHOCYTE ISLET COCULTURE

## 5.1 Introduction

Information on the mixed lymphocyte islet coculture (MLIC) and related tests can be obtained from a relatively small number of papers as shown in Tables 3.1 - 3.18 at the end of Chapter Three. A number of authors have reported the use of human islets in the MLIC, including Behamou(271), Demidem(267), Harrison(261), Liu(285), Roth(255), Stein(130), Ulrichs(269) and Zeevi(273) but at the start of this study, none had used fresh human islets in the same condition as would be used in clinical transplantation. Recently published accounts describing the use of appropriate volumes (to give islet equivalents - IEQ) from isolated islet preparations of varying purity(273) (285) and studies using  $\gamma$ -irradiated disassociated islet cells as stimulators(271), have added to work described by Ulrichs(269) using pure handpicked islets and islet preparations of varying purity (freeze-thawed rather than fresh). Harrison used fresh human islets in the MLIC but with HLA-DR matched rather than fully allogeneic responder PBLs(261).

The MLIC as described in the reports listed above, was adapted from the mixed lymphocyte reaction (MLR), an assay that has been widely used to predict the extent of HLA-D mismatch between two lymphocyte populations(225). Because of the association between HLA-D mismatching and renal graft outcome(220), the mixed lymphocyte reaction (MLR) has been used as an indication of a beneficial donor recipient match. However, the duration of five to six days has precluded its use prospectively for cadaveric transplantation and the MLR is used instead as one of the tests of renal transplant compatibility for living related donors and of matching between bone marrow recipients. Recently, shorter duration assays have been developed which measure the IL-2 produced (using IL-2 sensitive CTLL cells) as an early indicator of activation and subsequent proliferation of T helper cells *in vitro* (213) (247).

In contrast to the short time interval between retrieval of cadaver kidneys and subsequent renal transplantation, isolated human pancreatic islets can be maintained in culture before use. This culture period allows completion of islet viability and function tests, microbiological testing for sterility(117), potential reduction of immunogenicity(129) and potentially allows selection of the most suitable recipient. In our laboratory we aim for the transplantation of MHC class II matched fresh human islets, on a 1:1 donor recipient ratio, within four days of isolation.

The initial impetus for these studies was to determine whether meaningful results could be obtained from the MLIC within this putative four day pretransplant culture period and so encourage an investigation of the relationship between the MLIC and islet graft immunogenicity.

Chapter Five describes a series of experiments which investigate the parameters and techniques necessary to enable use of the MLIC as a pretransplant assay and includes the potential development of a short duration MLIC using CTLL cells to measure IL-2 production. Briefly, the allogeneic MLIC comprises lymphocytes, such as PBLs (responders), incubated under tissue culture conditions together with islets (stimulators), in microwell plates for a period of time dependent on the optimum response for this combination of cell populations(253) (256). After incubation, the wells are pulsed with tritiated thymidine which is taken up in the DNA of the proliferating T helper cells. The cells are harvested some 18 - 24 hours later and the level of incorporated tritium label counted. Thus, the counts per minute reflect the incorporation of tritiated thymidine and also the amount of proliferation by the responder cells stimulated by the allogeneic islets(215). The CTLL assay makes use of the fact that IL-2 is produced immediately prior to the proliferation of the responder population so showing a result in a shorter time. IL-2 is detected using a cell line (CTLL) that proliferates in the presence of IL-2 and dies without it(247).

Thus in summary, this work was started because of the imminent implementation of the human islet programme in Leicester. It was thought to be important to set up an assay to measure immunogenicity of human islets and the possibility of using such an assay as a pretransplant model of donor recipient matching was considered an immediate priority. Therefore a series of preliminary experiments was designed to gain familiarity with

the techniques involved and to indicate which parameters could be used as a basis for the work before attempting to reduce the time course of the MLIC to within the four day culture period allowed by the human islet transplant protocol.

5.1.1 A study of the basic methodology of the MLIC

Three experiments were planned (Expts 1 - 3) based on work described by Ulrichs(269), in which a duration of six days and a stimulator islet number of 40 islets was used for the human allogeneic MLIC. Several of the parameters used by Ulrichs were changed for this study, including a decrease in the number of responder cells used, the use of 10% FCS rather than 10% ABS in the medium, the use of fresh islets rather than freeze-thawed islets, and an increase in the number of repeated samples from three to four. The reasons for these changes were as follows: a) Ulrichs reported 1 x 10<sup>6</sup> PBLs per well but this would have involved considerable quantities of blood for the assays planned.  $1 - 2 \times 10^5$  PBLs have been used successfully by others(267) and so were used here. b) 10% FCS was used as a growth supplement in the RPMI medium in place of 10% ABS as used by Ulrichs as this was the usual growth supplement used for islets in Leicester.

c) For this study, the human islets were not treated with mitomycin C, as used by Ulrichs, or  $\gamma$ -irradiation which is normally used to inhibit stimulator cell growth in the MLR. This was because adult human islets do not substantially proliferate in culture(282) and future experiments were planned to use the MLIC to test immunomodulation protocols which included low dose irradiation.

d) quadruplicate rather than triplicate samples were used to reduce variation.

The controls for this study included Con A which has been used as positive control for non specific stimulation, and negative controls for islets, PBLs and medium.

Thus in summary, the basic methodology was  $1 - 2 \ge 10^5$  HLA mismatched PBL responders and 40 (optimum number as found by Ulrichs) fresh human islets. Incubation of the coculture was for six days in tissue culture conditions at 37°C using RPMI + 10% FCS as a serum supplement. Samples, in quadruplicate, included negative and positive control wells. The cocultures

were pulsed with  $[^{3}H]$ thymidine at day five, harvested on day six and the beta emission counted.

#### 5.1.2 Results of the first MLIC experiments

The results of the first three MLIC experiments (Expts 1-3) showed a proliferative response of  $1 - 2 \times 10^5$  cryopreserved PBLs to Con A and to 40 HLA mismatched cryopreserved islets at days five to six. Negative control counts for medium alone were <300 cpm (counts per minute). The means of quadruplicate samples are given in Table 5.1.

 Table 5.1. The lymphocyte proliferative response and control levels (cpm) in the five to six day MLIC

Expt	No. PBLs	Con A + PBLs	40 Islets + PBLs	Islet control	PBL control
		(cpm)	(cpm)	(cpm)	(cpm)
1	1 x 10 <sup>5</sup>	2,689 <u>+</u> 162	1,120 <u>+</u> 337	7,605 <u>+</u> 1,287	574 <u>+</u> 147
	2 x 10 <sup>5</sup>	4,096 <u>+</u> 1,950	702 <u>+</u> 275	5,031 <u>+</u> 2,432	693 <u>+</u> 227
2	1 x 10 <sup>5</sup>	2,123 <u>+</u> 2,055	3,048 <u>+</u> 1,747	1,200 <u>+</u> 643	402 <u>+</u> 267
	2 x 10 <sup>5</sup>	1,167 <u>+</u> 1,274	2,346 <u>+</u> 2,942	617 <u>+</u> 919	197 <u>+</u> 521
3	1 x 10 <sup>5</sup>	8,119 <u>+</u> 13,914	8,689 <u>+</u> 5,832	4,951 <u>+</u> 5,946	2,371 <u>+</u> 2,086
	2 x 10 <sup>5</sup>	11,078 <u>+</u> 1,161	8,829 <u>+</u> 7,266	5,785 <u>+</u> 7,275	1,987 <u>+</u> 2,287

Expts 1 - 3 used NUNC plates, 1 - 2 x  $10^5$  cryopreserved responder PBLs and HLA mismatched cryopreserved islets. Figures given represent means <u>+</u> S.E.M. (cpm) of quadruplicate samples.

These first three results showed:

a) variable incorporation of  $[^{3}H]$  thymidine in all groups with no increase represented by using a higher number (2 x 10<sup>5</sup>) of PBLs,

b) a low level of incorporation by the positive controls (Con A stimulated PBLs) and this may have represented a post peak (three days) response,c) although a measurable lymphocyte proliferation occurred against the islets, this was variable between the three experiments andd) the background cpm of islets alone was unacceptably high as a negative control.

# 5.1.3 Reduction of the $[^{3}H]$ thymidine incorporation by islet controls

The incorporation of [<sup>3</sup>H]thymidine by islets alone was thought to be due to outgrowth of fibroblasts from the islets seen during the course of the experiment. This was investigated by using different types of microwell plates to contain the lymphocytes and islets resulting in an outgrowth of fibroblasts seen from islets in tissue culture treated plates (NUNC, Primaria), which was not seen in the non treated (Linbro) plates. The result of incubation of islets for six days on tissue culture treated or non treated plastic is shown in Figures 5.1a and 5.1b.



**Fig 5.1a and 5.1b.** 1a shows an islet incubated in tissue culture treated plastic for six days with a vigorous outgrowth of fibroblasts which may contribute to high background cpm of islet controls. 1b shows an islet from the same pancreas and for the same length of time in non tissue culture treated plastic with minimal fibroblast outgrowth and no adherence to the plastic.

One MLIC experiment (Expt 4) was carried out using different types of microwell plate to determine whether the non tissue culture plates could support a PBL proliferative response comparable to that obtained in tissue culture plates. The results are shown in Table 5.2.

Table 5.2. The three day MLIC using different culture plates

plate type	Con A + PBLs	40 Islets + PBLs	Islet control	PBL control
	(cpm)	(cpm)	(cpm)	(cpm)
NUNC	35,391 <u>+</u> 5,618	2,263 <u>+</u> 1,707	968 <u>+</u> 280	925 <u>+</u> 598
Linbro non TC	31,357 <u>+</u> 2,245	1,011 <u>+</u> 490	863 <u>+</u> 379	590 <u>+</u> 126
Primaria	44,726 <u>+</u> 1,970	745 <u>+</u> 310	592 <u>+</u> 280	520 <u>+</u> 128

Expt 4 used 2 x  $10^5$  cryopreserved responder PBLs and HLA mismatched cryopreserved islets. Numbers given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples.

The [<sup>3</sup>H]thymidine incorporation was found to be similar in each group when using different plates and the response to Con A was significantly higher (p < 0.05) than that to islets or control groups in each type of plate. No other comparisons were significantly different, although a trend of correlation was seen between the incorporation by islets alone and by islets + PBLs.

When the microwells were viewed under the microscope on day three, all wells containing islets had limited fibroblast outgrowth. In addition, the levels of  $[^{3}H]$ thymidine recorded, although low in the islet controls, were also low for the islet + PBLs samples when compared with the results in Table 5.1.

It was decided to extend the duration of the MLIC from 3 to 6 days in order to compare the MLIC with fibroblast outgrowth, to the MLIC without fibroblast outgrowth. One experiment (Expt 5) was carried out to compare the six day MLIC incubated using different plates as shown in Table 5.3.

The results showed a low background response for the islet control in Linbro non tissue treated plates after six days but there was also a low PBL response to islets in these plates. A low response to Con A at day six combined with the high response obtained at day three, as shown in Table 5.2, supported the suggestion that low incorporation of PBLs in response to Con A at day six was due to a post peak response. From these experiments, a trend was seen towards reduction in the levels of thymidine incorporation by islets alone in Linbro plates, which was supported by finding restricted fibroblast outgrowth by microscopic examination.

Table 5.3. The six day MLIC using different culture plates

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40 islets+PBLs	Islet control (cpm)	PBL control (cpm)
(cpm)		
2103 <u>+</u> 374	1730 <u>+</u> 942	113 <u>+</u> 46
449 <u>+</u> 191	540 <u>+</u> 247	104 <u>+</u> 65
2898 <u>+</u> 459	4438 <u>+</u> 1498	88 <u>+</u> 57
	40 islets+PBLs (cpm) 2103 ± 374 449 ± 191 2898 ± 459	40 islets+PBLs         Islet control (cpm)           (cpm)

Expt 5 used 1 x  $10^5$  cryopreserved responder PBLs and HLA mismatched cryopreserved islets. Numbers given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples.

Thus in summary, Linbro non tissue culture plates were determined to be the most suitable for the MLIC. This was due to their ability to restrict fibroblast outgrowth from human islets and subsequent attachment to the microwells while retaining the ability to support a Con A response.

# 5.2 Studies to determine the suitability of the MLIC as a pretransplant assay

A comparison between the level of allogeneic lymphocyte proliferative response to islets at three and six days was made. As a four day period between isolation and transplantation was envisaged for the human islet transplant programme, the response at three days would need to be sufficient to indicate *in vitro* immunogenicity in place of the five to six day duration normally used for the clinically related MLR. The results are shown in Table 5.4.

The results show a small proliferative response of allogeneic PBLs to islets detected at day three which reflected an increasing response up to day six. As previously, the response to Con A was shown to be decreasing by day six.

Table 5.4. A comparison between the three day and six day MLIC

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Duration	Con A + PBLs	40 Islets + PBLs	Islet control	PBL control
	(cpm)	(cpm)	(cpm)	(cpm)
3 days	35,391 <u>+</u> 5,618	2,263 <u>+</u> 1,707	968 <u>+</u> 280	925 <u>+</u> 598
6 days	11,078 + 1,042	8,829 + 325	5,785 + 2,531	1,987 + 636

Expts 4 (3 days) and 3 (6 days) used NUNC plates,  $1 - 2 \times 10^5$  cryopreserved PBLs and HLA mismatched cryopreserved islets. Figures given represent means <u>+</u> S.E.M. (cpm) of quadruplicate samples.

Further experiments using Linbro plates and a duration of three days were used to estimate the repeatibility of the low lymphoproliferative response to islets.

Table 5.5. Variation between experiments in the three day MLIC

Expt No.	Con A + PBLs	40 Islets + PBLs	Islet control	PBL control
	(cpm)	(cpm)	(cpm)	(cpm)
7	36,146 <u>+</u> 4,432	1,188 <u>+</u> 257	861 <u>+</u> 292	1,235 <u>+</u> 464
8	21,950 <u>+</u> 5,149	980 <u>+</u> 235	997 <u>+</u> 207	776 <u>+</u> 221
9	34,050 <u>+</u> 5,913	332 <u>+</u> 159	643 <u>+</u> 47	6,007 <u>+</u> 1,575

Expts 7 - 9 used Linbro plates,  $2 \times 10^5$  cryopreserved PBLs and HLA mismatched fresh islets. Figures given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples.

As can be seen in Table 5.5, there was a vigorous Con A response at 3 days and, as recorded in previous experiments, a small PBL response to allogeneic islets which did not appear to correlate with either the control islet or control PBL cpm.

Thus in summary, a comparison between the three day and six day MLIC showed that the response at three days was low and did not always exceed the background  $[^{3}H]$ thymidine incorporation of the controls.

## 5.3 Early detection of the T cell proliferative response by IL-2 presence in the MLIC

Other authors(213) (247) have reported the detection of IL-2 using IL-2 sensitive CTLL cells in proliferation assays as an early indication of the T helper cell proliferative response. In order to enhance the ability to detect the PBL response to allogeneic islets after three days incubation, three experiments (Expts 6, 7 and 8) were carried out in which samples of supernatant from the cocultures were added to IL-2 starved CTLL cells. The subsequent proliferation of CTLL cells recorded at day two and three was compared with the T cell proliferation of the MLIC at day three. The results are shown in Table 5.6.

**Table 5.6.** CTLL cell proliferation (cpm) in response to IL-2 at day two and three compared with the T cell proliferative response to islets (cpm) in the three day MLIC

#### CTLL Day Two

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Expt	PBL No.	Con A + PBLs	40 Islets + PBLs	Islet control	PBL control
		(cpm)	(cpm)	(cpm)	(cpm)
6	1 x 10 <sup>5</sup>	8,071 <u>+</u> 3,673	997 <u>+</u> 94	614 <u>+</u> 77	671 <u>+</u> 400
7*	2 x 10 <sup>5</sup>	11,156 <u>+</u> 1,741	12,884 <u>+</u> 1,411	16,333 <u>+</u> 1,591	10,885 <u>+</u> 1,243
8	2 x 10 <sup>5</sup>	3,993 <u>+</u> 589	1,083 <u>+</u> 599	515 <u>+</u> 407	335 <u>+</u> 83

Expts 6-8 used Linbro plates,  $1 - 2 \times 10^5$  cryopreserved PBLs and HLA mismatched fresh islets. Figures given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples. CTLL cells were  $1 \times 10^3$  per well (Expt 6) and  $1 \times 10^4$  per well (Expts 7 and 8).

#### CTLL Day Three

Expt	PBL No.	Con A + PBLs	40 Islets + PBLs	Islet control	PBL control
		(cpm)	(cpm)	(cpm)	(cpm)
6*	1x 10 <sup>5</sup>	9,144 <u>+</u> 637	14,512 <u>+</u> 7,303	9,279 <u>+</u> 1,312	9,570 <u>+</u> 1,779
7	2x 10 <sup>5</sup>	9,735 <u>+</u> 455	768 <u>+</u> 291	609 <u>+</u> 259	358 <u>+</u> 64
8*	2x 10 <sup>5</sup>	45,643 <u>+</u> 12,303	14,905 <u>+</u> 4,027	13,457 <u>+</u> 1,553	14,737 <u>+</u> 1,588

Expts 6-8 used Linbro plates,  $1 - 2 \ge 10^5$  cryopreserved PBLs and HLA mismatched fresh islets. Figures given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples. CTLL cells were  $1 \ge 10^3$  per well (Expt 6) and  $1 \ge 10^4$  per well (Expts 7 and 8). \*Using CTLL cells after overnight IL-2 depletion was found necessary as shown by Expt 6 day three and Expt 7 day two for which the cells were depleted of IL-2 after a weekend in medium, yet subsequent absence of IL-2 for two to three hours was

insufficient, as high background levels were recorded. Overnight IL-2 starvation of CTLL cells did not always reduce the background counts as seen in Expt 8 day three.

MLIC	Day	Thre	e		
Expt	PRI	No.	Con	A +	p

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Expt	PBL No.	Con A + PBLs	40 Islets + PBLs	Islet control	PBL control
		(cpm)	(cpm)	(cpm)	(cpm)
6	1x 10 <sup>5</sup>	44,258 <u>+</u> 18036	2,493 <u>+</u> 403	1,509 <u>+</u> 328	2,156 <u>+</u> 344
7	2x 10 <sup>5</sup>	36,146 <u>+</u> 4432	1,188 <u>+</u> 257	861 ± 292	1,235 <u>+</u> 464
8	2x 10 <sup>5</sup>	21,950 <u>+</u> 5149	980 <u>+</u> 235	997 <u>+</u> 207	776 <u>+</u> 221

Expts 6 - 8 used Linbro plates,  $1 - 2 \times 10^5$  cryopreserved PBLs and HLA mismatched fresh islets. Figures given represent means <u>+</u> S.E.M. (cpm) of quadruplicate samples.

This limited number of experiments showed that the regular culture of CTLL cells and careful control of the IL-2 level in the medium was necessary to obtain consistent results. Such a routine was found to be incompatible with the irregular pattern of work caused by the intermittent supply of human islets.

The results shown in Table 5.6 indicated that the high proliferative response (cpm) of the Con A + PBL cultures corresponded to relatively high IL-2 levels as indicated by CTLL cell proliferation (cpm). Similarly the relatively small increase of PBL proliferation against islets compared with the controls was mirrored by the low level of CTLL cell proliferation. Therefore, the results of the day two CTLL assay reflected the results of the three day MLIC but the detection of the proliferative immune response to islets was not sufficiently enhanced to give a significant result.

### 5.3.1 Dose response curves for IL-2 and CTLL proliferation

As part of Expt 5 (day 3), a CTLL IL-2 dose response curve was set up in three types of plate (Nunc, Linbro and Primaria). The results in the Nunc and Linbro plates showed a steep, almost straight line correlation between 0.2 units/ml and 1 unit/ml IL-2 and 4,000 - 11,000 cpm, representing the incorporation of  $[^{3}H]$ thymidine by the CTLL cells, as shown in Figure 5.2. The CTLL cells did not proliferate in the Primaria plates.



**Fig 5.2.** Proliferation of IL-2 sensitive CTLL cells in response to recombinant human IL-2. Above 0.2 units/ml of IL-2, there was a strong correlation between IL-2 concentration and CTLL proliferation in Nunc and Linbro microwell plates, but no CTLL proliferation in the Primaria plates.

Dose response curves were also set up comparing three different methods of CTLL cell treatment at  $1 \times 10^3$  and  $1 \times 10^4$  cells per well. The results of these treatments (listed below) of CTLL cells before and during the IL-2 detection assay can be seen in Figures 5.3a and 5.3b.

Treatment a = CTLL cells + Il-2 18 - 24 hours, then no IL-2 for 3 - 4 hours, test concentration of IL-2 added to the CTLL cells for 6 hours before pulsing with  $[^{3}H]$ thymidine for 18 - 24 hours.

Treatment b = CTLL cells + Il-2 for 6 hours, then no IL-2 for 18 - 24 hours, test concentration of IL-2 added to the CTLL cells for 6 hours before pulsing with  $[^{3}H]$ thymidine for 18 - 24 hrs.

Treatment c = CTLL cells + ll-2 given 3 days previously, then test concentration of IL-2 added to the CTLL cells for 18 - 24 hrs before pulsing with [<sup>3</sup>H]thymidine 6 hours.



Fig 5.3a and 3b. Three treatments of CTLL cells caused little difference in proliferation of  $1 \times 10^4$  CTLL cells per well (5.3a). At  $1 \times 10^3$  CTLL cells per well (5.3b) proliferation was less pronounced and treatment a was less effective than treatments b and c.

As can be seen in Figure 5.3a, different treatments of CTLL cells did not alter the dose response curve above 20,000 cpm with an initial number of 1 x  $10^4$  CTLL cells per well. A reduction in initial number of CTLL cells (Figure 5.3b) led to a variable response which was lower using treatment a. One difficulty of IL-2 detection for the MLIC was that the low proliferative response induced by allogeneic islets appeared close to the limit of sensitivity of the method.

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Thus in summary, the detection of IL-2 production using CTLL IL-2 sensitive cells was found to be dose related and can be applied to the MLIC. However, the low level of proliferative PBL response to allogeneic human islets and low level of IL-2 production was insufficient to allow detection within three to four days as would be required for a pretransplant assay.

5.3.2 The effect of  $\gamma$ -irradiation and cell disruption on the immunogenicity of human islets in the three day MLIC

In an attempt to enhance the detection of lymphocyte proliferation against allogeneic human islets, two different treatments were considered. Firstly, the effect of  $\gamma$ -irradiation (3,000 rads) was studied, which is a common treatment for stimulator lymphocyte populations to arrest their growth and prevent a two-way MLR proliferation. The results are shown in Table 5.7.

Table 5.7 Proliferative lymphocyte response (cpm) to fresh or  $\gamma$ -irradiated islets in the three day MLIC

Expt	Islets + PBLs	Islet control	γ-irrad islets +	γ-irrad islet
	(cpm)	(cpm)	PBLs (cpm)	control (cpm)
7	1,188 <u>+</u> 257	861 <u>+</u> 292	1,168 <u>+</u> 708	355 <u>+</u> 68
9	332 <u>+</u> 159	643 <u>+</u> 47	517 <u>+</u> 359	632 <u>+</u> 208

Expts 7 and 9 used Linbro plates,  $2 \times 10^5$  cryopreserved PBLs and HLA mismatched fresh islets. Figures given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples. Linbro plates were used and 40 islets per well.

The results of these two experiments indicated no evidence to show that  $\gamma$ -irradiation modulated the stimulatory capacity of human islets in the three day MLIC.

An additional method was tried involving the disruption of islets by repeated freeze-thawing which was thought to make the islet antigen more accessible to the responder PBLs. The results are shown in Table 5.8. **Table 5.8** Proliferative lymphocyte response (cpm) to fresh islets or islet antigen in the three day MLIC

Expt	Islets + PBLs	Islet control	lslet antigen +	lslet antigen
	(cpm)	(cpm)	PBLs (cpm)	control (cpm)
9	980 + 235	997 + 207	887 + 302	335 + 177

Expt 9 used Linbro plates, 2 x  $10^5$  cryopreserved PBLs and HLA mismatched fresh islets. Figures given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples. Linbro plates were used and 40 islets or antigen from 40 islets per well.

The results of this experiments indicated that there was no evidence to show that disrupting the islets as antigen increased the stimulatory capacity of human islets in the three day MLIC.

Thus in summary, two different treatments of the stimulator islets,  $\gamma$ -irradiation, which is used as a treatment for stimulator cells in the MLR, and disruption to form islet antigen, which might make the islet antigens more accessible to antigen presenting cells, did not increase the immunogenicity of human islets in the three day MLIC.

# 5.4 Effect of the number of responder PBLs on proliferation levels in the MLIC

Three experiments compared two different PBL concentrations (1 x  $10^5$  and 2 x  $10^5$ ) per well. The results are shown in Table 5.9.

 Table 5.9. Proliferative lymphocyte response (cpm) using variable numbers of responder PBLs in the five to six day MLIC

Expt	PBL No	Islets + PBLs	PBL control	PBL No	Islets + PBLs	PBL control
	(cpm)	(cpm)	(cpm)	(cpm)	(cpm)	(cpm)
1	1 x 10 <sup>5</sup>	1,120 + 337	575 + 147	2 x 10 <sup>5</sup>	702 + 275	693 + 227
2	1 x 10 <sup>5</sup>	3,048 +1363	402 + 104	2 x 10 <sup>5</sup>	2,326 + 1084	197+ 113
3	1 x 10 <sup>5</sup>	8,689 + 2593	2,371 + 60	2 x 10 <sup>5</sup>	8,829 + 325	1,987 + 636

Expts 1 - 3 used Nunc plates, 1 - 2 x  $10^5$  cryopreserved PBLs and 40 HLA mismatched cryopreserved islets. Figures given represent means  $\pm$  S.E.M. (cpm) of quadruplicate samples.

No significant difference was seen between the results using  $1 \times 10^5$  or  $2 \times 10^5$  PBL responders in the coculture wells over a five to six day incubation.

Another experiment (Expt 20) relating to the use of different responder PBL concentrations  $(1 \times 10^3, 1 \times 10^4 \text{ or } 1 \times 10^5)$  was carried out using Con A or 10 islets for stimulation. The results are shown in Figure 5.4.



**Fig 5.4.** Effect of increasing the responder cell population on lymphocyte proliferation ( $a = 10^3$  PBLs,  $b = 10^4$  PBLs,  $c = 10^5$  PBLs), All three groups - the MLIC, positive control (Con A) and negative control (PBLs), showed a trend of correlation between initial PBL concentration and final cpm.

