"IN VITRO ALTERATION OF RAT PANCREATIC ISLET IMMUNOGENICITY IN AN ALLOGENEIC TRANSPLANT MODEL"

by

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ABSTRACT:

Allograft rejection remains the fundamental stumbling block to tissue transplantation. Traditional assumption has been that transplanted tissue alone provides an antigen source (alloantigen), which directly stimulates a host response resulting in graft rejection; accordingly, traditional attempts at circumventing the allograft response have focussed on techniques of recipient immunosuppression. Recently, increasing attention has been given to a subset of non-parenchymal, bone marrow derived lymphoid cells (characterized by their surface expression of class II MHC antigen) which are carried passively with the allograft into an immune competent recipient. A current hypothesis is that these cells, called antigen presenting cells (APCs), participate in the sensitization of the immunologically naive but responsive host to the transplanted tissue, leading ultimately to graft rejection. Therefore, it has been suggested that depletion of APCs from donor tissue prior to transplantation may permit allogeneic transplantation to occur, without host immunosuppression. In contrast to solid organs, pancreatic islets are well suited to this type of immunomodulation prior to transplantation, since they can be maintained in a functional ex vivo state by cell culture.

The purpose of this thesis was to evaluate donor islet APC depletion by pre-transplant cell culture and APC-ablative photodynamic therapy (PDT), and to see whether either in vitro technique could prevent rejection in a rat, allogeneic transplant model.

Briefly, a donor (Sprague Dawley, RT1u) -recipient (Wistar Furth, RT1a) pair with a major histo-incompatible barrier was selected. After collagenase digestion of donor pancreata, islets were isolated from the digested tissue by centrifugation through a discontinuous dextran gradient followed by hand picking using a dissecting microscope. Once isolated, the islets were either used fresh, placed in tissue culture (Ham's F-12 media, 11 mM glucose, 5% CO2/room air at 37 C) for variable periods, or subjected to APC-ablative PDT.

Islet APC depletion was assessed by fluorescent immunocytochemistry. Fresh, cultured and PDT treated islets were frozen in liquid N2 then cryostat sectioned and stained for class II MHC + cells (APCs), using an anti-class II mouse monoclonal antibody (OX-6), followed by a fluorescent (fluorescein indothiocyanate) labelled anti-mouse monoclonal. Using this technique, APCs could be identified by fluorescent microscopy on the basis of their enhanced surface staining. While fresh islets demonstrated between 1 and 5 APCs per cryostat section, a culture period of at least 10 days resulted in complete islet APC elimination. Islet allograft studies with fresh and cultured islets were then performed to determine: 1) if pre-transplant islet culture could sufficiently reduce donor tissue immunogenicity to allow successful allografting in immune-competent recipients, and if so, 2) what duration of culture was necessary to permit consistently successful allografting. Allografts of fresh and cultured (4, 7, 10, 14, and 21 day) islets were placed under the renal capsule of immune-competent, recipient rats and after 12 days the grafts were removed and studied histologically for evidence of rejection. While all grafts which were cultured for 10 days or less prior to transplantation were rejected, 4/10-14 day cultured islets, and 4/5-21 day cultured islets demonstrated engraftment. In vivo function of 21 day cultured islet allografts was demonstrated by transplantation of islets via the portal vein, into recipients which had been rendered hyperglycemic by IV streptozotocin. This resulted in an immediate and sustained reversion to euglycemia (as assessed by daily plasma glucose determinations using a glucose analyser) over a 30 day period of study. In contrast, streptozotocin "diabetic" recipients of fresh and 14 day cultured islet allografts demonstrated a brief (7-10 day) period of graft function (euglycemia) prior to a return of hyperglycemia, consistent with graft rejection.

Photodynamic therapy (PDT) achieves selective cell ablation by the stimulated emission of singlet oxygen from a light-activated compound (benzoporphyrin) which has been delivered to the cell target. In these experiments, APC elimination was attempted by in vitro islet treatment with OX-6, followed by a specific, secondary antibody (RAMIg) to which BPD had been conjugated. After UV light

activation the treated islets were frozen, cryostat sectioned and immunostained for Class II MHC + cells. In contrast to control islets which underwent a secondary incubation with either BPD alone or BPD conjugated to an irrelevant secondary antibody, islets which underwent PDT using the specific RAMIg-BPD conjugate demonstrated elimination of APCs as assessed by immunocytochemistry. When syngeneic and allogeneic transplants were performed using islets which had undergone APC "photoablation", the histologic appearance of the grafts was compatible with either inflammation in response to non-viable tissue, or allograft rejection.

The temporal disparity between the duration of tissue culture necessary to deplete islet APCs and that required to allow successful islet allografting can be variably explained. One possibility is that failure to stain APCs after a 7-10 day period of culture is not proof that these cells have been destroyed. It is conceivable that culture alters the surface of the APC such that it is no longer identified by anti-Class II MHC immunostains, but nevertheless retains its ability to present alloantigen. Alternatively, one can hypothesize that in vitro culture causes some donor tissue alteration other than APC depletion which renders it less immunogenic. The failure of PDT to permit successful syngeneic or allogeneic transplantation despite its apparent ability to eliminate islet APCs suggests that the treatment itself may cause irreversible islet injury, and that the inflammatory reaction observed is merely in response to non-viable transplanted tissue.

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INTRODUCTION

1) TRANSPLANTATION IMMUNOBIOLOGY: HISTORICAL ASPECTS

1.1 Contribution from Studies of Tumor Immunity

The "immunity theory" of graft rejection was postulated by several authors during the first decade of the twentieth century, based on histologic studies of rejected tumor homografts from immunologically naive but competent recipients. Popular belief at the time was that all immune reactions were the work of circulating antibody, however the inability to demonstrate antibodies in hosts of allografts of normal tissue (versus tumor allografts), and failure to confer allograft immunity passively with serum led to questioning of this theory (1). A number of investigators, led by Murphy (2), recognized the consistent presence of the "small lymphocyte" in host tissues surrounding rejecting transplanted tumors, and the concept of a cellular response in homograft rejection evolved. Mitchison (3,4), observed that immunity to tumor homografts could be transmitted between identical mouse strains by the transfer of lymph nodes draining the site of the graft. This resulted in a second set response (an accelerated rejection phenomenon) when the sensitized recipient received its first tumor homograft. Billingham and colleagues termed this phenomenon "adoptive transfer of immunity" (5).

Further experiments sought to characterize this transferable factor. It was initially proposed that the lymph nodes were transferring living tumor cells or at least tumor antigens (isoantigens). The immunizing activity of transferred lymph nodes seemed to be dependent on the interval between administration of the tumor implant and harvest of immunizing lymph nodes. Activity was maximum at 5-10 days, but had disappeared by 15-20 days. It was later shown that transfer of immunity could be accomplished not only by regionally draining lymph nodes, but also by spleen and remote lymph nodes as well (6). Weaver et al investigated the growth of transplantable tumors in diffusion chambers (permeable to body fluids but not cells), placed intraabdominally, and found that tumor homografts were killed rapidly only if the diffusion chamber contained pieces of immune spleen (7). These experiments, along

with the empiric histologic observation of lymphocyte penetration of homografts prior to destruction suggested an intimate interaction between immune lymphocytes and target tumor cells.

In 1937, a humoral homograft response was recognized when Gorer reported that mouse sera from recipients of a rejected tumor homograft was capable of agglutinating red blood cells from the donor (8). This also confirmed a belief that red cells and tumor cells of the donor shared a common antigen. However, when transfer of tumor immunity to a secondary host with sera from a sensitized recipient was attempted, it was apparent that the growth of subsequent tumor homograft was facilitated rather than inhibited, compared to non-immunized controls (9,10). Further experiments revealed that lyophilized tumor and other non-living tissues (including spleen) from sensitized recipients were capable of enhancing tumor growth.

It was speculated that perhaps the "enhancing effect" was confined to tumor homograft models on the basis of abnormal tumor growth potential, however the same effect was demonstrable with homografts of normal skin in both mouse and rabbit models (11,12). In most cases, homograft survival was prolonged relative to controls, yet there were only occasional reports of permanent graft survival (13). Billingham (12), postulated that antisera either prevented or more likely, delayed exposure of effective homograft antigens to regional lymph nodes, and referred to this phenomenon as "afferent inhibition" of the homograft reaction.

1.2 Snell's Passenger Leukocyte Hypothesis

In a departure from traditional doctrine which stated that the host response to tissue homograft was incited by isoantigens associated with the fixed, parenchymal cell population of the graft, Snell theorized that passively transported, donor lymphoid cells were responsible for the immunogenic stimulus that invoked the cellular homograft response. He demonstrated that addition to the tumor inoculum of normal lymphoid tissue of the same genotype as the tumor, would counteract the enhancing effect and result in tumor rejection (14). He suggested that the added lymphocytes were able

to escape the afferent inhibition imposed by the presence of specific antisera, and reached regional lymph nodes where they could initiate a cellular immune response. This concept was supported by the known amoeboid motility of leukocytes and the abundant lymphatic supply of the skin, providing a mechanical basis for the rapid passage of lymphocytes from subcutaneous or intracutaneous grafts to regional lymph nodes. Additional support for this hypothesis came from Hardin and Werder, who noted that survival of skin homografts was prolonged by irradiation of the donor as well as the host, a treatment that would selectively eliminate lymphoid cells (15).

1.3 Passenger Cells and Graft versus Host Reactions

The concept that donor lymphoid cells were capable of mediating cellular immune responses was also supported by some early classic studies of graft versus host reactions (16,17,18). It was shown that a local response could be incited in guinea pig skin by intracutaneous injection of lymphocytes pre-sensitized to host tissue antigens (the so-called Immune Lymphocyte Transfer [ILT] reaction), and to a lesser extent by the innoculation of lymphocytes from unsensitized donors (Normal Lymphocyte Transfer [NLT] Reaction).

Initially, there was some confusion as to whether the cellular immune response was donor or host in origin. Brent, Brown and Medawar assumed that the donor lymphocytes attacked constitutive cells in the host guinea pig's skin. Their finding that preirradiation of guinea pig hosts with doses of up to 1500 rads did not prevent the development of NLT reactions, supported this hypothesis (18).

Subsequent work by other investigators using different animal models suggested that host lymphocytes were responsible for the cellular response in both NLT and ILT reactions. An elegant series of experiments done by Ramseier and Billingham, demonstrated that although non-lethal, total body irradiation of hamster hosts prior to intracutaneous injection of allogeneic, sensitized lymphocytes markedly impaired the subsequent ILT reaction, this effect could be negated by the addition of an equal number of viable host lymphocytes to the normal or sensitized donor lymphocyte pool prior

to injection into irradiated hosts (19, 20). Elkins innoculated the renal subcapsular space of F-1 hybrid rats with parental strain splenocytes and found that the resulting immune response could be prevented by total body irradiation or administration of leukopenic drugs such as cyclophosphamide and amethopterin to the host animal (21, 22).

Similarities were noted between local GVH reactions and the recently described in vitro Mixed Lymphocyte Interaction System (23), prompting Wilson and Elkins to suggest that in vivo mixed lymphocyte interactions were responsible for the development of GVH reactions (24).

1.4 Lafferty's Contribution to the Allograft Reaction

Conventional belief in the time of Thomas and Medawar, was that transplanted tissue cells possessing allogeneic histocompatibility antigens were attacked by host lymphocytes because they were mistakenly identified as tumor cells. Implicit to this concept of immune surveillance was the notion that alloantigen alone directed the final differentiation of specific immunocyte clones. According to Medawar, the solution to allograft rejection involved immune manipulation of the host in attempts to dampen or eliminate completely the host's response.

