THYMIC INOCULATION DOES NOT RESULT IN DEVELOPMENT OF TOLERANCE TO ALLOGENEIC THYROID GRAFTS IN THE OUTBRED RABBIT

BY

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ABSTRACT

More than 30 studies have demonstrated that allograft tolerance can be achieved in inbred rats and mice through intrathymic injection of donor cells or cell antigen and a single treatment with antilymphocyte serum. Additional experiments have also achieved induction of tolerance in similar animal models with low dose, short duration immune suppression and donor-specific whole blood transfusions. In outbred rat and dog models, xenografts, and inbred species with major MHC antigen difference (six studies in total) tolerance could not be induced using intrathymic inoculations. The focus of our study was to determine whether allogeneic thyroid graft tolerance could be achieved in outbred rabbits. In the experimental group (n=5), recipient rabbits (NZW) received an intrathymic injection of donor (California) lymphocytes (5×10^7) and a single treatment of 165 mg of antilymphocyte serum (ALS). Controls (n=5) received intrathymic cell culture medium and ALS treatment. Donor-recipient allogenicity was monitored with mixed lymphocyte culture (MLC) before and at one, seven, and eighteen weeks following intrathymic injection. Donor thyroid tissue was placed into recipient gluteal muscle fibres one week following the last MLC measurement. A third group of rabbits (n=4) received thyroid autografts without any other treatment. Biopsies of the thyroid grafts were taken one and six weeks following graft placement. One experimental group donor-recipient pair was lost due to late death of the recipient (respiratory complication). There were no differences in MLC stimulation indices between the control and experimental group. MLC did not change within groups over the eighteen week monitoring period. All thyroid autografts survived over a two week monitoring period and demonstrated normal thyroid

follicles on histologic examination. All thyroid allografts resulted in severe acute rejection reactions noted on the one week biopsy, with rare intact follicles visible. No intact follicles were seen in any allograft recipient at six weeks following grafting. The reason for the failure to induce tolerance in the outbred rabbit model is unknown. Further studies using outbred animals examining the role of thymic inoculation should be undertaken to determine whether similar techniques might be successful in the human.

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LIST OF ABBREVIATIONS

AIDS - acquired immune deficiency syndrome

ALS - antilymphocyte serum

APC - antigen processing cell

CD - cluster of differentiation

CML - cell-mediated lympholysis

CsA - cyclosporine A

DST - donor-specific transfusion

FK-506 - tacrolimus

GVHD - graft versus host disease

HLA - human leukocyte antigen

IFNγ - interferon gamma

IL - interleukin

IT - intrathymic

IV - intravenous

MAb - monoclonal antibody

MHC - major histocompatibility complex

MLC - mixed lymphocyte culture

RBC - red blood cell

TCR - T-cell receptor

TGF - tumor growth factor

WBC - white blood cell

1. INTRODUCTION

Extended survival of allogeneic grafts in rats has occurred following intrathymic injection of a suspension of donor cells or cell antigen and a single treatment of the recipient with antilymphocyte serum (ALS). The success of this method has been widely confirmed in grafts of kidney, heart, skin, bowel, and pancreatic islets. There are also many other reports of achieving similar increased survival following intravenous treatment with donor-specific blood or short term perioperative immune suppression alone. All of the successful experiments were achieved in highly inbred rat or mouse strains. A single publication of a similar experiment in mongrel dogs demonstrated no prolonged survival of grafts with intrathymic inoculation and antilymphocyte serum. Two xenograft experiments (rat/mouse and rat/hamster) have had similarly poor graft survivals. The purpose of this project was to produce a model of prolonged survival of allogeneic thyroid grafts in the rabbit following intrathymic injection of donor lymphocytes and a single treatment with antilymphocyte serum. Outbred rabbits were chosen as the experimental model because of the similarity between rabbit and human immune systems and donor-recipient genetic relationships. If this protocol for tolerance induction was successful, the potential benefits would be significant. If similar intrathymic injection in humans could increase the survival of allografts, without the necessity of chronic immune suppression, the cost for transplant programs would decrease dramatically. The financial impact from drugs such as Cyclosporine A or rapamycin is significant in direct drug costs as well as the costs related to increased patient morbidity and mortality resulting from extended immune suppression.

2. REVIEW OF THE LITERATURE

2.1 The Thymus

The thymus is a bilobed lymphatic organ overlying the great vessels of the heart in the mediastinum. It develops embryologically from the epithelium of the endodermic lining of the third branchial pouch as well as the underlying mesenchyme into which the pouch invaginates and enlarges (1). The thymus is not fully developed at birth and appears to reach its greatest size in early puberty. Beyond this period, involution of the organ begins to occur. By adulthood, the thymus is often difficult to differentiate from surrounding adipose tissue (2). Histologically, the thymus is composed of two distinct areas, the cortex and the medulla. Blood vessels supplying the thymus enter the medulla along a series of septae and provide blood to the cortex by capillary branches. The medulla is a lighter staining area containing blood vessels, lymphocytes, and macrophages. The cortex stains darker and contains macrophages that phagocytose T-lymphocytes destined for destruction as well as small lymphocytes or thymocytes. The cortex is separated from the medullary septa by a row reticular epithelial cells forming a cellular reticulum in which T-cells mature (3).

Until recently it was thought that in the adult, the thymus was not functional or had minimal function. The basis of this was the observation that patients having thymectomies for tumors or other diseases do not demonstrate significant changes to the

function of their immune system. More recent studies (4) indicate that the most significant involution in the adult thymus occurs in the non-functional structures including perivascular spaces. In adolescence these areas are filled with all types of leukocytes but they become progressively filled with fatty tissue after puberty. However, the epithelial cell network and its associated lymphocytes are only marginally reduced in size and continue to remain active, secreting hormones and T-cells but at a reduced level compared to childhood.

At the present time four thymic hormones have been well studied (5). Thymosin $\alpha 1$ and thymopoietin are secreted by epithelial cells and are required for the differentiation of T-cells in the thymus. In patients with malignancies, thymosin $\alpha 1$ serum concentrations are reduced. Thymopoietin serum levels are higher than normal in patients with autoimmune thymic hyperplasia seen in myasthenia gravis. Thymulin (also known as FTS) is also a product of epithelial cells and requires zinc for its functional activity. In children with AIDS, decreased serum levels of thymulin are seen before any changes in peripheral lymphocytes are visible. Elderly patients often have reduced serum zinc levels and it may be possible that this causes a reduction in activated thymulin and alterations in the immune system. In mice, dietary supplements of zinc have restored the normal secretion of thymulin in aged thymus tissue in culture (6). Thymic hormonal factor, the least well characterized of the thymus hormones appears to participate in clonal expansion and maturation of individual T-cell subsets.

The main role of the thymus is to provide a specialized environment for the development and differentiation of T-cells. T-cells originate from bone marrow haematopoietic stem cells which preferentially migrate from the bone marrow into the thymus. In the thymus, the stem cells differentiate into two lineages - dendritic cells and T-cell prototypes (7). Total thymic lymphocyte counts have been estimated from thymic tissue biopsies collected from patients undergoing open heart surgery (8). In children the thymocyte count was estimated at 10^{10} compared to adults at 10^{5}. The function of the thymus is affected by many physiologic and iatrogenic agents including sex hormones, steroids, radiation, growth hormones, antineoplastics, and other specific drugs including Cyclosporine A and streptozotocin (9).

2.2 T-Lymphocytes

T-lymphocytes (T-cells) make up approximately 70-80% of the total normal circulating peripheral lymphocytes, 90% of thoracic duct lymphocytes, and approximately 30% of lymphocytes found in the spleen and lymph nodes. T-cells originate from stem cells located in the bone marrow (or in the liver in fetal life) and preferentially travel to the thymus gland. Once in the thymus, these pro-T-cells undergo a series of rearrangements and productive expression of T-cell receptor genes. The thymus epithelial cells, macrophages, and dendritic cells assist the T-cell in development and selection of surface antigen receptors. These receptors allow T-cells to recognize and react with foreign, but not normally, self antigens.

During T-cell development, a series of surface proteins are expressed and deleted providing a phenotypic method for defining specific T-cell subgroups. The most often reported proteins are the CD4, CD8, and CD3. Helper T-cells, which make up about 70% of the total T-cell population are identified by their CD4+/CD8-/CD3+ surface protein pattern. Killer or cytotoxic T-cells, make up about 25% of the T-cell population and have a CD4-/CD8+/CD3+ phenotype. The remaining 5% of the T-cell population includes CD4-/CD8-and CD4+/CD8+ phenotypes. T-cells mature in distinct stages matching histologically identifiable areas of the thymus: outer thymic cortex - CD4-8-, cortex CD4+8+, and medulla CD4-8+ and CD4+8- (6). Most of the cells that do not exit the thymus are active against self antigen and are destroyed. The majority of immature T-lymphocytes are negatively selected before or during the development of single positive phenotypes (CD4+8- or CD4-8+)(10).

From recent research it appears that each of the cells of the thymus has a specific role to play in T-cell maturation. The thymic epithelial and dendritic cells play a larger role in the positive selection process for T-lymphocyte self major histocompatibility complex (MHC) restricted cells. Epitopes of the T-cell receptor (TCR) gene products also appear to assist in the positive selection (11). In transgenic mice, thymic cortex cells bearing class II MHC molecule mediate and assist in the positive selection of CD4+ phenotypic cells (12). In mouse thymus organ culture, if the thymus was preferentially depleted of its MHC II antigen expressing cells (using monoclonal antibodies -MAb), the available macrophages and dendritic cells could process antigen, express it with MHC I antigen

and initiate the proliferation of T-lymphocytes (13). These antigen presenting cells can also exert cytostatic and cytotoxic properties within the thymus providing a method for cell deletion. MHC class II antigen, while required for CD4+ phenotypic T-lymphocytes, is not the only cell surface factor necessary for T-cell maturation. In another thymus culture system, the addition of MAb against CD4 reduced the development of CD4+8- cells but the further addition of MAb to MHC class II antigen did not increase this inhibition (14). In organ culture, MHC I surface antigen positive thymus cells can induce MHC I thymocyte tolerance without the need for macrophages or dendritic cells (15).

Thymic dendritic cells differentiate from the same stem cells as T-cell prototypes within the confines of the thymus (7). A thymic dendritic cell remains in the thymus for less than 30 days. In this way, new T-cells in the thymus are formed simultaneously with new dendritic cells. This would help to limit the effects of peripheral dendritic cells that might enter the thymus and present foreign antigens as self during T-cell development, resulting in unwanted tolerance. Bone marrow (BM) transplants provide a new pool of stem cells and therefore thymic dendritic cells and T-cells. BM grafts can result in the formation of a chimera.