The results showed that the level of proliferation of PBLs increased according to the initial concentration of PBLs per well and the length of incubation. High [<sup>3</sup>H]thymidine incorporation for the MLIC at day 7 appeared anomalous and led to an examination of the Beta Counter which showed an irregular fault which produced high cpm, this was subsequently repaired.

Thus in summary, the findings from Expt 20 showed that the initial concentration of  $1 \times 10^3 - 1 \times 10^5$  PBLs in the MLIC, Con A and PBL control wells correlated with the total [<sup>3</sup>H]thymidine incorporation over a 3, 5, 7 and 9 day culture period. As the earlier experiments (Expt 1-3, Table 5.9) had shown no differences in results using  $1 \times 10^5$  or  $2 \times 10^5$  PBLs per well

and it was preferable that the number of PBLs used per well be kept to a minimum to conserve resources, then  $1 \times 10^5$  PBLs per well was used in the later standardisation of the assay. This figure is within the range identified by other workers as can be seen in Tables 3.1 - 3.4.

# 5.5 Overall summary of the preliminary MLIC experiments

The experiments described in this chapter were designed to elucidate the basic parameters of MLIC methodology. A reduction in fibroblast outgrowth by using non tissue microwell plates for a six day MLIC incubation period was established. Meaningful results could not be obtained from the MLIC within the three to four day pretransplant culture period of human islets intended for clinical allogeneic transplantation. The lymphoproliferative response to HLA mismatched islets was low compared with the PBL response to Con A at day three and lower than the minimal MLIC response at six days. Enhancement of detection of proliferation in the three day MLIC was attempted using IL-2 detection (measured using IL-2 sensitive CTLL cells), the release of which precedes T cell proliferation, but this did not sufficiently increase the MLIC results. Attempts to enhance the ability of islets to stimulate allogeneic PBLs, including y-irradiation of the islets and disruption of the islets as antigen, were ineffective in these experiments. Finally, a limited investigation of the effect of varying the numbers of responder PBLs was carried out and showed that 1 x 10<sup>5</sup> was an optimal number, taking into consideration the need to conserve resources of human tissue typed PBLs.

In conclusion, an allogeneic lymphocyte proliferative response to human islets was observed in the MLIC but the lymphoproliferation at three days was insufficient for accurate determination of a positive response. This meant that the assay could not be completed within the three to four day pretransplant period available between human islet isolation and clinical transplantation.

## The Next Chapter...

Using the findings of the preliminary experiments as a starting point, Chapter Six examines the first titration and kinetic studies for the human allogeneic MLIC and MLAC.

# CHAPTER SIX

## CHAPTER SIX CONTENTS

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# ESTABLISHING PARAMETERS FOR THE HUMAN ALLOGENEIC MLIC AND MLAC

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## CHAPTER SIX

# ESTABLISHING PARAMETERS FOR THE HUMAN ALLOGENEIC MLIC AND MLAC

### 6.1 Introduction

This series of experiments was based on the findings of the preliminary studies described in Chapter Five. The common parameters so far included the use of Linbro non tissue plates for the cultures, the use of fresh islets normally within 48 hours of isolation, and incubation in RPMI medium containing 10% FCS as well as the usual growth supplements described in the methods in Chapter Four. The islets were picked as representative samples of the tissue separated by density centrifugation, so reflecting the purity of human islets intended for transplantation.

In this chapter, the parameters for the MLIC were investigated and established. Firstly, experiments to determine the titration and kinetics of the MLIC were attempted using digest tissue, as described below, in place of islets which were not available at the time (Expts 10, 11, 12). The resultant low lymphoproliferative response was unexpected as some reports have described impure islet preparations as being more immunogenic than purer islet preparations(269) (273).

These findings led to a comparison of islets, digest tissue pieces and acinar cell clumps (Expts 13, 15, 17, 18, 19) using titration and kinetic parameters in cocultures (MLIC, MLDC and MLAC). One experiment (+ one which was discarded due to infection), was used to investigate the effect of using dithizone on proliferation in the MLIC (Expts 21 - 22). Dithizone has been found to identify islets with a red stain by reacting with the zinc in the folded insulin molecule(280).

The problem of high background PBL control responses was then addressed by a series of lymphocyte response assays in which different sera were used in culture medium. Four experiments (LRA 1 - 4), then examined the proliferative responses of PBLs from different individuals, and were used to batch test human serum samples (LRA 6 - 8). One experiment was made possible by the availability of human islets which were subsequently used for clinical transplantation (Expt 16) and is described at the end of this chapter.

### 6.2 Titration and kinetics of digest tissue

Islets were not available during this period and digest tissue was used as a substitute. For this, disassociated pancreatic tissue was taken immediately after digestion with collagenase and maintained in culture medium (RPMI + 10% FCS). Pieces of this dispersed pancreatic tissue of approximately the same volume as the islets used (an average of 150  $\mu$ m diameter), were picked out from the culture medium using a fine pipette under the dissecting microscope. Initially digest tissue was not stained with dithizone and the selected tissue pieces were expected to include a cross section of all cell types in the digest, including islets in the proportion found in the pancreas. This tissue will be referred to as digest pieces or, after dithizone staining for accurate selection, acinar cell clumps.

Three experiments (Expts 10, 11, 12) were carried out using  $1 - 2 \times 10^5$  responder PBLs per well with a titration of 20, 30, 40 and 50 pieces of digest tissue per well as the stimulator population and a duration of 3, 5, 7 and 9 days to measure the kinetics of the response. The results indicated that the MLR peaked at seven days and had a reduced rate of proliferation by nine days. The PBL control values were relatively low at day five but by day seven had risen almost to the MLR peak cpm. In contrast the responses to digest pieces were low, and some negative values were obtained after subtracting the control values for digest (the stimulator population). The results are shown in Figure 6.1.



Fig 6.1. Titration and kinetics of the PBL response to splenocytes (MLR) and medium (PBL) compared with that to 10-40 pieces of digest tissue. For each point, the stimulator population, but not the responder population, controls were subtracted from the test cpm. Each point represents the median of 3 experiments each with quadruplicate samples (Expts 10-12).

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These results posed two immediate problems. One concerned the unacceptably high background levels of the (negative) PBL controls beyond five days duration which indicated that much of the proliferative response in the MLR could be accounted for by the response of PBLs to 'self' antigen. The other concerned the response to digest which was very low and did not reflect a higher level of immunogenicity than islets as suggested by the work of Ulrichs(269) and Zeevi(273). In addition, the PBLs + digest tissue coculture (MLDC) did not proliferate as much as the PBL controls, suggesting an inhibitory effect of the digest tissue on the PBL response.

These problems were addressed by two series of experiments: a) to compare the PBL proliferative response to digest or acinar tissue and islets from the same donor pancreas using titration and kinetic studies and, b) to investigate ways of reducing the high background PBL response using lymphocyte response assays.

# 6.3 Titration and kinetics of digest/acinar tissue and islets

In the five experiments (Expts 13, 15, 17, 18, 19) described here, a titration of 10, 20, 30 and 40 islets was compared with the same number of digest or acinar tissue pieces which were handpicked to be the same average size (150  $\mu$ m). Splenocytes ( $\gamma$ -irradiated 1 x 10<sup>5</sup>) were used as the stimulator population for the MLR and 1 x 10<sup>5</sup> HLA mismatched cryopreserved PBLs were used as the responder population in each coculture. The cultures and cocultures were incubated for 3, 5, 7 and 9 days.

The results of these five experiments indicated that the peak response of the MLR was on day seven (using Linbro plates) and that there was a similar but lower response of PBLs alone. The virtual absence of a lymphocyte proliferative response to digest tissue described earlier (Expts 10 - 12) (Figure 6.1), was repeated in the results shown here. These results also showed that despite a lack of response to digest tissue, there was a proliferative response to human islets from the same source. The reason for the low response of lymphocytes to allogeneic digest tissue was not clear and an investigation of this phenomenon is described in Chapter Eight. The results are shown in Figures 6.2a - 6.2c.

Thus in summary, the experiments described in this chapter so far (Expts 10 - 12 and Expts 13, 15, 17 - 19) showed that the lymphoproliferative response of human lymphocytes to allogeneic HLA mismatched digest tissue was lower than that to islets from the same source.



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**Figs 6.2a, 2b and 2c.** Difference in PBL response to splenocytes (MLR) (2a) and medium (PBL control) (2a), 10-40 pieces of digest tissue (2b) and 10-40 islets (2c). For each point the stimulator population, but not the responder population, control was subtracted from the test cpm. Each point represents the median of 5 experiments each with quadruplicate samples (Expts 13, 15, 17-19).

# 6.4 Effect of dithizone on islets and acinar cell clumps in the MLIC and MLAC

Although the initial concept behind these experiments was to work with the islet preparation in the same condition as used for transplant purposes, the experiments described so far have showed low and variable immunogenicity of islets and digest and the identification of the contribution of both the islet and acinar cell types was thought to be necessary. Latif(280) previously has reported a concentration of dithizone that could be used to stain islets without impairing their function so making the positive identification of islets and negative identification of acinar tissue possible. The next experiment described here was to investigate the use of dithizone and its effect on the MLIC.

Three different formulae to dissolve the dithizone were used as described in the methods and are briefly as follows: a) 0.026 mM DTZ in 1% ethanol in MEM b) 2.4 mM DTZ in 10% DMSO in MEM c) 0.13 mM DTZ in 1% ethanol.

Islets were handpicked after being identified by one of the 3 methods above, or without any staining, and cocultured with  $1 \ge 10^5$  HLA mismatched cryopreserved PBLs for 3, 5, 7 or 9 days duration. Human AB serum (10%) was used as the growth supplement in RPMI medium. The results are shown in Figures 6.3a and 3b.



**Fig 6.3a and 3b.** The results of (3a) two controls (Con A and PBL alone) and (3b) the proliferative response to islets handpicked without staining or with positive identification using 3 different formulae for DTZ solutions. For DTZ (a) compared to islet control p<0.05, the other combinations were not significant. Each point represents the mean  $\pm$  sem of quadruplicate samples (no subtraction of control cpm) for one experiment (Expt 22).

These results illustrate a peak response of PBLs to Con A stimulation at day five, and a low background response of the PBL control. The three DTZ treatments of islets showed little difference from each other except at day nine when the highest response (islets handpicked using 0.026 mM DTZ) and lowest two responses (islets handpicked without staining and the islets stained using a 0.13 mM solution of ethanol dissolved dithizone) were significantly different (Students t test).

Thus in summary, it was found to be possible to handpick islets using dithizone and still induce a response in the MLIC. The lowest concentration of dithizone (0.026 mM) was chosen for subsequent work based on the findings of Latif who has shown that this concentration of dithizone does not affect islet function.

# 6.5 Reduction of high background levels of PBL proliferation

The next series of experiments described in this chapter were designed to reduce the background proliferation of PBLs in medium which were intended as negative controls for responder PBLs. Other researchers using the human MLIC have all chosen to use human AB serum as a medium supplement (see Tables 3.1 - 3.18) without indicating an excessive background PBL response. For the next series of experiments it was decided to test the effect of human AB serum in culture medium to determine whether this would support cell growth and proliferation but at the same time, reduce the level of background PBL proliferation.

6.5.1 Lymphocyte response assays

The lymphocyte response assays (LRAs) were carried out using human PBLs unstimulated in medium alone, or stimulated with Con A and maintained in medium RPMI containing 10% FCS or 10% AB serum as described in the methods, Chapter Four.

#### 6.5.2 Variation in proliferative responses of PBLs

Four experiments (LRA 1, 2, 3 and 4) compared the proliferative response of PBLs from three sources, with or without stimulation by Con A. The results illustrated the variation in control response and response to Con A stimulation between PBLs from three different individuals such that number one (6.4a) showed a vigorous response to Con A at day three, number two at day five (6.4b), whereas individual number three showed little response throughout (6.4c). The PBL control response was below 3,000 cpm in two cases (6.4b and 6.4c) but rose to 6,000 cpm at day nine in one individual (6.4a).



Fig 6.4a, 4b and 4c. Proliferative reponse of PBLs from three individuals to Con A or medium alone. Each point represents the median and IQ range of 3 results with quadruplicate samples (LRAs 1-4).

### 6.5.3 Effect of different sera on PBL proliferation

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One experiment was carried out to compare the efficiency of different sera as growth supplements for PBL proliferation (LRA5). PBLs from three different individuals were incubated with or without Con A stimulation for 3, 5, 7, and 9 days. The results are shown in Figures 6.5a, 5b, 5c and 5d.



**Fig 6.5a and 5b (see also Fig 6.5c and 5d).** Different proliferative responses of PBLs alone or after Con A stimulation in medium containing different sera as growth supplement. 5a) 10% FCS, 5b) 10% autologous human serum. Each point represents the median and IQ range of 3 individuals each with quadruplicate samples.



Fig 6.5c and 5d (see also Fig 6.5a and 5b). Different proliferative responses of PBLs to Con A or alone in medium containing different serum as growth supplement. 5c) a batch of pooled human serum, 5d) human serum from an individual with AB blood group. Each point represents the median and IQ range of 3 individuals each with quadruplicate samples.

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The graphs in Figures 6.5a, 5b, 5c and 5d show the results of PBL proliferation either unstimulated or to Con A stimulation in the presence of medium containing 10% FCS (5a), 10% autologous serum (5b), 10% of a pooled human serum batch (5c) or 10% AB serum (5d). One striking result can be seen in Figure 6.5c which shows that this particular batch of human serum did not support PBL growth. All of the other three sera supported

Con A stimulated growth of PBLs and Figure 6.5d showed the lowest PBL control proliferation.

To further investigate the use of human AB serum as an alternative growth supplement to FCS, three experiments (LRA 6, 7 and 8) were used to test three different batches (A1, A2 and A3) of human AB serum. The results shown in Figures 6.6a, 6b and 6c, illustrate the different levels of PBL proliferation with or without stimulation by Con A in RPMI medium containing 10% serum from three different batches of AB sera. Each point represents the median  $\pm$  IQ range of three experiments (LRA 7, 8 and 9) using PBLs from three different sources, each carried out in quadruplicate. Serum batches A3 and A2 showed that the serum could support proliferation of Con A stimulated lymphocytes while maintaining low background levels of proliferation from PBLs alone. These two batches were considered suitable for use as growth supplements for the MLR and cocultures and A1 was discarded.

Thus in summary, this series of LRA experiments showed variability in response between PBLs from different individuals (Figure 6.4a - c), between four different sera (Figure 6.5a - d) and between batches of human ABS (Figure 6.6a - c). A trend towards lower proliferation of the negative PBL control after culture in RPMI containing 10% human ABS was seen. Batch testing of human AB serum with PBLs from three different individuals to identify suitable batches led to the use of 10% ABS in preference to FCS as a growth supplement for the cocultures.





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# 6.6 Experiments to measure the viability of islets in human ABS

Before the introduction of 10% ABS as the growth supplement for RPMI medium in the mixed lymphocyte cultures, an estimation of the effect of the human AB serum on islet, acinar cell and cultured PBL viability was made using the microfluorimetry test as described in the methods, Chapter Four. Briefly, the cellular material from the microwell cocultures was aspirated onto a glass slide, incubated with a mixture of fluorescein diacetate (which accumulates in the cytoplasm of cells with an intact cell membrane as a green stain) and propidium iodide (which passes through dead or dying cell membranes to give a red nuclear stain), the fluorescence of the islets or acinar cell clumps measured and the viability calculated(283). The proportion of viable leucocytes in the cultures and cocultures was calculated using trypan blue and a haemocytometer.

The results of two viability experiments (VA1 and VA2) are shown in Figures 6.7a, 7b, 7c and 7d.

The viability of islets and acinar tissue alone or in combination was found to be similar using the FDA/PI stains and the average of the three combinations to range from  $84 \pm 17\%$  (day 3) to  $94 \pm 3\%$  (day 9) (Figures 6.7a and 7b). The addition of PBLs to islet and acinar tissue did not significantly alter the percentage viability although a trend towards lower viability at day three was noted. Using trypan blue, the viability of the lymphocytes in the controls showed a trend towards reduction over the nine day period from 85% to 68% although none of the differences were statistically significant due to low numbers per group (Figure 6.7c). This reduction at nine days was not seen when PBLs were cultured with islets or acinar tissue (Figure 6.7d).

Thus in summary, using the microfluorimetry method, islets and acinar tissue were found to have high viability with and without PBLs present and PBLs showed good viability in the presence of islets and acinar tissue but a trend towards lower viability during a nine day incubation period when cultured alone, with Con A or splenocytes.


Fig 6.7a and 7b. Percentage viability of islet and acinar tissue as measured using FDA/Pl in control (7a) and coculture (7b) wells. Each point represents the mean of two samples each from two experiments.

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Fig 6.7c and 7d. Measurement of lymphocyte viability over a nine day period using the trypan blue dye exclusion test. Each point represents two samples from each of two experiments, no differences were statistically significant.

### 6.7 Human islet transplantation and the MLIC

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One aim of these studies was to provide an *in vitro* model of the potential *in vivo* immune response to transplanted human islets. To date, three human islet transplants have been carried out in Leicester and only the third involved islets from a single donor. During the course of the titration and kinetic series of experiments to compare the lymphocytic response to digest/acinar tissue and islets, human islets were isolated from one pancreas in sufficient quantity to allow a patient to be transplanted (the third patient to receive a human islet transplant at Leicester). The patient, who had been diagnosed with Type I diabetes 35 years previously, was a four antigen match with the donor as shown in Table 6.1 and received a kidney from the same donor four days prior to islet transplantation.

Table 6.1. Tissue type of recipient and donor for human islet transplant patient

Stimulator islets	Blood	MHC class I		мнс	
	group			class II	
HP237	O+ve	A2,31	B7,15	DR4,4	
Responder PBLs					
HLA mismatch control	O+ve	A1,1	B8,12 <sup>44</sup>	DR3,7	
Recipient	O+ve	A2,2	B15,40	DR4,4	
HLA matched non recipient	O+ve	A2,2	B15,15	DR4,4	

Some of the islets became available to set up an MLIC (Expt 14) which is described below.

## 6.7.1 MLIC of donor islets and recipient PBLs from a transplant patient

This experiment used 10% FCS as a growth supplement for the culture medium and although the islets were selected without prior DTZ staining, the isolated islet preparation was of particularly high purity (95%).

For this experiment (Expt 14), the MLIC used 10, 20, 30 and 40 islets against HLA-mismatched responder PBLs, as in previous experiments. PBLs from the islet transplant recipient were used as an additional responder

population as well as using PBLs from a potential recipient (who did not receive the islet transplant because of a slightly positive cytotoxic cross match) but who was also a four antigen HLA match with the donor. As limited numbers of islets were available, the duration of the MLIC was for seven days only. A titration of acinar tissue using 10, 20, 30 and 40 pieces per well with HLA mismatched responder PBLs and a duration of 3, 5, 7 and 9 days was also carried out. Negative PBL controls for each source of PBLs were included.

The results of the PBL control proliferation, the MLR positive control and the MLAC using digest tissue as the stimulator population are given in Figures 6.8a and 8b.



**Fig 6.8a and 8b.** Control response (8a) of HLA mismatched PBLs with splenocytes (MLR), from the pancreas donor, to medium alone (PBL). Titration and kinetics (8b) of HLA mismatched PBLs to 10-40 pieces of digest tissue from the donor pancreas. Each point represents the mean of one experiment with quadruplicate samples (Expt 14).

The MLR of Expt 14 gave a particularly low response, contrasting with the high response of the HLA mismatched PBLs alone, suggesting that the splenocytes had inhibited lymphocytic proliferation. Figure 6.8b shows that 10 and 20 acinar tissue pieces produced a proliferative response at seven days, more than obtained in the earlier experiments which may have been due to the high background response of the HLA mismatched PBLs.

The results of the human MLIC using the three different sources of responder PBLs (HLA mismatch, recipient [four antigen match] and another individual who was a four antigen match) are shown in Figure 6.9.



**Fig 6.9.** Proliferative response (day 7 only) of HLA mismatched, recipient and HLA 4 ag matched PBLs to 10-40 donor islets. The PBL negative control of each group is shown to the right of each group. Each bar represents the mean of quadruplicate samples for one experiment (Expt 14).

The background proliferative levels of the PBL controls appeared to bear some relationship to the levels of proliferative response to the stimulator islets, however they did not entirely account for the high response obtained with the HLA mismatched responder-stimulator combination and the low response obtained with the two HLA matched responder-stimulator cocultures.

As a further illustration of the difficulties of data interpretation, Figures 6.10a and 10b show two methods of incorporating the negative control cpm responses (responder PBLs in medium and islets or irradiated splenocytes in medium) into the final MLIC or MLR result.



Fig 6.10a and 10b. The same data as shown in Figure 6.9 (Expt 14), is represented here as (10a)  $\Delta$ cpm(test cpm - sum of responder and stimulator cpm controls) or (10b) stimulation index (test cpm /sum of responder and stimulator cpm controls).

The results of calculations shown in Figures 6.10a and 10b, raise an additional problem of interpretation in that the results can be alternatively represented either as  $\Delta$ cpm, for which the sum of the responder and stimulator negative controls are subtracted from the test cpm, or stimulation index, for which the test cpm is divided by the sum of the responder and stimulator negative controls. This is further examined in Chapter Seven.

### 6.7.2 Clinical aspects of the islet allograft recipient

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It is of interest that the human islet transplant recipient, who received a relatively low number of islets (3,000 IEQ/Kg compared with the recommended number of 6,000 IEQ/Kg)(126), although having not attained insulin independence, has continued to produce C-peptide for more than two years post transplantation. The levels of C-peptide were measured by radioimmunoassay (CIS UK Ltd, High Wycombe) and are shown in Figures 6.11a and 11b, as fasting rates for the first 50 days (11a) and as both fasting and stimulated rates for the entire period to end of September 1994 (11b). C-peptide levels were measured after overnight fasting and 90 minutes after stimulation with 6 ml/Kg bodyweight of Sustacal (Mead Johnson, Hounslow). Normal C-peptide levels taken from ten healthy volunteers were  $3.71 \pm 0.87$  ng/ml (fasting) and  $7.57 \pm 1.50$  ng/ml (stimulated).



Fig 6.11a and 11b. C-peptide levels pre and post transplantation up to 50 days (fasting levels) (11a) and >26 months (stimulated levels) (11b). Pretransplantation samples of recipient PBLs and donor islets were as used in Expt 14.

Induction immunosuppression was with OKT3 and a reducing dose of prednisolone. The OKT3 was continued for 14 days, azathioprine for days 10 to 67 (discontinued due to neutropenia) and cyclosporin A at 15 days. Maintenance immunosupression continued with prednisolone and cyclosporin A, and there have been no kidney rejection episodes to date (September 1994).

Thus in summary, a single donor simultaneous islet-kidney transplant carried out in Leicester provided an opportunity to carry out MLIC experiments using four HLA antigen matched recipient PBLs and donor islets. Controls included the MLIC using PBLs from another four HLA antigen matched individual and from a complete HLA mismatch individual as well as an HLA mismatched MLDC (Expt 14). The results suggest that the HLA matching leads to a reduced *in vitro* response of the MLIC, this is discussed further in Chapter Ten.

# 6.8 Overall summary of studies to establish parameters for the human allogeneic MLIC and MLAC

In summary, the work described in Chapter Six provided the basis for an understanding of the MLIC and for planning subsequent experiments. The use of digest tissue as the stimulator population did not lead to a proliferative PBL response and a subsequent comparison between pancreatic digest tissue and islets confirmed a lower allogeneic lymphoproliferative response to the former. Because of the difference in allogeneic PBL response to islets and digest tissue, accurate identification of islets was found necessary and one experiment was used to compare the effects of using dithizone to handpick islets prior to coculture, leading to the subsequent use of 0.026 mM dithizone in 1% ethanol as described by Latif(280). The extension of the experiments to 9 days duration, identified a considerable proliferative response of the PBL controls and this was reduced by changing the serum in the culture medium from 10% FCS to 10% AB human serum. Human AB serum was shown to vary between batches, and tests involving the stimulation of PBLs with Con A were used to identify suitable ABS batches for use in the coculture experiments. Finally, one experiment relating to a human islet transplant carried out at Leicester was possible using donor islets cocultured with recipient PBLs, as well as two

controls in the form of HLA 4 antigen matched PBLs and totally HLA mismatched PBLs. This showed a difference in response between HLA mismatched and well matched combinations, which may have some clinical significance, but also identified difficulties in interpretation of the data, both merit further investigation.

In conclusion, the parameters and conditions for the MLIC were identified and found to include use of 10% human AB serum as a growth supplement in the culture medium, titration and kinetic studies to identify peak responses, the use of 0.026 mM dithizone to handpick islets and the need to have both pure islets and acinar tissue as stimulator cell populations.

The Next Chapter...

The findings of the studies to establish parameters for the human allogeneic MLIC form the basis for the standardisation of the MLIC which is described in Chapter Seven.

# CHAPTER SEVEN

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## CHAPTER SEVEN

### STANDARDISATION OF THE MLIC AND MLAC

### 7.1 Optimisation, titration and kinetics

#### 7.1.1 Introduction

Few reported studies have investigated the MLIC model beyond a close copy of the standard MLR, so that a single duration and a single number of islets or islet cells have normally been used (five or six days) (see Tables 3.1 -3.4). Shizuru has studied a titration of rat islets(264) and has shown an increasing proliferative response at five days that is related to the number of stimulator islets with a peak of 100 islets. Rabinovitch(263) has shown in his classic studies that the allogeneic canine MLIC peaks at day nine with 100 islets. Ulrichs, using dithizone to identify human islets, has found that 40 islets stimulates a peak response at a single duration of six days(269).

The studies described in this chapter were intended to optimise the human MLIC using titration and kinetic experiments based on the work of others described above and particularly on the preliminary studies described in earlier chapters. The parameters identified earlier were brought together for this series of experiments and included the use of:

a) non tissue culture treated microwell plates

b) the use of fresh (within 48 hrs) non cryopreserved or irradiated or mitomycin C treated islets or acinar cell clumps as stimulators

c) the use of  $\gamma$ -irradiated (3,000 rads) splenocytes as positive control stimulators

d) 10% ABS as a RPMI medium growth supplement

e) the use of 0.026 mM DTZ -ETH for handpicking islets and acinar tissue

f) a duration of 3, 5, 7 and 9 days for kinetic studies

g) a titration of up to 40 islets or acinar cell clumps

h) 1 x  $10^5$  HLA mismatched (at HLA-A, -B and -DR loci) PBLs as the

responder cell population.