Lafferty (25), proposed that allogeneic responses are the result of a blood cellular interaction in which donor cells of the lymphocyte/macrophage lineage provide a stimulus for activation of specific receptor-bearing host immunocytes. He hypothesized that stimulator-responder cell interactions result in activation and proliferation of an effector cell clone if a histoincompatibility exists between the stimulator and responder cells. Respecting the premise that self stimulation of cellular immune responses is forbidden, Lafferty suggests two models of stimulator-responder cell incompatibility. In the first instance, donor stimulator cells which accompany the graft are incompatible with the host responder cells, thus permitting responder cell activation. In the second instance, if both stimulator and responder cells are host in origin, modification of

the stimulator cell "self" antigen by some foreign agent (such as alloantigen), is necessary.

These two basic concepts can be expressed algebraically:

(1) $S^B + H_RA$ ----------> $H_{R'A}$ (initiation of immune response)

(2) $S^A + H_RA$ ----------> negative (resting situation)
$S^A + ag$ ----------> $S^A{}_{ag}$ and if
$S^A{}_{ag}$ is not equal to S^A then
$S^A{}_{ag} + H_RA$ ----------> $H_{R'A}$ (initiation of immune response)

where S = stimulator cell of phenotype B (donor) or A (host), H_RA = host responder cell, $H_{R'A}$ = activated host responder cell, **ag** = foreign tissue (allo) antigen, $S^A{}_{ag}$ = altered host stimulator cell.

1.5 The "Two Signal" Theory of T-cell Activation

Central to Lafferterian theory is a two signal model of T-cell activation. Foreign tissue antigen (alloantigen) is processed and presented on the surface of a stimulator or "antigen presenting" cell (APC), and is engaged by the responder T-cell receptor. This constitutes "signal 1". Also present on the surface of the APC are regulatory MHC antigens. Lafferty believes that it is the engagement of APC "non-self" MHC antigens by the responder T-cell, which triggers "signal 2", also known as "costimulator activity": the release of interleukin-1 by the APC. Thus, only after ligand binding (signal 1), has occurred in conjunction with release of an inductive molecule by the APC (signal 2), does responder T-cell activation with specific clonal effector cell expansion occur. It is assumed that this antigenic property of MHC antigen is distinct from that of alloantigen, as it has been suggested that MHC antigens by themselves, may only be weakly immunogenic (26).

It is now conceptually possible to propose mechanisms of responder or "host" T-cell activation by either donor (allogeneic) or host (syngeneic) APCs. If the APCs are of donor origin, MHC

incompatibility will facilitate costimulator activity by the APC and result in the initiation of an allograft response. This premise provides rationale for experimental depletion of APCs from donor tissue prior to transplantation, in an attempt to circumvent allograft rejection. Alternatively, if the APCs are host in origin, some modification of surface MHC antigen (perhaps through binding with alloantigen), is necessary to produce the requisite stimulator/responder incompatibility for the allograft response to occur. This mechanism has become known as the "Alternate Pathway of Alloantigen Presentation."

2) THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) AND ALLOANTIGEN PRESENTATION

The major histocompatibility complex (MHC), is that part of an organism's genome which encodes for the production of cell surface proteins called MHC antigens, which are believed to play a regulatory role in cell mediated immune responses. The MHC has been best characterized in the mouse, where there appear to be distinct histocompatibility loci located on virtually every chromosome. One of these loci designated H-2, exerts a particularly strong effect on allograft rejection, and is called a major histocompatibility locus while the others are referred to as minor histocompatibility loci. An important characteristic of the H-2 locus is the enormous genetic polymorphism that exists due to allelic diversity in outbred species, as well as the not infrequent chromosomal recombination that occurs during meiosis.

The availability of inbred mouse strains, alloantisera and monoclonal antibodies has permitted mapping of the MHC genes. The MHC is divided into two major subclasses: (a) the classically defined H-2 complex and (b) the T1a complex. The H-2 complex contains four regions: K,I,S and D, while the T1a complex contains three regions: Qa-2,3, T1a and Qa-1 (27, 28).

MHC antigens are divided into two classes: Class I and Class II. The class I molecules include the transplantation antigens K, D and L, and consist of a transmembrane glycoprotein which is noncovalently linked to a ß-2 microglobulin on the cell surface. These molecules

have five distinct regions: three globular domains above the cell membrane, one transmembrane domain and one intra-cytoplasmic domain. Carbohydrate residues are attached to the external domains. Class I MHC antigens are found on virtually every nucleated somatic cell and provide the essential context of self in which foreign cell surface antigens (such as those produced by a viral infection), can be recognized and destroyed by cytotoxic T-cells (designated CD8+ cells). This phenomenon, which results in direct activation and proliferation of a clone of effector cytotoxic T-cells is termed class I MHC-restricted alloantigen presentation.

The class II genes Aa, Aß, Ea and Eß are located in the I region (29). Class II molecules (also known as Ia for I region "associated"), consist of two polypeptide chains (a,ß) held together by non-covalent interactions. These molecules are also transmembrane globular glycoproteins with two external domains, one transmembrane domain and one intra-cytoplasmic domain. Class II antigens are found primarily on bone marrow derived lymphoreticular cells (activated B and T cells, macrophages and dendritic cells), as well as on vascular endothelium. These molecules provide self-recognition elements that allow macrophages and dendritic cells ("antigen presenting" or "accessory" cells) to interact, in the presence of processed, foreign antigen, with responder T-cells. The result is the generation of activated T-cells of the helper or CD4+ subset, which subsequently participate in the production of either antibody-secreting plasma cells or cytotoxic T-cells. This type of alloantigen presentation is termed class II MHC-restricted presentation.

These MHC molecules show structural homology with the immunoglobulin receptor of the B-cell, the T-cell receptor and the thy-1 molecule (T-cell differentiation antigen), expressed on the surface of mouse T cells. This homology suggests that the genes encoding these different molecules share a common ancestor, and that marked changes have occurred after divergence of the genes to fulfill different functions. These genes are referred to collectively as "the Supergene Family."

The MHCs of mice and other species differ fundamentally only in the organization of their genes and the descriptive nomenclature

(30). In rats, the MHC consists of four major class I loci and two class II loci, and carries an RT1 designate (31). The MHC of humans is referred to as the HLA complex and is located on the short arm of chromosome 6 (32). Human class I transplantation antigens are designated A, B, and C and the class II antigens are designated DP, DQ and DR.

3) ALLOGRAFT REJECTION: CURRENT CONCEPTS

3.1 The "Afferent Arc"

Host recognition of immunogenic determinants on allografted tissue initiates an immune response. The first phase of this response, called the "Afferent Arc", begins with an encounter between graft alloantigen (which may be present in blood, lymphoid tissue, or within the graft itself), and the appropriate host helper T-cell. Donor antigen presenting cells (APCs) called dendritic cells, carried passively with the graft are capable of self-processing alloantigen and can efficiently present antigen to host lymphoid cells. (33). Alternatively, alloantigen can be processed and presented by host APCs (34, 35), as is the usual case for antigen present on parenchymal cells of transplanted tissue. The mechanism by which an APC presents antigen to a responsive lymphocyte is unclear. Most evidence suggests that antigen is somehow modified by the APC prior to presentation (36). As suggested by Lafferty, if the APC is host in origin, antigen processing must impose an alteration in surface MHC antigens to facilitate a stimulator / responder incompatibility, which results in responder-cell activation.

The route of host sensitization depends on whether or not the graft is vascularized. With vascularized grafts, host cells within the blood compartment will be the first to encounter graft antigens, whereas in the case of skin grafts, cells within draining lymph nodes are first to contact antigen.

The most striking aspect of the immune response is its specificity. For each immune stimulus, a distinct population of antibodies or immune cells are elicited, which suggests that there must be specific antigen receptors (with structural similarity to immunoglobulin) on the surface of naive responder T-cells. How

these recognition molecules develop is unknown. The clonal selection theory suggests that clones of lymphocytes specific to an antigen probably arise by somatic mutation prior to antigen encounter. The subsequent encounter between antigen and specific, dedicated clone stimulates proliferation and maturation of that clone.

Clonal response to alloantigen can be either B or T cell in origin. Sensitized B cells proliferate and differentiate into plasma cells that actively secrete antibody, while sensitized T cells proliferate into a clone of T cells capable of inflicting damage to the graft by virtue of their close range. The activation of resting small lymphocytes of both types occurs in regional lymph nodes and the spleen.

3.2 Allograft Rejection: Effector Mechanisms

Early investigators studying the mechanism of graft rejection examined histologic sections, and the presence of a specific type of cell was taken as evidence of its role in allograft rejection (37). The recent availibilty of monoclonal antibodies against specific T-cell subsets has facilitated identification of the relative proportion of cytotoxic/suppressor (CD8+) cells to helper/inducer (CD4+) cells in stable grafts, as well as those undergoing rejection.

The different types of rejection reactions have been best characterized in the kidney. Classic, acute rejection involves a lymphocytic infiltration of the renal interstitium and blood vessels. Small lymphocytes are seen in contact with peritubular capillary and venular endothelial cells within hours of transplantation. Within three days, large lymphocytes appear adjacent to the endothelial cells lining intertubular capillaries and venules, and endothelial injury becomes evident. The lymphocytes infiltrate diffusely throughout the interstitium, with progressive disruption of peritubular capillaries and venules and interstitial fluid accumulation, leading to a fall in renal blood flow and further cellular damage on an ischemic basis.

Hyperacute rejection is antibody mediated and is seen in recipients with antidonor antibodies at the time of transplantation. This is most likely to occur in humans when exposure to blood transfusions, pregnancy, or a previous transplant has induced the

formation of antibody to class I antigens. Preformed ABO antibodies can also result in hyperacute rejection of most incompatible organs, whereas antibodies to class II antigens do not (38). Typically, antibody binds specific antigen on the vascular endothelium, with resulting complement activation and massive intravascular activation (39). Biopsies of hyperacutely rejected kidneys show deposits of IgG and C3 on the glomerular and peritubular capillary walls, with luminal occlusion by platelet-fibrin aggregates.

Hyperacute rejection can also occur following transplantation between phylogenetically distant species, such as a kidney graft from a pig to a dog (40). Apparently the recipient has natural antibodies against the donor species without previous antigenic exposure.

Chronic low-grade rejection occurs in most allograft recipients and results in gradual loss of organ function over months or years. Histologically, this involves interstitial fibrosis and chronic vascular changes with arteriolar narrowing and thickening of capillary basement membranes caused by deposition of antibody and complement, with secondary fibrosis.

Cytolytic T-cells have always been considered to be the primary effectors in allograft rejection because of their demonstrable activity in vitro (41, 42). Anti-donor specific cytolytic cells have been retrieved from human renal, hepatic and cardiac allografts, as well as many animal transplant models (43, 44, 45).

Monoclonal antibodies directed against mouse T-cell subsets have been used to determine the relative contributions of these subsets to graft rejection. Cobold and Waldmann have found that when anti-L3T4 monoclonal antibody (directed against surface markers on helper cells), was administered early to skin grafted mice, significant graft prolongation was achieved, while anti-Lyt 2 antibody (directed against markers on cytolytic T-cells), had no effect. Both anti-L3T4 and anti-Lyt 2 significantly prolonged graft survival when administered later (46). These experiments support the central role of the T-helper cell early in the allograft response, and suggest that the cytolytic T-cell is more important later on.

Recent attention has been given to the role of Interleukin 2 (Il-2) receptor- bearing cells in allograft rejection. Administration of

monoclonal antibody against mouse Il-2 receptor significantly prolonged vascularized cardiac allograft survival in two separate H-2 incompatible strains (47), and similar experiments with comparable results have been reported in the rat (48). The Il-2 receptor is expressed on all activated T-cells, and the production of Il-2 (which is a lymphokine mediator of T-cell activity) by activated T helper cells (49), suggests a central role for the T helper cell as the initiator of cytolysis.