Information about thymus T-lymphocyte kinetics has been obtained from murine models either from pulse or continuous cell labeling techniques. Pulse thymidine labeling demonstrated that of the total thymic T-lymphocyte (thymocyte) population, 20% are dividing cells while 80% are non-dividing product cells. While over 86% of all the

dividing cells are CD4+8+ phenotype cells the CD4+8- and CD4-8+ cells are derived from a population of nondividing precursor cells (10). The average time a thymocyte spends in the thymus is 12 days. The process of entry and exit is not a defined FIFO (First-In-First-Out) transport (16), but a more random distribution with the actual resident time of any one cell being highly variable. The number of mature single phenotypically positive T-lymphocytes leaving the thymus is represents 3% of the total original double positive thymocytes. The total lifespan of CD4+8+ thymocytes in the thymus is 3.5 days regardless of whether they are positively selected or not (17). It has been demonstrated in mice that peripheral T-cells can reenter the thymus (18). However the vast majority of these are peripheral activated cells (blast cells) rather than resting T-cells. It appears that these returning cells have an intrathymic lifetime of at least 30 days.

T-cells recognize foreign antigen when it is associated with self major histocompatibility complex (MHC) cell surface molecules (19). MHC Class I molecules are expressed on virtually all cell types whereas MHC Class II molecules are found on B cells, dendritic cells, and macrophages. Other cell types can be induced to express MHC Class II antigen when exposed to interferon gamma (IFNγ). An antigen processing cell (APC) such as a macrophage or B-lymphocyte can phagocytose the antigen and break large sections into fragments. After processing, the antigen is irreversibly bound to the cell surface MHC of the APC (20) providing a means of long lasting memory for future T-cell interaction. As an example, a B-cell can continue to stimulate T-cells 10 days after the original phagocytosis of the antigen has occurred (20). It appears that at least 10% of resting T-

cells are extremely sensitive to foreign MHC antigen alone, resulting in T-cell activation apparently without the need for antigen processing (19). The T-cell receptor (TCR) complex on the surface of the T- cells is the major docking mechanism to the MHC and antigen. Many CD proteins also assist in this recognition and adhesion. MHC Class I antigens (with foreign antigen) bind with CD4-8+ (cytotoxic) T-cells while MHC Class II antigen binds with CD4+8- (helper) T-cells. Each type of cell initiates a different immune response sequence.

The ability of T-cells to recognize a huge variety of antigens comes from the potential variability in the T-cell antigen receptor (TCR) chain production. The TCR is a heterodimer composed of one α and one β chain. The α chain has approximately 50 variable gene segments and more than 100 junctional segments, while the β chain has 60 variable segments, 2 diversity segments, and 13 junctional segments. The products of these gene segments are spliced together during TCR production providing a total combination potential of approximately 8×10^6 (21,22,23). Diversity similar to that of TCR is also seen in MHC complexes. This diversity occurs not only between species but is also demonstrated between strains within a species. MHC antigens from rat to human demonstrate a homology of approximately 70% (24). The current MHC antigens (also referred to as Human Leukocyte Antigen, HLA) specifically identified in humans consist of Class I, HLA-A, HLA-B, and HLA-C of which there are 80 different types, and Class II DP, DQ, and DR with 35 different permutations. An additional DO group has been initially identified (25).

Immune tolerance describes a state where an organism is not able to develop an immune response against an antigen. This is most critical for preventing reaction against self-antigens. There are three major mechanisms that can be involved with the development of tolerance including peripheral T-cell suppressors, clonal deletion, and clonal anergy (26). There is a specific population of T-cells that have been identified as suppressor cells, the majority of which are CD8+. The exact functional mechanism of suppression is not well understood but it is believed that autoreactivity might be limited through the CD8+ cell secretion of cytokines including tumor growth factor (TGF) β_1 a known immune system down-regulator. Clonal deletion mechanisms of tolerance refer to the process of destruction of self reactive T and B cells during their development. For T-cells, the process occurs in the thymus as already discussed. B-cells appear to undergo a similar sorting in the bone marrow where cells reacting to membrane antigens are deleted. However, for B-cells at least, the deletion process is not perfect since we know that there are B-cells in circulation in normal individuals that are reactive to normal tissue components including DNA and collagen. The final method of developing tolerance is clonal anergy. This occurs if signals that would normally allow for T-cell activation are not completed. T-cells require not just antigen+Class II MHC recognition but a number of other APC signals. If one of these co-stimulator signals is lost, the T-cell will not become activated. Anergy can also occur if an antigen is located on the surface of a cell but that cell does not also express MHC II. Finally, B-cells can undergo clonal anergy if they are exposed to antigen before they are mature. In this case, the antigen is endocytosed but because of immaturity, the B-cell can never produce their immunoglobulin receptors.

2.3 Transplantation and Rejection

Human allogeneic transplantation has become one of the front-line treatments for a variety of incurable medical conditions most notably renal failure. Transplantation of heart and/or lung, cornea, liver, bone marrow, pancreatic islet cells, and small bowel have been used in both pediatric and adult patients with varying degrees of success. By carefully matching HLA (MHC) phenotypes, many grafts have survived for extended periods of time providing the recipient with an extended lifetime and also enhanced quality of life. Good graft survival can be achieved if donor and recipients have at least Class II similarities, especially in the DR antigens. Typing for all antigens is not practical or possible.

When the first transplant programs began, morbidity associated with rejection and graft versus host disease (GVHD) was controlled medically using large doses of steroids and other immune suppressants such as azathioprine. The side effects from these drugs were considerable and more often than not chronic rejection resulted in loss of graft function. In the early 1980's, Cyclosporine A (CsA) was marketed by Sandoz Corporation as an immune suppressant under the trade name of Sandimmune. This non-polar cyclic oligopeptide is a fermentation product of a group of fungi imperfecti. CsA could be administered intravenously or orally and appeared to control cases of acute or chronic graft rejection and GVHD. The mode of action of CsA appears to be related to helper T-

lymphocyte activation processes where it prevents the production or release of interleukin-2 (27,28).

CsA treatments can also cause side effects especially in the kidney (29) and liver (30). This risk is especially high for pediatric patients since they may face many decades of CsA therapy for control of rejection and prevention of graft loss. Many newer immune suppression agents have become available in the past few years including tacrolimus (FK-506), and rapamycin. Like Cyclosporine A, they also exert their effects along the T-lymphocyte pathways. Aside from renal and hepatic toxicities the patient that is immune suppressed to a donor graft is likewise immune suppressed to any other foreign antigens. Transplant patients have a known increased risk for serious infections including cytomegalovirus, Pneumocystis carinii, and Candida and Aspergillus organisms. These patients also have significantly greater risk of developing tumors especially cancer of the cervix, skin, and lips, and lymphomas (31,32).

There are significant costs associated with CsA therapy, not including that for treatment of related morbidities. Recent reviews of transplantation cost-effectiveness indicate that over 10,000 renal transplants are performed annually in the US. The cost of immune suppression per transplant is typically $4,000/yr for CsA alone. Treatment of serious infections due to immune suppression costs $17,000 per case while rejection treatment adds another $20,000. The average surviving graft costs $100,000 in total for the first year but saves $25,000/yr in dialysis costs. However, almost 25% of renal grafts are

rejected within the first year despite immune suppression (33,34). Similar costs have been identified in lung transplantation where the survival of patients with grafts is no longer than patients waiting for grafts (35). Estimated figures for the United States (1984) for use of CsA in kidney, liver, and heart transplants combined was $412,000,000 (36). These figures were reported in terms of 1984 dollars.

During the process of acute rejection, graft cellular antigens are recognized by the hosts helper or killer T-lymphocytes after APC processing (37). Graft cells are then destroyed by B- lymphocyte secreted antibodies (T-helper initiated humoral response) or by direct cytotoxic attack from the cellular immune response (killer T-cells). Acute cellular rejection is seen as interstitial infiltration of mononuclear cells (mostly lymphocytes) whereas acute humoral rejection shows neutrophil infiltration and necrotizing vasculitis. Occasionally a subacute intimal thickening caused by macrophages and fibroblasts is seen. Chronic rejection shows mononuclear infiltrations typically with plasma cells and the presence of basophils. A less common phenomena, hyperacute rejection, literally takes place within minutes of graft anastomosis. It results from the host, for any number of reasons, having preexisting antibodies to graft antigens. This reaction is so swift that is often seen by the surgeons during completion of surgery. Depending on the specific genetic discrepancies between the donor and recipient the rejection reaction can be quite mild requiring only short term, low level treatment, or may quickly become graft and life-threatening requiring intensive therapy. Acute rejection is seen within the first few days to months following grafting. Rejection may also occur after a number of years of good graft

function and apparent immune tolerance. Chronic rejection is demonstrated by long term changes to the graft resulting from prolonged or multiple therapies for episodes of acute rejection.

Graft versus host disease (GVHD) results from donor lymphocytes reacting with and destroying (foreign) recipient cells (38). These lymphocytes may have been intentionally transferred in bone marrow transplantation or they may have been passengers in a solid organ graft such as in liver or kidney. The GVHD syndrome often presents symptomatically with a rash and diarrhea or intestinal ileus and may lead to severe immune compromise. Cyclosporine and methotrexate have been used to treat such episodes of GVHD with some success. A number of experimental treatments are currently being considered including antilymphocytic antibodies but as yet, 30% of patients with severe GVHD will succumb to this complication of transplantation (39).

One of the newer agents being used to combat acute rejection in solid organ transplants and GVHD in bone marrow transplants is Muromonab CD-3 (OKT-3) manufactured by Ortho Diagnostics. This murine derived monoclonal antibody has specific binding capacity to CD-3 positive T-cells (mature T- cells) (40). OKT-3 is administered daily as an single intravenous bolus. The antibody clings to the T-cells making them available for opsonization by the reticuloendothelial system. Within minutes of injection, the peripheral CD3+T-cell population decreases. This process is analogous to the effects of the ALS treatment used as part of the experimental protocol in our current experiment.

During chronic therapy with OKT-3, the patient may develop antibodies to the murine MAb which reduce its effectiveness. OKT-3 has proven useful in treatment of acute episodes of rejection and GVHD and is also being tested for induction therapy before grafting to reduce immediate postoperative immune suppression therapy requirements. Side effects of OKT-3 include fever, headache, dyspnea, and hypertension, but may also include life threatening aseptic meningitis, pulmonary edema, and increased risk of infection by opportunistic organisms (41). An additional cost of $58M per year would be added to US transplant costs if OKT-3 therapy was used for renal transplants alone (34).