### 7.1.2 Titration and kinetics of 10 - 40 islets and acinar tissue pieces

The studies described here comprised three experiments (Expts 23, 24 and 26) using a titration of 10, 20, 30 or 40 islets and acinar tissue pieces. The results are shown in Figures 7.1a and 1b and indicate a) that the response of HLA mismatched lymphocytes to islets and acinar tissue pieces decreased with increasing stimulator population numbers, and b) that the response to islets was higher than to acinar tissue pieces. In addition, the response to both tissues appeared to increase over time but the response to acinar tissue was delayed compared with the response to islets. None of these differences were found to be significant (Mann-Whitney U test), which may have been principally due to the low numbers (n = 3).

# 7.1.3 Titration and kinetics of 1 - 10 islets and acinar tissue pieces

The decrease in proliferative response to increasing amounts of islet and acinar tissue led to the next experiments in which the amount of stimulator tissue was further reduced. For one experiment (Expt 25), only islets were available, not acinar tissue, and were from a particularly pure preparation in which the islets were well cleaved with compact and well rounded morphology. The duration used for the coculture was five to nine days. The results shown in Figure 7.2 indicated a positive correlation between the proliferative response to increasing numbers of islets which was significant using two or ten stimulator islets (p < 0.05 Student's t test).

The subsequent series of experiments (Expts 27, 28 and 29) was planned to investigate further the use of one to ten islets and corresponding acinar tissue as the stimulator populations. The results of the titration of 1, 2, 5 and 10 islets and acinar cell clumps are shown in Figures 7.3a and 3b and indicated a trend towards a lower proliferative response to acinar cell clumps compared to that against islets, and that the proliferative response to acinar tissue was delayed. However, all differences were found to be non significant (Mann-Whitney U test) which may have been due to the low numbers (n = 3).



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Fig 7.1a and 1b. Titration (1a) of 10-40 islets or acinar cell clumps (peak response - day 7-9) and the kinetics (1b) between days 3 and 9 of ten islets or acinar cell clumps in the MLIC and MLAC (Expts 23,24 and 26). Differences were not significant Mann Whitney U test). Each point represents the median  $\pm$  IQ range of 3 experiments each with quadruplicate samples.



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Fig 7.2. Titration of 1-10 islets (Expt 25) over nine days. Each point represents the mean  $\pm$  S.E.M. of quadruplicate samples. The difference between proliferation against 2 or 10 islets was statistically significant at day nine (\*p <0.05) (Student's t test).



Fig 7.3a and 3b. Titration (3a) of 1-10 islets or acinar cell clumps and the kinetics (3b) of 10 islets or acinar cell clumps over 3-9 days. Each point represents the median  $\pm$  IQ range of 3 experiments (Expts 27, 28 and 29) each with quadruplicate samples. All comparisons were found to be non significant (Mann-Whitney U test).

### 7.1.4 Titration and kinetics of 5 - 20 acinar free, intact islets

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In order to clarify whether the high purity and cleaved, well rounded nature of the islets used could account for the results of Expt 25 in which a positive correlation between proliferation and islet number up to ten islets was seen at day nine, three further experiments (Expt 33, 34 and 36) were planned. For these experiments a titration of 5, 10 and 20 islets was used and only well cleaved islets with well rounded and compact morphology. The results are shown in Figure 7.4.



**Fig 7.4a and 4b.**Titration (4a) of 5-20 islets ( $\Delta$ cpm), peak response day 7 -9, and kinetics (4b) of ten islets at 7 and 9 days (Expts 33, 34 and 36). Each point represents the median  $\pm$  IQ range of 3 experiments each with quadruplicate samples.

The results indicated an increasing proliferative response to an increasing number of islets between 10 and 20 per well, but not between 5 and 10. The level of response against 20 islets in these experiments was considerably higher than obtained in most of the earlier work, although the proliferation was not found to be significantly different from the proliferation against 20 islets shown in Figure 7.1a. The kinetics of the response to 10 islets was found to be well within the range of previous experiments.

The kinetics of the lymphoproliferative response against allogeneic islets and acinar tissue as shown in Figures 7.1b, 7.3b and 7.4b, indicated a continuation of proliferation up to nine days which was the maximum duration used.

#### 7.1.5 Extended kinetics of the MLIC

The following series of experiments (Expts 30, 32 and 35) was planned to extend the duration of the cocultures in order to reach the T cell resting or memory stage which has been found to follow the peak proliferation and has been described by others(230). The results of the extended kinetic experiments with a duration of 7, 9, 11 and 13 days with ten islets or acinar cell clumps as the stimulator population, are shown in Figure 7.5. The peak proliferation of the MLIC was found to be at day nine and to decline afterwards at days eleven and thirteen. The MLAC, which showed minimal proliferation up to day nine, showed a trend towards increased proliferation by day thirteen.

Thus in summary, these series of experiments showed a number of trends (statistically non significant) in the optimisation of the titration and kinetics of the MLIC and MLAC. Using 2 - 20 islets or acinar cell clumps per well as the stimulator cell populations, the optimal responses were found after coculture with 1 x  $10^5$  HLA mismatched PBL responder cells for seven to nine days duration. Extending the duration of the cocultures to day thirteen showed that the continuing proliferation seen at day nine represented the peak proliferation for the MLIC and MLR but that proliferation in the MLAC commenced at day eleven.





#### 7.1.6 'Back-proliferation' of untreated islets in the MLIC

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The final experiments in this series formed an investigation of the contribution of the islets to the overall proliferation in the allogeneic coculture. In the experiments described so far, the stimulator cell population was  $\gamma$ -irradiated (3000 rads) for each MLR, to prevent splenocyte proliferation in response to the allogeneic responder PBLs. For the MLIC combinations, because adult human islets do not proliferate in culture(282), and an investigation of the immunomodulatory effect of  $\gamma$ -irradiation was planned for the future, the islets were not pretreated. Two experiments were planned to show whether the islet stimulator cells substantially contributed to the overall [<sup>3</sup>H]thymidine incorporation recorded by the MLIC. The results (Expt 39 and 41) are shown in Figure 7.6. The results indicated that the cells of islets (non irradiated) did not proliferate against HLA mismatched PBLs (irradiated and non proliferating).

Thus in summary, proliferation of the non irradiated stimulator islet population in response to the allogeneic lymphocytes was found not to contribute to the overall proliferation in the coculture.





### 7.2 Compilation of data from each series of experiments

The isolation of human islets is a time consuming and laborious procedure, usually outside normal working hours. The resultant irregular supply of good quality human islets meant that the experiments described in this thesis were limited in number for each series. Although trends have emerged from the data, few results were found to be statistically significant when analysed. For this reason, the remaining part of this chapter utilises results of parameters common to each series of experiments in order to accumulate sufficient data for comparison and determination of the optimum titration and kinetics of the MLIC and MLAC.

### 7.2.1 The titration of islets in the MLIC

The combined results of the titration of 1 - 40 islets in a 3 - 13 day MLIC are shown in Figure 7.7a. The number of experiments represented at each point varied (n = 3 - 22). Peak proliferative responses are shown such that the peak of nine experiments was on day seven, of twelve experiments on day nine, and of two experiments on day eleven. The mean of quadruplicate samples for each experiment was used to calculate the median  $\pm$  IQ range of multiple experiments represented by each point on the graph.

Ten islets (average 150  $\mu$ m diameter each) per well was the optimum number causing a peak proliferation by 1 x 10<sup>5</sup> HLA mismatched PBLs in the MLIC. All subsequent kinetic experiments were carried out using ten islets per well. It is interesting to note that a measurable proliferative response was generated even to a solitary islet.

Thus in summary, the optimum allogeneic lymphoproliferative response was found to be against ten human islets in the MLIC.

7.2.2 The titration of acinar tissue pieces in the MLAC

The results of the titration of 1 - 40 acinar tissue pieces per well in a 3 - 13 day MLAC are shown in Figure 7.7b. The number of experiments represented at each point varies (n = 3 - 17). Peak proliferative responses rather than proliferation on a single day are shown such that the peak of two experiments was on day seven, of eleven experiments on day nine, of one experiment on day eleven and of three experiments on day thirteen. The mean of quadruplicate samples for each experiment was used to calculate the median  $\pm$  IQ range of multiple experiments represented by each point on the graph.

Ten acinar tissue pieces (average 150  $\mu$ m diameter each) per well was the optimum number, causing a peak proliferation of 1 x 10<sup>5</sup> HLA mismatched PBLs in the MLAC. All subsequent kinetic experiments were carried out using ten acinar cell clumps per well.

Thus in summary, the optimum lymphoproliferative response was found to be against ten acinar tissue pieces in the MLAC.



**Fig 7.7a and 7b.** Titration of the MLIC and MLAC using combined data ( $\Delta$ cpm median  $\pm$  IQ range) such that the number of experiments at each point varies. In the MLIC (7a), for 1 islet n = 3, 2 islets n = 4, 5 islets n = 7, 10 islets n = 22, 20 islets n = 6, 30 islets n = 3, 40 islets n = 3. In the MLAC (7b), for 1 acinar cell clump n = 3, 2 acinar cell clumps n = 3, 5 acinar cell clumps n = 4, 10 acinar cell clumps n = 17, 20 acinar cell clumps n = 4, 30 acinar cell clumps n = 3, 40 acinar cell clumps n = 3.

7.2.3 The kinetics of the response to splenocytes in the MLR

The results of the kinetics of the response to splenocytes in the MLR, using  $1 \times 10^5 \gamma$ -irradiated splenocytes per well for a 3 - 13 day period, are shown in Figure 7.8a. The number of experiments represented at each point varies (n = 3 - 20). The mean of quadruplicate samples for each experiment was

used to calculate the median  $\pm$  IQ range of multiple experiments which is represented by each point on the graph.

Nine days duration was found to be optimal for a peak proliferative response when 1 x  $10^5$   $\gamma$ -irradiated splenocytes were used as the stimulator population for 1 x  $10^5$  HLA mismatched PBLs per well. The similarity of the results at day seven led to the use of a duration of seven and nine days for subsequent experiments.

Thus in summary, the optimum duration of the MLR for the peak allogeneic lymphoproliferative response was found to be nine days.

#### 7.2.4 The kinetics of the response to islets in the MLIC

The results of the kinetics of the response to ten islets per well for a 3 - 13 day period in the MLIC are shown in Figure 7.8b. The number of experiments represented at each point varies (n = 3 - 22). The mean of quadruplicate samples for each experiment was used to calculate the median  $\pm$  IQ range of multiple experiments which is represented by each point on the graph.

Nine days duration was found to be optimal for a peak proliferative response when 10 islets (average 150  $\mu$ m diameter each) were used as the stimulator population for 1 x 10<sup>5</sup> HLA mismatched PBLs per well. The similarity of the results at day seven led to the use of a seven to nine days duration for subsequent experiments.

Thus in summary, the optimum duration of the MLIC for the peak lymphoproliferative response was found to be nine days.

7.2.5 The kinetics of the response to acinar tissue in the MLAC

The results of the kinetics of the response to ten acinar tissue pieces per well for a 3 - 13 day period in the MLAC are shown in Figure 7.8c. The number of experiments represented at each point varies (n = 4 - 15). The mean of quadruplicate samples for each experiment was used to calculate the median  $\pm$  IQ range of multiple experiments which is represented by each point on the graph.



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**Fig 7.8a, 8b and 8c.** Kinetics of the MLR (8a), MLIC (8b) and MLAC (8c) using  $1 \times 10^5$  HLA mismatched PBLs and combined data ( $\Delta$ cpm). Each point represents the median  $\pm$  IQ range of a variable number of experiments such that for the MLR (8a), day 3 n = 10, day 5 n = 12, day 7 n = 20, day 9 n = 20, day 11 n = 3 and day 13 n = 3. In the MLIC (8b), day 3 = 6, day 5 = 7, day 7 n = 22, day 9 n = 21, day 11 n = 3 and day 13 n = 3. In the MLAC (8c), day 3 n = 6, day 5 n = 6, day 7 n = 15, day 9 n = 14, day 11 n = 4, day 13 n = 4.

Nine days duration was found to be optimal for a proliferative response when 10 acinar cell clumps (average 150  $\mu$ m diameter each) were used as the stimulator population with 1 x 10<sup>5</sup> HLA mismatched PBLs per well. The similarity of the results at day seven led to the use of a seven to nine days duration for subsequent experiments.

Thus in summary, the optimum duration of the MLAC for the peak lymphoproliferative response was found to be nine days.

### 7.3 Summary of the optimal MLIC and MLAC

From the results shown in Figures 7.7a - 7b, 7.8a - 8c, using conditions which included use of non tissue culture 96 well plates, RPMI containing 10% human AB serum, fresh islets and acinar tissue handpicked with 0.026 mM dithizone and  $1 \times 10^5$  HLA mismatched PBLs per well, the optimal parameters for the MLIC and MLAC were identified. The optimal allogeneic proliferative response was obtained against ten islets or ten acinar cell clumps (average 150 µm diameter each) with a duration of culture for seven and nine days.

7.4 A comparison of the peak level of response in the MLIC, MLAC and MLR.

The peak  $\Delta$ cpm results of all MLIC, MLAC and MLR experiments utilizing ten islets, ten acinar tissue pieces or  $1 \times 10^5 \gamma$ -irradiated splenocytes as the stimulator population with a duration of seven or nine days coculture are shown in Figure 7.9. This data represents a collation (16 - 22 experiments per group) of which the median responses ( $\pm$  IQ range) were found to be as follows:

MLR =  $25013 \pm 21803 \text{ } \Delta \text{cpm}$ ,

MLIC =  $10135 \pm 3474 \Delta cpm$ ,

MIAC = 3993 ± 2486 Δcpm.



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Fig 7.9. Comparison between collated data ( $\Delta$ cpm) for the MLR, MLIC and MLAC using 1 x 10<sup>5</sup> HLA mismatched responder PBLs with 1 x 10<sup>5</sup> stimulator splenocytes, 10 islets or 10 acinar cell pieces. The median ± IQ range for the MLR = 25013 ± 21803, the MLIC = 10135 ± 3474 and the MLAC 3993 ± 2486. Statisical significances were calculated using the Mann-Whitney U test.

Each group was significantly different (Mann-Whitney U test) from the others (p < 0.02 - < 0.001) as shown in Figure 7.9. The allogeneic lymphocyte proliferative response was found to be 40% (islets) and 16% (acinar tissue) of that induced by splenocytes from the same source.

Thus in summary, the combination of results shown here has led to statistically significant findings that the lymphoproliferative response to HLA mismatched splenocytes (MLR) was greater than that to islets (MLIC) which was greater then that to acinar tissue (MLAC). In smaller series of experiments, this was reflected as a trend without statistical significance.

#### 7.5 Variability within and between experiments

The extent of inter-experiment variability is illustrated in Figure 7.10 using the raw data of quadruplicate samples (cpm - without control values subtracted). The mean  $\pm$  S.E.M. are shown separately for each experiment of the MLR (10a), MLIC (10b) and MLAC (10c) and indicated a wide range of variability within (error bars) and between (points) experiments.

For ease of comparison between the MLR, MLIC and MLAC, the variability within each experiment of quadruplicate samples, was calculated as a percentage variation from the mean (standard deviation /mean x 100). The mean for the series of experiments together with the range of percentages are shown in Table 7.1.

Table 7.1. Average variation within each experiment of the MLR, MLIC and MLAC

Expt	No. of expts	S.D./mean x 100	Range
MLR	20	37%	4 - 106%
MLIC	22	41%	16 - 96%
MLAC	16	52%	16 - 84%

Thus in summary, the variability between and within experiments was considerable regardless of the stimulator cell type, so that combined data from multiple experiments were needed to obtain statistically significant results.



Fig 7.10a, 10b and 10c. Variability within each experiment by the error bars (mean + S.D. of quadruplicate samples of raw data on day 9, without subtraction of negative controls), and between experiments by the separate points for the MLR (10a) the MLIC (10b) and the MLAC (10c).

# 7.6 Comparison between FCS and ABS as a growth supplement

Part of the work described in Chapter Six related to the use of FCS or human ABS in RPMI medium which was used as a growth supplement and was shown to maintain a lower background proliferative response of the PBL control. Having completed a number of experiments using ABS as a growth supplement, a comparison was made here to check that the reduction in background response of the PBL negative control had proved to be lower using ABS, so supporting the results obtained earlier (Lymphocyte Response Assays - Figures 6.4 - 7).

Figure 7.11 shows the combined results of PBL controls from individual experiments with three to nine days incubation (median  $\pm$  IQ range) in which 10% FCS (Expts 10 - 20) or 10% ABS (Expts 22 -24, 26 - 29) was used as the growth supplement. Each point on the ABS line was found to be significantly lower than the equivalent point on the FCS line (p <0.02 - <0.005) using the Mann-Whitney U test.

The results confirmed the LRA test results that there was less background PBL proliferation recorded using ABS rather than FCS as the growth supplement for RPMI tissue culture medium.

Thus in summary, the combined data of PBL control results supported the findings of the LRA tests that the use of ABS in the RPMI medium led to lower background PBL responses compared with those obtained using FCS.



Fig 7.11. Background proliferation of PBL controls cultured for 3-9 days in RPMI containing either 10% FCS or 10% human ABS. Each point represents the median  $\pm$  IQ range of 11 experiments (FCS) and 7 experiments (ABS) each with quadruplicate samples. Proliferation in the ABS group was significantly lower than in the FCS group (day 3 and 5 \*p <0.005, day 7 \*p <0.01, day 9 \*p <0.02 Mann-Whitney U test).

# 7.7 Correlation between background cpm of PBL control and MLR, MLIC or MLAC

The PBL background response has been found to be due to the presence of growth factors in serum supplement(235) and more specifically to the autologous response of T cells to 'self' B cells in the PBL population(286). In the comparison presented here, the magnitude of the PBL background was compared with the magnitude of the PBL response to allogeneic stimuli - splenocytes (MLR), islets (MLIC) and acinar tissue (MLAC). This correlation is illustrated in Figures 7.12a - c.

The results shown in Figures 7.12a - c compare the cpm of the background PBL response and the cpm of the PBL response to allogeneic cells (coculture cpm - background cpm for the stimulator population only) after coculture in RPMI containing 10% ABS. The correlation coefficient between the two experimental groups is given (R), as well as the significance (p). The relationship between the proliferation of PBLs alone to proliferation to an allogeneic stimulus is shown by the slope of the line (y =). Only the results of experiments using ABS were included as too few



**Fig 7.12a, 12b and 12c.** Relationship between the PBL controls and the MLR (12a), MLIC (12b) or MLAC (12c). Each point represents the mean of quadruplicate samples for one experiment and the line of best fit is shown. No correlation (12a) between the PBL control and MLR response (n = 16) was found but there was a statistically significant correlation between the PBL control and the MLIC (12b) (p <0.001, n = 18) and the MLAC (12c) (p <0.001, n = 13).

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results were available using FCS to allow meaningful comparison (numbers available for FCS comparison were MLR = 9, MLIC = 6, MLAC = 6).

The cpm results used for the graphs were obtained after nine days incubation in medium containing ABS (Figure 7.12a - c) and represent PBL background responses compared with the MLR (12a) (n = 16), the MLIC (12b) (n = 18) and the MLAC (12c) (n = 13).

Thus in summary, a positive correlation was found between the autologous background PBL response and the level of the allogeneic response to different cell types (splenocytes, islets and acinar tissue) which was statistically significant for the MLIC and MLAC but not the MLR.

# 7.8 Relationship between allogeneic proliferation to splenocytes, islets and acinar tissue

This comparison was intended to demonstrate whether the peak allogeneic lymphoproliferative responses ( $\Delta$ cpm) to splenocytes, islets and acinar tissue were related. Each point represents one experiment in which the stimulator cell populations were from a single donor. The results are shown in Figures 7.13a, 13b and 13c. The correlations demonstrate a wide scatter of points ( $\Delta$ cpm) about the line of best fit and all three correlation coefficients were non significant (Mann-Whitney U test).

# 7.9 Comparison of different calculations for expressing MLR, MLIC and MLAC results

Two methods for expressing the results have been used by others (see Tables 3.9 - 3.14) and comprise either  $\Delta$ cpm which is the test cpm minus appropriate controls ( $\Delta$ cpm = coculture cpm - [stimulator control + responder control]) or the stimulation index (S.I. = coculture cpm / responder cpm + stimulator cpm)(267) (153). For the purpose of comparison, the results of the experiments (Expts 23 - 44) previously shown in Figure 7.9, have been expressed both as  $\Delta$ cpm and S.I. and are illustrated in Figures 7.14a - f. Note that the MLR, MLIC and MLAC have different scales for the y axis, this is to allow visual comparison of the pattern of results for the whole series of experiments. The mean results of



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Fig Ch7 13a, 13b and 13c. Correlation between  $\Delta cpm$  results of the MLR and MLIC (13a) (n = 20, non significant), the MLR and MLAC (13b) (n = 16, non significant) and the MLIC and MLAC (13c) (n = 16, non significant). Each point represents a comparison of the means of quadruplicate samples and the line represents the linear best fit.

quadruplicate samples are represented by  $\Delta cpm$  or S.I. and show considerable difference in the magnitude of individual results.

Two further comparisons were made comparing stimulation index and  $\Delta$ cpm calculations. The correlations shown in Figures 7.15a, 15b and 15c, represent the same original data as used in Figures 7.13a, 13b and 13c but using instead the peak stimulation index for each experiment. As previously, the correlation between the MLR and either the MLIC (15a) or the MLAC (15b) was found to be non significant. However the correlation between the MLIC and MLAC (15c) was statistically significant (p <0.01, Mann-Whitney U test).

The final comparison was made using data taken from that represented in Figure 7.9 in which the median  $\pm$  IQ ranges of the peak MLR, MLIC and MLAC responses were illustrated as a dot plot. The data were calculated as  $\Delta$ cpm and as S.I. and are shown in Tables 7.2a (data) and 2b (statistical significance, Mann-Whitney U test).

Table 7.2a. Comparison of stimulation index and ∆cpm results from the MLR, MLIC and MLAC

Coculture	МІ	R	MI	.IC	ML	AC
Results	۵cpm	S.I.	۵cpm	S.I.	۵cpm	S.I.
Median	25,013	9.24	10,135	5.05	3,993	2.20
+ IQ range	21,803	5.72	3,474	1.87	2,486	1.25

Table 7.2b. Statistical significance of comparisons between stimulation index and Acpm results from the MLR, MLIC and MLAC

Comparison	p value ∆cpm	p value S.I.
MLR:MLIC	p <0.02	p <0.05
MLR:MLAC	p <0.001	p <0.001
MLIC:MLAC	p ⊲0.001	p ⊲0.002









The data shown in Tables 7.2a and 2b indicate that although the numbers vary according to the method of calculation used for the data for individual or small groups of results, this had little effect on the overall findings of the combined data.

Thus in summary, the expression of results, which differs between published authors as either  $\Delta$ cpm or S.I. does make a difference to comparisons between individual experiments but makes little difference to larger studies which together show a statistical significant difference between the MLR, MLIC and MLAC.

# 7.10 Overall summary of the standardisation of the MLIC and MLAC

The optimum lymphocytic proliferative response of  $1 \ge 10^5$  HLA mismatched PBLs in the titration experiments, was found to be against ten islets (150 µm diameter) in the MLIC and against ten acinar cell clumps (150 um diameter) in the MLAC. The optimal duration of coculture for the peak response was found to be nine days for the MLR and MLIC and nine to eleven days for the MLAC. The parameters included the use of non tissue culture treated 96 well plates, RPMI containing 10% AB human serum and the use of 0.026 mM dithizone to handpick the islets. Three experiments per series showed trends in the results but these were insufficient for statistical significance and a combination of results from experiments with the same parameters was necessary to provide sufficient data for statistically significant findings. 'Back proliferation' of untreated stimulator islets in response to allogeneic lymphocytes in the MLIC was found to be insignificant. Combined data confirmed that the MLR showed the greatest lymphoproliferative response and the MLAC, the lowest. The variability within and between experiments was found to be considerable over the course of the work without any clear reasons for this. Other findings from the combined data included the low background PBL response using human ABS as a growth supplement in the coculture medium, a positive correlation between the control autologous PBL response and the allogeneic response to islets and acinar cells. Lastly, others have chosen to represent their data as either Acpm or as a
stimulation index, so a comparison of the two types of calculation was made. The results illustrated the ambiguity of data representation for individual experiments.

In conclusion, the MLIC and MLAC showed peak lymphoproliferative responses to ten HLA mismatched islets or acinar cell clumps after nine days of coculture. The variation within and between experiments suggests that single responder stimulator combinations or small series of experiments do not necessarily provide sufficient data for statistical analysis.

### The Next Chapter...

The combined results showed that there was a reduced proliferative response to HLA mismatched acinar compared with the response to islets. Some possible explanations of this phenomenon are examined in Chapter Eight.

# CHAPTER EIGHT

### CHAPTER EIGHT CONTENTS

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### CHAPTER EIGHT

### AN INVESTIGATION OF THE IMMUNOGENICITY OF ACINAR TISSUE IN THE MLAC

#### 8.1 Introduction

This chapter describes the last part of the coculture work in which the relatively limited proliferation of HLA mismatched lymphocytes to acinar tissue was investigated.

Following published reports by Gotoh(21) (139) on the increased rate of rejection in mice caused by non islet pancreatic tissue, which is normally found as a contaminant of islet preparations, others have described such impure islet preparations as particularly immunogenic in the human MLIC(29) (269) (273). Although the original intention for the MLIC studies was to use islet preparations of varying purity, as obtained after density gradient centrifugation and as used for transplantation, the low levels of proliferation and variability of results led to separation of the islets and acinar tissue stimulator populations. As described in Chapter Seven, these studies showed that the PBL proliferative response to HLA mismatched human acinar tissue was lower than that to islets. The apparent discrepancy between this finding and the work of others which has shown that impure islet preparations, led to an investigation of the low immunogenicity of acinar tissue.

#### Three approaches were taken:

a) to determine whether pretreatment of the stimulator cell population to arrest cell growth had any effect on the responder cell proliferative response,

b) to determine the effect of the soluble products from acinar cells on T cell proliferation, using nucleopore filters to separate the acinar tissue from the MLR,

c) to determine whether pretreatment protocols for inhibition of stimulator cell proliferation, had any effect on amylase production by the acinar tissue, d) to upregulate the MHC antigens on a cinar tissue using IFN- $\gamma$  and TNF- $\alpha$ , to determine whether this could lead to increased immunogenicity.

Thus in summary, others have reported that the impure islet preparations, compared with pure islets, have an increased immunogenicity in the MLIC. This is in disagreement with the observed low immunogenicity of acinar tissue in the MLAC, and studies were undertaken to investigate reasons for the reduced allogeneic lymphoproliferative response to acinar tissue.