Cell killing in allograft rejection can be accomplished by a specific cytolytic effector clone or none-specifically, through the release of a variety of inflammatory mediators by activated helper cells, resulting in a Delayed-Type Hypersensitivity (DTH), reaction. Support for the latter is provided by studies which have shown that T-cell deprived rats can reject skin, heart or renal allografts when reconstituted solely with helper T-cells (50, 51). The identification of rat lymphotoxin in rejecting rat renal allografts has led to its incrimination as the actual injurious agent in DTH reactions (52), while others have suggested that the cytotoxic effect of lymphotoxin is augmented in the presence of gamma-interferon secreted by stimulated helper cells (53). Since gamma-interferon also induces expression of class II antigens on parenchymal cells, lymphotoxin and gamma-interferon may have synergistic deleterious effects on transplanted tissue.

Tests of in vitro cytotoxicity against various tumor targets has shown that lymphoid cells from non-sensitized animals can be highly cytotoxic to certain targets (54) . This activity shows no evidence of target cell specificity or memory, and has been attributed to the activity of NK cells. NK cells are nonadherent, nonphagocytic and do not express surface immunoglobulin; nor is their interaction with other cells under MHC restriction. Any stimulation of an animal's immune system seems to result in an increase in NK activity, likely through lymphokine (gamma interferon) release. Since NK cells will only lyse a limited range of target cells, their in vivo significance remains unclear.

Antibody-mediated allograft damage, apart from hyperacute rejection is of uncertain significance, although it has been identified

in a number of models (55, 56). The effect exerted by the antibodies involves a number of non-specific pathways, including complement, antibody-dependent cytotoxicity, clotting and generation of kinins and chemotactic factors.

4) IMMUNOLOGIC TOLERANCE

4.1 Passive and Active Tolerance

Tolerance is any specifically altered state of reactivity that results in the failure of the animal to express an immune response to the tolerizing antigen, while leaving responses to unrelated antigens intact. Burnet's clonal selection theory postulated that tolerance to "self" antigens occurs during the development of the immune system, as a result of deletion of self-reactive clones (57). This theory is supported by neonatally induced, (passive) transplantation tolerance, in which induction of tolerance in strain A mice results from neonatal injection of (A x B) F1 bone marrow. Adult mice treated in this way will accept skin grafts from strain B mice, but reject skin grafts from third party animals in a normal manner. Similarly lymphoid cells from such tolerized animals will not respond to strain B cells in a mixed lymphocyte reaction, yet respond normally to third party cells. An identical form of tolerance occurs when adult animals are lethally irradiated, and then reconstituted with F1 bone marrow; so-called "Radiation Chimeras." Tolerance of this form cannot be transferred from one animal to another, and is most likely due to deletion of a clone of responder T-cells; a form of "Passive Tolerance," (58).

A second form of tolerance can be induced by exposure of the immune system to soluble antigen, either during neonatal life or, in some cases following appropriate antigen administration to the adult animal. Neonatal tolerance is maintained as long as the tolerizing antigen, which is mistakenly identified as self, is present. Tolerance to that antigen can be reversed by its withdrawal, suggesting a clonal deletion mechanism. Tolerance in adult animals can be induced by injection of very low or very high doses of soluble antigen. This mechanism of tolerance induction is referred to as "active" tolerance

and involves T-suppressor cells, and can be transferred to a naive animal by T-lymphocytes from a tolerant donor (59).

The exact mechanism of T-suppressor cell induction and function is unclear. It has been postulated that induction involves an MHC-restricted cellular interaction between a naive suppressor cell and an antigen-activated accessory cell (APC), or T-helper cell. The result is an activated "effector" T-suppressor cell which acts directly, or via secreted suppressor proteins to suppress antigen-specific immune responsiveness (60).

It should be recalled that there are other ways of suppressing the immune response, one being the presence of antigen specific antibody, which removes antigen and thereby diminishes host reactivity, in both specific and non-specific (DTH) immune responses.

4.2 Induction of Allograft Tolerance in Adult Animals

A successful allograft of tissue which has undergone pretransplant modification to remove donor accessory cells, will undergo prompt rejection when the recipient is actively immunized with donor accessory cells. However with time, the graft enters into a stable interaction with the host and can no longer be rejected by active immunization of the recipient. This stable graft-host interaction results from the induction of a state of specific altered immune reactivity ("allograft tolerance"), that allows acceptance of a graft that would otherwise be rejected.

The mechanism of this tolerant state is unknown, but could involve either passive (clonal deletion), or active (suppression) mechanisms alone, or in combination (61). The kinetics of spontaneous graft stabilization vary considerably according to the tissue studied; thyroid allografts stabilize more slowly than islet allografts (350 days and 120 days, respectively), (61).

Graft stabilization could conceivably occur in one of two ways. Either there is some adaptation of the graft such as loss of antigenicity, or the reactivity of the host is altered. The latter hypothesis is supported by the observation that retransplanted (cultured) thyroid allografts from spontaneously stable animals into naive recipients, are promptly rejected upon host immunization with

"original" donor accessory cells. This illustrates that the long-established, cultured allograft still demonstrates antigenicity in a non-adapted host.

This form of specific tolerance has been demonstrated in animals carrying both stable islet and thyroid allografts (61). These animals accept a second, uncultured graft of donor type, but reject a third-party graft transplanted at the same time. The acceptance of a graft that would otherwise be rejected and the specificity of the graft acceptance reflect the *specific* state of tolerance that has been induced in the recipient of a cultured graft. Although tolerant animals are hyporesponsive in vivo they retain normal mixed leukocyte reactivity in vitro, suggesting that a clonal deletion mechanism is not responsible for tolerance induction under these circumstances (61). Some active mechanism must be inhibiting in vivo graft rejection.

5) EXPERIMENTAL REDUCTION OF GRAFT IMMUNOGENICITY

5.1 Thyroid Transplantation Studies

The concept of organ graft pretreatment in an effort to modulate its immunogenicity and prevent rejection has found its major experimental success in endocrine transplantation, due largely to the fact that endocrine grafts do not require immediate vascularization for continued survival.

The initial reports of prolonged allograft survival after a period of pre transplant culture came from Lafferty, who maintained Balb/c mouse thyroid lobes in 95% O2, culture for variable periods, and then transplanted them under the renal capsule of H-2 incompatible, non-immunosuppressed $C_{57}B_1$ recipient mice (62, 63). Graft function was followed by measuring the level of ^{125}I uptake by directed scintillation counting over the graft. These initial experiments clearly showed that thyroid tissue maintained in organ culture prior to transplantation survived far longer in an allogeneic host than did non-cultured tissue. Additional experiments by Lafferty showed that brief pretreatment of the host with cyclophosphamide prior to thyroid allografting allowed a significant reduction in the period of

organ culture (from four weeks to one), required to effect prolonged allograft survival (25).

Lafferty concluded that culture conditions of high oxygen concentration were selectively toxic to the vascular bed and to lymphoreticular elements of the graft, and proposed that organ culture may have removed from the graft those cells capable of providing an allogeneic stimulus (64). Sollinger and associates complemented the findings of Lafferty with the discovery that addition of high oxygen tension to the conditions of culture resulted in markedly prolonged thyroid xenograft survival without host immunosuppression (65).

Further support for the concept of alloengraftment facilitated by culture depletion of donor lymphoreticular elements was provided by Talmadge and colleagues (66), who demonstrated that injection of only 1000 donor-type peritoneal cells into a mouse host carrying a cultured thyroid graft restored its immunogenicity, and led to prompt allograft rejection.

5.2 Pancreatic Islet Transplantation: Early Experience with In Vitro Culture

After success with prolonged mouse and rat parathyroid allograft survival after pretransplant culture under conditions of high oxygen tension was reported (25, 67), many investigators turned their attention to the possibility of transplantation of pancreatic islets with a view towards eventual application to the treatment of diabetes mellitus.

Kedinger et al, reported prolonged recipient survival, with biochemical evidence of graft function after transplantation of 4 day cultured rat islets directly into the liver of recipients rendered glucose intolerant by treatment with IV streptozotocin (68). One of the early problems with culture of islet tissue was the apparent sensitivity of the parenchymal tissue to conditions of culture (most notably high oxygen concentrations), and the loss of endocrine function. To reduce islet oxygen toxicity, Bowen and colleagues (69), cultured mouse pancreatic islets in clusters of approximately 50 islets (so-called "megaislets"), and reported prolonged, functional

allograft survival. Lacy's group (70), introduced a technique of collagenase digestion of rat pancreas, with hand-picking of individual islets which were maintained in 7 day, high oxygen culture prior to transplantation as xenografts into mice. The addition of a single dose of anti-rat lymphocyte serum to the hosts prior to transplantation resulted in a significant prolongation of xenograft survival.

5.3 Temperature as a Variable in Pancreatic Islet Culture

The discovery that lymphocytes which were cultured at low temperature (22C), lost their ability to stimulate in an MLR, but retained their ability to respond to non-cultured allogeneic lymphocytes, suggested that low temperature culture adversely affects the immunogenicity of lymphoreticular cells (71). With this information, Lacy and his colleagues (72), performed allogeneic rat islet transplants following 7 day culture at 24C in room air, and demonstrated 85% graft survival beyond 100 days. Thus, it appeared that low temperature had the same deleterious effect on the lymphoreticular elements in the islet cultures, as high oxygen concentration, but spared the parenchymal cells. Lacy also proved that the endocrine cells retained their antigenicity, by inducing acute rejection of functioning, tolerated allografts by the injection of donor peritoneal exudate cells (73).

5.4 In Vitro Use of Class II MHC Antisera

With the advent of monoclonal technology, attempts were made to eliminate immunogenic lymphoreticular cells from organ allografts with sera directed against class II MHC molecules. Faustman and colleagues demonstrated that class II MHC molecules were not expressed on the surface of mouse pancreatic B cells, but rather were on the passenger leukocytes present within the donor tissue, termed "dendritic" cells (74, 75). Faustman then showed that anti-class II (anti-dendritic cell) antibody and complement treatment of donor mouse pancreatic islets resulted in 100% survival of mouse islet allografts for more than 200 days, following transplantation across a major histocompatibility barrier(76). Gores and colleagues attempted to reproduce these findings, but could not, and instead

described an elegant in vitro, mixed islet-lymphocyte coculture model which demonstrated that in addition to donor cells, recipient cells and even third party antigen presenting cells were capable of alloantigen presentation. (77)

Another application of class II antisera to the elimination of Ia bearing antigen presenting cells has been the recent development of "immunotoxins", which are highly toxic proteins, such as ricin or diptheria toxin, that have been covalently coupled to monoclonal antibodies. Shizuru et al (78), have shown that pretreatment of islets with an anti-Ia monoclonal antibody covalently conjugated to purified ricin toxin, results in the elimination of the allostimulatory properties of islets in mixed lymphocyte islet cell cultures (as assessed by proliferative indices of responder lymphocytes), without damage to the hormone secreting cells.

5.5 Donor Tissue Irradiation

The rationale for UV irradiation of donor tissue was the recognition of the importance of passenger leukocytes (specifically dendritic cells), in the allograft response, and evidence that these cells were exquisitely sensitive to UV light inactivation. Hardy and colleagues irradiated isolated Lewis rat islets, "rested" them in tissue culture for 24 hrs and then transplanted them into glucose intolerant, immunocompetent ACI hosts. With this treatment regime, he found that irradiated islet allografts corrected hyperglycemia for longer than one year in over 70% of recipients. Furthermore, he was subsequently able to induce rejection by the administration of donor-type rat dendritic cells (79).

RATIONALE:

As outlined above, there is already a substantial body of experimental evidence which suggests that the donor antigen presenting cell (APC) plays an important role in the initiation and regulation of allograft rejection. The recent availability of inbred small animal strains and monoclonal antibodies has permitted mapping of the major histocompatibility complex (MHC), and identification of its gene products -called MHC antigens, on cell surfaces. APCs are composed of a number of bone marrow derived lymphoreticular cells (activated B and T cells, macrophages and dendritic cells), which are characterized by their unique expression of class II MHC (also known as Ia) antigen; consequently, selective cell surface enhancement seen on flourescent microscopy after treatment with fluorescein-labelled anti-Ia antibody is now synonomous with the presence of antigen presenting cells.