2.4 The Thymus and Allogeneic Graft Immunity

A number of recent publications have described the potential role of the thymus in preventing allogeneic graft rejection. In the first type of experiment, the thymus offered a physically protected environment for transplanted cells to mature, divide, and maintain their normal physiologic role. Much of this work has centered on pancreatic islet cells. In the second type of experiment, recipient immunity to grafts placed outside the thymus appears to be maintained after intrathymic injection of donor cells or antigen in the recipient.

2.4.1 Protected Environment

In diabetes prone (DP) BioBreeding (BB) rats 70% of individuals develop a form of

autoimmune diabetes (42). They are often used as a model to study juvenile diabetes. In the first published experiment considering the role of the thymus (43), BB rats received an intrathymic injection of pancreatic islets from normal Lewis strain donors. These allogeneic islets reversed hyperglycemia in the BB rats for >120 days. An intraportal injection of islets given to a second group of the BB rats, resulted in 100% graft failure. In a third group, BB rats receiving Lewis (LEW) islets transplanted under renal capsule showed a 50% failure (n=6) at 40-50 days while three grafts maintained normoglycemia for >120 days. No group received immunosuppression of any kind. BB rats have a naturally occurring T-cell lymphopenia which may explain, in part, the easy acceptance of islet transplants (44). In a second set of experiments (43), WF rats were sensitized to LEW antigen by skin grafting (without immunosuppression) before intrathymic islet cell transplants. Every WF rat rejected the skin graft and became sensitized to LEW antigens. These same sensitized WF rats were then made diabetic with injections of streptozotocin (islet cell cytotoxin) followed by intrathymic injection of LEW islets. All islet grafts were rejected. Rejection also occurred if the WF rats were treated with ALS just prior to transplantation.

Posselt (45) also studied WF rats made diabetic by injection of a single dose of streptozotocin. After 2 to 3 weeks, the hyperglycemic rats were given LEW islet cells in one of a number of locations: liver, renal capsule, thymus, testicle. The testicle was used as a control immune protected environment. One-half of each organ group received no immunosuppression while the other half received (ALS) before transplantation. In rats

that did not receive ALS, liver, kidney, and testicle located islet grafts suffered rejection at 8-10 days while grafts located in the thymus survived 17 days. In groups that received the ALS, islets located in the liver survived 29 days, kidney 47 days, and in the thymus group, 10 out of 13 rats remained normoglycemic for greater than 200 days. In these experiments the ALS reduced the total number of peripherally circulating T-lymphocytes to 10% of normal values. After the 120 days of normoglycemia intrathymic islet transplanted rats received renal subcapsular islet cells from LEW to examine whether the immunity was only realized within the thymus. All these grafts survived. When third party DA rat islets cells were transplanted (renal subcapsular) into these long term survivors, the DA islets were rejected immediately. The indefinite survival of the LEW grafts in the renal subcapsular space after intrathymic injection lead to the suggestion that the thymus may be producing T-lymphocytes that now recognize donor LEW antigens as self.

Similar promising results have been obtained in NOD (nonobese diabetic mice) strains. In this model, the diabetes is a result of a bone marrow level defect in T-cell responses to normal islets. Recipients (newborns) received intrathymic grafts of syngeneic islets (46). This prevented the later occurrence of diabetes normally seen in maturing NOD mice. The experimental animal native pancreatic tissue was normal while control animals demonstrated severe insulitis. This experiment also confirmed that the intrathymic injection, in some way, altered the normal T-cell maturation process preventing the destruction of islets. Goss (47) had simlar success grafting endocrine cells. Parathyroid

tissue from LEW rats was transplanted into Buffalo recipient thymus or renal subcapsular tissue. Recipients also received a single ALS treatment. In the same study, renal situated grafts survive for 37 days and thymus placed grafts 67 days (50% of them indefinitely). All renal grafts placed in animals previously having thymus grafts survived indefinitely.

However, all but one of the many experiments reported in the literature using the thymus primarily for an immunologically protected site have utilized inbred mice or rats. Weide designed an experiment using Lewis rat donors and outbred Wistar rats, providing a less genetically cohesive group (48). The experimental group recipients were treated with islets grafted under the renal capsule with or without simultaneous ALS injection, or intrathymic islet grafts with subcapsular renal grafts and ALS. There was no difference in grafts survival between any of the groups.

2.4.2 Intrathymic Injection for Immunity

These experiments expand on defining the role of intrathymic donor cell inoculation in the development of tolerance to allogeneic grafts.

The first experimental evidence that modifying the environment of the thymus could provide tolerance to alloantigens came from Vojtíšková in 1965 (49). Two strains of mice with a difference in only the H-2 locus were used in a model of skin grafting. The recipients had thymectomies followed by syngeneic thymus grafts. The thymus grafts had

been injected with donor splenocytes. Some recipients also received 400 rad total body irradiation just prior to thymus grafts. Groups that received the donor thymus grafts (with or without irradiation) had extended survival of donor skin grafts. It was not until about 1990 that similar investigations about thymic inoculation began to be published.

Remuzzi et al (50), isolated glomeruli from Brown Norway (BN) rats and injected them intrathymically into LEW rats. The LEW rats were pre-treated with CsA 2 days before intrathymic injection and with dexamethasone. The effect of the drug treatment on T-lymphocyte population or on thymus histopathology was not evaluated. Ten days following intrathymic injection, a bilateral nephrectomy was performed and a BN renal allogeneic graft was implanted. No post graft immune suppression used. Controls groups received intrathymic placebo media and the same course of pretreatment immunosuppression. All of the control animals died with anuria within 6 to 8 days. Those that received the intrathymic injection of glomeruli lived for 70 days (experimental endpoint). The creatinine clearances of the intrathymic treatment group remained comparable to that of sham operated controls.

Remuzzi's group later demonstrated that the tolerance effect was not dependant on cell type but was strain dependant (51). Glomeruli from Sprague Dawley (SD) rats were injected into recipient LEW rats thymus. Following this, BN donor renal allografts were transplanted into the LEW rats. All renal grafts rejected in less than 14 days. BN white cells injected intrathymically induced tolerance in LEW receiving BN kidney. As an

alternate site of inoculation, intraperitoneal donor glomeruli failed to provide any tolerance to donor kidney.

The intrathymic injection of donor specific blood was used in a heterotopic heart transplant model to demonstrate induction of allogeneic immunity (52). In this case, several DA rats out of a group receiving LEW transplants and intrathymic LEW whole blood injection had graft survival extended. Animals in this study received no immunosuppression. A rat cardiac allograft model has also been used to pinpoint the role of the thymus in this process of tolerance development (53). Lewis rats were recipients of ACI rat cardiac allografts. Recipients received total body irradiation at a sublethal dose of 200 rad before transplantation and either either intrathymic T-lymphocytes or B-lymphocytes. The T-cell group had indefinite survivals of their cardiac allografts while those receiving intrathymic B-cell injection all rejected their allografts. Subcutaneous, intraperitoneal, or intratesticular injection of T-lymphocytes did not induce tolerance to the cardiac allografts. In the intrathymic T-lymphocyte non-rejecting animals, if the thymus was removed before 21 days post transplant, all animals rejected grafts. Removal of the thymus in non-rejecting animals after 21 days post-op did not alter extended graft survival. These experiments suggest that this tolerance is dependant on the production of a new clonal population of thymus derived T-cells.

Using a slightly different approach, Posselt (54) gave neonatal BB rats subtherapeutic numbers of MHC compatible WF adult male rat islets (60-80 islets) intrathymically. The

number of islets required for a therapeutic transplant is usually in the range of 1000-1500 islets. Controls received only intrathymic saline. No immunosuppression was given to either group. Rats that received the islets did not developed diabetes while 50% of the controls became diabetic by 120 days. Intrathymic treated rats remained normoglycemic for greater than 70 days after removal of the thymus demonstrating that the islets in the thymus were not maintaining the normoglycemia. Pancreatic islet cells were healthy on histologic examination in this group. However, the associated thyroiditis typically seen in the BB rats occurred in control and experimental groups showing the specificity of protection towards only the islet cells. These experiments indicate a deletion or inactivation of specific T-cell clonal population.

Importance of the role of thymic tissue has been confirmed by an experimental murine model using athymic nude BALB/c mice that received embryonic thymic rudiments (contained no haematopoietic cells) from non-matched MHC donors (55). The chimeras that resulted had the normal number of T-lymphocytes. Spleen and lymph nodes contained the appropriate number and subpopulations of T-cells. The recipients showed no immunity to third party skin grafts but they permanently accepted skin grafts from thymic epithelium donor strains. The allogeneic thymus had attracted the syngeneic stem cells and produced tolerance. No autoimmune diseases or symptoms appeared in the mice suggesting that the stem cells originating in syngeneic bone marrow in some way were transformed into both self and non-self tolerant types in the thymus.

Table 1- Studies utilizing whole cells as inoculant for intrathymic injection

Ref.	Species	Donor, Recipient	Innoculant	Other Treatment	Grafted Organ	Result
58	rat	LEW, WF	splenocytes	ALS	heart	> 120d graft survival (control 5d)
59	rat	ACI, WF	myocytes	ALS	liver	83 d - all grafts rejected
60, 59	rat	OA, PVG (high responders)	splenocytes	ALS	heart	13d (control 7d)
			splenocytes	ALS + CsA	heart	26d
			none	ALS	heart	13d
61	rat	ACI, LEW	splenocytes	ALS	heart	50% to >100d (control 7d)
		LEW, ACI	splenocytes	ALS	heart	75% to >100d
62	rat	LEW, Buffalo	splenocytes	ALS	bowel (7d post ALS)	18d (control 7)
			splenocytes	ALS	heart (7d post ALS)	indefinite
63	rat	ACI, Buffalo	splenocytes	ALS	heart (21d post ALS)	50% (n=2) extended survival
		LEW, Buffalo	splenocytes	ALS	heart (21d post ALS)	80% (n=2) extended survival

Table 1 (cont)- Studies utilizing whole cells as inoculant for intrathymic injection

Ref.	Species	Donor, Recipient	Innoculant	Other Treatment	Grafted Organ	Result
64	mice	Class I mismatched	splenocytes	ALS	skin	115 d
		Class I and Class II mismatched	splenocytes	ALS	skin	24 d
		Fully mismatched	splenocytes	ALS	skin	24 d
65	rat	ACI, LEW	T-cells	200 rads	heart	>300 d (control 6 d)
			T-cells	200 rads	small bowel	>150 d (control 6d)
66	rat	Major and minor MHC incompatible	splenocytes	ALS	heart	15 d (control 6 d)
		Major MHC incompatible	splenocytes	ALS	heart	165 d
		Partial major MHC incompatible	splenocytes	ALS	heart	163 d
67	rat	ACI, LEW	bone marrow	ALS	islets	130 d (control 8d)

Table 1 (cont)- Studies utilizing whole cells as inoculant for intrathymic injection

Ref.	Species	Donor, Recipient	Innoculant	Other Treatment	Grafted Organ	Result
68	rat	BN, LEW	lymphocytes	none	kidney	63 d (control 10 d)
			lymphocytes	CsA	kidney	8 d
			none	CsA (3d)	kidney	8 d
			none	CsA (100d)	kidney	>40 d
69	rat	ACI, LEW	splenocytes	200 rads	intraportal islets	>200 d (control 9 d)
70	rat	BN, LEW	bone marrow	ALS	liver	>150 f (control 13 d)
			bone marrow	none	liver	no extended survival
71	rat	LEW, ACI	T-cells	200 rads	islets	extended survivial
72	rat	BN, LEW	splenocytes	ALS	heart	no extended survival

Many other studies have complimented the result of the earlier work and demonstrate that the inoculation of the recipient thymus with donor cells (in inbred rodents) can provide tolerance to allogeneic donor tissue. A summary of the results is provided in Table 1. In most studies, rats and mice will accept allogeneic grafts provided they are pretreated with antilymphocyte serum and intrathymic inoculation of donor cells. However, in cases where there were major MHC mismatches between donor and recipient, graft survival was not extended.