# 8.2 Determination of the effect of stimulator cell pretreatments on the responder proliferative response

In the standard one-way MLR, the proliferation of the stimulator population (normally lymphocytes) has been prevented by pretreatment with  $\gamma$ -irradiation or mitomycin C. The negligible proliferation by islets and acinar cells in culture and an ultimate intention to use the coculture model for immunomodulation studies, led to the decision not to use stimulator cell pretreatments for the MLIC and MLAC. However, in order to investigate any differences between this work and that of others, an experiment was designed to detect any substantial difference of immunogenicity caused by treating the islets, digest and acinar cells with  $\gamma$ -irradiation and mitomycin C. In addition, the effect of Trasylol was investigated as it has been used to inhibit the production of enzymes which, in the cocultures, might have a detrimental effect on the proliferating T cells.

The effect of  $\gamma$ -irradiation has been found to be ionisation resulting in the generation of free radical ions and neutral free radicals. Therefore in a system of which the major component is water then water can be ionised to form a free radical ion (H<sup>+</sup>) and a neutral free radical (OH·) with one unpaired electron in the outer orbit.

 $H_20 \longrightarrow H^+ + OH^-$ 

These reactive components have been found to cause DNA damage including breaks in DNA, unscheduled DNA synthesis and alteration of the DNA bases or protein damage by a direct ionising effect(287). Lymphocytes have been shown to be particularly sensitive to irradiation damage (288) and for this experiment 3000 rads was used, an equivalent dose to that used to inhibit growth of stimulator splenocytes in the MLR. The other common stimulator cell pretreatment, mitomycin C, has been found to inhibit cell proliferation (289) and in addition to its direct cytotoxic effects, which involve cross-linkage of DNA (290), it can also induce expression of MHC class II antigen(287). Mitomycin C has been used as a growth inhibitor in the MLIC (269) at 12.5  $\mu$ g/ml of suspended islets over 20 minutes incubation followed by 3 x washing. The third pretreatment used for the stimulator population was Trasylol (Aprotinin), which has been found to be a protease inhibitor(146) and was used at 28  $\mu$ g/ml.

The effects of the three different stimulator population pretreatments on the MLIC, MLAC and MLDC (MLDC = digest as stimulator population) are shown in Figure 8.1. The experiment included the use of 10% FCS as a growth supplement in the RPMI medium and untreated or pretreated stimulator cell populations including 20 islets, 20 pieces of tissue from pancreatic digest and 20 acinar tissue pieces cocultured with  $1 \times 10^5$  HLA mismatched human PBLS. The peak lymphoproliferative response over a 3 - 9 day incubation is shown.

The results demonstrated a low proliferative response to islets and comparatively higher response to digest tissue pieces. The response to  $\gamma$ irradiation treatment of the stimulators was positive in every case and the response to mitomycin C treated stimulators was negative in each case, possibly due to toxicity of residual mitomycin C in the cocultures. In the light of previous work showing the difficulties of interpretation of single experiments, little information was gained from this experiments except that no single stimulator cell pretreatment led to a clear enhancement of stimulatory capacity for islets, acinar cells and digest tissue.

The results were compared statistically, including combined results using the three different tissues, as shown in Table 8.1, and found to be non significant (Mann-Whitney U test).





 Table 8.1. Coculture experiments comparing different treatments of pancreatic

 tissue (islets, acinar tissue and digest - combined results).

Treatment	Untreated	γ-irradiated	Mitomycin C	Trasylol
Acpm median +	93 + 6623	1285 + 5597	-4159 + 334	-2737 + 2527
IQ range				一开业学校开始。

Acpm represents the median (peak day) of three experiments, each with quadruplicate samples.

Thus in summary, different pretreatments of the stimulator cells, two of which have been used by others to arrest cell growth ( $\gamma$ -irradiation or mitomycin C) and one which can inhibit the activity of proteases (Trasylol), were used in the MLIC, MLAC and MLDC. No significant difference of immunogenicity between the three treatments was shown. These results did not point to any definite reasons for the higher levels of stimulation by acinar tissue in cocultures observed by others.

# 8.3 Effect of acinar cell soluble products on the MLIC and MLR

Additional experiments were planned to investigate whether the soluble products of the acinar tissue might have a direct effect on responder cell proliferation in cocultures. These experiments involved suspending acinar cell clumps in nucleopore filter cones in the medium over cocultures (MLR or MLIC) such that the soluble products of the acinar cells but not the acinar tissue itself was in direct contact with the cocultures. The first experiments (Expts 31, 37, 38, 40) were the usual combination of ten islets (MLIC) or  $1 \times 10^5$  y-irradiated splenocytes (MLR) combined with HLA mismatched PBLs. The second experiments used  $1 \times 10^5$  y-irradiated PBLs (MLR) as stimulators combined with  $1 \times 10^5$  HLA mismatched PBLs per well. Amylase concentrations were measured in the coculture supernatant in each well as a marker for the presence of soluble products from the acinar cells in the filter cone.

8.3.1 The effect of soluble products of acinar tissue pieces on the MLIC

The results (median  $\pm$  IQ range) of four experiments (Expts 31, 37, 38, 40) in which ten acinar tissue pieces were suspended in cones in medium above mixed lymphocyte cultures and islet lymphocyte cocultures, are shown in Figure 8.2.

The results indicated a slight decrease in lymphocyte proliferation in the presence of acinar cell products, from  $34,500 \pm 18,488 \,\Delta \text{cpm}$  to  $31,869 \pm 17,994 \,\Delta \text{cpm}$  for the MLR and from  $9,155 \pm 16,930 \,\Delta \text{cpm}$  to  $8,143 \pm 3,188 \,\Delta \text{cpm}$  for the MLIC (median  $\pm \text{IQ}$  range), but neither of these comparisons were statistically significant (Mann-Whitney U test). The amylase levels were 197 units/litre (day seven) and 199 units/litre (day nine) in wells containing ten acinar cell clumps in nucleopore filter cones (n = 3), and <27 units/litre (day seven and nine) in wells without acinar and cones (n = 3). Note that 27 units/litre represents the lower detection limit of the amylase assay. The amylase levels in wells where the acinar tissue pieces were part of the coculture i.e. not separated by the cones, were 186 units/litre (day seven) and 203 units/litre (day nine) (n = 3), so comparable with the levels where the acinar tissue was confined to the cones.

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**Fig 8.2.** Effect of soluble products of acinar tissue on the MLR and MLIC. Each bar represents the median of four experiments (Expts 31, 37, 38 and 40) each with quadruplicate samples.

In order to extend these observations, additional four MLR experiments were planned. These used  $1 \times 10^5$  PBLs as the responder population, as usual, combined with  $1 \times 10^5$  HLA mismatched  $\gamma$ -irradiated PBLs as the stimulator cell population in place of splenocytes. The results of these four experiments (MLR 1a and b, MLR 2a and b) as well as the previous four experiments (Expts 31, 37, 38 and 40) are shown in Figure 8.3.

The results indicated that in seven of eight cases the lymphocyte proliferative response to allogeneic lymphocytes in the presence of soluble products from acinar cells was reduced compared with the controls (each bar represents the mean of quadruplicate samples). The median + IQ range for the MLR group =  $41,946 \pm 10,550$   $\Delta$ cpm and for the MLR + acinar cell soluble products =  $23,808 \pm 84,98$   $\Delta$ cpm, p <0.06 (non significant, Mann-Whitney U test). However it is clear that the result obtained in Expt 37 was unusually low and without this one result the comparison was statistically significant. Using the group of seven results, the median  $\pm$  IQ range for the MLR group =  $43,251 \pm 9,310$   $\Delta$ cpm and for the MLR + acinar cell soluble products =  $24,208 \pm 8,211$   $\Delta$ cpm, p <0.05 (Mann-Whitney U test). The average proliferation in the presence of acinar cell soluble products was 65% of the normal MLR (range 42 - 88%, n = 7).



MLR
 MLR + soluble products of acinar tissue

**Fig 8.3.** Effect of soluble products from acinar tissue on the MLR. Each point represents the mean  $\Delta$ cpm of quadruplicate samples at day seven.

The amylase levels were measured in experiments MLR 1a and 1b and were found to be 126 units/litre (day 7) and 116 units/litre (day 9) in the wells containing acinar tissue pieces in cones (n = 6), and <27 units/litre (day seven and day nine) in wells without acinar tissue or cones (n = 6).

Thus in summary, a trend towards decreased allogeneic lymphocyte proliferation in the presence of soluble products from acinar cells was found to be statistically significant after removal of one low result. This suggests that soluble products of the acinar cells inhibit allogeneic stimulated lymphocyte growth.

8.3.2 The effect of soluble products from acinar cell clumps on the PBL control response

A comparison of the PBL controls alone or in the presence of acinar cell clumps in nucleopore cones, showed that in all eight experiments, there was a slight increase in the mean cpm of the latter group. The median  $\pm$  IQ range of the combined results of these eight experiments were: PBLs alone = 1,967  $\pm$  1,932 cpm and PBL + acinar soluble products = 3,104  $\pm$  3,639 cpm. This trend was found to be non significant (Mann-Whitney U test). The mean cpm results for individual experiments are shown in Figure 8.4.





Thus in summary, a trend indicating a stimulatory effect of the nucleopore cones containing acinar cell clumps on the PBL controls was found to be non significant.

# 8.3.3 Amylase as an indication of functional activity of acinar tissue pieces in nucleopore cones

As described above, estimations of amylase levels were carried out in Expt 40 and MLR 1a and 1b, and showed that wells with acinar cell clumps in nucleopore filter cones contained amylase in the medium while those without showed minimal amylase levels. The presence of amylase in the MLR supernatant confirmed that soluble products of the acinar cells in the nucleopore cones were able pass from the cones into the medium containing lymphocytes or coculture. The next series of experiments was planned to measure the persistence and continued production of amylase by acinar tissue in culture.

# 8.4 Determination of the effect of acinar tissue pretreatments on amylase production

8.4.1 Persistence of amylase in supernatant of cultured acinar tissue

The first experiment studied the persistence of amylase in supernatant removed by centrifugation (800g for 2 minutes) of 10 mls culture medium containing 30  $\mu$ l of digest. This concentration was similar to that used for tissue culture of digest tissue or islets. Samples of supernatant were taken after centrifugation (Day 0) and the same supernatant incubated until used for amylase estimation on day 2, 5, 8 and 9. The results are shown in Table 8.2.

Day	0	2	5	8	9
Amylase	61,000	55,675	47,625	48,425	52,925
units/litre	+ 22,316	+ 3,724	+ 11,393	+ 2,533	+ 12,048
mean + sem					

Table 8.2. Persistence of amylase in culture after removal of acinar tissue

n = 4/day.

Little difference was found between the day zero and day nine amylase measurements (statistically non significant, Students t test) demonstrating that amylase can persist in culture. The levels of amylase were very high involving 1:100 dilution of the sample before accurate measurement could be made.

# 8.4.2 Experiments to measure the effect of different pretreatments of acinar tissue on amylase production

For these experiments fresh handpicked acinar cell clumps were used untreated or treated with 3000 rads  $\gamma$ -irradiation, 12.5 µg/ml mitomycin C (for 20 minutes followed by three washes) or light fixation using 0.05 -0.1% glutaraldehyde for one minute. The experiments used increasing concentrations of acinar tissue in medium which is described under each section. Amylase concentration in the supernatant was measured at intervals between day zero and day nine.

#### 8.4.2a Experiment Aci 1

In this experiment (Aci 1), ten acinar cell clumps, as in the MLAC and experiments involving nucleopore cones, per 4 ml medium were used. The culture medium containing acinar cell clumps was centrifuged (200g for 2 minutes) and a sample of the supernatant taken for amylase measurement. Fresh medium was added to the acinar tissue for further incubation. The amylase results (single samples) are shown in Table 8.3.

Table 8.3. Amylase production following pretreatment of acinar tissue

Treatments	Day 0	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7	Day 9
Untreated	214	<27	<27	<27	<27	<27	<27	<27
γ –irradiation	118	<27	<27	<27	<27	<27	<27	<27
Mitomycin C	93	<27	⊲27	⊲27	⊲27	⊲27	⊲27	<27

N.B. 27 units/litre represents the minimum detection level of this assay.

The loss of amylase production after the day zero sample can be seen from the results. It was decided to increase the amount of acinar tissue per ml of medium for the next experiment and to increase the interval between samples to 48 hrs.

#### 8.4.2b Experiment Aci 2

In this experiment (Aci 2), 50 acinar cell clumps per 4 ml medium were used. The culture medium containing acinar cell clumps was centrifuged (200g for 2 minutes) and a sample of the supernatant taken for amylase measurement. Fresh medium was added to the acinar tissue for further incubation. The amylase results (single samples except where stated) are shown in Table 8.4.

Table 8.4. Amylase production following pretreatment of acinar tissue

Treatment	Day 0	Day 2	Day 4	Day 6	Day 8
Untreated	78	98	<27	<27	<27
$\gamma$ -irradiation (n=2)	61	51	<27	<27	<27
Mitomycin C	67	71	27	<27	<27
Glutaraldehvde (n=2)	66	27	<27	<27	-

N.B. 27 units/litre represents the minimum detection level of this assay.

The results show that the initial level of amylase was considerably lower than that seen in the previous experiment (Aci 1) despite increased quantities of acinar tissue. A loss of amylase production between the day two and day four samples can be seen.

It was decided to again increase the concentration of acinar tissue and to study samples of accumulated supernatant (three and six days) as well as samples with replenished medium.

#### 8.4.2c Experiment Aci 3

In this experiment (Aci 3), 30 acinar cell clumps per 200  $\mu$ l medium (in a microwell) were used. Supernatant for amylase measurement was taken from the well and in the cases of replenished wells, new medium added. Samples were taken at day three and day six starting with fresh medium on day zero. The amylase results (n = 2) are shown in Table 8.5.

Table 8.5. Amylase production following pretreatment of acinar tissue

Treatment	Sample	Day 3	Day 6
Untreated	accumulated	79	340
	replenished	381	<27
γ -irradiation	accumulated	480	354
	replenished	608	180
Mitomycin C	accumulated	139	183
	replenished	277	<27
Glutaraldehyde	accumulated	750	701
	replenished	750	177

N.B. 27 units/litre represents the minimum detection level of this assay (varies with batch number of the assay).

No clear pattern of amylase production was identified in the results, apart from the high levels seen after light glutaraldehyde fixation. This result was not seen in earlier experiments however, the loss of amylase following medium replacement was evident in each group of replenished but not accumulated samples. It was decided to compare samples from different pancreata to determine whether this could identify a trend towards differences between groups. Transwells were used to facilitate the removal of tissue from supernatant.

#### 8.4.2d Experiment Aci 4

transwells

In this experiment (Aci 4), acinar tissue from three different pancreata with 100 acinar cell clumps per 4 mls medium in a small petri dish or, 30 acinar cell clumps per 2 mls medium (transwell) were used. Acinar tissue in transwell filters (4  $\mu$ m pore size) were inserted in the top of a well in a 24-well plate to facilitate supernatant removal. Supernatant was replaced by fresh medium on day -2 (HP357 and HP358) or day -3 (HP359) then samples of medium taken for amylase measurement and the medium replaced on day zero then day two to three and day four to six. The amylase results are shown (single samples) in Table 8.6.

Pancreas No.	HP 357		HP 358		HP 359		
Treatment	Day 0	Day 3	Day 0	Day 3	Day 0	Day 2	Day 4
Untreated							
microwells	319	75	925	<34	7480	2890	211
transwells	670	57	2460	132	5580	378	97
γ -irradiation							
microwells	366	75	7487	<34	7480	2580	192
transwells	774	83	3780	236	7220	1120	179
Mitomycin C							
microwells	343	34	644	<34	8500	1214	122
transwells	825	<34	5140	53	8500	230	64
Glutaraldehyde							
microwells	355	97	875	<34	8250	1307	159

Table 8.6. Amylase production following pretreatment of acinar tissue

N.B. 34 units/litre represents the minimum detection level of this assay (varies with batch number of the assay).

850 <34 4690 34 7480 224

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The results show that the length of time that acinar tissue was cultured prior to setting up the assay reflected the total amount of amylase released at day 0 (HP357 = 12 days, HP358 = 7 days and HP 359 = 0 days). However, this

did not affect the overall comparisons and no clear trend of a difference between treatment groups was seen.

The data from this series of experiments was pooled to compare the control and treatment groups, (numbers below the minimum level were taken as zero for the purpose of calculation). As shown in Figure 8.5, amylase production was found to fall to lower levels, but not to zero, by day four in all groups. Each point represents the mean of two to ten results.



**Fig 8.5.** Effect of three different treatments used to arrest cell growth, without impairing antigen expression, on the amylase content of acinar cell cultures. Each point represents the median of 2-10 samples (Aci 1-4),

As can be seen from the combined results, all three different treatments and untreated acinar tissue yielded similar levels of amylase production. This suggests that culture and medium replacement has more effect on the level of soluble amylase than treatments which are designed to inhibit acinar cell function.

Thus in summary, amylase was shown to persist in supernatant for nine days after removal of acinar tissue by centrifugation. In addition, acinar cell clumps handpicked before placing in culture medium and after replacement of medium following supernatant sampling (24 - 72 hrs),

showed rapid but incomplete loss of amylase production within four days of culture. This rapid loss was not affected by treatment of acinar tissue with  $\gamma$ -irradiation, mitomycin C or glutaraldehyde. However the low levels of residual release of amylase were within the range found in the supernatant from microwells containing acinar cell clumps in experiments Expt 40 and MLR1a and 1b (described earlier).

# 8.5 Effect of cytokine pretreatment on acinar cell MHC class II expression and the MLAC response

This series of experiments was designed to investigate the effect of upregulation of MHC antigens on acinar cell stimulation of an allogeneic proliferative response in the MLAC, based on the findings of Steinman that MHC class II antigen is primarily involved in the initiation of an immune response(234). The cytokines TNF- $\alpha$  and IFN- $\gamma$  when used in combination, have been found to induce MHC class II antigen on human islets(70) and might be expected to a exert similar effect on acinar tissue.

Three experiments involving the MLAC using 10 acinar cell clumps per well which were untreated or treated with a combination of the two cytokines (TNF- $\alpha$  and IFN- $\gamma$ ), were carried out. The duration of treatment and the concentration of the cytokines was increased after the first experiment (Expt 42). Details of the 3 experiments are listed in Table 8.7.

Expt No.	Pancreas	Post	Cytokine	IFN-y	TNF-α
		isolation	incubation	units/ml	units/ml
Expt 42	HP327	1 day	2 days	50	10
Expt 43	HP333	0 days	6 days	500	100
Expt 44	HP340	2 davs	4 davs	500	100

Table 8.7. Experiments to measure the effect of cytokines on MLIC

N.B. The responder population was  $1 \ge 10^5$  HLA mismatched PBLs, duration was for seven and nine days.

Immunocytochemical staining was carried out on acinar tissue from the same preparation and with the same post isolation interval and duration of cytokine pretreatment as the stimulator population in the cocultures. This allowed comparison between the level of MHC expression (immunocytochemistry) and allogeneic T cell proliferation (MLAC). The relevant pancreas number for Expt 42 was HP327, for Expt 43 - HP333 and for Expt 44 - HP340.

#### Expt 42, 43 and 44

As shown in Table 8.7 above, acinar cell clumps were incubated with or without the presence of cytokines TNF- $\alpha$  (10 or 100 U/ml) and IFN- $\gamma$ (50 or 500 U/ml). The results of the immunocytochemistry are described in Chapter Nine.

No increase of lymphoproliferative response was seen in the allogeneic MLAC following cytokine pretreatment of the stimulator acinar tissue. In fact as shown in Table 8.8, there was a trend towards a decrease in the lymphocytic proliferative response ( $\Delta$ cpm) after cytokine treatment of the acinar tissue, but this was found not to be statistically significant.

**Table 8.8.** The effect of cytokine treatment on the immunogenicity of acinar cell

 clumps in the MLAC

Experiment	∆cpm (Median + IQ range)				
MLAC	10,997 + 2,072				
MLAC + cytokines	3,328 + 3,382				

Acpm represents the median (peak day) of three experiments, each with quadruplicate samples.

Thus in summary, pretreatment of acinar tissue with two cytokines known to induce MHC class II expression on human islets (TNF- $\alpha$  and IFN- $\gamma$ ), did not increase the stimulatory capacity of acinar tissue in the MLAC. The parallel immunocytochemical studies are described in Chapter Nine.

# 8.6 Effect of islet cell soluble products on proliferation in the MLAC and MLR

The majority of experiments described earlier in this chapter were carried out as part of the studies on the low proliferative responses to acinar tissue. The availability of islets from some of the same pancreata enabled similar studies using human islets (Expts 37, 38 and 40 - effect of soluble products from the stimulator cell population on lymphoproliferation in cocultures, Expts 42, 43 and 44 - effect of cytokine pretreatment of the stimulator population on lymphoproliferation in cocultures).

Similar to the experiments (Expts 37, 38 and 40) as described earlier, nucleopore filter cones containing ten islets were inserted into the top of microwells containing cocultures of ten acinar cell clumps and  $1 \times 10^5$  HLA mismatched lymphocytes. The proliferative response was recorded in lymphocyte-acinar cell coculture and appropriate control wells together with the insulin content of each well.



The results (median  $\pm$  IQ range of peak result) are shown in Figure 8.6.

**Fig 8 .6.** Effect of soluble products from islets (in Nucleopore filter cones) on the MLR and MLAC compared to the normal MLR and MLAC. Each bar represents the median and IQ range of four experiments (Expts31, 37, 38, 40).

The results indicate that there was a decrease in peak lymphocyte proliferation, median  $\pm$  IQ range  $\triangle$ cpm (from 34,500  $\pm$  18,488  $\triangle$ cpm to 13,964  $\pm$  28,241  $\triangle$ cpm in the presence of soluble products from islets) for the MLR and for the MLAC (from 2,729  $\pm$  642  $\triangle$ cpm to -3,607  $\pm$  5,470  $\triangle$ cpm in the presence of soluble products from islets), but neither of these comparisons were statistically significant (Mann-Whitney U test). The insulin levels were measured for Expt 40 and showed similar insulin levels (four wells pooled to give a single result), regardless of whether the ten islets were with lymphocytes in the bottom of the wells or were in the nucleopore cones. The level of insulin was found to be minimal in wells without islets. The results are shown in Table 8.9.

Table 8.9. Insulin levels (ng/ml) of islets in the MLIC or suspended in nucleopore cones

insulin per well	insulin ng/ml				
location of islets	10 islets in cones	10 islets in well	zero islets in wells		
Day 7	770	830	<7		
Day 9	850	> 900	<7		

Thus in summary, the effect of soluble products on the MLAC was not clear but there was a trend towards a lower  $\Delta cpm$  result of the MLAC in the presence of islet soluble products (statistically non significant).

### 8.7 Effect of cytokine pretreatment of islets on MHC class II expression and the MLIC response

In addition to the comparison between MHC class II expression and the lymphocyte proliferative response in the MLAC, human islets were also used in the same three experiments (Expt 42, 43 and 44). Details for the three experiments were as described in Table 8.7 above, but using ten islets instead of ten acinar cell clumps as the stimulator population. Samples of islets were snap frozen for immunocytochemical staining the same day as the cocultures were set up. The immunocytochemical staining pattern of control and treated islets is described in Chapter Nine.

The lack of availability of islets towards the end of this project meant that additional experiments on the effect of islet soluble products on lymphocyte proliferation in the MLR could not be carried out.

The results showed that there was no increased proliferation in the allogeneic MLIC following cytokine pretreatment of the stimulator islet population. As shown in Table 8.10, there was a trend towards a decrease in

the lymphocyte proliferative response ( $\Delta$ cpm) after cytokine treatment of the acinar tissue, but this was found not to be statistically significant (Mann-Whitney U test).

Table 8.10. The effect of cytokine treatment on immunogenicity of islets in the MLIC

Experiment	∆cpm (Median + IQ range)
MLIC	20,614 + 5,372
MLIC + cytokines	15,563 + 5,555

Acpm represents the peak proliferation as a median of three experiments, each with quadruplicate samples.

Thus in summary, treatment of human islets with two cytokines (TNF- $\alpha$  and IFN- $\gamma$ ) was not found to increase in immunogenicity as measured in the MLIC. The effect of these two cytokines on MHC class II expression is described in Chapter Nine.

# 8.8 Overall summary of the investigation into the low immunogenicity of acinar tissue in the MLAC

Acinar tissue was found to stimulate a lower lymphocyte proliferative response than islets using in vitro cocultures (MLAC < MLIC). This result is contrary to findings by others which show that impure islet preparations can stimulate a higher response than pure islet preparations. The results of one coculture experiment and a series of experiments using acinar tissue as the stimulator population, showed that pretreatments of acinar tissue (y-irradiation or mitomycin C), as used by others to inhibit stimulator cell growth, had no effect on in vitro immunogenicity. These treatments did not significantly affect the level of amylase release from acinar cells although there was a rapid but incomplete loss of amylase within four days following replacement of supernatant with fresh medium. The effect of Trasylol, a protease inhibitor, on the MLR, MLIC and MLAC was inconclusive. A series of experiments showed statistically significant inhibition of proliferation by the soluble products of acinar cells in seven MLR experiments, although the effect was inconclusive in three MLIC experiments. The effect of insulin secretion on the MLR and MLAC showed a trend towards inhibition of the lymphocytic proliferative response but

this was not statistically significant. The last experiments described in this chapter showed that pretreatment of acinar tissue and islets with cytokines IFN- $\gamma$  and TNF- $\alpha$ , did not increase their stimulatory capacity in the MLIC and MLAC.

### The Next Chapter...

The purpose of pretreating the islet and acinar cell stimulator populations with cytokines IFN- $\gamma$  and TNF- $\alpha$ , was to modulate the expression of MHC class II antigen which is involved in the initiation of the immune response. An immunocytochemical investigation of the expression of MHC antigens on islets and acinar tissue in the pancreas, after isolation, culture and treatment with cytokines, is described in Chapter Nine.