As a caveat, one might infer that the inability to demonstrate selective cellular fluorescence with anti-Ia immunofluorescent techniques is evidence of the absence of APCs within the tissue specimen. This rationale allows objective evaluation of donor tissue immunomodulation in which the aim of the treatment is depletion or complete elimination of APCs from the donor tissue.

In Vitro Donor Islet Culture:

The immunomodulatory effects of in vitro islet culture may be assessed in two ways. First, culture depletion of isolated rat islet APCs can be evaluated by fluorescent anti-Ia islet staining after increasing periods of in vitro culture. In this manner, one might determine the "critical period" of culture required to "remove" all islet APCs. Next, fresh and cultured islets are transplanted into allogeneic hosts, and the grafts are subsequently evaluated for evidence of engraftment versus rejection. If cultured islets are shown to engraft preferentially over non-cultured controls (as assessed by histologic criteria), one might infer that pre-transplant, in vitro culture imposes changes in the donor tissue that facilitates allotransplantation. If the duration of in vitro culture necessary for successful allotransplantation is comparable to that necessary for

islet APC depletion, this would provide strong supportive evidence for the role of the "donor" APC as the sole mediator of the allograft rejection response.

Elimination of Donor Islet APCs by Photodynamic Therapy (PDT):

Benzoporphyrin derivative monoacid ring A (BPD-MA), is a tetrapyrrole ring with photosensitizing properties. When the molecule is stimulated by light in the ultraviolet range (600nm), singlet oxygen is released causing destabilization and lipid peroxidation of nearby cell membranes, resulting in cell lysis and death. If the BPD molecule could be conjugated to an anti-Ia monoclonal antibody, then theoretically, isolated islets can be "purged" of donor APCs by incubation with the BPD-anti-Ia conjugate, followed by photoactivation of BPD with ultraviolet light. If the allograft response were mediated solely by donor APCs, this in vitro technique could be performed on isolated islets and may then allow successful islet allotransplantation.

PURPOSE:

The purpose of this thesis is to evaluate two techniques of in vitro immunomodulation of rat pancreatic islets in an allogeneic transplant model: 1) donor islet culture and 2) donor islet APC ablation by Photodynamic therapy (PDT). Finally, based on the results, an assessment of the role of the donor APC in the allograft response will be made.

MATERIALS AND METHODS:

1) ANIMALS:

Two inbred strains of rats differing at both major and minor loci of the major histocompatibility complex were used. Sprague Dawley (SD) rats (RT1u), were utilized as pancreatic islet donors, and Wistar Furth (WF) rats (RT1a), as transplant recipients.

2) ISLET HARVEST TECHNIQUE:

Male SD rats (200-250 gm), were anaesthetized with intraperitoneal urethane (100 mg/kg), and through a midline laparotomy, cardiorespiratory arrest was induced with bilateral pneumothoraces. The proximal common bile duct was cannulated with a fine polyethylene catheter and the duct was occluded distally, at the ampulla of Vater. The pancreas was then distended in a retrograde fashion with cold (4 C) collagenase (Type XI, Sigma Chemicals) in sterile Hanks' balanced salt solution (HBSS), at a concentration of 0.42 mg (650 U) per ml. After in situ collagenase distension, a total pancreatectomy was performed. The glands were digested for 22 minutes in a 37 C waterbath, and then the digestion process was terminated by the addition of sterile, cold HBSS. The digested glands were dispersed by trituration through a sterile, siliconized pipette. The crude tissue slurry was passed through a 200 μm screen filter to remove undigested ducts, blood vessels and lymph nodes, and was then centrifuged through a discontinuous dextran (Sigma Chemicals) gradient consisting of two monolayers of specific gravity 1.065 and 1.031 respectively. The less dense islet tissue was then aspirated from the monolayer interface, washed with sterile HBSS, and then further purified by hand picking under a dissecting microscope. Using this technique, 200-400 morphologically intact islets were isolated per pancreas.

3) ISLET CULTURE TECHNIQUE:

After isolation, islets were either used immediately or subjected to in vitro culture (prior to subsequent use), for 4-21 days in Ham's F-12 medium (Gibco, [glucose] =11mM), supplemented with

25% fetal calf serum, 15 mM Hepes buffer and 1%pen/strep/ fungizone, in a 5% CO_2/room air incubator at 37 C. The islet suspensions were agitated daily with a Pasteur pipette to prevent islet clumping, and the media was changed weekly for those islets cultured longer than 10 days.

4) ISLET APC DEPLETION BY PHOTODYNAMIC THERAPY (PDT), USING A BPD-MA -ANTIBODY CONJUGATE:

4.1 Antibody Conjugation Protocol:

Benzoporphyrin derivative monoacid ring A (BPD-MA, to be subsequently abbreviated as BPD), was produced by Quadra Logic Technologies, Vancouver, B.C, and was stored frozen as a stock solution in dimethylsulfoxide (DMSO), at 1 mg/ml. Immediately prior to conjugation, the stock solution was diluted to 200 μg/ml in sterile phosphate-buffered saline (PBS), then was mixed with a known quantity of monoclonal antibody. For pilot experiments, BPD was conjugated directly to mouse anti-rat Ia (OX-6), however subsequent experiments demonstrated better photoablation of APCs when BPD was conjugated to a secondary antibody (Rat anti-mouse Ig = RAMIg).

The affinity between BPD and antibody is a non-covalent association between hydrophobic moieties of the 2 molecules, and relies on an aqueous milieu to maintain "binding." All conjugations were carried out in low light conditions to avoid photoactivation of BPD.

Two specific RAMIg-BPD conjugates were prepared: one with a calculated BPD : Ab, molecular ratio of 15:1, and the other with a calculated ratio of 40:1. (Molecular weights: BPD-MA = 718 g/mole, antibody ≈ 150,000 g/mole). These molecular ratios were selected on the basis of previously performed photoablative experiments using a chronic granulocytic leukemia (CGL) cell line, in the laboratory of Dr J Levy (Dept of Microbiology, UBC). In addition, conjugates of BPD to an "irrelevant" monoclonal antibody (goat anti-7s ribosomal protein Ig = GA7sIg), were prepared at the same two relative molecular ratios to serve as controls. The antibody-BPD

conjugates were allowed to incubate for 1 hr at room temperature after mixing, and were then dialyzed for 36 hours in eppendorf tubes (covered with a dialysis membrane impermeable to molecules of molecular weight > 14,000 g/mole), against 3 litres of PBS with a stir bar, at 4C. In this manner, any unbound BPD would be dialysed away on the basis its size relative to the dialysis membrane pores.

4.2 Assessment of RAMIg Retention of BPD after Dialysis using ^{14}C labelled BPD:

Liquid scintillation counting of ^{14}C-BPD in a known quantity of dialysed conjugate was carried out to determine the actual antibody : BPD molecular ratio in the conjugate following the 36 hour dialysis period. RAMIg-BPD conjugation was carried out as described above using ^{14}C-labelled BPD with a known specific activity of 134 disintegrations per minute (dpm) per nanogram (ng). After dialysis, 0.1 ml samples of each conjugate (with **calculated** molecular ratios of 15:1 and 40:1, respectively), were mixed with 5 ml of aquasol (scintillation liquid), and each sample was counted in triplicate over a 5 minute period using a scintillation counter (Phillips Instruments). From the total number of counts, the amount of 14C-labelled BPD (in ng), in the aliquot of dialysed conjugate could be calculated using the formula:

$$\frac{\text{total \# of counts (dpm)}}{\text{activity of BPD-MA (134 dpm/ng)}} = \text{amount of 14C-BPD-MA (ng)}$$

Knowledge of the quantity of RAMIg in the conjugate aliquot thus allowed calculation of the **actual** molecular ratio of BPD to antibody after dialysis. The calculated (pre-dialysis) and actual (post-dialysis) molecular ratios of the two RAMIg-BPD conjugates, are shown in Table 1.

Table 1:

Pre-dialysis Molecular Ratio (RAMIg : BPD)	Post-dialysis Molecular Ratio (^{14}C-BPD)	% BPD Retained
1 : 40	1 : 18	45%
1 : 15	1 : 6.5	43%

4.3 Islet Treatment with BPD-antibody conjugate:

Sprague Dawley islets were isolated in the usual fashion and subjected to overnight culture in complete Ham's F12 medium at 37C in a 5% CO2, room air incubator. The following morning the islets were resuspended and deposited in 96 well Costar plates (200 µl/well), at a density of approximately 50-75 islets per well. Using a micropipette with the assistance of the dissecting microscope, the islets were washed under direct vision by 2 complete volume exchanges with sterile PBS, to remove extraneous protein. The islets were then resuspended in the 200 µl wells with 11mM Ham's F-12 + 1% pen/strep/fungizone, (no fetal calf serum), and incubation with primary antibody was carried out.

The primary incubation was performed using purified OX-6 Ig obtained from an ammonium sulfate-cut, ascites preparation (Dr R McMaster; Dept of Microbiology, University of B.C.). Using a light spectrophotometer the absorbance of the purified OX-6 Ig was measured, and the protein (antibody) concentration was calculated to be 20.25 mg/ml. The isolated islets were then incubated with purified OX-6 Ig (at a concentration of 0.2 mg/ml) in 200 µl wells for 2 hours at room temperature. After the primary incubation, the islets were again washed by 2 complete volume exchanges with sterile PBS.

All secondary incubations were carried out in duplicate, and in low light conditions. The islets were incubated with each of two RAMIg-BPD conjugates of different relative molecular ratios. In addition, the islets were also incubated with the "irrelevant" conjugate, with BPD alone, and with media alone, as controls. The secondary incubations were carried out for 2 hours, at room temperature. The primary and secondary islet-antibody incubations are summarized in Table 2:

Table 2:

1 ° Incubation (2 hr)	**2 ° Incubation** (2 hr)
OX-6 Ig (0.2 mg/ml)	RAMIg-BPD (1 : 6.5)
"	RAMIg-BPD (1 : 18)
"	GA7s Ig-BPD
"	BPD alone
"	11mM Ham's F-12 media alone

4.4 Photoactivation of BPD-bound Islets:

Upon completion of the secondary incubation, the islets were again washed with sterile PBS and resuspended in 11 mM glucose Ham's F-12 for light exposure. The light source was a bank of four fluorescent tubes (General Electric F20T12- cool white), and the spectrum of light emitted ranged from 300-750 nm. The intensity of the light was measured by a YS1- Kettering Model 65 RAdiometer and was 1.5 milliwatts per cm^2. The islets were exposed for 1 hour at a distance of 11.0 cm from the light source, where temperatures were measured and did not exceed 25 C. After the photoactivation period, the islets were washed, resuspended in complete Ham's F-12 media and cultured overnight. The following morning, immunocytochemistry was performed on the treated islets to assess APC depletion.