The intrathymic injection of nonimmunogenic cells does not induce tolerance in the rats model as demonstrated by Moore (56,57). The two reported studies were identical and used rat F344 donors and WF diabetic (streptozotocin treated) recipients. Pancreatic islets were collected from adult and neonatal donor animals and injected intrathymically into recipients (with or without ALS treatment). The neonatal islets had negligible amounts of Class II antigens. After intrathymic inoculation, recipients received renal subcapsular donor strain adult islet grafts. The group with adult cell thymic inoculation had prolonged normoglycemia whereas those that received neonatal islets all continued to have hyperglycemia and islet graft failure.

To better determine the factors involved in the induction of tolerance with intrathymic inoculation of donor cells more recent studies have refined the material used for injection. T-cell and other cell membrane soluble antigens (MHC Class I or II) can be purified using a KCl dissolution process with ultracentrifugation (73). In addition, synthetic peptide

Table 1- Studies utilizing whole cells as inoculant for intrathymic injection

Ref.	Species	Donor, Recipient	Innoculant	Other Treatment	Grafted Organ	Result
a27	rat	LEW, WF	splenocytes	ALS	heart	> 120d graft survival (control 5d)
a22	rat	ACI, WF	myocytes	ALS	liver	83 d - all grafts rejected
a21 a22	rat	OA, PVG (high responders)	splenocytes	ALS	heart	13d (control 7d)
			splenocytes	ALS + CsA	heart	26d
			none	ALS	heart	13d
a17	rat	ACI, LEW	splenocytes	ALS	heart	50% to >100d (control 7d)
		LEW, ACI	splenocytes	ALS	heart	75% to >100d
a16	rat	LEW, Buffalo	splenocytes	ALS	bowel (7d post ALS)	18d (control 7)
			splenocytes	ALS	heart (7d post ALS)	indefinite
a15	rat	ACI, Buffalo	splenocytes	ALS	heart (21d post ALS)	50% (n=2) extended survival
		LEW, Buffalo	splenocytes	ALS	heart (21d post ALS)	80% (n=2) extended survival

Table 1 (cont)- Studies utilizing whole cells as inoculant for intrathymic injection

Ref.	Species	Donor, Recipient	Innoculant	Other Treatment	Grafted Organ	Result
64	mice	Class I mismatched	splenocytes	ALS	skin	115 d
		Class I and Class II mismatched	splenocytes	ALS	skin	24 d
		Fully mismatched	splenocytes	ALS	skin	24 d
65	rat	ACI, LEW	T-cells	200 rads	heart	>300 d (control 6 d)
			T-cells	200 rads	small bowel	>150 d (control 6d)
66	rat	Major and minor MHC incompatible	splenocytes	ALS	heart	15 d (control 6 d)
		Major MHC incompatible	splenocytes	ALS	heart	165 d
		Partial major MHC incompatible	splenocytes	ALS	heart	163 d
67	rat	ACI, LEW	bone marrow	ALS	islets	130 d (control 8d)

Table 1 (cont)- Studies utilizing whole cells as inoculant for intrathymic injection

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68	rat	BN, LEW	lymphocytes	none	kidney	63 d (control 10 d)
			lymphocytes	CsA	kidney	8 d
			none	CsA (3d)	kidney	8 d
			none	CsA (100d)	kidney	>40 d
69	rat	ACI, LEW	splenocytes	200 rads	intraportal islets	>200 d (control 9 d)
70	rat	BN, LEW	bone marrow	ALS	liver	>150 f (control 13 d)
			bone marrow	none	liver	no extended survival
71	rat	LEW, ACI	T-cells	200 rads	islets	extended survivial
72	rat	BN, LEW	splenocytes	ALS	heart	no extended survival

chains representing epitopes of the intact antigen have been produced. Both materials have been used as thymic innoculant in a number of studies, the results of which are summarized in Table 2.

The use of intrathymic injection for developing immunity to allografts in the rat may be a promising technique but the rat is perhaps unique among other species in its ability to also readily accept allogeneic grafts when treated using other nonthymic protocols. A number of these experiments are described below.

2.5 Development of allogeneic graft tolerance in the rat - nonthymic

Hutchinson (74) has experimented with the use of donor specific transfusion to produce tolerance in allogeneic grafts. Lewis rats receiving ACI heterotopic hearts had significantly enhanced graft survival if they were treated with both donor specific transfusion (DST) and CsA (5 mg/kg/d subcutaneously from time of transfusion to 14 days after transplant) in comparison to CsA treatment alone (>98 days compared to 50 days). In the control group that received no CsA or donor specific transfusion death occurred within 10 days. In this experiment, the 2 week course of CsA alone increased survival. This may be attributable to a reduced T-lymphocyte population or CsA damage to the thymus. If CsA was not be given at the time of the DST, rejection occurred at 14 days comparable to the control group. There were no benefits seen with multiple DST treatments. Results comparable to this were also demonstrated in renal transplants in rats

Table 2 - Studies utilizing thymic inoculation of cell antigen

Ref	Species	Donor, Recipient	Innoculant	Other Treatments	Grafted Organ	Result [control]
77	rat	LEW, WF	T-cell antigen (2-4 mg)	none	islets	prolonged survival
			T-cell antigen (0.5-1.9 mg)	none	islets	no extended survival
			T-cell antigen (0.5-1.9 mg)	ALS	islets	marginally extended survival
78	rat	RT1b and fully mismatched RT1L	splenocyte antigen	ALS	heart	153 d
					kidney	14 d
					skin	12 d
79	rat	ACI, LEW	splenocyte antigen	ALS	islets (renal capsule)	>40 d
80	rat	ACI, LEW	multiple donor splenocyte Ag	ALS	mixed donor islets	>150 d
73	rat	LEW, WF	T-cell antigen	ALS	heart	prolonged survival
			T-cell antigen	ALS	islets (renal capsule)	prolonged survival

Table 2 (cont) - Studies utilizing thymic inoculation of cell antigen

Ref	Species	Donor, Recipient	Innoculant	Other Treatments	Grafted Organ	Result [control]
81	rat	WF, LEW	splenocyte Ag	ALS	heart	prolonged survival
82	rat	LEW, WF	LEW + BN splenocyte Ag	ALS	LEW heart	prolonged survival
		ACI, WF	ACI + BN splenocyte Ag	ALS	ACI heart	no survival
		LEW, WF	none	ALS+LEW splenocytes IV	LEW heart	prolonged survival

(LEW to DA), where the pretransplant sharing of donor blood, in groups with either minor or major histocompatibility antigens, lead to extended graft survival (58).

Ahmed et al (75) compared the efficacy of RBC, lymphocytes (peripheral), and whole blood intravenous injections in LEW recipients of ACI heart grafts. Their protocol included 14 days post graft CsA treatment. Control (CsA alone), whole blood, and RBC groups had survivals of approximately 50 days while those receiving lymphocytes survived 90 days. Brunson (76) used a 5 days CsA regimen (post graft) and gave a single ACI whole blood injections to recipient LEW rats. Control animals survived 5 days, those on CsA alone 10 days, and those on CsA plus donor blood 24 days. Many of the studies noted in Table 1 used IV injection of donor cells as thymus injection controls and found no extended survival. Light et al (72) recently published results contradictory to this. They used BN donors and LEW recipients that received either IT or IV injection of donor splenocytes. In the IV group, 50% of animals had extended survival while no animals survived in the IT group.

Similar donor-specific transfusion treatment has been used in humans with some success. Anderson et al (83) and Newton et al (84) have reported results from patients receiving donor specific blood cell inoculation and continuous immunosuppression (azathioprine) before receiving living-related-donor kidneys. The donors had HLA (MHC) that was at least one haplotype different from the recipients. No clinically significant sensitization occurred in the recipients. There was a 92% renal allograft survival which is a rate similar

to that found in HLA identical sibling transplants. A more recent treatment (85) has been to include bone-marrow infusion with cadaveric renal grafting. The rationale is that the donor bone marrow cells will mature within the recipient producing a form of microchimerism and enhanced survival of cadaveric allografts. However, bone marrow infusion can also result in GVHD. There is evidence that in some human transplant cases, microchimerism develops simply from the presence of the donor graft (86,87).

Two studies in mice have demonstrated good results in allogeneic graft tolerance using ALS injection alone. Bishop (88) used DBA/2 donors and C57BL/6 recipients, giving recipients anti-CD4 serum one day before and one day after cardiac grafts. No other treatment was given. Over half of the grafts continued to function after 60 days. Song (89) used A/J and B6 mice in a similar experiment but compared anti-CD4 and anti-CD8 (and both) serum. The two sera combined provided extended survival of grafts whereas anti-CD4 alone did not. Anti-CD8 alone provided only a minor delay in the onset of rejection. These results can also be seen in some ALS control groups of studies highlighted in Table 1 and Table 2.

A study in rats by Yoshimura (90) designed to test the immunoprotective role of amniotic fluid provides interesting information. Female LEW rats were mated with male BN rats. Amniotic fluid was removed from the pregnant rats at 14 days. Other LEW rats then received renal grafts from BN donors and either intravenous amniotic fluid or saline. Amniotic fluid treated animals rejected grafts in 20 days whereas saline treatment resulted

in rejection in 8 days. The rats received no other immunosuppression during the study period. Treatment with intravenous maternal blood for 5 days in the LEW rats did not increase graft survival beyond controls. The amniotic fluid injections suppressed LEW lymphocyte, allokiller T-cell, interferon and IL-2 production.