# CHAPTER NINE

### CHAPTER NINE CONTENTS

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## CHAPTER NINE

### MHC CLASS I AND CLASS II ANTIGEN EXPRESSION IN HUMAN PANCREAS, ISLETS AND ACINAR TISSUE POST ISOLATION AND CULTURE

#### 9.1 Introduction

The expression of MHC antigens by cells in the diabetic pancreas has been controversial(64) and relates to the appearance of aberrant MHC class II antigens on beta cells prior to insulitis(51) or as a result of engulfment of beta cells by macrophages seen in BB rats(58) and recurrent human autoimmune diabetes(5). In the normal human pancreas, less diverse evidence exists and it is generally accepted that MHC class I antigens are expressed on individual cells in islets and acinar tissue of the human pancreas which include vascular endothelium, macrophages/dendritic cells, duct epithelium and nerve fibres. In addition, the parenchymal cells of the islet, but not acinar tissue, weakly express MHC class I antigens (22) (23) (51). MHC class II antigens are not expressed on either parenchymal islet or acinar tissue but are found on individual cells including lymphocytes, macrophages/dendritic cells and human vascular endothelium (22) (23) (28) (29). The expression of MHC class I and II antigens on human isolated islets and acinar tissue has not been fully studied but as MHC antigen expression is considered to be fundamental to the generation of an alloimmune response, it has important implications for the potential transplantation of isolated islet preparations.

The focus of this study has been to develop an *in vitro* assay as a model for the *in vivo* rejection response to human islets. The *in vitro* MLR allogeneic lymphocyte proliferative response has been found to principally be mediated by T helper cells(230) for which the expression of MHC class II antigens are necessary. Thus, it was considered important to study MHC antigen expression of human islets, and of the major contaminant, acinar tissue. In this chapter the results of immunocytochemical staining of pancreas, isolated islets and acinar tissue (at day zero and day seven post isolation) are presented.

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### 9.2 Preliminary studies

#### 9.2.1 Study of antibodies used for immunocytochemical staining

For these results, some preliminary work was necessary to establish the techniques to be used. A panel of primary antibodies was chosen:

a) to identify the two major cell types of interest HB124 insulin IgG2a LDS8 acinar cells IgM

b) to identify MHC class I and II antigens W6/32 HLA-A,B,C (I) IgG2a HB120 HLA-A,B,C IgG1 (I)HB55 HLA-DR (II) IgG2a HB145 HLA-DR (II) IgG1

c) It is appropriate to record here that a much larger panel of antibodies than finally chosen was initially used. This panel included the following:

MCA 532 CD54/ICAM-1		IgG1	
Li8	IFN-α	IgG1	
CB1375	TNF-α	IgG1	
HB8291	IFN-y	IgG1	
MC4243	Hsp65	IgG1	

Firstly the panel of antibodies (a - c) was tested against normal pancreas using the routine method of immunocytochemical staining used in this laboratory. This is a 3-layer method as described in Chapter Four, briefly comprising a first layer of primary antibody (overnight), a second layer of the appropriate biotinylated antisera (45 minutes), incubation with extravidin conjugated to alkaline phosphatase (20 minutes) and colour development with Fast Red and napthol phosphate.

Examples of the pattern of staining obtained with antibodies to insulin, acinar cells, MHC class I and II on normal human pancreas, can be seen in Figures 9.1a - d.



**Figure 9.1a.** Expression of insulin in human pancreas. Immunocytochemical staining used HB124 (mouse anti human insulin) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification =  $x \ 160$ .



Figure 9.1b. Expression of an acinar cell antigen in human pancreas. Immunocytochemical staining used LDS8 (mouse anti human acinar cell) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160.



Figure 9.1c. Expression of MHC class I antigen in human pancreas. Immunocytochemical staining used W6/32 (mouse anti human HLA-A,B,C) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160.



Figure 9.1d. Expression of MHC class II antigen in human pancreas. Immunocytochemical staining used HB55 (mouse anti human HLA-DR) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160. The positive staining patterns seen using the antibodies to insulin, MHC class I and II antigens, acted as a control for the finding that acinar tissue showed variable expression of the antigen detected by LDS8 in different pancreata. The remaining antibodies (panel c anti CD54/ICAM-1, anti IFN- $\alpha$ , anti TNF- $\alpha$ , anti IFN- $\gamma$  and anti Hsp65) gave either results that were difficult to interpret or no staining. ICAM-1 was the clearest of these with individual cells (vascular endothelium) identified in eight of ten pancreata used. Other tissues such as human spleen and heat-shocked human lymphocyte cytospin smears were used in an attempt to identify a positive control for the antibodies but with little success. In addition, the use of this panel (c) on serial islet and acinar sections post isolation showed that the panel was too ambitious and outside the scope of this part of the project. For the remainder of this chapter, the results of antibodies to insulin, acinar tissue, MHC class I and MHC class II only will be described.

Thus in summary, using a 3-layer biotin avidin staining technique with alkaline phosphatase and Fast Red as the colour development, primary monoclonal antibodies were incubated with frozen sections from normal human pancreas. The panel of antibodies (all mouse anti human) chosen for further work included HB124 (anti insulin), LDS8 (anti acinar cell), W6/32 (anti HLA-A, B, C) and HB55 (anti HLA-DR).

#### 9.2.2 An investigation of double staining techniques

The purpose of including the IgG1 antibodies to MHC class I and II antigens was to enable a double staining technique to be developed using the isotype of the primary antibodies to differentiate two colour markers.

Using HB124, LDS8, W6/32 and HB55 as the standard antibodies, the results from two different pancreata showed that HB145, although weaker than HB55, identified the same cell types and was suitable for use in the double staining technique. HB120 was found to reflect the staining normally seen using W6/32 but was weaker and resulted in poor morphology of the sections.

For the double staining, initially a method using BCIP/NBT ( 5-bromo-4chloro-3-indolyl phosphate and nitroblue tetrazolium) (dark purple) as the colour development for alkaline phosphatase was intended as a contrast to

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AEC (3-amino-9-ethylcarbazole) (red), a colour development for HRPO (horseradish peroxidase). The two standard antibodies HB124 and W6/32 were separately used with a 3-layer method developed with BCIP/NBT which produced good stain but the negative control nonspecifically stained individual cells in the pancreas. The reaction was caused by the BCIP/NBT itself and as this could not be resolved even after blocking the alkaline phosphatase with levamisole, it was discontinued.

Next, a combination of the colour development of antisera conjugated to HRPO with DAB (3', 3' diaminobenzidine) (brown) was attempted in combination with the colour development of antisera conjugated to alkaline phosphatase with Fast Red and napthol phosphate (red). Although both these systems were successful when used alone with LDS8 (IgM) (alkaline phosphatase) and HB145 (IgG1) (HRPO), in combination the second colour used did not show specific staining. Sequential immunostaining and simultaneous application of the primary antibodies were both used, the latter resulted in poor morphology. One further combination was used to attempt to resolve the double staining technique. HB145 (IgG1) was used as the first primary antibody with colour development using DAB (brown) then W6/32 (IgG2a) as the second primary antibody using Fast Red (red) as the colour marker. Again, although both antibodies were successful when used alone, when used sequentially, only the first primary antibody showed specific staining.

Fluorescent methods of double staining which have been described by others(52) (70) (291) were not used because the morphological identification of non stained tissues was felt to be important. As an alternative it was decided to use serial sections.

Thus in summary, an attempt was made to use a double staining technique involving primary antibodies of different isotypes and appropriate secondary antisera and colour development using alkaline phosphatase with nitroblue tetrazolium or horseradish peroxidase with DAB. Neither was successful despite considerable effort and so the use of the well tested 3-layer biotin avidin method developed with alkaline phosphatase and Fast Red was used in conjunction with appropriate antibodies for identification on serial sections.

#### 9.2.3 Serial sections and the final panel of antibodies used

Problems were encountered with the positive identification of isolated islets using HB124 (anti insulin) and LDS8 (anti acinar cell), so serum from a patient with Stiff Man's Syndrome (SMS) containing anti GAD antibodies was additionally used. The final panel of antibodies was as follows:

HB124	mouse anti human insulin	(IgG2a)
LDS8	mouse anti human acinar cells	(IgM)
SMS	human anti human GAD	(IgG)
W6/32	mouse anti human (HLA-A,B,C)	(IgG2a)
HB55	mouse anti human (HLA-DR)	(IgG2a)

Serial frozen sections were used from ten different pancreata (HP257, HP258, HP259, HP260, HP261, HP263, HP264, HP266, HP267, HP270) to represent the following:



Thus in summary, the final panel of antibodies used was for identification of islet beta cells, by insulin content (HB124) or reactivity with Stiff Man Syndrome serum (SMS), acinar cells (LDS8) as well as MHC class I (W6/32) and II (HB55) antigen positive cells. Islets and acinar tissue in the pancreas, immediately after isolation and after seven days culture from ten different pancreata were used.

# 9.3 Presence of positive staining on pancreas as well as islets and acinar tissue on day zero and day seven

The immunocytochemical staining of islets, acinar cells and antibodies against insulin, acinar antigens, MHC class I and class II antigens on isolated islets and acinar cell clumps, can be seen in Figures 9.2a - f. The photographs include examples of the identification of islets and acinar cell clumps at day seven post isolation, and the staining pattern of MHC class I and II positive antigens on islets and acinar tissue at day zero and day seven post isolation.



Figure 9.2a. Expression of insulin on a positively stained human islet (upper cellular group) and a negatively stained acinar cell clump (lower cellular group) at seven days post isolation. Immunocytochemical staining used HB124 (mouse anti human insulin) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160.



**Figure 9.2b.** Expression of acinar cells on a minimally stained (individual cells only) human islet (upper cellular group) and a positively stained acinar cell clump (lower cellular group) at seven days post isolation. Immunocytochemical staining used LDS8 (mouse anti human acinar cells) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x = 160.



**Figure 9.2c.** Expression of MHC class I antigens on a human islet (upper cellular group) and an acinar cell clump (lower cellular group) at day zero post isolation. Both the islet and acinar cell clumps show positively stained individual cells and some parenchymal stain, the latter of which is weaker on the acinar cells than on the islets. Immunocytochemical staining used W6/32 (mouse anti HLA-A, B, C) in 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160.



**Figure 9.2d.** Expression of MHC class I antigens on two human islets (cellular groups on right) and an acinar cell clump (cellular group on left) at day seven post isolation. The islets and the acinar cell clump show positively stained parenchymal cells. Immunocytochemical staining used W6/32 (mouse anti HLA-A, B, C) in 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160.



**Figure 9.2e.** Expression of MHC class II antigens on a human islet (upper cellular group) and an acinar cell clump (lower cellular group) at day zero post isolation. Islet and acinar cell clumps show positively stained individual cells. Immunocytochemical staining used HB55 (mouse anti human HLA-DR) in 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x = 160.



**Figure 9.2f.** Expression of MHC class II antigens on a human islet (cellular group to right of photograph) and an acinar cell clump (cellular group to left of photograph) at day seven post isolation. The islet shows positively stained individual cells and the acinar cell clump shows positively stained parenchymal cells. Immunocytochemical staining used HB55 (mouse anti human HLA-DR) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160.

Immunocytochemical staining of human pancreas showed that HB124 bound to islets which contained insulin and LDS8 bound to parenchymal human acinar cells as well as individual cells in islets. A combination of these two antibodies was used to identify islets and acinar tissue in the pancreas and post isolation. The photographs above (Figures 9.1a - d) show serial sections from one human pancreas (HP260), however there was variation in staining patterns between each pancreas, the most prominent was the extent of LDS8 binding which ranged from 10% to 100% of the acinar tissue in the pancreas. This was less variable after isolation when the parenchymal staining of acinar cells was more frequent and islets were identifiable by the pattern of individual stained cells (Figure 9.2b). The morphology of the acinar tissue varied between preparations such that at day 0 the cells were frequently dispersed as a loosely bound cellular mass (40%) and the islets, although more frequently intact, not always separate from the acinar tissue. By day seven of culture, the acinar tissue was more usually seen as cellular clumps similar in size to islets and the islets were more rounded but often with less than 80% of cells expressing insulin. Within the series of ten pancreata, morphology of the tissue was too poor and made accurate identification impossible in up to 20% of cases.

The antibody W6/32 showed that MHC class I antigens were expressed on individual cells in islets and acinar tissue and weakly on parenchymal cells of islets immediately post isolation (day zero) but by day seven were expressed on all parenchymal cells of islets and acinar tissue and were not visible on occasional cells. The antibody HB55 showed that MHC class II antigens were expressed on individual cells immediately post isolation in both islets and acinar cell tissue and in islets after seven days post isolation culture. MHC class II antigens were expressed by acinar parenchymal cells in culture at day seven post isolation.

Thus in summary, isolated islets and acinar tissue were found to show an enhancement or induction of MHC class I antigens which were prominent on parenchymal cells after seven days post isolation culture. MHC class II antigens were expressed by individual cells in islets and acinar tissue immediately post isolation and by individual cells in islets after seven days in culture. MHC class II antigen expression was induced on parenchymal acinar cells by seven days post isolation culture but not on islets.

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# 9.4 Percentage of islets and acinar tissue pieces expressing MHC class I and II antigens

Difficulty was encountered in the unambiguous identification of human islets and acinar tissue after isolation. Islets and acinar tissue were separated by their differential density characteristics on density gradient centrifugation using a COBE 2991 centrifuge(114). The density gradient layers were stained red with dithizone and the layers containing most islets were pooled as the islet fraction which ranged from 60%-90% purity. Acinar tissue was taken from the lowest level of the density gradient and the purity of the acinar tissue fraction was estimated to be above 98%.

Islets were defined on the basis of positive anti insulin and negative anti acinar cell staining and vice versa for acinar tissue. There were a few instances of tissue being double negative or double positive with both antibodies. Tables 9.1 and 9.2 show the change in MHC class I and class II antigen expression of islets and acinar tissue in the pancreas, immediately after isolation (day zero) and after seven days culture.

 Table 9.1. Percentage of islets containing different cell types (individual or parenchymal) before and after isolation

MHC antigen	Cell Type	Islets in	Day 0 Islets	Day 7 Islets
		Pancreas		
class I	Individual cells	100%	93%	0%*
class I	Parenchymal cells	45%**	79%	100%
class II	Individual cells	100%	100%	58%
class II	Parenchymal cells	0%	0%	0%

n = 11-14 per group

\* This number (0%) may represent a lack of detection of individual cells against a strongly staining parenchymal background or an absence of individual cells. \*\*weak stain
Table 9.2. Percentage of acinar cell clumps containing different cell types

 (individual or parenchymal) before and after isolation

MHC antigen	Cell Type	Acinar	Day 0 acinar	Day 7 acinar
		tissue in	cell clumps	cell clumps
		Pancreas		
class I	Individual cells	100%	100%	0%*
class I	Parenchymal cells	0%	20%	100%
class II	Individual cells	100%	100%	55%
class II	Parenchymal cells	0%	20%	42%

n = 5-22 per group

\* This number (0%) may represent a lack of detection of individual cells against a strongly staining parenchymal background or an absence of individual cells.

For the data shown in Tables 9.1 and 9.2, strict criteria were used regarding the identification of islets and acinar cell clumps. Thus, islets and acinar cell clumps were included only if the same piece of tissue could be identified in sections which gave a double confirmation of source i.e. islets were positive with HB124 and negative or showing the islet cell pattern with LDS8, and both MHC class I and II staining was identified on some part of the section.

Tables 9.1 and 9.2 show that the levels of MHC antigen expression on cell populations in islets change after isolation and, in some cases, again after culture. As shown in Table 9.1, individual MHC class I positive cells were still present in over 90% of islets after isolation but could not be separately detected from the parenchymal cells after seven days in culture. MHC class I antigen expression on the parenchymal islet cells, which was detectable on 45% of islets in the pancreas, was more frequently detected after isolation (on 79% of islets) and present on 100% islets after seven days in culture. The number of islets containing individual cells (100%) expressing MHC class II antigen in the pancreas, did not change after isolation, but showed a reduction in frequency to 58% after seven days culture. Parenchymal expression of MHC class II antigen was not found on islets which were both positive using HB124 and negative using LDS8.

The change in expression of MHC antigens on cells in acinar tissue is shown in Table 9.2 and show that individual MHC class I positive cells were still present after isolation (day zero) but could not be separately detected after seven days in culture. There was no MHC class I antigen expression on acinar parenchymal cells in the pancreas, but this antigen was subsequently expressed in 20% of acinar tissue clumps immediately after isolation and detectable on 100% acinar cell clumps after seven days in culture. MHC class II antigen expression was seen on individual cells in the acinar tissue of the pancreas, and individual cells were still present immediately post isolation in 100% of cases but dropped to 55% at day seven. Parenchymal expression of MHC class II antigens were not seen in the acinar tissue in the pancreas but expression was seen post isolation in 20% of acinar clumps and this had increased to 55% by day seven of culture.

Thus in summary, strict criteria were used for the unambiguous identification of islets and acinar cell clumps in cryostat sections. Individual MHC class I positive cells, present in islets and acinar tissue before and after isolation, could not be detected after seven days in culture. The concurrent parenchymal expression of MHC class I antigens on islets and acinar cell clumps which was complete after seven days culture, gave rise to uncertainty whether individual Cells were absent or undetectable. The number of islets containing individual MHC class II positive cells was seen to reduce during culture to 58%. Parenchymal MHC class II expression was seen on 55% of acinar cell clumps after seven days culture but was not seen on any islets before or after culture.

# 9.5 Number of individual MHC class I and II positive cells in islets and acinar tissue pieces

In MLR studies, the number of immunostimulatory cells (particularly dendritic cells(234) have been shown to affect the level of the allogeneic lymphocytic proliferative response. As the individual MHC class II positive cells in islets are thought to be immunostimulatory(150), the number per islet and in the associated non islet pancreatic tissues would be expected to affect the response to these tissues. Several authors have counted MHC class II positive cells in islets which range 0 - 15 in rat and dog islets(27) (36) (37), 5 - 10 in mouse islets(265), from 7-20 macrophages/dendritic cells and 0.5 - 2 leucocytes in whole rat islets(32).

For this study, the number of MHC class I and II positive cells in frozen sections of human islets and acinar tissue were counted in the pancreas, immediately after isolation and after seven days in culture. This included both endothelial cells and leucocytes. The results are shown in Figure 9.3.



**Figure 9.3.** Number of individual MHC (I and II) positive cells in islets and acinar tissue (a)in the pancreas, (b), immediately after isolation and (c) after seven days culture. Each bar represents the median + IQ range of individual cells per islet or acinar tissue piece counted in a variable total number of islets or acinar tissue pieces such that (from left to right) n = 11, 13, 5 / 19, 5, 6 / 11, 14, 7 / 22, 5, 6. Samples were taken from three different pancreata. The number zero at day seven for MHC class I expression may represent a lack of detection of individual cells against a strongly staining

parenchymal background or an absence of individual cells. These results show the median number of MHC class I and II positive occasional cells found in single islets and similarly sized acinar cell clumps in the pancreas and at day zero and day seven post isolation. The number of MHC class I positive cells, both in single islets and acinar cell clumps, showed a tendency (non significant by Mann Whitney U test) to decrease in number after isolation and were undetectable after seven days culture. The number of MHC class II antigen positive cells, both in single islets and acinar cell clumps showed a tendency to decrease (non significant by Mann Whitney U test) after isolation but were still present after seven days culture. There was a higher number of MHC class II positive occasional cells in the acinar tissue both in the pancreas (p < 0.005) and immediately after isolation (p < 0.05) and a drop in number after seven days culture (p < 0.01) to be comparable with the number counted in islets at day seven. All other comparisons were non significant (Mann Whitney U test). Thus in summary, the numbers of individual/occasional MHC class I positive cells per single islet and acinar cell clump (mainly endothelial cells and leucocytes) were found to reduce to undetectable levels between day zero and day seven of post isolation culture. The number of individual /occasional MHC class II positive cells were higher in acinar cell clumps compared to islets at each stage (p < 0.05 - p < 0.01) and a trend to reduction of number of individual MHC class II positive cells after isolation and again after seven days culture in islets and acinar tissue, was significantly lower (p < 0.01) only for acinar tissue at day seven.

### 9.6 Effect of treatment of islets and acinar tissue with cytokines TNF- $\alpha$ and IFN- $\gamma$

Immunocytochemical staining of islets and acinar cell clumps which were incubated in medium containing IFN- $\gamma$  (50 - 500 units/ml) and TNF- $\alpha$  (10 -100 units/ml) was concurrent with Expts 42, 43 and 44 and the results of the coculture experiments were given earlier in Chapter Eight. Essentially the purpose of incubating islets and acinar tissue in cytokines was to determine whether MHC class II antigens were upregulated or induced on islets and acinar tissue and whether this made any difference to their immunogenicity *in vitro*. The results showed some induction of MHC class II antigens on islet parenchymal cells (from 0% to 37% of islets) and considerable enhancement on parenchymal acinar cell clumps (from 12% to 100%) but this did not result in an increased proliferative response in the MLIC or MLAC.

The results of the three immunocytochemical staining experiments (on pancreata HP327, HP333 and HP 340, which relate to Expt 42, 43 and 44 respectively) showed the incidence of MHC class II positive individual and parenchymal cells in islets and acinar tissue with or without treatment using TNF- $\alpha$  and IFN- $\gamma$ , and are given in Table 9.3.

**Table 9.3.** Effect of cytokine treatment on the expression of MHC class II antigen on islets and acinar tissue at days three to six

Tissue/Treatment	no. per	Tissue with positive	Tissue with positive
	group	individual cells	parenchymal cells
islets control	4	100%	0%
islets treated	11	100%	37%**
acinar control	48	87%	12%***
acinar treated	57	0%*	100%

\* This number (0%) may represent a lack of detection of individual cells against a strongly staining parenchymal background or an absence of individual cells.

\*\* The nature of the staining pattern seen on HP340 is shown in Figure 9.5 which, although featuring individual cells which are principally lymphocytes and macrophages or dendritic cells, could also represent the early stages of parenchymal expression of MHC class II antigens.

\*\*\*The 12% acinar cell clumps shown in Table 9.3 as having parenchymal staining, represents the total tissue from HP333 and HP340 with a total culture period of six days. The remaining acinar tissue came from a total culture period of three days when no parenchymal stain was seen.

The results shown in Table 9.3 describe MHC antigen expression on islets and acinar tissue at days three to six post isolation, including the cytokine treatment period. These can be compared with Tables 9.1 and 9.2 given earlier, which describe MHC antigen expression on islets and acinar tissue at days zero and seven post isolation. In Table 9.3 the cytokine treated and control islets showed that 100% of islets retained individual MHC class II positive cells up to day six whereas Table 9.1 showed that only 58% of islets retained individual MHC class II positive cells by day seven post isolation. The occurrence of MHC class II positive parenchymal cells in untreated islets was 0% at three to six (Table 9.1) and seven (Table 9.3) days in both series of experiments and this increased to 37% after cytokine treatment. Untreated acinar tissue showed a drop in the number of cell clumps containing individual MHC class II positive cells from 100% at day zero (Table 9.2) to 87% at days three to six (Table 9.3) and to 55% at day seven (Table 9.2), individually stained cells were not distinguishable from strong parenchymal staining after cytokine treatment. The number of untreated

acinar cell clumps with parenchymal stain was 20% at day zero (Table 9.2), 12% at days three to six (0% at day three and 100% at day six - Table 9.3) and 42% at day seven (Table 9.2), this increased to 100% after cytokine treatment. Despite the relatively small numbers involved, the controls for the cytokine study were found to reflect the trends seen earlier.

Examples of the immunocytochemical staining for the three cytokine experiments on islets from pancreata HP327, HP333 and HP340 as given in Table 9.3, are shown in Figures 9.4 and 9.5 below. After six days incubation, the control islet tissue showed the presence of individual MHC class II positive cells only (Figure 9.4 and Table 9.3), whereas at day six the cytokine treated islets showed an indication of early parenchymal MHC class II antigen expression (Figure 9.5 and Table 9.3).

Examples of the immunocytochemical staining for the three cytokine experiments on acinar tissue from pancreata HP327, HP333 and HP340 as given in Table 9.3, are shown in Figures 9.6 and 9.7 below. After three days incubation, the control acinar tissue showed the presence of individual MHC class II positive cells only (Figure 9.6 and Table 9.3), whereas at day three the cytokine treated acinar cell clumps showed parenchymal MHC class II antigen expression (Figure 9.5 and Table 9.3). These results can be compared with the results shown in Table 9.3 and 9.2 that after six and seven days culture, 100% and 42% (respectively) of the acinar cell clumps showed parenchymal MHC class II antigen expression.

Thus in summary, cytokine treatment using a combination of TNF- $\alpha$  and IFN- $\gamma$  caused induction of MHC class II antigens on human parenchymal islet cells (four to six days treatment at 100 units/ml TNF- $\alpha$  and 500 units/ml IFN- $\gamma$ ) and early appearance on parenchymal acinar tissue (three days treatment 10 units/ml TNF- $\alpha$  and 50 units/ml IFN- $\gamma$ ). The untreated controls showed that by day seven post isolation, over 40% of islets had lost individual MHC class II positive cells but this loss was not observed on day three or six. Over 10% of acinar tissue at days three and six, and 45% at day seven showed loss of individual MHC class II positive cells, however, it proved difficult to detect individual MHC antigen positive cells in the presence of generalised parenchymal stain.



Figure 9.4. Expression of MHC class II antigen on islets after six days total culture. Individual MHC class II positive cells can be seen in the islet. Immunocytochemical staining used HB55 (mouse anti human HLA-DR) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x = 160.



Figure 9.5. Expression of MHC class II antigen on islets after six days culture including four to six days treatment with TNF- $\alpha$  (100 units/ml) and IFN- $\gamma$  (500 units/ml). Although the MHC class II positive cells appear individual, the pattern resembles an early stage of parenchymal islet cell expression. Immunocytochemical staining used HB55 (mouse anti human HLA-DR) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 160.



**Figure 9.6.** Expression of MHC class II antigen on acinar tissue after three days culture. Individual MHC class II positive cells can be seen in the acinar cell clump. Immunocytochemical staining used HB55 (mouse anti human HLA-DR) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 320.



**Figure 9.7.** Expression of MHC class II antigen on acinar tissue after three days culture including two days treatment with TNF- $\alpha$  (10 units/ml) and IFN- $\gamma$  (50 units/ml). Parenchymal MHC class II positive cells can be seen throughout the acinar cell clump. Immunocytochemical staining used HB55 (mouse anti human HLA-DR) in a 3-layer method with alkaline phosphatase and Fast Red colour development. Magnification = x 320.

### 9.7 Identification of potentially immunogenic cell types in pancreatic digest

Samples of digested pancreas from ten different donors, one sample per donor, (HP255, HP257, HP258, HP260, HP 261, HP 263, HP264, HP266, HP267, HP270) were sectioned and stained with a panel of antibodies to identify the different cell types found in the pancreas. The essential aim of this immunocytochemical study was to investigate whether small lymph nodes of the type described by Gotoh(139), which have been shown to decrease the survival of transplanted islets, were present in human pancreatic digest. The range and proportions of cell types studied here included islets, acini, endothelial cells, leucocytes and ducts, all of which are potential immunogenic contaminants of isolated islets.