5) EVALUATION OF APC DEPLETION BY IMMUNOCYTOCHEMISTRY:

Immunocytochemistry was used to visualize Ia+ cells in cryostat sections of fresh, cultured and BPD-treated islets. After a thorough washing with HBSS to remove extraneous protein, 75-100 islets were deposited in polypropylene cassettes containing OCT medium, and were snap frozen with liquid N_2. The frozen cell block was mounted in a cryostat microtome (-20 C), and 6 μm islet sections were cut and mounted on glass slides. The cryostat sections were then fixed with dilute acetic acid and air dried, prior to rehydration with PBS and application of antibody. A 2-layer technique of antibody staining was used to visualize Ia+ cells: first a (mouse) anti-rat Ia MAb (OX-6 Seralab, 3.3 μg/ml) was applied and allowed to incubate with the cryostat sections for 1 hr at room temperature, or overnight at 4 C. After washing off excess primary antibody, a fluorescein indothiocyanate (FITC) labelled (goat) anti-mouse secondary Mab (Jackson Laboratories, 6.7 μg/ml) was applied and allowed to incubate for 1 hr at room temperature. After washing off unbound secondary antibody, 10% glycerol and a coverslip were applied to the islet sections, which were then examined in the dark using a fluorescent microscope (Zeiss instruments). With this technique, 1-5 APCs could be identified within each fresh islet section, by their enhanced fluorescent surface staining and characteristic dendritic morphology. APC depletion was evaluated qualitatively by a relative absence of fluorescent cell enhancement compared to fresh islet controls.

6) TRANSPLANT HISTOLOGY STUDIES:

Approximately 100 fresh, cultured or BPD-treated SD islets were transplanted under the renal capsule of a WF recipient using a micropipette. Twelve days later a recipient nephrectomy was performed, and the grafts were recovered, Bouins fixed, paraffin imbedded and stained with hematoxylin and eosin, then examined microscopically by a blinded observer, to determine whether engraftment or rejection had occurred. Rejection was determined histologically by numerical grading of endocrine cell integrity ($\leq$+1 on

a scale of 0 to +4) and inflammatory cell infiltration (≥ +3 on a scale of +1 to +4), and by the absence of graft neovascularization.

7) CULTURED ISLET ALLOTRANSPLANTATION OF STREPTOZOTOCIN-DIABETIC WF RECIPIENTS:

These experiments were performed with culture-modulated islets only. WF recipients were rendered glucose intolerant by intravenous treatment with streptozotocin, 75 mg/kg, and maintained without insulin for a minimum of three weeks prior to allotransplantation, to insure that native ß cell function and normoglycemia, would not return. Hyperglycemic recipients were then transplanted with approximately 1000 fresh or cultured SD islets via the portal venous system. Under ether anaesthesia, a recipient laparotomy was carried out, and with the aid of the dissecting microscope, vascular control was obtained on a cecal mesenteric vein. A fine polyethylene catheter was used to cannulate this vein, and a washed islet suspension in sterile HBSS was gently injected. Transplant recipients were treated with a single intraperitoneal dose of long acting insulin (9U/kg), for 4 days, and then plasma glucose determinations were commenced on Day 6, using a glucose analyser (Beckman II, Beckman Instruments). Allograft rejection was defined by consecutive plasma glucose determinations greater than 400 mg/dl.

RESULTS:

1) ISLET IMMUNOMODULATION BY TISSUE CULTURE:

1.1 APC Depletion Experiments:

Islet APC depletion experiments were performed to determine the duration of culture necessary to remove Ia positive cells from SD rat islets as detected by indirect fluorescent immunostaining. Cryostat sections of freshly isolated rat islets were observed to contain between one and five Ia+ cells per section. With increasing culture periods of four and seven days, a decrease in the number of staining cells per section was observed (Figure 1). After a minimum of 10 days in culture, Ia positive cells could no longer be demonstrated in islet cryostat sections (Figure 2).

1.2 Islet Allograft Histology Studies:

Islet allograft histology studies were carried out to determine the duration of pre-transplant culture necessary to allow consistently successful islet allografting. Islet allografts cultured for periods of up to 10 days prior to transplantation were uniformly rejected (Figure 3). Four of 10 allografts cultured for 14 days prior to transplantation demonstrated histologic engraftment, as did 4 of 5 allografts cultured for 21 days (Figure 4). Allograft endocrine viability was demonstrable by immunoperoxidase staining for insulin (see Figure 5). Table 3 summarizes the APC depletion and allograft histology studies.

Table 3:

	Duration of Tissue Culture (days)					
APC Depletion:	0	4	7	10	14	21
Presence of Ia+ cells	yes	yes	yes	no	no	no
Allograft Histology:						
Islet Integrity	0	0	0	0	+4	+4
Mononuclear Infiltrate	+4	+4	+4	+4	+1	+1
Neovascularization	no	no	no	no	yes	yes
# of Animals	5/5	5/5	5/5	5/5	4/10	4/5

1.3 In Vivo Allograft Function:

Based on the finding that islet allografting was consistently, histologically successful when islets were cultured for 21 days prior to transplantation, in vivo studies were carried out on streptozotocin treated, hyperglycemic but otherwise immune-competent WF recipients of fresh, 14 day and 21 day cultured SD islets. Pilot in vivo studies involved transplantation of islets under the renal capsule of the hyperglycemic recipient in an attempt to match the allograft histology studies, however it was apparent that the volume of islets required to effect euglycemia initially, could not undergo satisfactory revascularization at this location. Therefore, allografting of streptozotocin induced hyperglycemic recipients was carried out through the portal venous system, which allowed the islets to settle in the liver sinusoids, where they could easily acquire microvascularization.

Figure 6 shows the typical appearance of in vivo allograft rejection, after transplantation with uncultured islets. There is evidence of early graft function between day 6 to 10 post-transplant, followed by persistent hyperglycemia heralding graft rejection.

Figure 7 depicts allotransplantation with 14 day cultured islets, and demonstrates a similar phenomenon of graft rejection after early graft function.

In contrast, Figure 8 demonstrates 30 day function of 21 day cultured islet allograts in streptozotocin-rendered hyperglycemic recipients.

2) ISLET IMMUNOMODULATION BY PHOTODYNAMIC THERAPY (PDT):

2.1 APC Depletion Experiments:

These experiments demonstrated selective depletion of APCs in photoactivated islets which had been treated with the specific secondary RAMIg-BPD conjugate, at two relative molecular ratios (Figure 9). In contrast, islets treated with either an irrelevant secondary antibody conjugate (GA7sIg-BPD), BPD alone or media alone prior to photoactivation, all demonstrated preservation of islet APCs (Figure 10).

2.2 Transplant Histology Results:

Syngeneic (3) and allogeneic (4) renal subcapsular transplants were carried out using islets which had been treated by anti-class II-specific PDT. Subsequent histologic evaluation of both syngeneic and allogeneic grafts revealed complete replacement of graft by lymphocytic infiltrate without identifiable endocrine tissue.

DISCUSSION:

The aim of this thesis was to test Snell's "passenger leukocyte" theory, using a rodent pancreatic islet allograft model. According to this hypothesis, allograft rejection is mediated by specialized donor lymphoid cells called "antigen presenting cells" (APCs), which are carried passively into the host with the transplanted tissue. Antigen presenting cells consist of activated B and T lymphocytes, dendritic cells of Langerhans, macrophages and in some cases, capillary endothelial cells, and are characterized by their unique expression of class II MHC (Ia) antigen. Within the immune-competent allograft recipient, APCs process and present foreign tissue antigen or "alloantigen" to histoincompatible responder lymphoid cells, thus initiating the allograft response. The MHC disparity that exists between donor APCs and responder (host) T cells provides the stimulus for this antigen-specific clonal immune response; accordingly, the removal of APCs from the donor tissue prior to allotransplantation should prevent host recognition of alloantigen. Furthermore a permissive host environment should exist indefinitely, provided no donor-identical APCs are introduced. We sought to test this hypothesis using two in vitro techniques of donor APC depletion: 1) in vitro culture, and 2) monoclonal antibody-directed photodynamic therapy.

Isolated rat islets were subjected to variable periods of in vitro culture at specified conditions, then were examined with immunocytochemistry to determine the duration of culture necessary to deplete APCs. Although the technique used to evaluate APC depletion was qualitative, a clear trend of progressive APC depletion with prolonged culture was observed. Specifically, we found that a culture period of 7-10 days was required for APC removal from islet cryostat sections. The mechanism of APC depletion by tissue culture is thought to be related to the sensitivity of lymphoid cells to local culture conditions including pO2, pH and temperature.

After determining that a 7-10 day period of culture was necessary to deplete islet APCs, allogeneic transplants of fresh and

cultured Sprague Dawley (RT1u) islets were placed under the renal capsule of immune-competent Wistar Furth (RT1a) recipients. Twelve days after transplantation, the allografts were removed and histologic studies by a blinded observer employing strict criteria (degree of mononuclear infiltrate, endocrine cell integrity and graft neovascularization) were performed to determine whether rejection had occurred. Utilizing this technique, we found that allografts transplanted either fresh or after periods of culture of 4, 7 and 10 days were all rejected. In contrast, 4 of 10, 14 day cultured islet allografts, and 4 of 5, 21 day cultured allografts demonstrated engraftment. One explanation for the apparent disparity in culture period necessary to deplete islet APCs versus that required for successful allografting is based on the observation that class II MHC antigen expression is an inducible phenomenon (80). During the first 7-10 days of culture, expression of class II MHC antigen by the APC may be progressively down-regulated until antigen expression is below the limit of detection by immunocytochemistry. This could be due to an absence of lymphokine- (for example, gamma-interferon) supported or stimulated class II MHC antigen expression by APCs in culture. Thus, one could hypothesize that a culture "window" exists during which APCs are viable but cannot be detected by immunocytochemistry because they have ceased to express class II MHC antigen. If the islets remain in culture for longer periods (21 days), the APCs die as a result of their relative sensitivity to the conditions of culture (pH, pO_2, temperature) compared to endocrine cells. Allogeneic transplantation of cultured islets during this "window" period could result in host-lymphokine induced re-expression of class II MHC antigen by donor APCs, which permits a donor APC mediated allograft reaction to occur. Alternatively, one can hypothesize that elimination of donor APCs is not by itself, responsible for prevention of the allograft reaction; therefore in vitro culture must impose other changes on tissue (perhaps alteration of alloantigen or irreversible inhibition of APC function), which permit prolonged allograft survival after a requisite period of pre-transplant culture. Recent investigations have demonstrated a potential role for islet treatment regimes which alter or block donor

potential role for islet treatment regimes which alter or block donor specific MHC class I antigen. Stock et al (81) demonstrated that whole mouse islet pretreatment with anti-class I monoclonal antibody blocked the generation of allo-specific cytotoxicity against target cells following a mixed islet-lymphocyte co-culture period. This permits speculation that culture specific immunomodulation may occur through depletion or alteration of class I MHC antigen, which may also explain the observed disparity in culture time required to deplete APCs versus that necessary for successful allotransplantation.

Of perhaps greatest significance was the demonstration that islets were capable of in vivo function for at least 30 days when transplanted into non-immunosuppressed, allogeneic recipients following 21 days of in vitro culture. This was shown in both WF recipients of 21 day-cultured SD islets that had been rendered glucose intolerant by treatment with streptozotocin. In contrast, islet allografts cultured for 14 days prior to transplant (2 animals), or without any period of pre-transplant culture (1 animal) were all rejected. These transplant recipients demonstrated persistent hyperglycemia following a brief (7-10 day) period of graft function manifest by euglycemia, which correlates temporally with the period of time necessary to mount an ablative immune response.

The implications of these experiments are significant: using an in vitro islet culture technique of immunomodulation in a rodent model, we have demonstrated both histologically and functionally successful allogeneic transplantation across a major MHC barrier, without host immunosuppression.

The second set of experiments were done to test photodynamic therapy (PDT), as a possible immunomodulatory modality in islet transplantation. PDT has not yet found experimental application in transplantation, though its utility in the treatment of malignancy, namely tumors of the bladder, esophagus and bronchus is currently being evaluated in phase III comparative, controlled clinical trials (82). The success of PDT relies on the delivery of the porphyrin molecule to the target cell (in this case, the islet APC), by a specific carrier monoclonal antibody. Once bound to its cellular target, the

porphyrin molecule is photoactivated by UV light causing emission of singlet oxygen which results in lipid peroxidation, cell membrane distruption and ultimately cell death.