A variety of CsA regimens were compared in a study with BN and WAG rat strains receiving renal allografts and indicated that donor/recipient strain relationships are also critical to survival (91). WAG donor to BN recipients: controls 17d, 15 mg/kg CsA (one week) 20d, 25 mg/kg 3 times weekly until endpoint 93d. BN donor to WAG recipients: controls 12 d, 5/kg CsA (one week) 41d, 15 mg/kg CsA (one week) >200d. The WAG to BN is a much more difficult model to induce tolerance to than BN to WAG where permanent survival could easily be obtained with only moderate doses of CsA. High dose treatment with CsA in WAG rats caused significant toxicity and mortality. Vogt (92) tested LEW rats against a variety of donor strains. MHC mismatched grafts were rejected even if minor MHC groups were identical. Major MHC compatible grafts survived indefinitely as did groups with MHC minor incompatibilities. This experiment demonstrated that MHC Class II antigens were much more effective at inducing rejection than MHC Class I antigens and that antigens that were non-MHC associated usually did not induce rejection in these groups. Similar more recent results have also been published (66).

2.6 Other Animal Models

There are few published reports examining the use of intrathymic injection for development of immune tolerance using species other than rodents. Kenomochi et al (93) used young outbred NIH minipigs as donors and recipients of intrathymic islets. Recipients had previously had complete pancreatectomies (30% immediate post operative mortality rate). Out of a group of five animals, two grafts survived for >200 days while the remainder survived 7-30 days (as measured by occurrence of hyperglycemia). Merhav et al (94) used mongrel dogs as their model for cardiac and renal allogeneic grafts. They injected donor splenocytes into recipient thymus with concurrent ALS treatment but there was no extended survival of either graft type (median 16 days). Brief treatment (1 day pregraft and 4 days postgraft) with prednisone, CsA, and azathioprine did not improve survival. There have been two reports of the use of intrathymic injection in xenograft models. The first by Zeng (95), was a rat/mouse model in which C57BL/6J mice received WF rat synthetic MHC class II peptides intrathymically (with or without anti-CD3 serum). Islets were grafted into the recipients and grafts rejected before 40 days. The longest surviving grafts were found in those mice that received both ALS and intrathymic antigen. Shen (96) used one day old rats as recipients and hamsters as donors in a heterotopic heart graft model. Recipients were treated with intrathymic donor splenocytes but no extended survival was demonstrated.

2.7 Rat Versus Human Immune Systems

Inbred rats may be unique in their ability to develop immunotolerance toward a variety of allografts after treatments ranging from small doses of CsA alone to intrathymic injection of donor cells with ALS treatment. Simple tolerance induction is not usually seen in the human transplants where 25% of recipients of cadaveric grafts reject the grafts within one year despite intensive treatment with a variety of immunosuppressant systems (33,34). What are some of the significant interspecies differences that suggest that the rat is not the ideal model for deriving useful human clinical techniques?

Many of the rat experiments involved vascular grafts. Both rats and humans have prominent MHC Class I and MHC Class II antigens on passenger lymphocytes, as would be expected. Humans demonstrate MHC I and MHC II antigens within vascular endothelial cells and MHC I antigen within renal tubular cells. A similar distribution of antigens can be found on the cell's surface. In the rat, there is no MHC I or II antigens on the vascular endothelium or intertubular capillary cells. MHC II can be found in the cytoplasm of the proximal renal tubular cells but is nonimmunogenic since none of the MHC II antigens is found on the cell surface (97). The rat kidney has MHC Class I surface positive interstitial dendritic cells that have the ability to present antigens and stimulate the host's lymphocytes. These freely circulating cells have been found to be highly immunogenic but are not seen within transplanted grafts 3-4 days post surgery (98). In human grafts, MHC Class I antigens are found on epithelial cells of end

capillaries throughout the graft life and present a constant site for potential rejection reaction. The wash out effect seen in the rat may be one of the reasons that a short course of CsA alone is required to induce tolerance to grafts by simply carrying the recipient beyond the immunoreactive washout period (91,99).

Many early studies using intrathymic injection to produce graft tolerance (50,51) relied on immunosuppression (other than ALS) in the perioperative period as did a number of other rat transplant models (91,100). In a study of CsA effects on the thymus and immune system (101), rats receiving 45 mg/kg/d CsA all died in less than 14 days whereas 15 mg/kg/d was well tolerated for 14 days and provided a mean whole blood concentrations of CsA of 6 μg/ml (approximately ten times the recommenced human whole blood concentration). After a dose of 15 mg/kg/d for 14 days in rats, there was a complete disappearance of thymic medulla structure, including the microenvironment defined by medullary type epithelial cells, dendritic cells with MHC II, and mature T-lymphocytes. Two weeks after CsA treatment was stopped, the medulla began to reappear with epithelium, and appeared to have almost completely returned in about 4 weeks including the return of mature T-lymphocytes. However, after the CsA treatment and thymus recovery, there were still numerous gaps in the medulla filled with epithelium and dendritic cells. A similar study demonstrated that administration of CsA to mice at a dose of 15 mg/kg IP arrests the differentiation of CD4+8+ and CD4-8- cells in the thymus (102). An immuno-comparable dose of a newer generation immunosuppressant, FK-506, has the same effect on the rat thymus cells as CsA (103). It is not known how these

factors might affect tolerance induction.

2.8 The Rabbit as a Model for Allograft Transplantation

Significantly fewer studies of allogeneic transplantation have been reported in the rabbit model compared to the rat or mouse. From an animal management and surgical point of view, the rabbit can be much more fragile than the rodents. They are more costly to maintain and there are no readily available truly inbred strains. However, the rabbit is perhaps a more appropriate model with which to investigate clinically relevant transplant and immunology methods. The similarities between rabbit and human immune and transplant models are discussed below.

2.8.1 The Rabbit Immune System

The rabbit MHC Class II pattern of expression is in close harmony with that of humans (104,105,106). In relation to the human Class II gene regions (DR (25), DQ (107), DP, and DZ) the rabbit expresses similar counterparts for these genes in a variety of tissues including the spleen, bone marrow, appendix, and lymph nodes. The DQ, DR, and DZ genes are also found in the thymus. A more recently described human beta chain gene (DO) has also demonstrated in homology in the rabbit. Compared to the mouse, which has a more restricted expression of MHC II genes, the rabbit compares much more closely to the human expression or coexpression of genes from the three subloci (DQ, DP, DR)

(106). Genetic homology by nucleotide sequences indicate the rabbit to human values for the DP α1 genes is approximately 89% (105). Similarities between the human MHC Class I antigen expression and the rabbit have also been described in detail (108). In mixed lymphocyte culture technique for monitoring the allogenicity between donors the rabbit RLD locus provides the necessary cellular control, identical to that in human cultures dependent on the HLA-D region (109).

The distribution of the rabbit 8AC8+ cells (helper and cytotoxic T-cells) and 8AC8- cells (B-lymphocytes, null cells, granulocytes, and monocytes) is analogous to humans. Rabbit T-lymphocytes demonstrate close homology to human counterparts. A set of rabbit cells responding to MAb 8AC8, are similar in reaction to human OKT4+ cells (helper cells). They proliferate as a response to alloantigens and they can generate cytotoxic effector cells in a xenograft system (110).

Further indication of human-rabbit immune similarity has been demonstrated in B-lymphocytes (110). A MAb against human MHC class II was developed (MAb 44H10) and demonstrated reactivity to all human B-lymphocyte populations regardless of their HLA phenotypes. When tested against a variety of rabbit lymphoid tissues, the cells from appendix, lymph nodes (mesenteric), and spleen all demonstrated reaction. When these same cell suspensions (spleen and lymph node) were depleted of Ig+ cells, no reaction was seen with the MAb 44H10. There was no cross reactivity between the MAb and T-lymphocytes. Individual outbred rabbits had similar reaction positive cell distributions.

The reaction of lymphocytes during the immune process requires the adhesion to each other and to endothelial cells before specific reactions can occur. One of the most important of these adhesion complexes is the CD11/CD18 glycoprotein cell surface complex. Using a series of MAbs to CD18, it has been demonstrated that the rabbit glycoprotein complex (GP150/GP85) is analogous to human CD11/CD18 and is found on matching cells in the rabbit (lymphocytes, monocytes, and granulocytes). Although these rabbit and human glycoproteins had distinct molecular weight differences, an injection of human anti-CD18 into rabbits produced the expected circulating lymphocytosis, indicating loss of adhesion and reduction of cells homing in peripheral lymphoid tissue (111).

2.8.2 Rabbit as Transplant Models

The rabbit has been successfully used in traditional transplant models that rely on CsA for immune suppression (112). Rabbits have also been used in models for donor-specific transfusion transplant work. In a study to determine the value of donor specific transfusion and CsA, as well as the optimal regime for enhanced skin allograft survival, Smit et al (71) found that doses of CsA of 20 mg/kg (single dose at time of DST) were most beneficial. Higher doses (50 mg/kg) had reduced survival times as did those receiving smaller doses of either 5 mg/kg or 1 mg/kg. Survival of rabbits receiving only CsA was 50% shorter than those receiving CsA and DST therapy (113). This reduced

effect seen in the highest dose group may be a result of the destruction or inhibition of T-lymphocytes responsible for inducing (or mediating) immune tolerance to the skin grafts.

Similar CsA dose requirements were required to prevent rejection of allogeneic cultured fetal islet cell grafts in mature rabbits (114). No islet cell transplants survived in control groups receiving either no CsA, or CsA alone (30 mg/kg/d on days 0-3) with no donor cell UVB irradiation. In other CsA groups, islet cell cultures were treated with UVB radiation (900 J/m^2) prior to renal subcapsular transplantation. Survival with UVB and CsA at 10 mg/kg/d (short course) was only 16%, whereas increasing the CsA to 30 mg/kg/d (short course) resulted in an 80% graft survival. Rabbits treated with chronic lower dose CsA (10 mg/kg/d) from time of transplant to sacrifice also had prolonged survival. The ability for this ultrashort course CsA to induce tolerance is similar to that seen in the rat thymus models. No thymic histology or peripheral blood smears were examined in this series.

2.9 Mixed Lymphocyte Culture

MLC (mixed lymphocyte culture) is an assay that allows for a measure of one of two cell mediated immunity functions, namely proliferation (or recognition of immunologically foreign cells). Cytotoxicity can be quantified using cell-mediated lympholysis (CML)

assay. This measures the ability to produce cytotoxic T-cell resulting from the initial steps following antigen recognition. In humans and rabbits, the cellular control of the MLC is based on the MHC complex HLA-D region (109). In MLC assays, donor lymphocytes are treated with radiation or mitomycin C (a chemical agent that prevents DNA synthesis) to render them non-proliferative. Recipient and donor lymphocytes are then cultured together. A small population of recipient cells can proliferate in response to the presence of the donor cells. This specific proliferation is measured using a radioactive thymidine pulse technique.