Of the ten pancreatic digests studied by immunocytochemical staining, four showed the digest to principally contain dispersed single cells, the other six contained cellular groups with some intact morphology. The results showed that no lymph nodes or clumps of leucocytes were seen in the 10 pancreatic digest samples, four samples contained very few leucocytes and one sample contained a large number of single cells expressing CD45RO (common leucocyte antigen) but no evidence of lymph node formation. Endothelial cells were present in all samples and ductal cells (as single cells or small clumps of cells) in six samples.

This result was in addition to a search by eye and using a dissecting microscope for lymph nodes in and around the human pancreas. This search revealed large, easily identifiable lymph nodes in the fat adjacent to the head of the pancreas around the splenic artery and vein. A considerable number of lymph nodes 2 - 5 mm diameter were found, none in the 200  $\mu$ m range, and all were located in the fatty tissue close to, but not directly attached to, the blood vessels. No lymph nodes were visible around the inferior mesenteric artery and vein. All the fatty tissue was normally removed before processing each pancreas.

Thus in summary, no evidence of lymph nodes were found in 10 samples of pancreatic digest although the number of leucocytes detected varied between different pancreata. Four of the ten samples were comprised

mostly of dispersed cells and all samples included ductal and endothelial cells.

# 9.8 Overall summary of the distribution of MHC class I and class II antigens in human pancreas and islets and acinar tissue post isolation and culture

MHC class I and II antigen expression has been found with limited distribution in the pancreas and potentially could affect the immune response to islets following transplantation. For these studies, immunocytochemical staining techniques were used to identify MHC antigen expression on human islets and acinar tissue post isolation and culture. After some preliminary investigations, serial sections with a limited panel of primary antibodies was chosen and a method using a 3-layer biotin avidin method colour developed with alkaline phosphatase and Fast red. HB124 (anti insulin) and LDS8 (anti acinar cell) antibodies were used to identify islets and acinar tissue, and W6/32 (anti HLA-A, B, C) and HB55 (anti HLA-DR) were used to identify MHC class I and II expression respectively.

Human islets and acinar tissue were found to express MHC class I antigens on individual cells in the pancreas and on parenchymal cells (weakly) in the islets. The pattern of staining found in the pancreas was also seen at day zero, however, after seven days culture individual MHC class I cells were undetectable against a background of generalised parenchymal stain. MHC class II antigen expression was found to be different on islets and acinar tissue. Individual MHC class II positive cell were seen in 100% of islets at day zero but only in 58% of islets after seven days culture. No parenchymal MHC class II expression was seen post isolation or within seven days culture. Acinar tissue showed a similar reduction in the number of tissue pieces containing individual MHC class II positive cells (from 100% to 55%) and a corresponding rise in number of cell clumps with parenchymal stain (42%) after seven days in culture. The number of individual cells per islet or acinar cell clump followed a similar trend resulting in no detection of individual MHC class I positive cells by day seven and a reduction, but not to zero, of individual MHC class II positive cells by day seven. Treatment of islets and acinar cell clumps with TNF- $\alpha$ 

and IFN- $\gamma$  resulted in early expression of parenchymal MHC class II antigen on acinar cell clumps (day three vs day six to seven) and the early stages of parenchymal expression on islets by day six post isolation. No evidence of small lymph nodes in the human pancreatic digest was seen.

#### The Next Chapter...

Having completed the description of the experimental studies, Chapter Ten contains the discussion of all the experimental results.

## CHAPTER TEN

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### CHAPTER TEN

#### DISCUSSION

#### 10.1 Introduction

Exogenous insulin treatment for Type I diabetes started in 1923, following the work of Banting and Best, and has been particularly successful in reducing mortality by allowing control of the levels of glucose in the blood so that with careful attention to diet, patients can lead a relatively normal life. However, the increased life expectancy of Type I diabetic patients has led to the appearance of severe complications such as macro and microvascular disease, blindness, renal failure and neuropathy(8). While a considerable research effort has been directed towards early diagnosis and potential prevention of this autoimmune disease, approximately 45% of diabetic patients develop nephropathy with a peak incidence sixteen years after diagnosis, and death from renal failure or associated microvascular disease occurs in about half of these patients. In addition to the medical treatment required by diabetes management, this has placed a burden on surgical programmes, particularly renal transplantation (25% of the patients entering dialysis programmes in the USA are diabetic)(10).

The success of renal transplantation has led to investigations of other potentially transplantable organs and tissues which, in the pursuit of islet transplantation, has brought together the surgical, diabetological and immunological fields of research. As a treatment for Type I diabetes, islet replacement has many advantages over pancreas transplantation, including the potential for treatment soon after onset in childhood which could avoid the development of complications following long-term hyperglycaemia(1).

Neither pancreas or isolated islet transplantation has proved easy. Pancreas transplantation involves major surgery and long term maintenance immunosuppression and so is unsuitable for young diabetic patients prior to the development of complications. In addition, well matched transplants between siblings has shown that recurrence of disease(5) can also occur and so poses an additional problem to rejection.

Islet transplantation, while holding the greatest promise for treatment of Type I diabetic patients, has proved difficult, not in the technique of transplantation itself, but in the methods of islet isolation from the human pancreas and the maintenance of islet function after transplantation(3).

While the problems of isolation continue to be addressed by various groups including that at Leicester(3) (113) (114), the problems of post transplantation immunogenicity of human islets are largely unknown. As islet allografts with immunomodulation or recipient immunosuppression have survived in rodent models(121) (136) (292), the relatively low survival rate of human islet transplants in Type I diabetic patients(111) has been unexpected and there is a need for studies specifically of human islet immunogenicity. Two such models of human islet immunogenicity are potentially available. One is currently being developed as an *in vivo* model and involves the transplantation of human islets into an immune deficient mouse reconstituted with a human immune system (SCID-hu) (293). This model has yet to be truly resolved as low frequency and incomplete human T cell reconstitution has been recorded(185). The other model is an in vitro coculture assay (MLIC) in which the proliferative T helper cell response of lymphocytes, stimulated by allogeneic islets, is measured(263). The MLIC, based on the MLR for which a substantial body of data exists, particularly in connection with MHC class II antigen matching for renal transplantation(222) (225), has been described by a number of authors (see Tables 3.1 and 3.18).

Thus in summary, hypoglycaemia of Type I diabetes has been largely controlled by the administration of exogenous insulin, however, there can be severe complications in later life including renal damage and microvascular disease. Tight control of blood glucose levels, which has been shown to lessen the risk of complications, could be achieved by replacement of the patient's islets destroyed by autoimmunity. One technique is to transplant human islets isolated from cadaver donor pancreata but the outcome of human islet allotransplantation, although promising, has encountered difficulties including apparent rejection of grafts.

#### 10.2 The human allogeneic MLIC

The aim of this thesis was to develop the human MLIC as a reproducible *in vitro* model for determining the potential immunogenicity of human pancreatic isolated islets. Two applications were of immediate interest. Firstly, to determine whether the human MLIC could be used as a pretransplant model for potential prediction of beneficial donor recipient matches and secondly, as a model to measure the effects of islet pretreatment on immunogenicity.

This work involved five main areas of experimental study which are described in Chapters Five to Nine. Chapter Five describes preliminary studies on the human MLIC and investigates whether the MLIC would be suitable as a pretransplant assay that could be used to predict the outcome of clinical islet transplantation. Chapter Six describes several series of experiments in which the basic parameters and conditions of the MLIC and MLAC (using islets and acinar tissue as the stimulator populations respectively) are identified. Chapter Seven describes the core of the work to standardise the MLIC using a titration of the stimulator population number and the kinetics of the response by untreated lymphocytes to the stimulator populations. Chapter Eight describes the experiments which investigated the possible reasons behind the observed low response of allogeneic lymphocytes to acinar tissue in the light of contrary published reports(269) (273). Chapter Nine describes the immunocytochemical studies using frozen sections of islet and acinar tissue before and after isolation and after culture to identify the expression of some of the antigens potentially involved in the immune response.

#### 10.2.1 Studies described elsewhere related to the MLIC

The MLR, which was first described in 1964(214), has been the means for the determination of allogeneic (HLA-DR) disparity for many years(220). Additionally, the importance of HLA-DP and DQ as well as the HLA-DR disparity in the MLR, have been identified using HLA-D specific hybridomas(229). Although a low MLR result relates to increased renal allograft survival(222), the limited time between cadaveric organ retrieval and identification of a suitable recipient, has led to the use of HLA matching by tissue typing peripheral blood to chose beneficial donor

recipient combinations, a technique which takes a few hours to perform rather than the five to six days of the MLR(225).

The introduction of the MLIC in animal models, first described using dogs by Rabinovitch in 1981, was to determine whether additional tissue specific antigens existed on islets that would provoke a heightened MLIC response compared with the MLR, and help explain the limited survival of allogeneic islet grafts in some species(263). The presence of islet antigens in the autologous MLIC was seen (3,000 cpm autologous MLIC vs 6,000 cpm allogeneic MLIC vs <1,000 cpm autologous MLR), however, the allogeneic MLIC was lower than the allogeneic MLR (12,000 cpm allogeneic MLR) (results were given as one example of five pairs of dogs responding similarly). So, these results showed that islets do have immunogenic specific antigens causing autologous stimulation but, far from islets being more immunogenic than lymphocytes as was suspected in vivo, the converse was found to be true in vitro, at least for allogeneic dog islets. This has called into question the relevance of the MLIC to islet allograft outcome, which in the rat, appears unrelated(38). However, the relationship between the MLR, MHC class II antigen disparity and renal allograft outcome has been well established(195) (225).

Using human islets in the MLIC was intended to determine whether specific differences exist between the species and to develop a model to explore the *in vitro* immunogenicity of putative human islet antigens in allogeneic combinations(255), which then may allow the development of immunomodulation protocols(267) (269). Some reports have indicated that impure islet preparations are more immunogenic than pure islets(269) (273), which has fuelled the debate about the immunogenicity and role of exocrine tissue in rejection(133). The limited number of reports and the lack of a reproducible human MLIC which could be linked to clinical transplantation, formed a starting point for the work described in this thesis.

The basis of the MLIC is a proliferation of lymphocytes to an allogeneic or autologous stimulus, which is measured using the incorporation of tritiated thymidine into DNA by the proliferating lymphocytes. The subsequent beta emission of the radio-labelled DNA, measured as cpm, relates to the increase in DNA synthesis. This technique has been widely used for the

determination of cell proliferation but several factors may affect the relative incorporation of [<sup>3</sup>H]thymidine to misrepresent the true proliferative rate. These include the thymidine pool size in the cells and the cell density. The effects of cell density have been observed using lymphocytes, which at 2 x  $10^6$  cells per ml, in addition to a high rate of cell death, have shown blast transformation and [<sup>3</sup>H]thymidine incorporation but no increase of cell number or DNA content but at  $1 - 5 \times 10^5$  cells per ml divided readily and were found to have a low rate of cell death. Therefore the need to use good controls, a relatively low number of responder cells and kinetic studies have been recommended(294).

MLR assays are normally carried out in tissue culture treated plastic microwells which promote adhesion and cell to cell contact of the two lymphocyte populations. However, islets are usually maintained in non tissue culture plastic which avoids adhesion and spreading of the islets (282). Of the published MLIC reports, relatively few mention islet adhesion or fibroblast outgrowth from islets except in the case of insulinoma cell cultures (insulinoma cells can be separated from fibroblasts prior to culture)(267) and at least two groups have used tissue culture plates for the MLIC(158) (264). The majority of the published MLIC assays have been completed within five to six days (see Tables 3.1 - 3.4) and perhaps would be less affected by fibroblast outgrowth than the nine day assay used later in this work (see Chapter Seven).

In addition, the stimulator population of the MLR is normally  $\gamma$ -irradiated (2 - 3,000 rads) to prevent 'back proliferation' of the stimulator cells in response to the allogeneic antigens of the responder cells(237) (246), and this practice has been followed by some of those using the MLIC(153) (255) (268). In the rodent pancreas, adult islets have been found not to increase in number but appear to maintain a balance between a low rate of beta cell proliferation and cell death(295) (296). No mitoses have been found in the beta cells of adult human pancreas(297). Rabinovitch, who did not pretreat his stimulator islets with  $\gamma$ -irradiation, found that 100 dog islets contained no more than 4,000 lymphocytes (2% of number of cells in each islet) and that this number was insufficient to stimulate a primary proliferative response. The exact identity of passenger leucocytes in human islets has not been resolved but has been found to include small round leucocytes as well as cells with processes which could be dendritic cells, macrophages or

both. Steinman has found dendritic cells to be 50 - 100 times more potent in the MLR than B lymphocytes and  $1 \times 10^3$  and  $1 \times 10^4$  dendritic cells can stimulate an allogeneic proliferation equivalent to approximately 10,000 cpm and 100,000 cpm respectively(235). Passenger leucocytes are discussed in more detail, in connection with the immunocytochemical studies, later in this chapter.

Thus in summary, the starting point for studies on the MLIC is the mixed lymphocyte reaction or MLR, which has been used clinically to predict and monitor HLA-D disparity between potential donor recipient pairs. MLIC studies were first reported in 1981 using canine cells, as a means of investigating the role of islet specific antigens, and have continued with studies using rat and human allogeneic combinations. However, a reliable and reproducible MLIC using fresh human islets with the potential for clinical use has been lacking. In published studies, measurement of responder lymphocyte proliferation by [<sup>3</sup>H]thymidine incorporation has been most effectively used with 1- 5 x 10<sup>5</sup> cells per well. For the stimulator population, few reports have mentioned fibroblast contamination of islets in the coculture and in studies which have not irradiated the islets, the contribution of the passenger leucocytes to overall proliferation in the MLIC has been considered to be negligible.

#### 10.2.2 Preliminary studies using the MLIC and MLAC

In the preliminary series of experiments for this thesis, the need to use non tissue culture plastic microwells for the MLIC to avoid fibroblast outgrowth was identified. This was because the use of viable (non  $\gamma$ irradiated) islets as the stimulator population was preferred, to match the potential conditions *in vivo* and to make the projected immunomodulation studies using irradiation protocols, more meaningful. Fibroblast outgrowth from viable islets and collagen secretion, which cause adhesion of islets to plastic could make islet cell surfaces less accessible and could cause lymphocyte trapping, both resulting in low recovery of [<sup>3</sup>H]thymidine incorporated DNA.

Human fibroblasts have been found to inhibit production (but not cell content) of insulin by HIT-T15 cells(298) and are known to secrete IL-1 which adversely affects islet function(77) (78). Insulin secretion has been

found to enhance lymphocyte proliferation at low levels (0.1  $\mu$ g/ml similar to the levels measured in the canine MLIC), to have no effect at 1  $\mu$ g/ml and to inhibit lymphocyte proliferation at higher levels (10  $\mu$ g/ml)(263). The insulin levels found here varied between 0.17 - 0.18  $\mu$ g/ml in one experiment (Expt 37, day seven and nine respectively) and 0.7 - 0.9  $\mu$ g/ml in the other experiment (Expt 40, day seven and nine respectively) so would be expected to be either stimulatory or to have no effect. It is interesting that ten human islets in 0.2 mls RPMI medium from two different pancreata produced two different accumulative insulin levels and that the lower of these (0.17  $\mu$ g/ml) was comparable to the levels produced by 100 dog islets in 0.2 mls RPMI medium (71  $\pm$  8 ng/ml - 100  $\pm$  24 ng/ml at day seven and nine, n = 3 - 4)(263).

The reasons for the choice of six days as the endpoint for the MLR was illustrated by Kuntz who showed that although the allogeneic MLR increased beyond six days, the autologous MLR also increased and the stimulation index showed the greatest difference between the allogeneic and autologous response at six days duration(299). Lymphocyte proliferation in the MLR was found to be principally due to an expansion of the T cell population in response to the non T cell population(299). More recently, the responding population was described as principally a CD4+ve T helper cell population with an augmentation of proliferation by CD8+ve T cells(233).

In models of parasitic infection, proliferation of the Th1 cell subset was shown to be driven by IFN- $\gamma$  originating from NK cells, which may have been a response to IL-12 produced by macrophages(300). The presence of IL-2 and IFN- $\gamma$  in syngeneic islet grafts transplanted into NOD mice, was shown to correlate with an increased rate of rejection(178). The production of IL-2 in the MLR by the T cells clustered around dendritic cells and subsequently proliferating(230), has been exploited to shorten the duration of the MLR from a five to six day assay by the detection of an earlier peak of IL-2 production(247). For these studies, the use of a shortened MLIC assay was considered essential for the model to have any potential in determining pretransplant islet immunogenicity-in particular donor recipient combinations. The results described in Chapter Five showed that an allogeneic lymphocyte response could be stimulated by 40 untreated human islets in a six day MLIC but considerable variation within and between results was seen (Table 5.1). One possible source of variation was caused by the presence of fibroblasts in the wells which may have contributed to the relatively low cpm of the lymphocyte and islet coculture and high background cpm of the islet controls. This was later avoided by using non tissue culture wells for the cocultures, however, limited evidence for increased test cpm and decreased islet control cpm in the absence of fibroblast outgrowth was seen (Table 5.2 and 5.3). Microscopically, the islets were virtually fibroblast free and all cellular material was harvested from the wells. An attempt to develop the the three day MLIC was of immediate importance at the time because of the implementation of the human islet transplant programme.

A comparison of the MLIC at day three (Table 5.5) and the CTLL proliferation of the wells at day two and day three showed that although high IL-2 levels were recorded at day two (Expt 7) or day three (Expts 8 and 9) (Table 5.6), high levels of proliferation were also recorded in the controls. This was thought to be due to the condition of the CTLL cells prior to use which were insufficiently IL-2 depleted. The remaining results, although showing a slight increase over the controls, did not show a significant enhanced or early IL-2 production in the lymphocyte islet coculture wells. These results are similar to the findings of Bishara using the mouse MLR(247) which show IL-2 results (cpm CTLL proliferation) at day two similar to the MLR at day three (Acpm lymphocyte proliferation). However, the results of the human MLIC presented here do not appear to be in agreement with Bishara's human MLR results which show an enhancement of IL-2 production at day three, but not day two, compared to the results of the six day MLR. This could have been because the level of stimulation in the human MLIC was below the sensitivity of the IL-2 assay, so standard curves for CTLL proliferation were set up. The results of the standard curves showed a good correlation between CTLL proliferation and IL-2 concentration but only between 0.2 and 1 unit per ml IL-2, corresponding to a CTLL cell proliferation of 4,000-11,000 cpm (Figure 5.2a). These levels were higher than those obtained in the human MLIC and so would appear that the MLIC results were below the sensitivity limit of the assay.

In an attempt to increase the proliferative response to allogeneic human islets, alterations to the nature of the stimulating antigen were made and included  $\gamma$ -irradiation of the islets prior to coculture and disrupting the islets to form antigen which might be more accessible to the lymphocytes. Neither treatment was found to enhance the allogeneic lymphocyte response to human islets (Tables 5.7 and 5.8).

The number of responder cells used in MLIC assays has been  $1 - 5 \times 10^5$  per well (Table 3.1-3.4), encouraged by the need to avoid artefacts caused by ambiguity of DNA [<sup>3</sup>H]thymidine incorporation(294). A titration of the responder PBL number in the MLIC as well as the Con A stimulated PBL and PBL alone controls, showed  $1 \times 10^5$  PBLs per well to be optimum in the range  $1 \times 10^3 - 1 \times 10^5$  PBLs per well.

Thus in summary, the work of Chapter Five showed that an allogeneic lymphocyte response could be generated in the human MLIC using non tissue culture plates which allowed the use of viable islets without adhesion caused by fibroblast outgrowth. The human allogeneic MLIC response was found to be variable and low, and even after potential enhancement gained from measuring IL-2 levels in the cocultures, did not give meaningful results at day three.

#### 10.2.3 Establishing parameters for the MLIC and MLAC

Following the decision not to pursue further the use of the human MLIC as a pretransplant model, the next stage of the work was to explore some of the parameters of the assay including titration of the stimulator population and the kinetics of the cocultures, as described in Chapter Six. Using pieces of digest tissue as the stimulator cell population in cocultures, led to the identification of a need to compare the relative stimulatory capacity of the islet and acinar cell populations. In addition, the increased duration of the assay led to the need to reduce the levels of proliferation in the PBL controls.

As described above, there is some evidence that the non islet cellular material that contaminates isolated islet preparations, is immunogenic. Evidence *in vivo* has come from an increased rate of rejection for impure

islet grafts which may well have been a combination of inflammatory and immunogenic reactions(133) and from MLIC studies(269) (273). The results shown in Figures 6.1 and 6.2 indicated that digested pancreatic tissue, which only contains about 2% islet cells, was considerably less immunogenic than islets, were therefore unexpected. The original concept of the development of the human MLIC was to use tissue pieces from the islet preparation to represent its heterogenous nature and thus represent the material potentially used for human islet transplantation. However, the apparently different levels of response to islets and pancreatic digest meant that it was important to study the islet and acinar cell populations separately. This phenomenon was explored further and as it forms a major part of the work in Chapters Seven and Eight of these studies, is discussed later.

The remaining part of Chapter Six describes an investigation of background PBL proliferation. In the MLR, this proliferation has been found to be principally an autologous response to 'self' antigen as the stimulation of T helper cells by B lymphocytes or dendritic cells from the same source(286) (299). In the same way that the original intention was to use human islets in the condition potentially used for transplantation, the use of a mixed PBL population as a responder population representative of the cells potentially encountered by the allogeneic islet graft *in vivo*, was intended. The findings of studies on the autologous MLR suggest that little can be done to reduce the PBL control value and question the validity of an autologous PBL control for an allogeneic response. This latter question is discussed in connection with data from Chapter Seven.

However, autologous serum as a growth supplement in medium has been found to increase the proliferative response compared to pooled human AB serum, in the autologous MLR(299). Because the serum supplement can affect proliferation, the effects of using different sera in the medium were tested. The results shown in Table 6.5a - 6.5d, confirmed the findings of Kuntz, that the autologous response (PBL control) was higher in the presence of autologous serum(299). In addition, although both FCS and human ABS supported lower proliferative responses to Con A stimulation, the background PBL proliferation was lowest using human AB serum. Additional testing to select the most suitable batch of human AB serum was

carried out before using it as a growth supplement in the islet and lymphocyte cocultures.

Thus in summary, the negligible response of allogeneic lymphocytes to pancreatic digest tissue was unexpected and prompted further studies separating the islet and acinar stimulator cell types in the cocultures, which are discussed later. The background autologous proliferation of PBLs seen in the experiments described here, was reduced by using batch tested human AB serum. The validity of the PBL control for the allogeneic MLIC is discussed later in connection with the means of expression used for the results.

#### 10.2.4 Standardisation of the human allogeneic MLIC and MLAC

#### 10.2.4a Published studies on the MLIC

The first report of a human MLIC came from Roth in 1984(255), was followed in 1988 by Demidem(267) and then by Ulrichs(29). None of the reports have adequately described full titration and kinetics of the human MLIC using fresh untreated islets as the stimulator population. Ulrichs' studies give some idea of the titration using 5 - 50 mitomycin C treated, frozen-thawed human islets in a six day coculture with a peak response to 40 islets(269). Other clues to the titration and kinetics of the MLIC have come from animal studies. Shizuru, using a titration of 2 - 100 rat islets in a five day coculture, showed a peak allogeneic MLIC response of 100 islets. In addition, the increase in islet number was found to be related to a shorter duration of the response, so that the peak response to 100 islets was found to be at five days, the peak response to 50 islets at six days and the peak response to 2 and 20 islets continuing at day seven (mean of three. experiments each in triplicate). Two experiments showed a straight line correlation between stimulator islet number and [<sup>3</sup>H]thymidine incorporation in the MLIC(264). The titration of allogeneic dog islets in the MLIC by Rabinovitch has also shown a peak response to 100 islets (Rabinovitch 1981). Only Rabinovitch (dog) and Roth (human), who are from the same group(255) (263), and Shizuru (rat)(264) have used kinetic studies, which were 3, 5, 7 and 9 days, to help determine optimum responses. Although Roth used human tissue, this was described as islet enriched pancreas fractions and would be expected to have included considerable amounts of acinar tissue. Ulrichs and Zeevi have described the use of

human islet preparations of different purity showing that increasingly pure islet preparations have a decreased ability to stimulate allogeneic lymphocytes in the MLIC(269) (273). None of the authors using the MLIC describe the same assay using pure acinar tissue as the stimulator population, the MLAC.

There are few published reports(248) (255) (256) (276), which compare the capacities of different stimulator populations *in vitro*, and examples of the results of using these stimulator populations used are shown in Chapter Three, Tables 3.9 and 3.18. Using human or porcine cells, the allogeneic and autologous lymphoproliferative response in the MLKC (kidney cortical cells) has been found to be less than the MLR (leucocytes) although the canine allogeneic MLKC has been found to be greater than the equivalent MLR. The human MLIC (islets) and the porcine MLLC (liver cells) and human MLEC (endothelial cells) have all resulted in proliferative responses but these have been found to be less than the equivalent MLKC. Porcine fibroblasts, human dermal fibroblasts, smooth muscle cells and epidermal cells have all been found to result in minimal or no stimulation.

Ambiguity exists in the presentation of data for experimental MLR and coculture results. Some authors have expressed the results of mixed lymphocyte reaction or coculture as test cpm(229) (248) (264), test cpm with separate control cpm (255) (256) (263) (269),  $\Delta$ cpm (247) (249) or SI (248) or both (267) (268). Results which are used for clinical MLR assays have been expressed as the relative response (RR = test cpm minus autologous control cpm both divided by reference cpm minus reference autologous cpm)(301). In addition, some authors have illustrated coculture results as one or more 'representative results' from a series of experiments(248) (255) (263) (269).

Thus in summary, others have described the MLIC using islet tissue although none has used fresh untreated human islets for titration and kinetic studies. One interesting study in rats has shown that a peak response can be generated to 2 - 20 islets in seven days but only requires five days if 100 islets are used. Coculture experiments using non lymphoid and non islet cells as the stimulator population, have been described and emphasise the contribution of tissue specific antigens. Several different methods of expressing results have been used by other authors.