Our technique of APC photoablation by PDT required a primary incubation of islets with OX-6 (an anti-class II MHC monoclonal) followed by incubation of a specific secondary monoclonal antibody (RAMIg) which had been conjugated to BPD. After photoactivation, the islets were cryostat sectioned and immunostained for class II MHC + cells. The depletion of APCs by this technique was specific, and seen only in islets which had been exposed to the specific secondary antibody-BPD conjugate prior to photoactivation. In contrast, islets treated with an irrelevant porphyrin-antibody conjugate, or porphyrin alone showed relative APC preservation, at least as detected by fluorescent immunostaining. When specifically treated islets were transplanted under the renal capsules of immune-competent syngeneic and allogeneic hosts (3 and 4 transplants, respectively), and the grafts removed at 12 days, there was histologic evidence of both acute and chronic inflammation without signs of graft survival. The failure of even syngeneic grafts permits speculation that perhaps the rigors of photodynamic therapy injured the islets irreversibly, so that the histologic appearance of the harvested grafts represented a non-specific inflammatory response to necrotic tissue, rather than a specific immune response. This hypothesis seems the most probable, given the altered fluorescent microscopic appearance of BPD-treated islets compared to their fresh and cultured counterparts (compare figures 1 and 2 with figures 9 and 10). Freshly isolated islets and those maintained viably in tissue culture have a typical "honeycomb" appearance, based on a faint, fluorescent outline of individual endocrine cells. In contrast, islets undergoing photodynamic therapy with BPD did not demonstrate the same individual cell preservation when examined with fluorescent microscopy, suggesting a non-specific cellular injury. It is quite likely that further experiments such as immunostaining of treated islets for insulin production would help to clarify the viability and functional status of the endocrine cells after PDT. On the other hand, based on our own experience with failed syngeneic

transplantation of freshly isolated Wistar Furth islets into immune-competent recipients, and through personal communications (83), there is sufficient evidence to suggest that the Wistar Furth rat strain is not highly inbred. It is therefore possible that minor MHC incompatibilities exist within the strain which are responsible for apparent "syngeneic" graft rejection, and that the rejection of all three "isografts", (as well as all four allografts) following islet treatment by MHC class II specific PDT, merely reflects the inability of donor APC depletion alone, to prevent the allograft reaction.

Despite our inability to demonstrate a facilitation of allogeneic transplantation with PDT, it remains an exciting prospective application in the field of immunomodulation on the basis of its cellular specificity (by virtue of the carrier monoclonal antibody), and rapidity of action. Clearly, experimental refinements are necessary to maintain specificity of target cell destruction without injury to surrounding parenchymal cells.

In conclusion, the fundamental stumbling block to tissue transplantation, whether it be vascularized, solid organ or non-vascularized, cellular (such as pancreatic islet), is allograft rejection. Circumvention of allograft rejection requires either host immunosuppression, reduction of donor tissue immunogenicity, or the two in combination. Anti-rejection strategies in pancreatic islet transplantation have focussed on in vitro techniques which eliminate APCs or "passenger leukocytes" from the donor tissue. This thesis has described the evaluation of two techniques of in vitro donor tissue treatment , namely pre-transplant tissue culture and monoclonal antibody guided photodynamic therapy. Although both techniques demonstrated selective APC depletion, successful allogeneic transplantation was possible only after islets were cultured for a period of time significantly longer than that necessary to deplete APCs. This suggests that donor APC depletion alone cannot prevent allograft rejection, and that in vitro culture must reduce donor tissue immunogenicity by some other mechanism, such that allogeneic transplantation into an immune-competent host is permitted.

Bibliography:

1. Loeb L. The Biological Basis of Individuality. Charles C Thomas, Springfield IL, 1945.

2. Murphy JB. J Exp Med1914; 19: 513.

3. Mitchison NA. Proc R Soc Lond 1954; 142: 72.

4. Mitchison NA, Dube OL. J Exptl Med 1955; 102: 179-97.

5. Billingham RE, Brent L, Medawar PB. Proc R Soc Lond 1954; 143: 58.

6. Brncic P, Hoecker G, Gasic G. Acta Unio Intern Contra Cancrum 1952; 7: 761-64.

7. Weaver JM, Algire gh, Prehn RT. J Natl Cancer Inst 1955; 15: 1737-67.

8. Gorer PA, Lyman S, Snell GD. J Pathol Bacteriol 1937; 44: 691-7.

9. Casey AE. Cancer Research 1941; 1: 134-5.

10. Snell GD, Cloudman AM, Failor E et al. J Natl Cancer Inst 1946; 6: 303-16.

11. Allen et al. Ann Surg 1952; 135: 239-44.

12. Billingham RE, Brent L, Medawar PB. Transplantation Bull 1956; 3: 84-8.

13. Hardin CA, Werder AA. Ann NY Acad Sci 1955; 59: 381-3.

14. Snell GD. Transplantation Bull 1956; 3: 83-4.

15. Hardin CA, Werder AA. Plastic Reconstr Surg 1955; 15: 107-13.

16. Brent L, Brown JB, Medawar PB. Proc Roy Soc Ser B 1962; 156: 187.

17. Brent L, Medawar PB. Proc Roy Soc Ser B 1966; 165: 281.

18. Brent L, Medawar PB. Brit Med Bull 1967; 23: 55.

19. Ramseier H, Billingham RE. J Exp Med 1966; 123: 629.

20. Ramseier H, Streilein JW. Lancet 1965; 1: 622.

21. Elkins WL. J Exp Med 1964; 120: 329.

22. Elkins WL. J Exp Med 1966; 123: 103.

23. Bain B, Vas MR, Lowenstein L. Blood 1964; 23: 108.

24. Wilson DB, Elkins WL. In "Proceedings of the Third Annual Leukocyte Conference" (WO Ricke, ED) pp 31-407. Appleton Century- Crofts, New York, 1969.

25. Lafferty KJ, Woolnough J. Immunol Rev 1977; 35: 231-62.

26. Lafferty KJ, Andrus L, Prowse SJ. Immunol Rev 1980; 51: 279.

27. Klein J, Juretic A, Baxevanis CN et al. Nature 1981; 291: 455.

28. Hood L, Steinmetz M, Malissen B. Ann Rev Immunol 1983; 1: 529.

29. Steinmetz M, Minard K, Horvath S et al. Nature 1982; 300: 35.

30. Grotze D. Springer-Verlag, New York, 1977.

31. Gill TJ, Kunz HW, Misra DN et al. Transplantation 1987; 43: 773-85.

32. Thorsby E. Tissue Antigens 1978; 11: 321.

33. Singer A, Kruisbeck A, Andrysiak PM. J Immunol 1984; 1332: 2199.

34. Moller G (ed). Immunol Rev 1978; 40: 1-255.

35. Unanue ER, Cerotinni JC. J Exp Med 1970; 131: 711-25.

36. Unanue ER. Ann Rev Immunol 1984; 2: 395-428.

37. Jakobisiaki M. Transplantation 1971; 12: 364.

38. Williams GM, Hume DM, Hudson RP et al. N Engl J Med 1968; 279: 611.

39. Starzl TE, Boehmig HJ, Amemiya H et al. N Engl J Med 1970; 283: 383.

40. Way LW, May J, Perper RJ. Fed Proc 1965; 24: 572.

41. Berke G, Amos DB. Transplant Rev 1973; 17: 71.

42. Roberts PJ, Hayry P. Transplantation 1976; 21: 437.

43. Tilney NL, Garavoy MR, Busch GJ et al. Transplantation 1979; 28: 421.

44. Tilney NL, Strom TB, MacPherson SG et al. Transplantation 1975; 20: 323.

45. Engers HA, Glasbrook AL, Sorenson GD. J Exp Med 1982; 156: 1280.

46. Cobbold S, Waldmann H. Transplantation 1986; 41: 634.

47. Kirkman RL, Barrett LV, Gaulton GN et al. J Exp Med 1985; 162: 358.

48. Kupiec-Weglinski JW, Diananstein T, Tilney N et al. Proc Natl Acad Sci USA 1986; 83: 2624-7.

49. Farrar JJ, Benjamin WR, Hilfiker ML et al. Immunol Rev 1982; 63: 129.

50. Hayry P, Von Willebrand E, Parthensas E et al. Immunol Rev 1984; 77: 85-142.

51. Heidecke CD, Kupiec-Weglinski JW, Lear PA et al. J Immunol 1984; 133: 582.

52. Lowry RP, Marghesco DM, Blackburn JH. Transplantation 1985; 40: 183.

53. Aggarwal BR, Moffat B, Harkins RN. J Biol Chem 1984; 259: 686.

54. Ortaldo JR, Herbeman RB. Ann Rev Immunol 1984; 2: 359.

55. Dubernard JM, Carpenter CB, Busch GJ et al. Surgery 1968; 64: 752.

56. Stetson CA. Adv Immunol 1963; 3: 97.

57. Burnet FM. Aust J Sci 1957; 20: 67.

58. Nossal GJV. Ann Rev Immunol 1983; 1: 33.

59. McCullagh P. Aust J Exp Biol Med Sci 1973; 51: 445.

60. Dorf ME, Benacerraf B. Ann Rev Immunol 1984; 2: 103.

61. Lafferty KJ Prowse SJ, Simeonovic CJ. Ann Rev Immunol 1983; 1: 143.

62. Lafferty KJ, Cooley MA, Woolnough J et al. Science 1975; 188: 259.

63. Lafferty KJ, Bootes A, Kilby VA et al. Aust J Exp Biol Med Sci 1976; 54: 573.

64. Parr EL, Bowen KM, Lafferty KJ. Transplantation 1980; 30: 135.

65. Sollinger HW, Burkholder PM, Rasmus NR et al. Surgery 1976; 81: 74.

66. Talmadge DW, Dart GA, Radovich J et al. Science 1976; 191: 385.

67. Naji A, Silvers WK, Barker CF. Surg Forum 1979; 30: 109.

68. Kedinger M, Haffen K, Grenier J et al. Nature 1977; 270: 736.

69. Bowen KM, Andrus L, Lafferty KJ. Diabetes 1980; 29: 98.

70. Lacy PE, Finke EH, Janney CG et al. Transplantation 1982; 33: 588.

71. Opelz G, Terasaki PI. Science 1974; 184: 464.

72. Lacy PE, Davie JM, Finke EH. Science 1979; 204: 312.

73. Lacy PE, Davie JM, Finke EH. Transplantation 1979; 28: 415.

74. Faustman D, Hauptfeld B, Davie JM et al. J Exp Med 1980; 151: 1563.

75. Steinman RM, Nussenzweig MC. Immunol Rev 1980; 53: 127.

76. Faustman D, Hauptfeld B, Lacy P et al. Proc Natl Acad Sci (USA) 1981; 78: 5156.

77. Gores PF, Sutherland DER, Platt JL et al. J Immunol 1986; 137: 1482.

78. Shizuru JA, Ramakrishnan S, Hunt T et al. Transplantation 1986; 42: 660.

79. Lau H, Reetsma K, Hardy MA. Science 1984; 223: 607.

80. Calamai EG, Beller DI, Unanue ER. J. Immunol 1982; 128: 1692-94.

81. Stock PG, Ascher NL, Chen S et al. J Surg Research 1989; 46: 317-21.

82. Dougherty TJ. Oncology 1989; 3: 67-73.

83. Personal communication, Dr R McMaster.

Figure 1a:

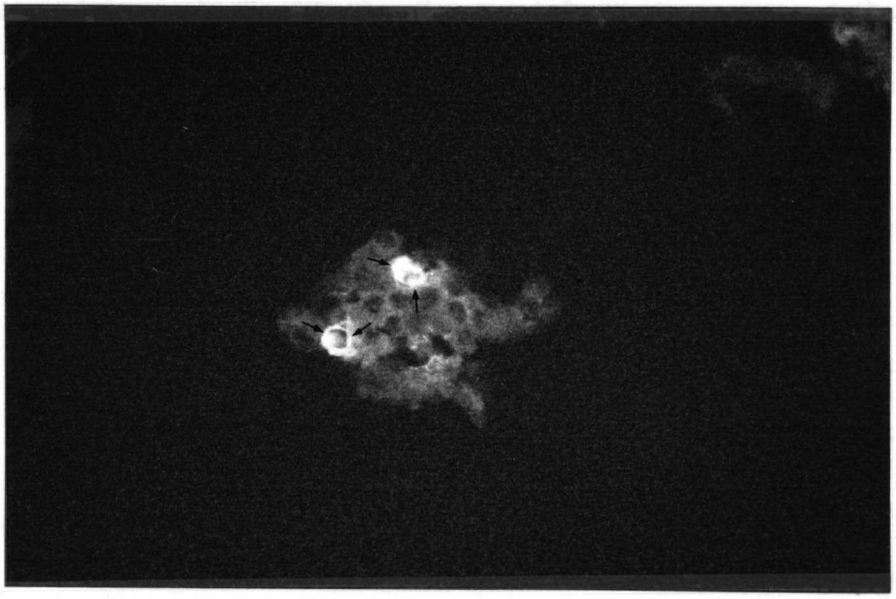

Figure 1b:

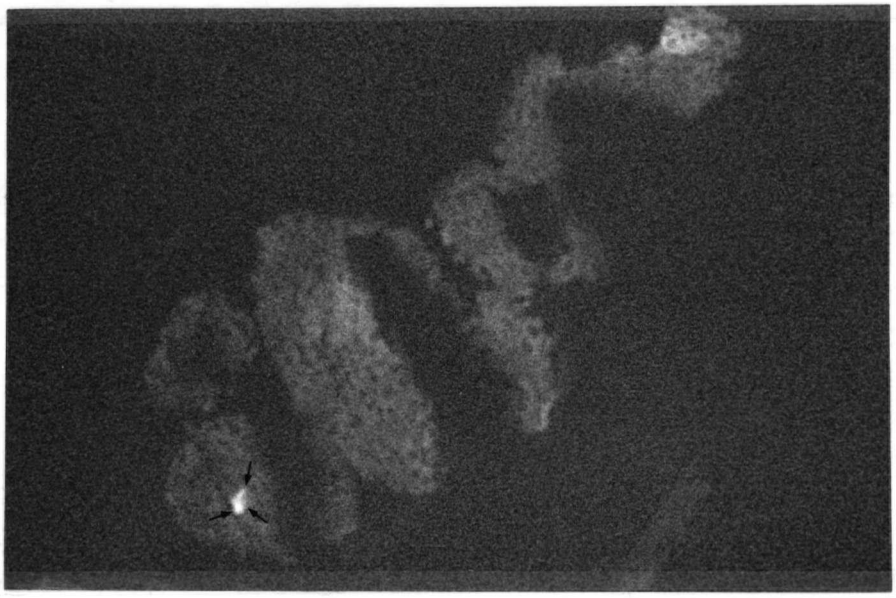

Figure 1:
Fluorescent micrographs of fresh (1a) and 7 day cultured (1b) Sprague Dawley islets after cryostat sectioning and 2 step fluorescent immunostaining for Ia + cells (arrows); (x 400 -1a, x 200 -1b).

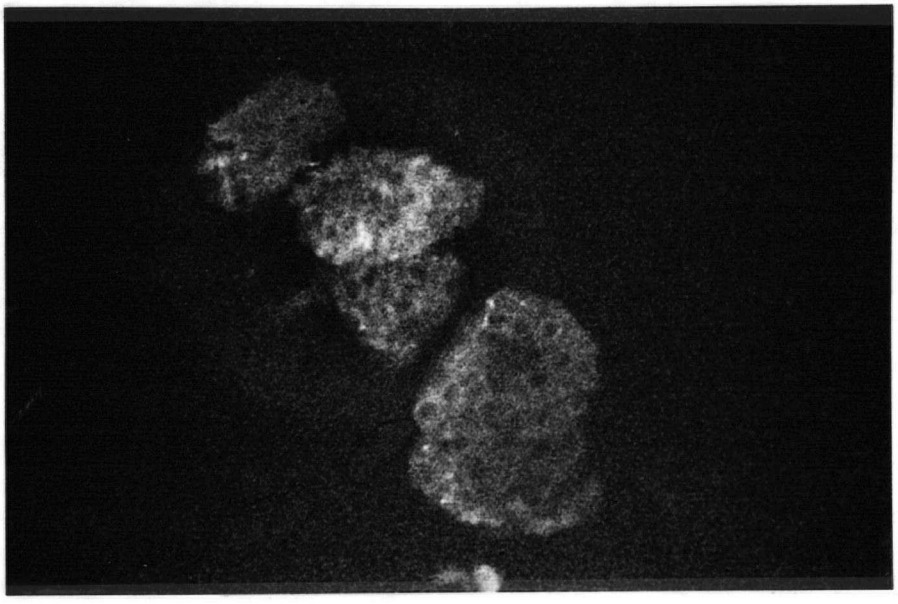

Figure 2:
Fluorescent micrograph of 14 day cultured Sprague Dawley islets after cryostat sectioning and 2 step fluorescent immunostaining for Ia + cells; (x 400). Note absence of fluorescent cellular staining (compared to figure 1).

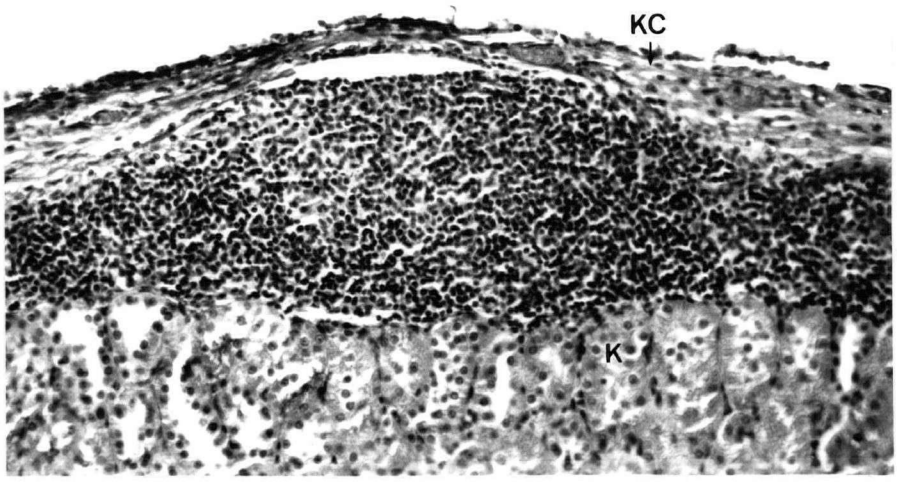

Figure 3:
Rejected, fresh Sprague Dawley islet allograft under renal capsule of Wistar Furth recipient, harvested 12 days after transplantation; (H & E stain, x 100). Note extensive mononuclear infiltrate between kidney capsule (KC) and kidney (K), which has completely replaced graft.

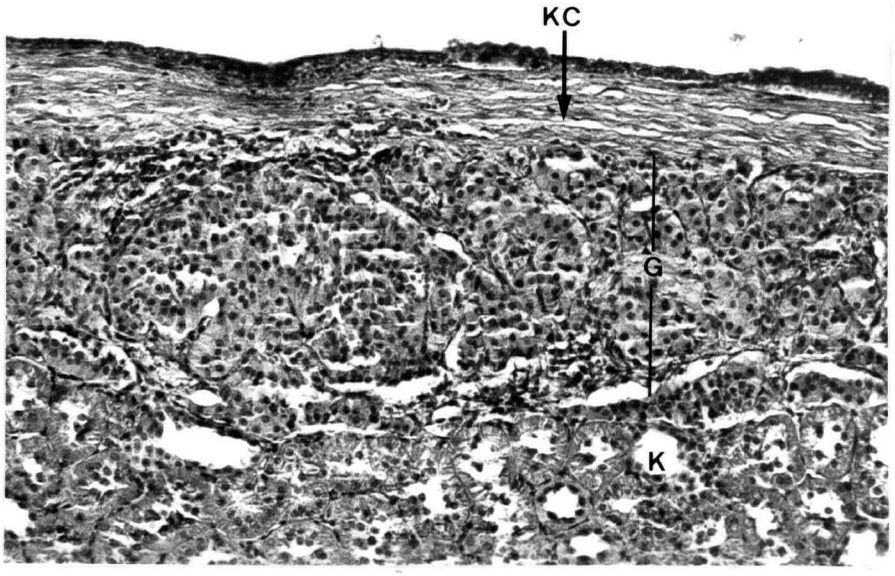

Figure 4:
14 day cultured Sprague Dawley islet allograft under renal capsule of Wistar Furth recipient, harvested 12 days after transplantation; (H & E stain, x 100). Note integrity of endocrine cells of graft (G), between kidney capsule (KC) and underlying kidney (K).

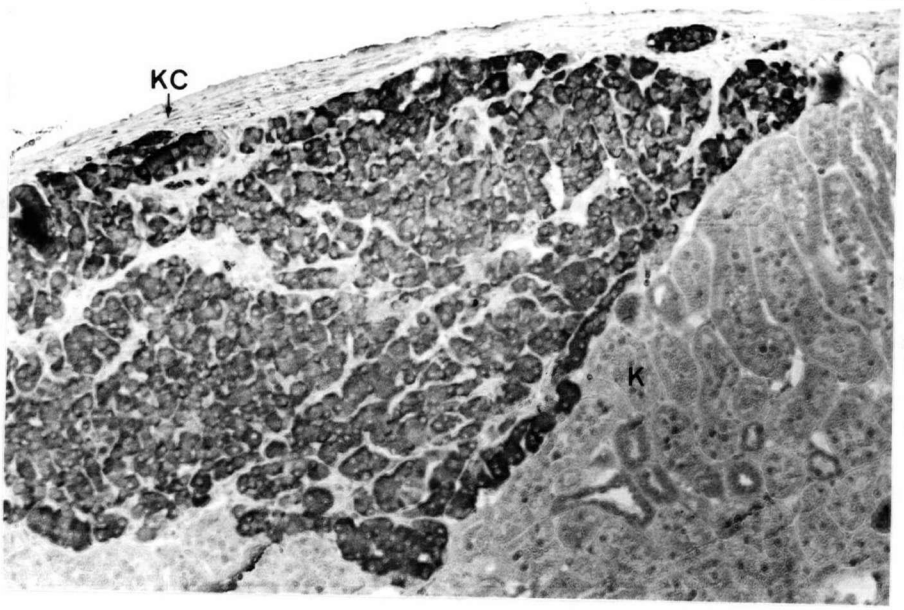

Figure 5:
Engrafted, 14 day cultured Sprague Dawley islet allograft under renal capsule of Wistar Furth recipient, stained with immunoperoxidase for insulin- confirming endocrine activity of transplanted islets after a period of in vitro culture; (H & E stain, x 100). KC = kidney capsule, K = kidney.