In clinical situations, MLC can be used as a predictor of donor-recipient compatibility. There is no set level at which compatibility/incompatibility can be specifically defined. However, the use of pooled blood MLC can be used as a reference to which experimental MLC results can be compared and rated. Methods for determining MLC results also include simply subtracting the radiated autologous cell levels (representing the spontaneous division of responder cells) from the values of the cells demonstrating the allogeneic response. The use of a simulation index (ratio of experimental to control counts) has been successfully demonstrated in rabbits by Maske R et al (115). They used MLC responses to provide a pregraft indication of allogenicity in rabbits undergoing corneal grafting. A stimulation index >1 indicates a greater than baseline level of recipient lymphocyte stimulation by donor lymphocytes.

2.10 Thyroid Transplantation

The transplantation of thyroid tissue without the need for vascular micro-anastomosis provided an ideal model for use in this matched donor-recipient long term study. Simple implantation or seeding of thyroid tissue has good clinical success. In 1963, Skolnick (116) reported on the case of a 30 year old female patient with an ectopic thyroid tissue mass located near the foramen cecum. Thyroid studies using radioactive iodine did not identify any thyroid tissue in the normal location in the neck. The mass required excision since it was interfering with normal glutation and breathing. At surgery, the bulk of the mass was excised and the remainder (along with contiguous tissue) was transposed into the neck. From the bulk of the excised mass, 1 mm slices were obtained which were placed into left and right rectus abdominus muscle sheaths. Similar slices were placed into the right pectoralis major muscle. Iodine studies six months later demonstrated good uptake in the abdomen and chest areas. Many other reports have since been published. Swan (117) described similar results in a 7 year old girl with histological evidence of intra-rectus thyroid autograft surviving eleven years post implant. Wertz (118) reviewed nine cases of thyroid autografts placed in the musculature in neck, abdomen, and chest. Functionality was limited in a number of the grafts but histological examination demonstrated normal appearing or hyperplastic thyroid tissue. Isolated follicle autografts have also been used successfully (119). Similar results have also been obtained with parathyroid tissue (120,121). This process of thyroid implantation has also bee used in animal models. Talmage (122,123) cultured mouse thyroid tissue and placed it under the

renal capsule in recipient mice with good results. Other groups have used slices of avascular thyroid in dogs (124) and isolated follicles in the rat (125) transplanted into muscle or retroperitoneal fat tissue.

2.11 Aim and Scope of Study

Many reports have been published that demonstrate achievement of tolerance to allogeneic grafts following intrathymic inoculation using inbred rat and mice models. Renal, cardiac, bowel, and skin grafts have all proven successful. However, it appears that this same success has not be achieved to date in the outbred or xenograft model. This present experiment was designed to determine whether the development of immune tolerance by intrathymic inoculation of donor lymphocytes can be achieved in the outbred rabbit model. Rabbits were chosen since this species provides an immunologic model closer to the human clinical situation compared to the rat or mouse. In addition rabbits were used to investigate whether this phenomenon could be demonstrated in a species other than the rat or mouse or whether development of this tolerance was truly species specific.

3. METHODS

3.1 Study Design

This study was approved by the University of Manitoba, Use of Animals in Research, Protocol Management Committee, as a class 'D' protocol. All animals were housed and cared for according to the Canadian Council on Animal Care Guidelines. Young adult (6 month old), outbred New Zealand White (NZW) rabbits (recipients) and California rabbits (donors) were obtained from Prairie Rabbitry (Manitoba). These rabbits were not specific pathogen free, so were kept in isolation and treated with enrofloxacin for one week to prevent Pasteurellosis outbreak. A number of rabbits developed acute-on-chronic episodes of mild to moderate respiratory Pasteurellosis which were treated with enrofloxacin. Recipient-donor pairs were assigned on a random basis and were maintained throughout the experiment. Donor animals could be reassigned to a second recipient if the first recipient died.

Rabbit pairs were divided randomly into experimental and control groups. The experimental protocol is outlined below:

Day -4	Blood samples for initial MLC assay
Day -1	Injection of ALS

Day 0 Intrathymic injection -

Control group - 1 ml of RPMI cell medium

Experiment group - 1 ml of RPMI with 5×10^7 donor lymphocytes

Day 7 Blood sample for MLC assay

Day 49 Blood sample for MLC assay

Day 125 Blood sample for MLC assay

Day 132 Thyroid transplantation

Day 139 Biopsy of graft site

Day 174 Biopsy of graft site and euthanasia

3.2 ALS Testing

An initial sample of whole blood was withdrawn from each animal (n=6) to determine total white cell and lymphocyte count. Counts were performed on dired blood smears processed with Wright's stain. Each rabbit then received a slow intravenous injection (marginal ear vein) of 0.5 ml (165 mg total protein) of goat anti-rabbit thymus lymphocyte antigen antiserum (Cedar Lane CL8800). The antiserum is specific for rabbit T-cell lymphocyte sub-populations. The rabbits were closely monitored for one hour for any immediate severe immune reaction. A second whole blood sample was collected the following morning at 0900-1000h.

3.3 Preparation of donor lymphocytes

Donor whole blood (10 ml) was collected by venipuncture of the marginal ear or jugular vein into a heparinized collection tube (Becton Dickinson, lithium heparin). The blood and an equal amount of RPMI-1640 (Sigma) cell culture media (22 C) were added to a 50 ml conical centrifuge tube (Falcon Brand). Using a 10 ml syringe with a 10 cm 14g needle, 5 ml of Histopaque-1077 (Sigma) was slowly added to the bottom of the tube. The layered solution was centrifuged at 400g for 30 minutes at 22C. The top layer of material was carefully removed using a Pasteur pipette. The next layer (lymphocytes) was similarly removed and added to 20 ml of ice cold Hanks buffered saline solution (HBSS, Sigma). The lymphocytes were centrifuged at 400g for 10 minutes at 4 C. The pellet was washed again with HBSS and centrifuged. The lymphocytes were resuspended in cold RPMI. The lymphocyte suspension was maintained on ice until the time of injection.

3.4 Intrathymic Inoculation

Recipient rabbits received ketamine and xylazine as preanaesthetics, were intubated, and maintained with mechanical respiration and isoflurane for the remainder of the procedure. Body temperature was controlled with a circulating water heating pad. After preparation of the surgical site, a 1.5 cm incision was made on the left chest wall between the second and third ribs beginning at the sternum. The incision was carried through the inter-costal

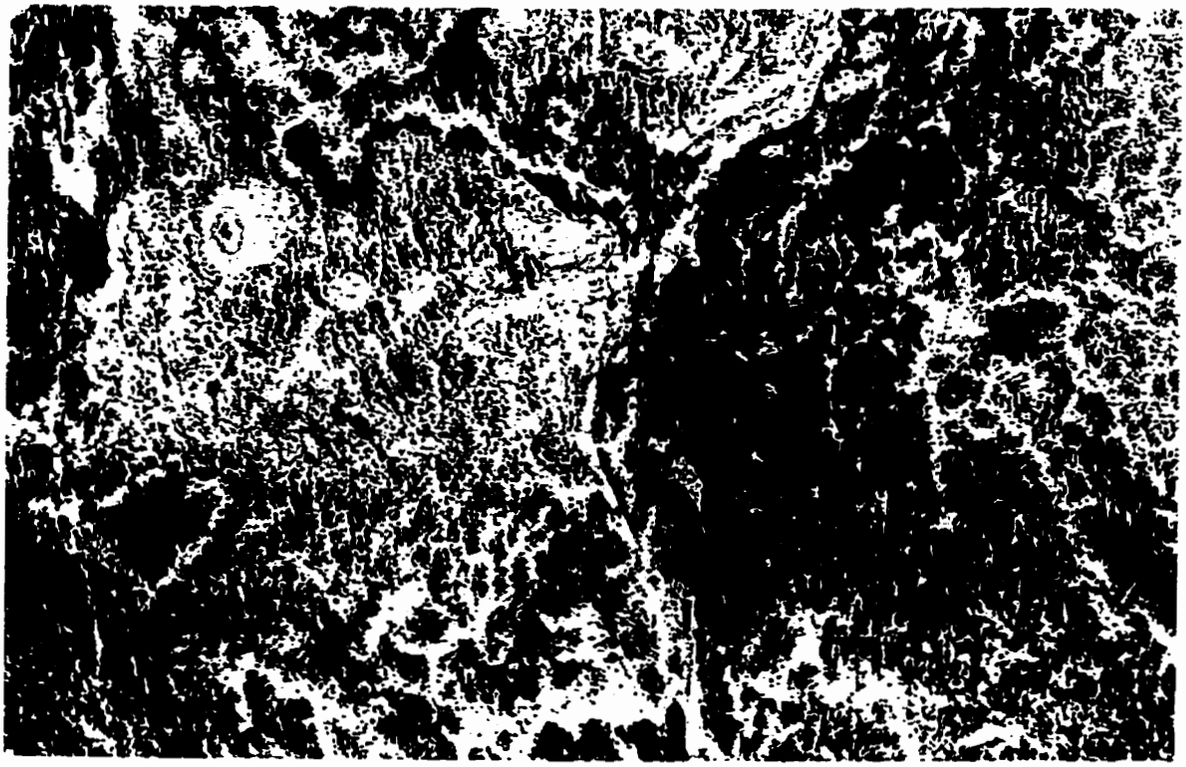
muscles taking care not to incise the lung. The second rib was bisected at the sternum and pivoted cranially to open a window into the chest cavity. The thymus was gently brought into the opening using a pair of forceps and held in place during injection. Intrathymic injection was completed using a 32g needle injecting into multiple sites. (Two additional non-paired rabbits received intrathymic injection of India ink for histological study and assurance that the thymus had been correctly identified. See Figures 1a and 1b) The thymus was replaced and the rib approximated using a figure-of-eight suture. One end of a small chest tube was placed into the pleural space and the opposite end exteriorized to an air-tight valve. Tissues were closed in layers and intermittent suction was applied to the chest tube. The tube was removed approximately 4 hours post operative once no further fluid or air could be removed. All animals received 48-72 hours of analgesia (torbugesic or buprenorphine) post operatively.