#### 10.2.4b Optimisation, titration and kinetics

In the studies described in Chapter Seven of this thesis, 40 islets per well (as Ulrichs' optimal number) were initially chosen for the stimulator population of the MLIC and the first titrations included 40 islets. The separation of the stimulator population into islets and acinar tissue (for the MLIC and MLAC respectively), involved the positive and negative identification of the two types of tissue. This was carried out using dithizone at a concentration that has been found not to impair islet function(280) and has been used in the MLIC by Ulrichs(29).

The results of the titration and kinetics of the human MLIC as described here, include an optimum allogeneic lymphocyte response ( $\Delta$ cpm) stimulated by ten islets after nine days of coculture (Figures 7.7a and 7.7b). The islet number was less than that described by others and may reflect the species difference as well as the size and quality of islets chosen (100 - 200 µm diameter). It is interesting to note that *in vivo*, rat isolated islet allografts have shown a large increase in fibrosis and visible destruction of the graft about 8 days post transplantation(132) suggesting some correlation with the peak *in vitro* T cell responses.

Under these conditions the MLR has also shown a peak at day nine (Figure 7.8a) which is later than that described for MLR by others(215) (299) and may be related to the nature of the microwells which, for these studies, were not tissue culture treated. The MLR stimulator population in these experiments was  $\gamma$ -irradiated following the practice of other authors, as described above, to prevent 'back proliferation'. Although non proliferating adult human islets were used as the stimulator population in these MLIC studies, the presence of passenger cells in islets(150) was a potential source of proliferating cells. The contribution of adult human islets to the overall level of [<sup>3</sup>H]thymidine incorporation was measured by having control 'islets alone' wells and including the data in the calculation of results. In addition, the results of experiments shown in Figure 7.6, indicated that there was no effective proliferation of cells from untreated islets against a  $\gamma$ -irradiated non proliferating allogeneic PBL population.

Thus in summary, the titration and kinetic studies of the MLIC and MLAC, using the parameters identified in the preliminary studies, was ten

fresh, dithizone stained but otherwise untreated, handpicked islets or ten acinar tissue pieces cocultured with  $1 \times 10^5$  allogeneic PBLs over a nine day period. Although the kinetics were similar to those described by others, the optimum number of stimulator islets was considerably less than that described by others.

#### 10.2.4c Statistical analysis of combined results

A trend in each series of experiments which indicated differences in the stimulatory capacities of allogeneic splenocytes, islets and acinar tissue in the cocultures, was not seen to be statistically significant until the relevant data was combined from all the series. The combined results (Figure 7.9) showed that the proliferative response to ten acinar tissue pieces in the MLAC was less than the response to ten islets in the MLIC and the lymphoproliferative response in the MLIC was less than the response to  $1 \times 10^5$  allogeneic lymphocytes in the MLR. The relationship between the MLIC and MLR was in broad agreement with data published by Rabinovitch in which the dog allogeneic response in the MLIC was lower than the response in MLR(263). The stimulatory capacity of human acinar tissue alone in the MLAC, which has not been reported elsewhere, was tested and compared with that of islets and splenocytes from the same source. The titration of acinar tissue in the MLAC did not lead to an increase in the proliferative response generated (Figure 7.7a and 7.7b) and there was a continuing minimal response up to day thirteen without any clear peak (Figure 7.8c).

The inherent variability of the results of mixed lymphocyte cultures has been shown in these experiments. The three different types of stimulator populations which constitute the MLR, MLIC and MLAC, have all shown wide variability in the results both as inter sample variation per experiment and as inter experiment variation (Figure 7.10a - 7.10c and Table 7.1). These two levels of variability suggest that both the responder and stimulator populations contribute to this phenomenon.

Variation in the capacity of cells from different donors to stimulate allogeneic lymphocytes could be due to events such as the initiation of autolysis in the pancreas due to warm and cold ischaemia after retrieval as well as the trauma of pancreas digestion and islet isolation which can enhance MHC antigen expression(3) (284). The variable quality of islets and acinar tissue produced may have been partially off-set in these standardisation experiments by careful handpicking of the islets and acinar tissue before use.

An additional source of variation was the selection of completely HLA mismatched responder PBLs, which was based on serological typing, as supplied by the Trent Regional Transfusion Centre. Improved HLA matching can be obtained for the HIA-D sites using oligotyping(225) and has replaced the MLC for matching donors and recipients for bone marrow transplantation in some centres(302). Another possible source of variation stemming from the responder population, is that wide differences have been found in the number of precursor Th lymphocytes (pThL) between individuals to HLA antigens(208) (213). This would effectively alter the percentage of reactive T helper cells within the initial responder population of  $1 \times 10^5$  PBLs per well and correspondingly affect the level of allogeneic proliferative response. Variability in Con A stimulated PBL responses from a single group of men under controlled conditions which have included both the PBL donors and the technical methodology, has suggested that a minimum of four carefully controlled repeat tests are necessary for reliable results(303) .

Data was pooled in order to illustrate the relative difference in background proliferation between PBLs cultured in RPMI containing 10% FCS or 10% AB human serum. The result is given in Figure 7.11 and shows that the background or autologous response was reduced using human AB serum and so justified its selection as a growth supplement for later experiments.

The contribution of the autologous response to the level of the allogeneic response, was examined by pooling results from all relevant experiments. Significant correlation between the PBL control and either the response to islets (MLIC test cpm minus islet control) or to acinar tissue (MLAC test cpm minus acinar control), is shown in Figures 7.12a - 7.12c suggesting that the allogeneic lymphoproliferative response is not independent of the autologous response.

The question of the most appropriate method of expressing the data of lymphoproliferation assays (mitogen stimulated), as raw data (test cpm),  $\Delta$ cpm (control values subtracted from test values) or as S.I. (test values

divided by control values), has also been addressed(304). Statistical analyses of six sets of published data, have shown that the test cpm (raw data) and the  $\Delta$ cpm lead to similar conclusions, but that use of S.I. values can lead to discrepancies in >20% of cases and the authors recommend that the S.I. is not used.

To investigate the appropriate use of either the  $\Delta$ cpm or S.I. values for these studies, correlation were made between groups of data. The correlation between results ( $\Delta$ cpm) of the MLR, MLIC and MLAC were plotted as shown in Figures 7.13a - 7.13c. None of these comparisons were statistically significant emphasising the variability between experiments and the individual nature of the response generated to each of the three stimulator cell types.

This lack of correlation prompted an additional analysis of the experimental results. This compared the  $\Delta$ cpm calculation with the stimulation index (S.I.) for which the test cpm was divided by the sum of the responder and stimulator control cpm. The advantage of the stimulation index is that it avoids negative results in instances where the test cpm did not exceed the control cpm. A comparison of the results using the two types of calculation is shown in Figures 7.14a and 7.14b (MLR), 7.15a and 7.15b (MLIC) 7.16a and 7.16b (MLAC). The two calculations ( $\Delta$ cpm and S.I.) gave a different profile for the results and only the values close to the baseline, were identified by both calculations. The results of Expt 14 given both as cpm with a separate responder control, as  $\Delta$ cpm and as S.I. (described at the end of Chapter Six), illustrate apparently different interpretations for the same data.

The routine clinical MLR assays appear to have solved the problem of expression of results by using the relative response (RR) which compares the test result to a reference result. However, using the MLR of each experiment as the reference result was not helpful in these studies as shown by the graphs in Figures 7.13a - 13c and Figures 7.17a-17c, in which neither the MLIC or the MLAC show any correlation with the MLR. The only significant correlation was found to be between the MLIC and MLAC but only using the S.I. values. This suggests that, in these experiments, the response to islets and acinar tissue was not entirely related to the

allogeneic response to PBLs, and may have reflected a partial tissue or antigen specific response.

Despite the variation within and between experiments as well as the ambiguous interpretation of data by different calculations, pooling data from multiple experiments showed that there was a difference in the allogeneic lymphocytic response to PBLs, islets and acinar tissue which was statistically significant, this is shown in Chapter Seven, Table 7.2b.

Thus in summary, the work described in Chapter Seven was aimed at standardisation of the MLIC and MLAC. This proved to be difficult and a considerable amount of data was accumulated which illustrated the wide variation at all stages of the work. However, pooling results did lead to the identification of the number of islets and acinar tissue pieces necessary to stimulate an optimal proliferation (ten islets or acinar cell pieces per well, average 150  $\mu$ m diameter) with an optimal duration of nine days. Combined data of the peak responses for each experiment showed that the allogeneic lymphoproliferative response in the MLIC was significantly reduced compared to the MLR and the response in the MLAC was statistically significantly reduced compared to the MLIC.

#### 10.2.5 Human islet transplantation and the MLIC

One of the problems of human islet transplantation has been an inability to obtain sufficient numbers of islets from a single donor pancreas to transplant one recipient. Multiple donor grafts have had considerable success(2), although the overall graft survival rate has been low. The experience at Leicester was that graft failure in two patients with multiple donor grafts coincided with the rise of the T cell count, however, in a third patient, a well matched graft from a single donor survived(3). This led to the idea that rejection could play a major part in loss of islet function following transplantation.

The MLIC is essentially a model for assessing the primary T helper cell response, which is MHC class II restricted, against islets. If rejection has a part to play in the immune response to islets then the MLIC response would be expected to be higher for HLA mismatched combinations and lower for HLA matched combinations. One such experiment was carried out during the course of this work using islets and acinar tissue from an isolated islet

preparation subsequently used for transplantation. Although this was a single experiment, the results were encouraging in that the PBLs from two HLA matched (but different) sources both showed a low lymphoproliferative response to the donor (stimulator) islets whereas the PBLs from a HLA mismatched source showed a high level of response to the donor islets. The low response of the recipient lymphocytes to the donor islets (four HLA matched) coincided with survival of islet graft function in the patient for over two years. The results calculated as a stimulation index showed less of a difference than the  $\Delta$ cpm results but there is little evidence that stimulation index has more relevance to transplant outcome than  $\Delta$ cpm. Therefore, it is not unreasonable to suggest that there may be some relationship between a low MLIC response and a favourable outcome of islet transplantation.

#### 10.2.6 Determination of acinar tissue immunogenicity in vitro

#### 10.2.6a Published studies on acinar cell immunogenicity

In Chapter Eight, the question of the reduced allogeneic lymphoproliferative response to acinar tissue compared with that to islets, was addressed. Some authors have investigated the contribution of acinar tissue, as a major contaminant of islet preparations and found a) that contaminating lymph nodes, rather than acinar tissue, can almost entirely account for the reduced graft survival time of impure islet preparations in the mouse(21) and b) that exocrine tissue has a local effect *in vivo*, inducing inflammation, functional islet loss(21) and impairment of implantation(134). However, Hegre has found increased histological evidence of rejection with non islet pancreatic components compared to islets in mice(138). In addition, others have suggested that the non endocrine cellular contamination of impure isolated islet preparations contributes substantially towards *in vitro* immunogenicity(269) (273). The results from these studies did not support the published *in vitro* findings and the reasons for this were examined.

Although the MLAC has not been reported elsewhere, several authors have studied the potential problem of acinar tissue in islet preparations and the indications are that isolated acinar tissue may not be long lived. Following intraportal transplantation, the majority of acinar tissue *in vivo* has been found to disappear although engraftment can occur in parenchymal

transplantation sites(3). This raises the question of whether complete removal of acinar tissue during the isolation of islets is necessary and whether it persists sufficiently long to cause a significant immune reaction.

Acinar tissue, to a certain extent, has been found to be dependent on the close proximity of islets and has been seen to atrophy around insulin deficient islets in pancreas sections from newly diagnosed Type I diabetic patients(69). Both insulin and pancreatic polypeptide have been found to contribute to acinar cell function by increasing protein synthesis and cell division (insulin) as well as DNA synthesis (pancreatic polypeptide)(17) (69).

Thus in summary, others have investigated the contribution of non islet cells to the allograft survival of islet preparations and found that small lymph nodes or leucocytes and acinar tissue can have some effect. Studies *in vitro* have suggested that impure islet preparations stimulate a greater lymphoproliferative response than pure preparations. This was in contrast to the findings of these studies.

### 10.2.6b Effect of acinar cell soluble products on allogeneic lymphoproliferation

Functional integrity of acinar cells is associated with the production of many enzymes including amylases, lipases and proteolytic enzymes, and these may have a direct inhibitory effect on the MLR or the cocultures. This was investigated by placing acinar cell pieces into cones made from filters with a 4  $\mu$ m pore size and inserting the cones into the top of microwells containing an MLR or coculture, such that only the soluble products but not the cells came into contact with the proliferating lymphocytes. The results, shown in Figure 8.2, indicated that the presence of soluble products from acinar tissue did not significantly affect the proliferative response. Bearing in mind the variable responses obtained during the titration and kinetic experiments, additional experiments were carried out to measure the effect of acinar cell soluble products on the MLR (as previous MLR experiments but using y-irradiated PBLs as the stimulator population). These results combined with the earlier MLR results showed a consistent but non significant reduction of proliferation in the presence of acinar tissue soluble products in seven of eight experiments. However with

the exclusion of one result, which showed background proliferation only, the data was statistically significant. The non proliferation of the MLR in that one instance was found to be similar to the PBL negative controls and showed (Figure 8.4), in the same way, a trend towards enhancement of proliferation in the presence of the acinar cell soluble products or the filter cones themselves.

Thus in summary, the findings, so far, showed that the soluble products of acinar cells directly inhibited lymphocyte proliferation. The next experiments were designed to measure amylase, as a representative acinar cell product, in order to determine the persistence of the soluble products in culture and the effects of stimulator cell pretreatments on acinar cell function.

10.2.6c Effect of stimulator cell pretreatments on acinar cell function Whether acinar function is maintained sufficiently long to affect the outcome of the MLAC is uncertain but it is possible that stimulator cell pretreatments may affect both longevity and function of acinar cells and could account for the differences in immunogenicity observed by others. Pretreatments used by others have included mitomycin C(269) and  $\gamma$ -irradiation(264), in addition, Trasylol (Aprotinin), which is a proteinase inhibitor(146) was used. The results shown in Figure 8.1 and Table 8.1 were variable but did not indicate that the treatments used substantially enhanced the proliferative response.

Others have measured amylase levels in relation to isolated islets in culture. Using dispersed human pancreatic samples from six individuals, amylase levels in culture medium have been found to fall to zero within four to six days (medium changed every two days)(145). Elsewhere, residual acinar cells have been found to disintegrate within one to two days in petri dishes(305). Matas(146) determined the insulin and amylase levels in medium containing 14 human, 5 dog or 50 rat pancreatic fragments over a 24 hour culture period and found amylase levels to decrease (3.1 to 0.19 mg/g human tissue, 3.8 to 0.24 mg/g dog tissue and 14 to 0.74 mg/g of rat tissue) but insulin levels to be relatively stable (120 to 136  $\mu$ g/g human tissue, 129 to 51  $\mu$ g/g dog tissue and 162 to 107  $\mu$ g/g rat tissue). Nomura has used acinar tissue from >60% pure human islets preparations (n = 14) and >40% pure porcine islet preparations (n = 9) with a daily change of culture

medium over a 4 day period, and found amylase levels to decrease from 868 to 36 SU/dL for the human preparations and from 4305 to 125 SU/dL for the porcine preparations(306). Together, these findings suggest that acinar tissue either disintegrates or loses considerable functional activity in culture even in the presence of islets.

Most of the studies described above have either been of short duration or included replacement of the medium every one to two days. Medium replacement in the MLAC is not possible during the coculture period and so the persistence of amylase in acinar cell culture supernatant, after removal of the cells, was measured. The results in Table 8.2, showed that high amylase levels can remain constant over a nine day period and so would exert an effect on lymphocyte proliferation throughout the duration of an MLAC assay. Stimulator pretreatments may be able to inhibit protein production from acinar cells before being used in the MLAC and this would avoid the inhibitory effect on lymphocyte proliferation, so the effect of such pretreatments on amylase production was studied. In addition to the effect of  $\gamma$ -irradiation and mitomycin C, the effect of glutaraldehyde pretreatment was investigated which was recommended (Londei - personal communication) as a means to inhibit protein production but preserve antigen integrity.

The experiments to illustrate the effect of acinar cell soluble products on the MLR showed that the supernatant from the wells containing 10 acinar cell clumps in filter cones, or as part of the MLAC, had a mean of approximately 200 U/L amylase whereas those without had a mean of <20 U/L amylase (data relates to Figure 8.3). The measurement of amylase levels in relation to stimulator cell pretreatments was carried out over a number of experiments and involved the measurement of amylase in medium containing acinar tissue with medium replacement after sampling. The first two experiments showed almost complete loss of amylase production by day two and four, in all groups including the untreated acinar tissue (Tables 8.3 and 8.4). One experiment in which accumulated samples (without medium replacement) and starting with fresh medium (zero amylase) were compared with replenished samples, showed that amylase persisted to day 6 in all groups (Table 8.5) of the accumulated but not the replenished samples. Lastly, one experiment compared acinar tissue from three different pancreata, which again confirmed a sharp fall in amylase

levels after the first sample was taken (medium replacement) and although there was some persistence of amylase to day three or four there was no clear difference between the groups. In an attempt to obtain a clear result, the data was pooled from these experiments and represented as Figure 8.5 to show that the sudden drop in amylase levels after changing the medium occurred in all groups but there was an indication that low levels of amylase could persist in the supernatant as shown at day six after stimulator cell pretreatment as well as in the control acinar cell clumps.

Thus in summary, these results showed that amylase can persist in culture but that the vast majority of amylase, present after digestion and islet isolation, was removed by replacing the supernatant with fresh medium. The effect of stimulator cell pretreatments on amylase levels, was found to be minimal compared with the effects of medium replacement. A minimal level of amylase was found to persist in the wells during coculture.

10.2.6d Effect of cytokine pretreatment on acinar and islet tissue As an alternative way of investigating the potential immunogenicity of the acinar tissue, cytokines were used in an attempt to upregulate the MHC antigens on the acinar tissue to determine whether this increased the stimulation of allogeneic lymphocytes in the MLAC.

Treatment using a combination of TNF- $\alpha$  and IFN- $\gamma$ , has been found by others to induce MHC class II antigen expression on human islets(70). This increase in antigen expression could contribute to the potential immunogenicity of the acinar cells by allowing them to act as antigen presenting cells, as has been found using thyroid tissue(307). More recently, Rabinovitch has reported that combination treatment with these two cytokines (TNF- $\alpha$  and IFN- $\gamma$ ) has resulted in DNA fragmentation and inhibition of insulin release(73). Markmann has found that cytokine treatment of rat islets (IFN- $\gamma$ ) despite an induction of MHC class II antigen, did not result in increased proliferation in the MLIC(39).

In these studies, combined TNF- $\alpha$  and IFN- $\gamma$  cytokine treatment of human acinar tissue (as described in Table 8.7) was found to enhance MHC class II antigen expression on acinar tissue compared to untreated controls (Figures 9.4 and 9.5), but this did not lead to an increased level of lymphoproliferative response to acinar tissue pieces. This result suggests

that parenchymal acinar cells cannot act as antigen presenting cells, which may be due to a lack of the necessary costimulatory molecules(240).

Thus in summary, pretreatment of acinar tissue with cytokines did not enhance the lymphoproliferative response *in vitro* despite upregulation of MHC antigens. This suggests that acinar cells lack costimulatory molecules for antigen presentation.

### 10.2.6e Effect of islet cell soluble products and cytokine pretreatments on islet tissue in cocultures

The same experiments carried out to determine the effect of soluble products from acinar tissue on the MLR and the MLIC, as well as the experiments designed to investigate the effect of MHC class II upregulation following cytokine treatment of acinar tissue, were also carried out using islets.

The principal soluble product of islets is insulin, which has been found to both stimulate and inhibit lymphocyte proliferation. Rabinovitch has measured the level of insulin in MLIC microwells and found it to be approximately 0.1  $\mu$ g/ml which corresponds with the level of added porcine insulin found to stimulate lymphocyte proliferation in the canine MLR. An intermediate level (1  $\mu$ g/ml) of porcine insulin was found to have no effect on the MLR and a higher level (10  $\mu$ g/ml) was found to be inhibitory(263).

The results of these studies, shown in Figure 8.6, indicated that the presence of soluble products from islets correlated with a decrease of lymphocyte proliferation in the MLR and MLAC. This result was not statistically significant. The insulin level in the wells containing ten islets in filter cones was approximately 800 ng/ml which is comparable to the level of insulin in the wells used by Rabinovitch containing 100 dog islets and which was found to stimulate lymphocyte proliferation. The difference between the two sets of results may relate to the different species used (Rabinovitch measured the effect of porcine insulin on the canine MLR) as well as the size and viability of islets. Once again, there was a problem with interpreting variable data from so few experiments.

The effect of cytokine treatment (TNF- $\alpha$  and IFN- $\gamma$ ) on islet cells was also examined by immunohistology and showed minimal induction of MHC class
II antigen compared with an untreated control (Figure 9.6 and 9.7). This did not lead to an increase of the lymphocyte response in the allogeneic MLIC as shown in Table 8.9. Although the MHC class II antigen expression was not as prominent on islets as on acinar cells following cytokine treatment, it is possible that islets may also lack the necessary costimulatory signals necessary for T cell activation.

Thus in summary, insulin released from islets did not enhance the MLR or MLAC in these experiments. Cytokine pretreatment, which showed some induction of of MHC antigens, did not enhance the lymphoproliferative response to islets suggesting that islet cells lack the capacity for antigen presentation.

Overall, the combined findings of the work described in Chapter Eight indicated that soluble products released from acinar tissue can inhibit lymphocyte proliferation. It is possible that pretreatment of acinar cells, and careful washing of the tissue, could have minimised the soluble products in the cocultures used by Ulrichs and Zeevi, which may have avoided the inhibitory effect on the lymphoproliferative response. It is also possible that effective reduction in the release of soluble products from islets, which would avoid the potential stimulatory effect of insulin on lymphocyte proliferation, might reduce the response in the MLIC. However, neither of these possibilities would account for substantially higher immunogenicity of impure islet preparations reported by others(269) (273).

One explanation could be that the increased immunogenicity of impure human islet preparations is related to the increased amount of tissue used to maintain the same number of islets in an impure preparation. If islets and acinar tissue have similar levels of immunogenicity, perhaps by containing similar numbers of MHC class II positive passenger cells, then increased stimulatory capacity of impure preparations, compared to pure islets, could be a result of an increased amount of tissue. Although the results presented in this thesis showed a peak proliferation to ten islets, others have show a higher number to be optimal. However, it must be borne in mind that the islet diameter has not always been defined by others and that this may make a substantial difference to the amount of functional islet tissue. Accordingly, cocultures using 100 canine islets(263) have been

found to secrete a similar amount of insulin to ten human islets of average diameter  $150 \ \mu m$  as reported here (Expt 40).

# 10.2.7 Immunocytochemical determination of MHC class I and II antigen expression in human islets and acinar tissue

The immunocytochemical studies described in Chapter Nine were originally designed to investigate the expression of a large number of molecules by cells in islets and acinar tissue. Technical difficulties with unambiguous islet and acinar cell identification led to the study concentrating on the expression of MHC antigens and the determination of whether islets and acinar tissue contained similar number of MHC class II positive cells, 'passenger leucocytes', which could confer similar potential immunogenicity on islets and acinar tissue pieces in the mixed lymphocyte coculture.

### 10.2.7a Background to the immunocytochemical studies

Immunocytochemical staining studies have shown that the human pancreas comprises many different cell types including the four endocrine cell types of the islet which produce insulin, glucagon, somatostatin and pancreatic polypeptide, and the acinar cells which produce enzymes such as amylase, lipase and proteolytic enzymes. These two tissues also contain vascular endothelium, duct epithelium, nerve fibres, a stromal network and, of particular importance to this study, leucocytes which are distributed throughout the pancreas(7).

In a study of the potential immunogenicity of human islet and acinar tissue, it has been appropriate to study the antigens involved in the generation of an immune response which are principally the MHC class I and II antigens. MHC class I antigen is expressed by most cells and has been found to be associated with the presentation of endogenous antigens, such as viruses, as peptides to cytotoxic T cells. MHC class II is expressed by a limited number of cells involved in immune responses, such as dendritic cells, B cells and macrophages. The molecule is associated with cell surface presentation of exogenous antigenic peptides to T helper cells(308).

The distribution of MHC class I antigen in the pancreas has been described by others and the molecule has been found to be expressed by vascular

endothelium, macrophages/dendritic cells, duct epithelium, nerve fibres and weakly by the endocrine cells of islets but not by acinar cells(22) (23) (51).

The vascular endothelium and associated MHC class I antigen expression is present immediately after isolation, as has been shown in the rat(26), but in culture, electron microscopy has detected the death of rat vascular endothelium within one to four days of high glucose and IFN- $\gamma$  treatment(25). Weak parenchymal MHC class I antigen expression on mouse islets, which has been found to be maintained after isolation, has been associated with increased susceptibility to the action of cytotoxic T cells(39) (309).

One reason that the expression of MHC class II antigen has been widely studied in islets, is because of the importance of this antigen in initiating the alloimmune response. The basis of the allogeneic MLR has been found to correspond to the number of dendritic or antigen presenting cells in the stimulator population(235), these are potent stimulators which have been found to carry a high concentration of surface MHC class II molecules(237), and it has been suggested that the basis of islet immunogenicity also lies with 'passenger leucocytes' which in the human islet may include dendritic cells(150). So, it is possible that the difference in response to leucocytes and pancreatic tissue is a function of the number of antigen presenting cells present.

Distribution of MHC class II antigen in the normal pancreas is restricted to dendritic cells, lymphocytes, macrophages and human vascular endothelium(22) (23) (28) (29). In the intact pancreas as well as isolated islets, the number of MHC class II+ve cells per islet has been reported as 0 - 15 cells in individual rat and dog islets with less in human islets(27), 5 - 10 cells in mouse islets, less than 2% in dispersed mouse islet cells(35) and 5 - 20 putative dendritic cells in Lewis rat islets(310). Looking at whole islets rather than immunocytochemical sections, Flesch has found that 38% of rat islets contain 10 or more MHC class II+ve cells(37) and this has been found to vary according to the rat strain such that 7 - 20 macrophages or dendritic cells and 0.5 - 2.2 leucocytes have been found per islet(32). MHC class II antigen has been found to be present on vascular endothelium, as well as leucocytes, in pig islets immediately after isolation, but after 10 days

culture, vascular endothelium has been found to disappear leaving the leucocytes intact(31). There have been few studies on the number of antigen presenting cells in acinar tissue although Ulrichs has reported that the majority of MHC class II+ve cells are isolated with the exocrine components of the pancreas(269).