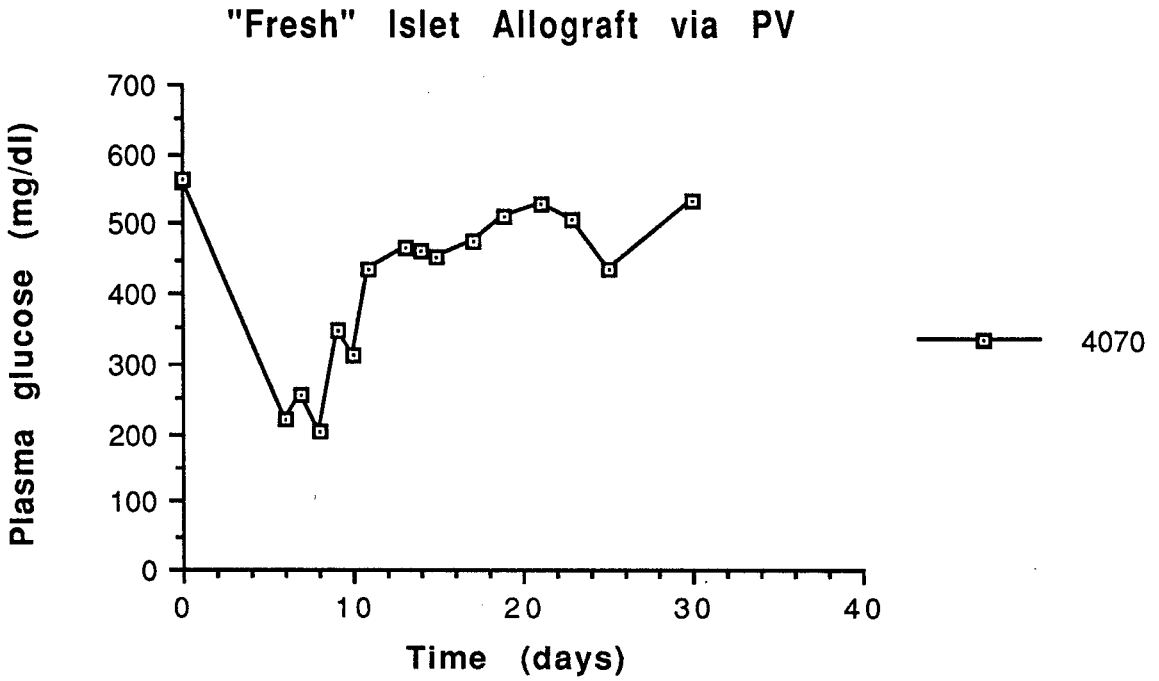

Figure 6:
A plot of plasma glucose vs time in an immune competent Wistar Furth recipient of a fresh Sprague Dawley islet allograft via the portal vein (PV). Persistent hyperglycemia (> 400 mg/dl) signifies graft rejection.

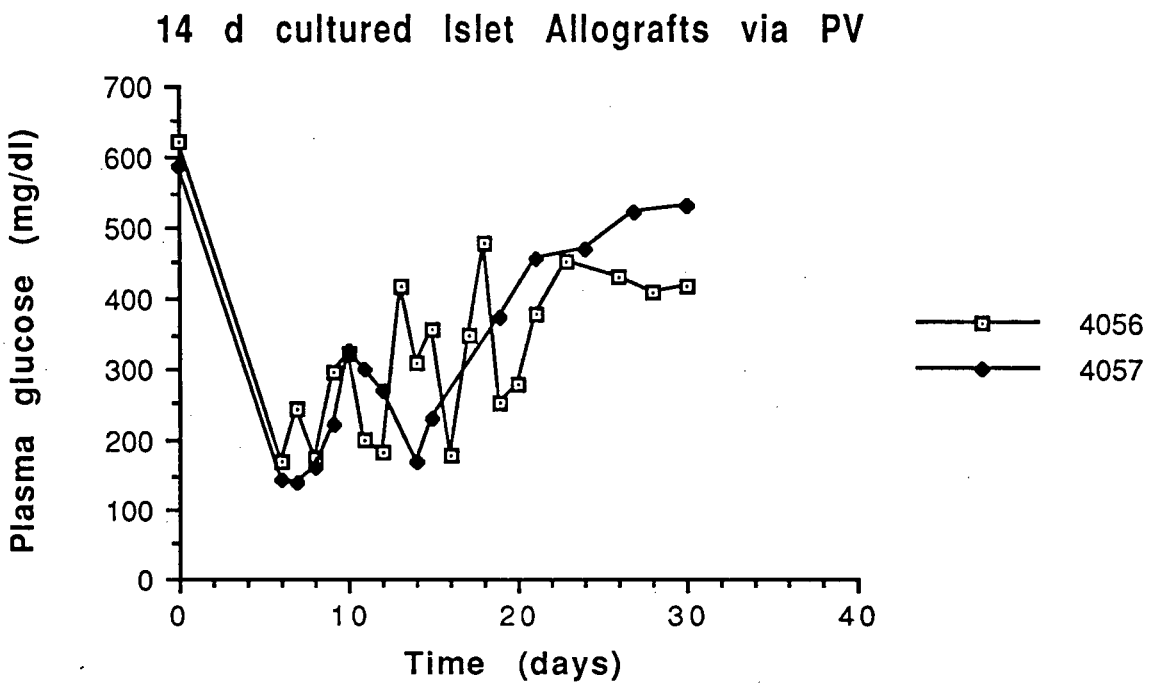

Figure 7:
A plot of plasma glucose vs time in immune competent Wistar Furth recipients of 14 day cultured Sprague Dawley islet allografts via the portal vein (PV). Early graft function has led to persistent hyperglycemia, signalling graft rejection.

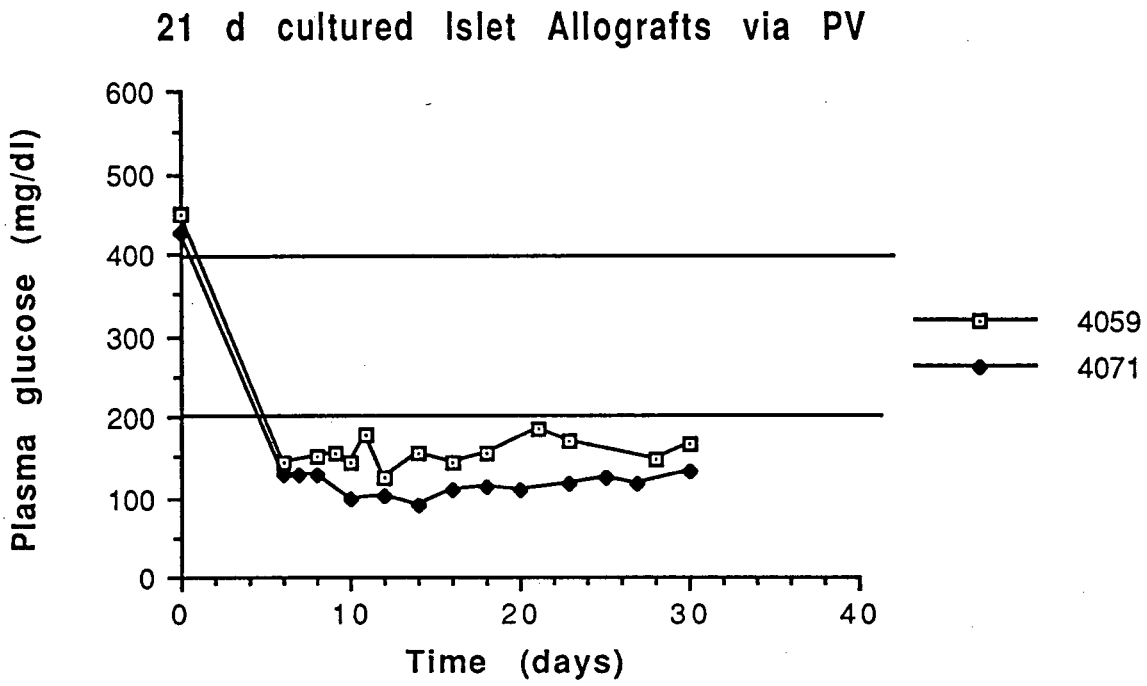

Figure 8:
A plot of plasma glucose vs time in immune competent Wistar Furth recipients of 21 day cultured Sprague Dawley islet allografts via the portal vein (PV), demonstrating 30 day in vivo allograft function.

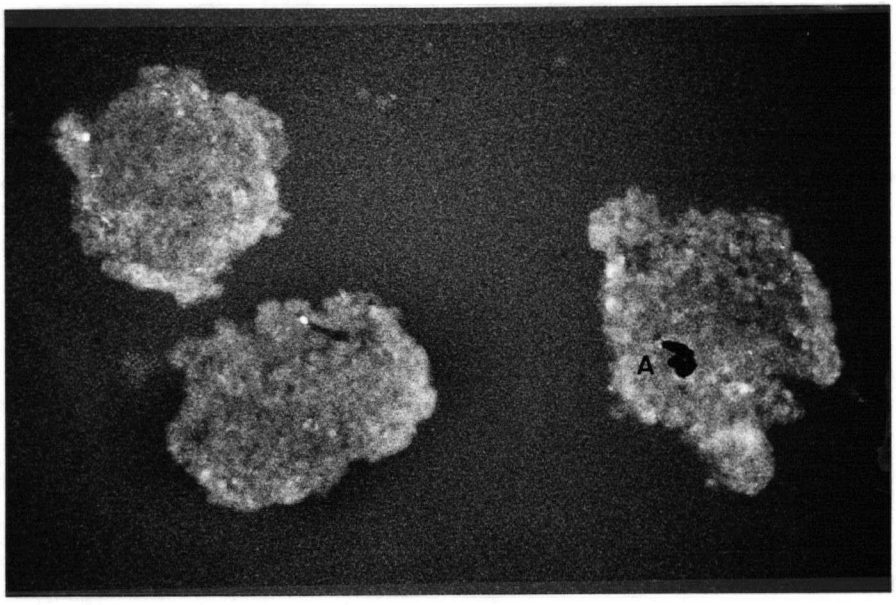

Figure 9:
Fluorescent micrograph of cryostat-sectioned Sprague Dawley islets immunostained for Ia + cells, following photodynamic therapy (PDT) with specific RAMIg-BPD conjugate; (x 400). Note absence of fluorescent cellular staining compared to fresh, untreated islets (figure 1a), and islets which have undergone PDT using an irrelevant antibody-BPD conjugate (figure 10). (A = artefact)

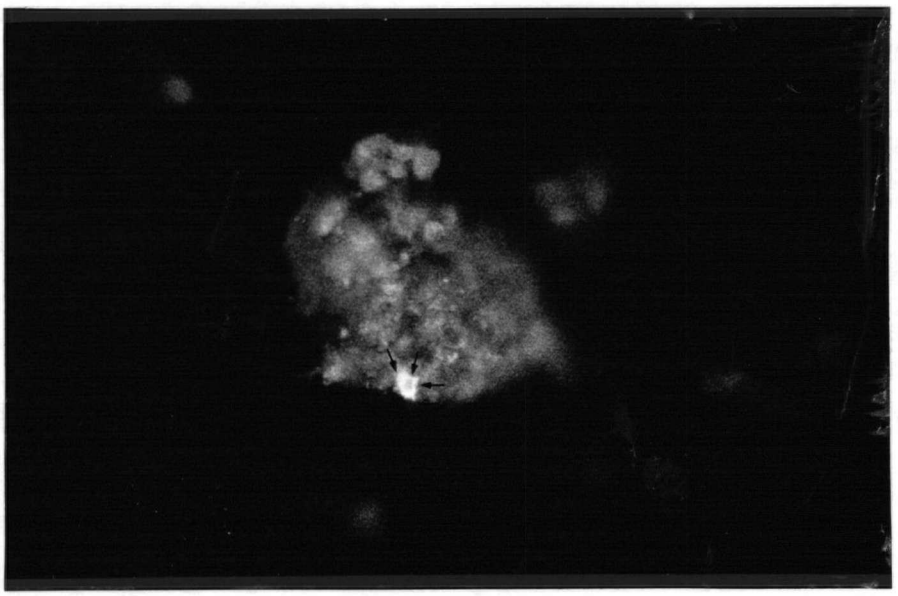

Figure 10:
Fluorescent micrograph of cryostat-sectioned Sprague Dawley islets immunostained for Ia + cells, following photodynamic therapy (PDT) with an irrelevant antibody-BPD conjugate; (x 400). Note preservation of Ia + cell, in contrast to figure 9.