3.5 Mixed Lymphocyte Culture Method

We used a modification of the methods described by Hutchinson (109) and Maske (115). Donor and recipient blood (10 ml) was collected into heparinized Vacutainers (Becton Dickinson, lithium heparin) by venipuncture of the marginal ear vein or jugular vein. All processing of samples was completed in a sterile laminar flow hood. Each sample of whole blood was centrifuged for 10 minutes at 1500g. The buffy coat containing white blood cells (normally contaminated with a small amount of red blood cells) was removed and diluted in approximately 5 ml of sterile normal saline (McGaw Ltd., Irvine, CA) at

Figure 1a:
Normal rabbit thymus tissue demonstrating the density of thymocytes.(160X)

Figure 1b:
Normal rabbit thymus. India ink injected as a trial to demonstrate inoculation of donor lymphocytes. (100x)



room temperature. The diluted cells were then carefully layered over 4 ml of Lympholyte-H (Cedar Lane, Hornby, ON) and centrifuged for 15 minutes at 800g at 22 C. The lymphocyte layer at the interface was removed using a 5 ml serological pipette. The cells were washed with saline and centrifuged at 400g for 10 minutes. The saline was removed and the cells carefully washed with 10 ml of RPMI-1640 + HEPES (Gibco). The mixture was centrifuged at 400g for 10 min. The lymphocyte pellet was resuspended in 3 ml of RPMI-1640 + HEPES with 5% normal rabbit serum (Sigma - treated at 56C for 1 hr) and 1% penicillin and streptomycin mixture (10,000 U/ml penicillin, 10,000 µg/ml streptomycin-Gibco). A 100 µl aliquot of the cell suspension was mixed with 100 ul of 2% trypan blue solution. A cell count was completed using an Improved Neubauer Brightline hemacytometer and the concentration of the original suspension adjusted to 2 x 10^6 cells/ml with the RPMI-1640 + HEPES culture media.

Donor (DX) cells were irradiated with 2500 rads over 9 minutes using a γ-irradiator (Beckman LS5801). An aliquot (typically 1 ml) of recipient (RX) cells was also irradiated for use as control cells. One hundred microlitres of untreated recipient (R) cells were mixed with 100 µl of DX cells in each of three wells. (Falcon, 96 wells, flat bottom). Similarly control cells were set up containing 100 µl of R and 100 µl of RX cells. This same cell mixture was used for every donor-recipient pair. The plates were incubated at 37C, 5% CO_2 for 48 hours. After preliminary incubation, 30 µl of ^{3}H-thymidine (Amersham Life Science, 185 Gbq/mmol, 37 Mbq/ml) was added. The cells were

incubated overnight under the original conditions. The contents of each well was removed for cell collection by filtration using a cell harvester (PHD 200, Cambridge Technology Inc.) and filter mats (Skraton). Six to eight washings of each well were also performed. All washings were processed through the same filter. The filter mats were then rinsed with 95% ethyl alcohol and dried for a minimum of 3 h at room temperature in individual scintillation vials. Before counting, 2 ml of scintillation fluid (Aquasol - Packard Ltd.) was added to each vial and the contents mixed by shaking. Each vial was counted for 1 minute. The mean count values for the triplicate samples were used in calculating the MLC stimulation indices.

3.6 Thyroid Transplantation

The donor was preanaesthetized with ketamine and xylazine and maintained on isoflurane. After appropriate skin preparation, a midline incision was made over the anterior neck and the muscles divided. The thyroid was isolated from surrounding tissue and approximately one-half of one lobe was excised and placed into normal saline. The incision was closed in layers. The thyroid tissue was diced into pieces approximately 1 mm x 1 mm and kept in saline. The recipient was similarly anaesthetized and the skin covering the right gluteal area was prepared for surgery. The skin was incised and two locations of the gluteal muscle fibres gently split with a surgical clamp. Four to five pieces of the thyroid were placed into each opening. The muscle was closed and a polyethylene suture was placed as a marker for biopsy locations and histological sections.

Tissues were closed in layers. All animals received postoperative analgesia. Remaining thyroid tissue pieces were submitted for confirmatory histology (see Figure 2). Four additional NZW rabbits received thyroid autografts using the same basic technique.

3.7 Histology

The muscle graft sites were excised under general anaesthetic. Tissue was placed into formalin until processing into produce paraffin blocks. Muscle blocks had 10 μm sections removed every 100 μm and were stained with hematoxylin and eosin. Sections were examined with light microscopy. Representative photographs were taken using 100ASA Kodak color print film. At the time of euthanasia, tissue samples were also taken from recipient thymus and spleen. All sections were examined by two independent observers both blinded to the experimental groups.

3.8 Statistics

Statistical analysis was performed using SPSS non-parametric tests as recommended by Lumley and Benjamin (126). Wilcoxon Matched Pairs testing was used to compare MLC values within one pair across time. Mann-Witney U testing was used to compare between control and experimental group MLC at each time interval.

Figure 2:

Sample of thyroid tissue used for graft implantation showing normal peripheral follicular cells (arrows) and intact follicle contents (arrow heads). (100X)

4. RESULTS

4.1 ALS Injection

Total white blood cell and lymphocyte counts before and 16 hours after ALS injection are shown in Table 3. There were no changes in the total white blood cell counts. The lymphocyte counts decreased significantly.

In order to determine if further decreases could be obtained in lymphocyte counts, two rabbits were given a second injection of 0.5 ml ALS approximately 24 hours following the first injection. Within minutes of injection, both animals displayed signs and symptoms of anaphylactic shock. These rabbits were treated with intensive resuscitation but both animals died. A single injection of the ALS was used throughout the remainder of the experiment.

4.2 Rabbit Donor-Recipient Pairing

Rabbit donor-recipient pairs are detailed in Table 4 as well as the randomization between control and experimental groups. The experiment was designed for a total of five animals in each group. Only four pairs in the experimental group were included in the data analysis due to the late death of a matched pair donor. Table 5 lists the rabbits that died during surgical procedures or were euthanized due to loss of matched pair.

Table 3: Comparison of white cell and lymphocyte counts immediately before and 18h after injection of 1ml of ALS (n=6) (Cells x 10^9/L, mean ± SD)

Time	White Cell Counts	Lymphocyte Counts
Pre ALS Injection	9.9 ± 1.6 *	6.6 ± 0.9 †
Post ALS Injection	9.9 ± 4.7 *	3.1 ± 0.8 †

* No significant difference ($p>0.05$)
†Significant difference ($p=0.043$)

Table 4: Identification of Donor (California) - Recipient Pairs (New Zealand White)

Group	Donor	Recipient
Control	96-13	96-14
Experiment	96-15	96-10
Experiment	96-21	96-20
Experiment	96-23	96-22
Control	96-27	96-26
Control	96-35	96-16
Control	96-37	96-24
Experiment	96-43	96-40
Control	96-45	96-42

Table 5: Experimental Animal Deaths

Animal Code	Time and Cause of Death
96-11	8 hr postoperative - respiratory complications
96-17	20 h postoperative - respiratory complications
96-19	intraoperative - heart failure
96-25	24 h postoperative - respiratory complications
96-31	presurgical - fx femur - euthanized
96-33	immediate postoperative arrhythmia
96-39	intraoperative - unknown
96-41	72h postoperative - respiratory complications
96-12	euthanized - loss of experimental pair
96-18	euthanized - loss of experimental pair
96-29	euthanized - loss of experimental pair

Table 6: Comparison of stimulation index† between control and experimental recipients
(mean ± SD)

Time	Control Recipients	Experimental Recipients
Preoperative*	2 ± 1 •	13 ± 12
Week 1 postoperative §	8 ± 12 •	7 ± 9
Week 7 postoperative §	4 ± 4 •	1 ± 1
Week 18 postoperative§	1 ± 0.5 •	2 ± 1

* Preoperative MLC's were performed on triplicate samples
• No significant difference between controls and recipients ($p>0.05$)
§ No significant difference between previous MLC test in control or experimental group
† Stimulation index (ratio of donor-recipient to donor control counts)

4.3 MLC Stimulation Indices

MLC stimulation indices for control and experimental animals over time are shown in Table 6. There was no statistical difference between controls or experimental animals. Comparisons of MLC indices between time periods within the same experimental group also demonstrated no statistical differences.

4.4 Thyroid Graft Survival

Animals that received autologous thyroid tissue demonstrated survival of the implanted tissue two weeks following the graft placements. The follicular epithelial cells appeared normal, follicles were filled with colloid, and there were only rare lymphocytes present in the grafts (See Figure 3a and 3b). There was no visible inflammatory reaction. The was no evidence of graft necrosis. The presence of colloid indicates some degree of heterotopic thyroid tissue response to TSH.

Control and experimental rabbits all demonstrated histological evidence of acute graft rejection in the biopsies of the heterotopic thyroid tissue one week following thyroid graft implant (See Figure 4a and 4b and Figure 5). These slides demonstrate massive infiltration of lymphocytes and virtually complete destruction of follicular cells and intrafollicular matrix. Control and experimental animal muscle biopsies showed continued histological evidence of chronic graft rejection and inflammation of the thyroid