The majority of estimates of MHC antigen positive cells in islets given above have been made by immunocytochemical staining in post isolation tissue, either using whole islets(32) (37), sections of whole islets(26) or monolayer culture(28). Others have estimated the number using FACS analysis(265) to give a percentage of MHC class II positive cells. Positive identification of human islets in relation to MHC antigens has been found necessary because of the variety of cell types found in the human pancreas(7) which are not necessarily distinct after human islet isolation(284).

Immunocytochemical staining by others has involved a number of techniques. These have included indirect immunofluoresence of whole rat or mouse islets which have revealed the presence of two types of MHC class II+ve cells with either round (lymphocytes) or stellate (dendritic cells or macrophages) morphology(27) (32) (37) (38) (39) (265) (310). Detection of MHC class II+ve cells in dog/rat islets by electron microscopy using indirect immunofluorescence has been able to localise MHC class II antigen expression in the vicinity of beta cells. Most MHC class II+ve cells were found to be monocytes (dendritic cells or macrophages) and other cells with scant cytoplasm were thought to be lymphocytes(27).

Cryostat sections of islets have been used, similar to the standard pancreas methods after snap freezing mouse or rat islets in OTC cryoprotectant, cutting sections and staining for MHC class I and II antigen with an immunoperoxidase method(26) (33).

Most authors described above have relied on the purity of rodent isolated islet preparations to allow identification of islets expressing MHC antigens. However human islets, in relatively less pure preparations, have required specific identification leading to the use of dithizone as a stain for whole islets, allowing immunofluorescent location of MHC class II antigen(29). For monolayers of islet cells, it has been possible to use immunofluorescent double staining(70) to link MHC expression to positive identification of beta

cells using anti insulin or c-peptide antibodies of different isotypes. Antibodies have been separately identified using an anti HLA-DR antibody with an IgM isotype, followed by an anti IgM-FITC and then anti pancreatic hormone antibodies of IgG isotype, including anti c-peptide, followed by an anti IgG-TRITC antibody(70). Other double staining combinations have been used on formalin fixed tissue and have included using primary and secondary antibodies separated by their Fc source (rabbit or goat) and similarly Fc separate tertiary antibodies, with one conjugated to alkaline phosphatase and the other to peroxidase(291). Primary antibodies have also been separated by Fc source (rabbit anti MHC I or II and guinea-pig anti insulin) and appropriate secondary antibodies conjugated to peroxidase or as a 3-layer method to fluorescein(52).

Thus in summary, others have investigated the range of cell types in the pancreas and, because of the potential importance of immunostimulatory molecules in Type I diabetes and islet allograft transplantation, have paid particular attention to the expression of MHC class I and II antigens. The presence of weak MHC class I expression on endocrine cells of islets, but not the adjacent acinar cells, as well as the expression of MHC class I and II antigens on vascular endothelium and 'passenger leucocytes' in the human pancreas have attracted wide interest. Although informative data has come from animal models, a full investigation of the expression in isolated human islets and the major non islet contaminant, acinar tissue, has not been previously reported. Other studies have used immunofluorescence on whole islets, FACS analysis or sequential or double immunocytochemical staining on cryostat sections to identify MHC antigens.

## 10.2.7b MHC antigen expression in the pancreas, human islets and acinar tissue, before and after culture

The immunocytochemical studies for this thesis were intended to determine any changes in the expression of MHC class I and II antigens on human pancreatic islets and acinar tissue before and after isolation and culture. During digestion and isolation, islets and acinar tissue suffer potential trauma from the collagenase digestion, centrifugation on density gradients and maintenance in culture medium(3). Any resulting change in MHC class II antigen expression potentially could affect the outcome of the MLIC and MLAC.

These studies commenced with identification and testing of the primary antibodies to be used and included antibodies to insulin for the detection of beta cells in islets (HB124) and to acinar cells (LDS8) as well as antibodies to MHC antigens including W6/32 , HB120 (HLA-A,B,C) HB55 and HB145 (HLA-DR). Although satisfactory immunocytochemical staining was obtained using the 3-layer method with Ig-biotin, avidin-alkaline phosphatase and Fast Red development, all attempts to double stain human pancreas using these isotypically different antibodies with a normal light visible detection system were unsuccessful. The reasons for this were not clear as Pujol-Borell has successfully double stained with immunofluorescence on monolayer islet cells for insulin and MHC class II antigen using isotype specific antibodies(70). It was possible that the detection markers, although having been successfully used to identify different cell types on the same section(291) might interfere with each other which would explain why only the first marker and not the second marker used in each attempt at double staining, was successful in each case.

The normal light visible detection markers were preferred because of the ability to simultaneously detect the morphology of the unstained tissues, so it was decided to use sequential sections(69) with the sensitive 3-layer method using Ig-biotin, avidin-alkaline phosphatase with Fast Red development. This in turn restricted any potential increase in the size of the panel of antibodies used because of the difficulty in identifying specific islets or acinar cell clumps in a large number of sequential sections.

A series of ten human cadaveric pancreata which were processed and resulted in the separation of islets and acinar tissue, formed the basis of the study in providing pancreas, islets and acinar cell clumps after isolation as well as islets and acinar cell clumps after seven days culture in RPMI medium +10% FCS at  $37^{\circ}$ C. The islets varied from approximately 60% - 90%purity, the acinar tissue, which forms the majority of the pancreas, was estimated to be at least 98% pure after removal of the islets.

The four photographs in Figures 9.1a - 9.1d, showing consecutive sections with positive staining of islet beta cells, acinar cells, HLA-A, B, C and HLA-DR illustrate some of the difficulties experienced, in that (9.1a) islets in frozen sections of human pancreas were not very easy to identify by

morphology alone and (9.1b) the acinar cell antibody did not stain all acinar cells (although it does in some pancreata). Sections of the same islet, shown in Figures 9.1c - d, illustrate that the exact identification of particular single cells as MHC class I and class II positive was not always possible.

Studies to count the number of MHC class I and II positive cells in islets and similarly sized acinar cell clumps, were difficult for the reasons described above. An additional antibody used to help identify islets, was serum from a patient with Stiff Man Syndrome which has been found to bind to GAD, an antigen expressed by islets. Although this serum satisfactorily identified islets in human pancreas sections, it was less convincing on isolated islets and acinar tissue where there was a relatively high background stain on acinar tissue.

Thus in summary, immunocytochemical staining was used to test the efficacy of a panel of antibodies to islets, acinar cells, MHC class I and II antigens and other molecules involved in the immune response. Double staining procedures, using light visible detection systems, to unambiguously identify islets and acinar tissue, were unsuccessful and as an alternative, the 3-layer biotin-avidin system was used on sequential cryostat sections to determine the expression of MHC class I and II antigens in these tissues. A series of ten human pancreata with the isolated islets and acinar tissue from the same donor, immediately after isolation as well as after seven days culture, were designated for the subsequent studies.

10.2.7c Distribution of MHC positive cells in human islets and acinar tissue Representative patterns of immunostaining using HB124 (anti insulin) and LDS8 (anti acinar cells) as the means of identifying isolated islets and acinar tissue pieces are shown in Figures 9.2a and 9.2b. The figures illustrate how weak anti insulin staining and cross reactive acinar cell stain added to the difficulties. In addition, the day 0 post isolation tissue was frequently (40% of cases) either single cells or small groups of cells, making identification of cell types across adjacent sections almost impossible. At day zero and day seven, discrete cellular clumps were not always identifiable (e.g. positive using HB124 and negative using LDS8) although morphologically islet or acinar cell in appearance. The results presented here do not take account of, for example, islet tissue not reactive with anti insulin antibody which might differ in its expression of MHC antigens from islet tissue actively producing insulin, and only represent a small sample of the general population from which the donors are drawn.

The pattern of staining is shown for MHC class I antigen on individual cells of fragmented tissue at day zero (Figure 9.2c) and on parenchymal cells of intact cellular clumps at day seven (Figure 9.2d). The intensity of the endocrine stain which was relatively weak in the pancreas and at day zero, so allowing the identification of individual positive cells within islets, was, by day seven of culture, much more intense and identification of individual positive cells, such as any remaining vascular endothelium and leucocytes, was not easily distinguishable (Table 9.1). Acinar tissue showed no parenchymal expression of MHC class I antigen in the pancreas but this was induced after isolation and upregulated to all cells by day seven (Table 9.2). MHC class I positive individual cells in the pancreas and at day zero, were apparently absent from acinar tissue at day seven, but, as for islets, there was a lack of separate identification against strong parenchymal cell stain. A similar picture can be seen in relation to the number of MHC class I positive cells in each tissue (Figure 9.3) where a relative loss of individual cells per islet can be seen as a trend at day zero and apparently complete by day seven. In addition, a trend can be seen (statistically non significant) of higher numbers of MHC class I positive cells in acinar tissue compared to the equivalent area of islet tissue.

Others have detected parenchymal MHC class I expression on rat islets after isolation(26) (39) and an increase in MHC class I expression has been observed on the exocrine tissue in with rejecting rat islet allografts(24). Using electron microscopy, the disappearance of MHC class I +ve vascular cells but not leucocytes after culture has also been observed elsewhere(25).

The pattern of staining is shown for MHC class II positive cells at day zero, in Figure 9.2e, which identified single positive cells in otherwise negative islets and acinar cell clumps. The section shows positive cells with dendritic-like morphology in the islet and vascular endothelial morphology in the acinar tissue although in general, MHC class II positive leucocytes and vascular endothelium were found in both tissues. After seven days culture, as shown in Figure 9.2f, MHC class II antigen was found to be expressed on parenchymal acinar cells but not on islet endocrine

cells. Individual MHC class II positive cells continued to be present after seven days in culture. Table 9.1 shows that all islets in the pancreas and immediately after isolation, at day zero, contained MHC class II positive cells, but that the number reduced to 58% of islets at day seven with no parenchymal expression. The equivalent amount of acinar cell tissue, as can be seen in Table 9.2, also showed a reduction in the percentage containing MHC class II positive cells from 100%, in the pancreas and at day zero, to 55% at day seven. In contrast to islets, acinar tissue showed an induction of parenchymal MHC class II antigen expression both after isolation (20%) and after seven days culture (42%). A similar picture can be seen in relation to the number of MHC class II positive cells in each tissue (Figure 9.3), where a relative loss of individual cells per islet was seen at day zero and continued at day seven but without complete loss. Again the cell number per tissue was found to be higher in acinar tissue than islets. The latter result is interesting because this would represent a higher number of immunoreactive cells in the MLAC compared to the MLIC but, as described earlier in Chapter Eight, acinar tissue pieces stimulated a reduced allogeneic lymphoproliferative response compared to the equivalent number of islets.

Others have found that individual MHC class II positive cells persist in rat islets after isolation(26) and for two days in culture(37), and there is some debate as to whether they persist for longer than fourteen days in culture(149) (32) (39). The number of islets (from Lewis rats) containing MHC class II positive cells has been found to decrease from 93.5% to 10.4% after five days culture(38) and the number of individual MHC class II positive cells per rat islet has been found to decrease from approximately 20 to 7, and 7 to 4 dependent on the species(32). Weak endocrine cell expression of MHC class II antigen has been observed on 20% of isolated adult rat islets(32) but not human islets(29) and little or none on mouse islets(33). Canine islets have shown a reduction in the number of MHC class II positive leucocytes per islet from five to nineteen to a maximum of three, and similarly, after ten days culture, leucocytes have been found in porcine islets(31).

Thus in summary, some difficulties were encountered with the unambiguous identification of isolated intact islets and acinar tissue pieces especially immediately after isolation. However it was possible to accurately

identify islets and acinar tissue pieces such that MHC class I expression was seen on individual cells in the pancreas and both tissue types at day zero, and on the parenchymal islet and acinar cells after seven days culture. MHC class II antigen expression was seen on individual cells in the pancreas and both tissue types at day zero. At day seven, although no MHC class II antigen expression was seen on the islet endocrine cells, it was seen on individual cells in 55 - 58% of islets and acinar tissue. Taken together, these results support the findings by others in rat, canine and porcine islets, that low numbers of MHC class II positive leucocytes persist in culture but vascular endothelium does not.

### 10.2.8 Effect of cytokine treatment on MHC antigen expression by human islet and acinar cells

Cytokine treatment as a means of enhancement or upregulation of MHC antigens on human islet cells(70) and the potential to increase the antigen presenting capacity of parenchymal cells(307), have both been described. The strong expression of parenchymal MHC class I antigen on human islets and acinar cells observed in this work, suggested that further enhancement was unnecessary, however the lack of MHC class II antigen expression by islets (Table 9.1) and the incomplete expression on acinar tissue (Table 9.2) led to the use of cytokines to determine whether MHC class II antigen could be induced or upregulated and whether this affected the outcome of the lymphoproliferative response in the MLIC and MLAC.

The results of the MLIC and MLAC were discussed earlier and indicated no enhancement of the allogeneic lymphocyte response to islets or acinar tissue. The results of the immunocytochemical staining are shown in Table 9.3. Cytokine treatment of islets did induce parenchymal expression of MHC class II antigen after three to four days on acinar tissue, as shown in Figures 9.4 and 9.5. However, spontaneous induction of parenchymal MHC class II expression was previously noted on acinar tissue after one week in culture without cytokine treatment (Figure 9.2f), suggesting that cytokine treatment caused an early enhancement of an on-going process. A limited expression of MHC class II antigen on islet endocrine cells was seen after cytokine treatment, as shown in Figures 9.6 and 9.7, which was not seen spontaneously after one week in culture. Others, who have investigated the induction of MHC antigens by IFN- $\gamma$ , have shown the enhancement of MHC class I antigen expression a) on rat islet endocrine cells after a combination of seven days culture and two days IFN- $\gamma$  (500 U/ml) treatment(39) and b) using human islet cell monolayers with IFN- $\gamma$  (50 - 500 U/ml)(28).

As was seen in the results above, others have also found MHC class II antigen difficult to induce on islets, and this has led to the use of a) high concentration of cytokine - IFN- $\gamma$  treatment (1,200 U/ml) for two days on rat islets(25), b) a combination of seven days culture and at least a further six days IFN- $\gamma$  (500 U/ml) treatment(39) or c) a combination of IFN- $\gamma$  and TNF- $\alpha$ . The latter work used human islet cell monolayers in which the combination of IFN- $\gamma$  (50 U/ml) and TNF- $\alpha$  (10 U/ml) treatment for three days was found to induce MHC class II antigen expression on 50% beta cells(70).

Thus in summary, the expression of MHC class II antigen was found to be induced on fresh human islets and enhanced on fresh human acinar tissue by a combination of two cytokines, IFN- $\gamma$  and TNF- $\alpha$ . Comparison with the data described in Chapter Nine and discussed earlier, showed that this increased MHC class II expression did not lead to an increased stimulatory capacity of islets and acinar tissue in the MLIC and MLAC, probably due to the lack of costimulatory molecules on islet and acinar cells.

### 10.2.9 Identification of different cell types in pancreatic digest

Increased understanding of the nature and relative immunogenicity of the acinar components of the pancreas and its contribution towards the alloresponse, has been gained from the findings of the work presented here. Others have suggested that it is the leucocyte population, originating from small lymph nodes rather than the islet 'passenger cell' population that contributes considerably towards the immunogenicity of impure islet preparations(21). Although small lymph nodes might be processed with the pancreas from mice, no evidence of this type of contamination was found in human islet preparations where the lymph nodes are removed with the adjacent fatty tissue before pancreas distention with collagenase. In addition, no evidence of small lymph nodes was found in the tissue after

digestion. There was some variation in the number of individual leucocytes between digest samples, but these cells were not isolated with the islets on the density gradient and so could not have contributed to the immunogenicity of the isolated islet preparation.

Thus in summary, no evidence was found for lymph node contamination of human isolated islet preparations and so did not appear to contribute to the immunogenicity of human islet preparations.

### 10.3 Overall summary of the discussion

The studies for this thesis encompassed five areas of experimental investigation. These included: a) preliminary work on the MLIC and an investigation of its potential as a pretransplant assay which might help predict outcome of human islet allografts, b) an establishment of basic parameters of coculture assays, c) standardisation of the conditions and parameters of the MLIC and MLAC, d) the relative *in vitro* immunogenicity of functional acinar tissue and e) immunocytochemical studies to determine the expression of MHC antigens on the components of human isolated islet preparations. Overall, the work showed that the MLIC response in non tissue culture plates takes nine days to develop and is optimally stimulated using ten islets. The duration makes the assay unsuitable, in this form, for potential pretransplant prediction of islet graft outcome.

One MLIC experiment in which donor islets used for transplantation were combined with recipient and control PBLs, showed that a low proliferative response was obtained for two different HLA matched (four antigens in common) combinations (recipient and potential recipient) but a high proliferative response was obtained against the same islets with completely HLA mismatched PBLs. This suggests that HLA matching may be important in islet transplantation and the MLIC could be used to investigate this further. In these studies, responses obtained in the allogeneic MLIC were different from those of the MLR suggesting that the MLIC may be a more appropriate model for human islet transplantation. In addition to the presence of islet antigens, the differences may be due to the limited number of antigen presenting cells in the islet and involve the indirect as well as direct method of antigen presentation in the immune response against islets.

Acinar tissue, as the major contaminant of human islet preparations, was found to contain slightly more MHC class II positive cells (passenger leucocytes) than islets but stimulated a reduced allogeneic lymphoproliferative response in the MLAC compared to that of islets in the MLIC. This effect was found to be principally due inhibition of lymphocyte proliferation by the soluble products of acinar cells. An increase in parenchymal MHC class II antigen expression induced by cytokines, did not appear to enhance the stimulatory capacity of either human islets or acinar tissue.

# CHAPTER ELEVEN

### CHAPTER ELEVEN CONTENTS

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### CHAPTER ELEVEN

# SUMMARY, CONCLUSIONS, ORIGINAL WORK AND SUGGESTIONS FOR THE FUTURE

### 11.1 Summary

Human islet transplantation is a surgical procedure with relatively few safety risks that has the potential to provide normoglycaemia in Type I diabetic patients and ultimate avoidance of life-threatening complications. However, graft survival rates have not been as good as hoped and models to study the specific immunogenicity of human islet allografts have been investigated.

The aim of this thesis was to investigate the *in vitro* human allogeneic lymphoproliferative response to untreated human islets (MLIC). Following the implementation of the clinical human islet transplantation programme in which islet allografts are given intraportally into Type I diabetic patients, the possibility of developing a short duration MLIC was studied. Given the time limitations between islet isolation and transplantation, this proved to be unsuccessful as a pretransplant model. Subsequently, the parameters and conditions of the MLIC were determined. These were found to include; the use of non tissue culture microwell plates to reduce fibroblast outgrowth and islet adhesion in the MLIC, the introduction of human AB serum as a growth supplement in the RPMI medium to reduce the levels of background responder cell proliferation and the use of dithizone staining to unambiguously identify islets.

Having determined the basic parameters of the model, and observed a trend of reduced stimulatory capacity of pancreatic digest compared to islets alone, conditions for the optimal proliferation to islets (MLIC) acinar tissue pieces (MLAC) were investigated and compared with the MLR. These optimal conditions were found to include the use of ten human islets or acinar tissue pieces (average 150  $\mu$ m diameter) cocultured with 1 x 10<sup>5</sup> HLA mismatched responder PBLs for a duration of nine days. Comparisons between the MLIC (ten islets) the MLAC, (ten acinar tissue pieces), and the MLR (1 x 10<sup>5</sup> splenocytes) in which the stimulator populations were from

the same donor source and the responder populations were  $1 \ge 10^5$  HLA mismatched PBLs, showed that, in terms of stimulatory capacity, the MLR was greater than the MLIC which in turn was greater than the MLAC.

An investigation of the acinar tissue showed that soluble products of untreated acinar cells inhibited lymphocyte proliferation in the MLR and so could explain the reduced response to acinar tissue compared with the response to islets. Immunocytochemical investigations to determine the number and distribution of MHC class I and II antigen positive cells in human isolated islets and acinar tissue compared to the pancreas, showed that MHC class I antigen was expressed by parenchymal islet and acinar cells after culture, whereas MHC class II antigen expression was confined to parenchymal acinar cells and passenger leucocytes which persisted in culture. Upregulation of MHC class II antigen expression on acinar cells (and induction on islets) by treatment with a combination of IFN- $\gamma$  and TNF- $\alpha$ , did not enhance the stimulatory capacity of either islet or acinar tissue so suggesting that these cells lack costimulatory factors which would allow them to present antigen.

This work was successful in allowing the development and standardisation of the human allogeneic MLIC, however, the model was found to have inherent variability both within and between experiments. For these studies, data was pooled to allow statistical comparison and this stressed the importance of using more than three experiments to obtain meaningful results. The establishment of this model gives the opportunity to study the immunogenicity of human islets further particularly treatments designed to immunomodulate islets prior to transplantation.

### **11.2 Conclusions**

This study aimed to determine whether an *in vitro* alloresponse could be generated to human islets and the major contaminant of human islet preparations, acinar tissue. Human islets are an irregular and scarce resource resulting in the need to maximise the information obtained from each experiment. Using the conditions defined in this thesis, ten human islets or acinar tissue pieces per well were able to generate an allogeneic lymphoproliferative response and showed peak stimulation of  $1 \times 10^5$  HLA

mismatched PBLs, after a duration of nine days coculture. When compared with each other, the response in the MLR was greater than that in the MLIC and both were greater than that in the MLAC. The low response to pancreatic cells, compared with the response to splenocytes in the MLR, may have been related to the number of antigen presenting cells in islet and acinar tissues. This stimulatory capacity was not increased by induction or enhancement of the MHC class II antigen expression after cytokine treatment. In addition, soluble products of the acinar cells were found to inhibit lymphocyte proliferation, so reducing the response in the MLAC.

The MLIC assay, in the optimised form described here, was not found to be suitable as a pretransplant assay to help predict islet graft survival because of its duration, variability and limited response. However the MLIC could be used to monitor the efficiency of passenger leucocyte removal by immunomodulation techniques and offers the potential for further study of the *in vitro* alloimmune response to human isolated islets.

# 11.3 Original work derived from this thesis and suggestions for future studies

This thesis has focused on a model to investigate the immunogenicity of human islets with relation to clinical allotransplantation into Type I diabetic patients. The MLIC has been previously reported using animal cells(263) (264) and more recently, using handpicked frozen-thawed human islets(269) or aliquots of human islet preparations(273). However, this is the first report of the MLIC using freshly isolated handpicked islets and represents the first report of the MLAC in which acinar tissue pieces have been used as the stimulator population. Optimisation of the MLIC by titration and kinetic parameters, means that a comparatively small number of human islets can be used per assay, so maximising this scarce resource for future studies.

The limited survival of islet grafts *in vivo* may be due to many factors including rejection, non specific immune responses, an inability of the islets to revascularise and poor islet viability. In rodent models, there is evidence that local inflammation is involved and in humans, impure islet

preparations have shown increased *in vitro* immunogenicity compared to pure islets. In these studies, the role of acinar tissue was investigated further. One method was to use filters to separate the acinar cells, but not the soluble products, from MLR cultures, and this showed that the soluble products of viable acinar cells had an inhibitory effect on lymphoproliferation in the MLR. This inhibition was thought to account for the reduced response of the MLAC compared to the MLIC. The published accounts of heightened immunogenicity found to impure human islet preparations, probably reflected an overall increase in volume of tissue used (of different purity but with the same number of islets present) as the stimulator population.

Immunocytochemical studies of islets by others, have focused on the decrease in number of MHC class II positive 'passenger leucocytes' during culture and acinar tissue post isolation. Others have shown that the number of cells in rat and dog islets expressing MHC class II antigens decrease during culture and such studies have supported the findings described here for human islets. In addition, these studies have shown that the reduction in number of MHC class II positive cells during culture also extends to acinar tissue but that neither islet or acinar tissue is totally devoid of such cells after seven days culture at 37°C.

One author only has noted that the majority of MHC class II+ve cells are isolated with the exocrine components of the pancreas(269). In these studies, counts of the number of MHC class II positive cells showed that acinar tissue contains more MHC class II positive cells after isolation then islets, although the potential increased stimulatory capacity of acinar tissue appears to have been outweighed by the local inhibitory effect of the soluble products on lymphocyte proliferation.

The optimal duration for a peak proliferative response in the MLIC excludes its use prior to transplantation and efforts to reduce the time scale in these studies were unsuccessful. However, the use of more sensitive methods to determine IL-2 upregulation could be investigated, particularly PCR detection of IL-2 gene activation. In addition, other early markers of T cell proliferation, such as CD28, could also be utilised. Potentially the most successful outcome of these studies of the standardisation of human allogeneic MLIC, is in its application as a model for immunomodulation studies using human islets. Future work to investigate the effect of immunomodulatory treatments on the immunogenicity of islet preparations would have direct application to the human transplant programme. Published reports have investigated the effect of removing passenger cells from the islets by using low temperature culture(129), UV irradiation(153), γ-irradiation(121), cryopreservation(311) or anti MHC antigen treatment with antibody + complement(29). These treatments, tested in rodent models, are difficult to study in human systems and the MLIC offers a potential model to assess immunomodulatory protocols for human islets. In addition, the reduction in expression of MHC class I antigen(165), which is upregulated on islets and acinar tissue after isolation, could also be investigated. Transplantation of immunomodulated islets would be expected to lead to increased graft survival because of limited recognition by the recipient immune system. This may also allow pooling of islets, to increase the number transplanted for successful reversal of diabetes, without jeopardising graft survival. Finally, immunomodulated islets may survive with minimal immunosuppression which could allow islet transplantation in young newly diagnosed diabetic children.

#### 11.3.1 Summary of original work from this thesis

1. Standardisation of the conditions required for the human allogeneic MLIC by titration and kinetic parameters using freshly isolated handpicked islets as the stimulator population.

2. Demonstration that soluble products of viable acinar tissue, which is a major contaminant of human islet preparations, can inhibit lymphoproliferative responses.

**3.** Demonstration that acinar tissue contains more MHC class II positive cells than islet tissue, post isolation and that the number of these cells are reduced in both islet and acinar tissues after seven days culture.

#### 11.3.2 Summary of suggestions for future studies

1. Investigation of other techniques to reduce the duration of the MLIC in order to use it as a pretransplant assay. This could include PCR methodology to detect early markers of T cell proliferation such as the IL-2 or CD28 gene activation.

2. In order to reduce the immunogenicity of human islets, which may allow wider application in transplantation, the MLIC could be used to measure the effects of immunomodulatory treatments such as UV irradiation,  $\gamma$ -irradiation, cryopreservation and low temperature culture. The effect of these protocols on removal of MHC class II positive passenger leucocytes and reduction of parenchymal MHC class I antigen expression could also be followed using techniques such as immunocytochemistry, confocal microscopy or PCR.

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