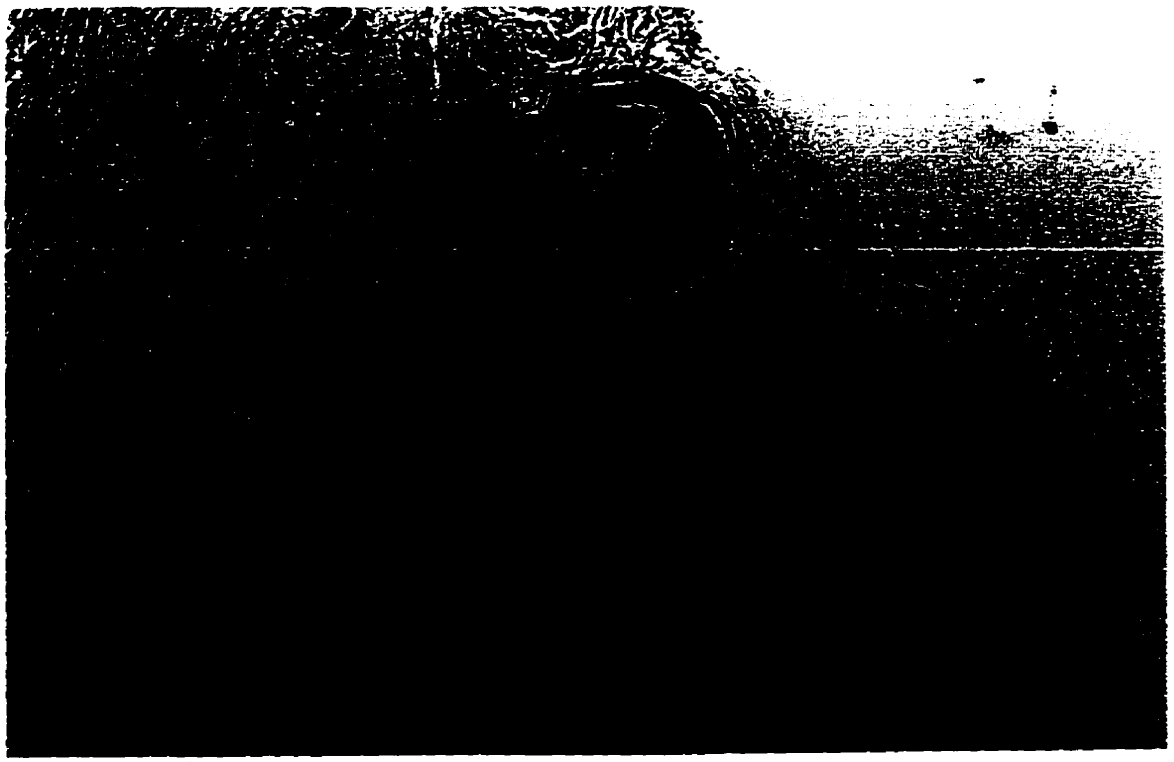
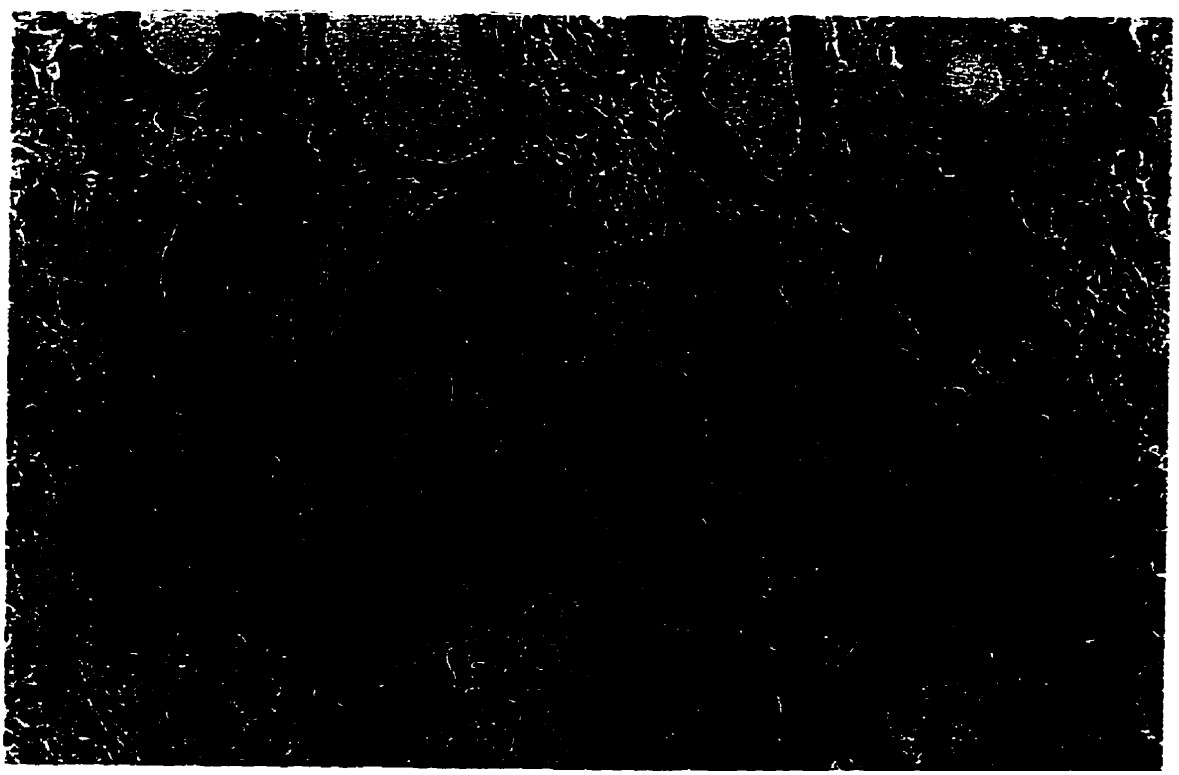


Figure 3a:
Biopsy of implanted thyroid autograft demonstrating normal follicle structure (2 wk post implant)(40x).

Figure 3b:
Biopsy of implanted thyroid autograft demonstrating normal follicle structure (2 wk post implant)(200x).
Follicular epithelial cells: arrows
Follicle colloid: arrow heads



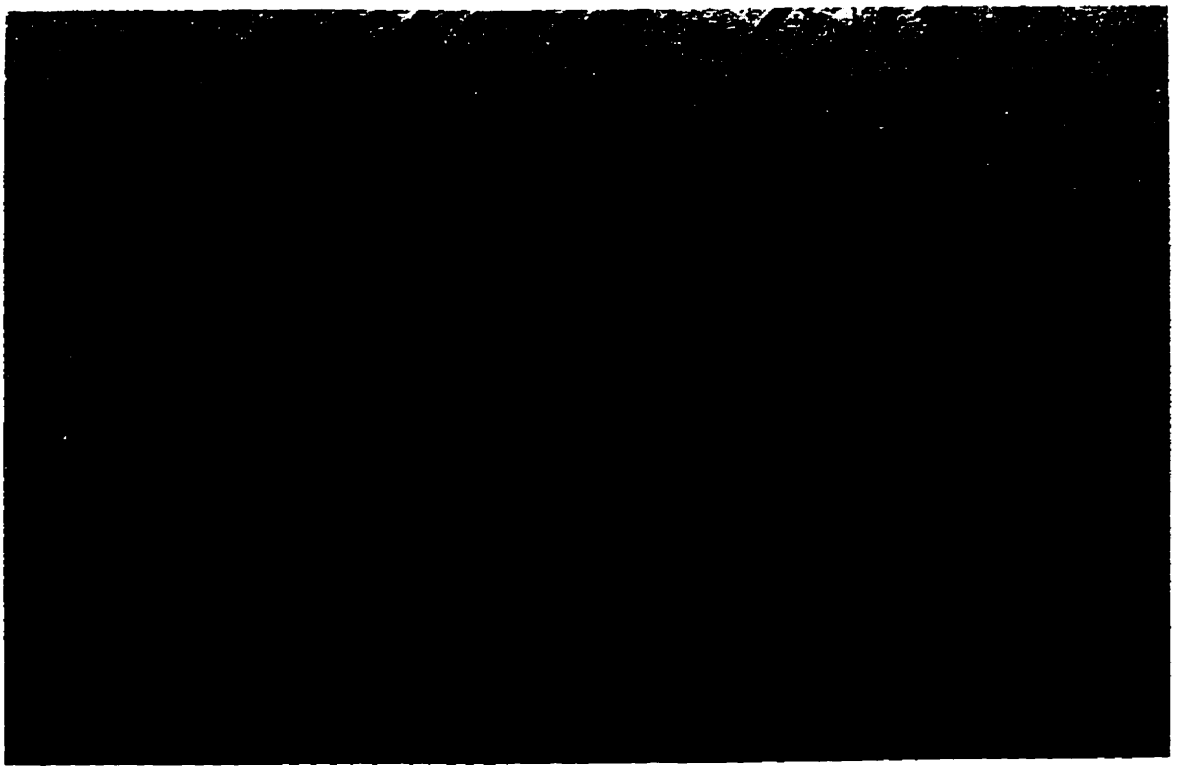
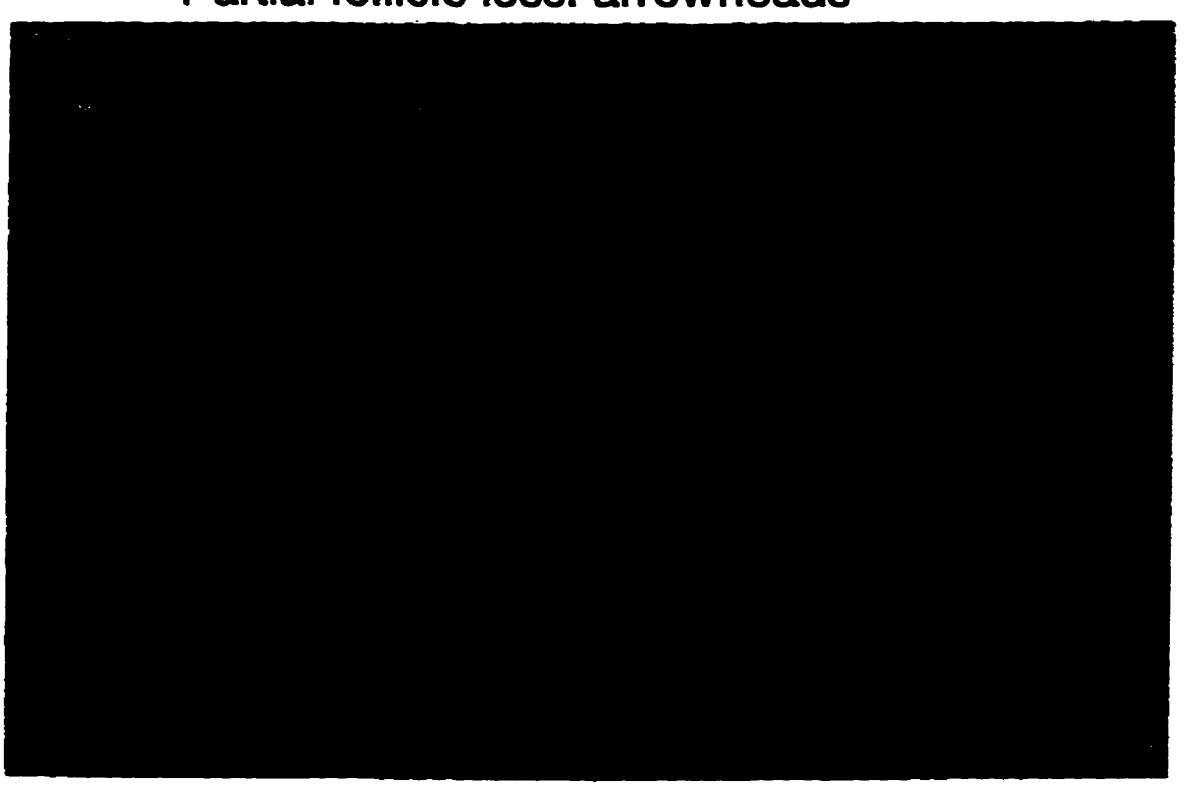


Figure 4a:
One week biopsy of implanted allogeneic thyroid graft demonstrating acute rejection.(40x)

Figure 4b:
One week biopsy of implanted allogeneic thyroid graft demonstrating acute rejection with diffuse mononuclear cell infiltrate.(200x)
Complete follicle loss: arrow
Partial follicle loss: arrowheads



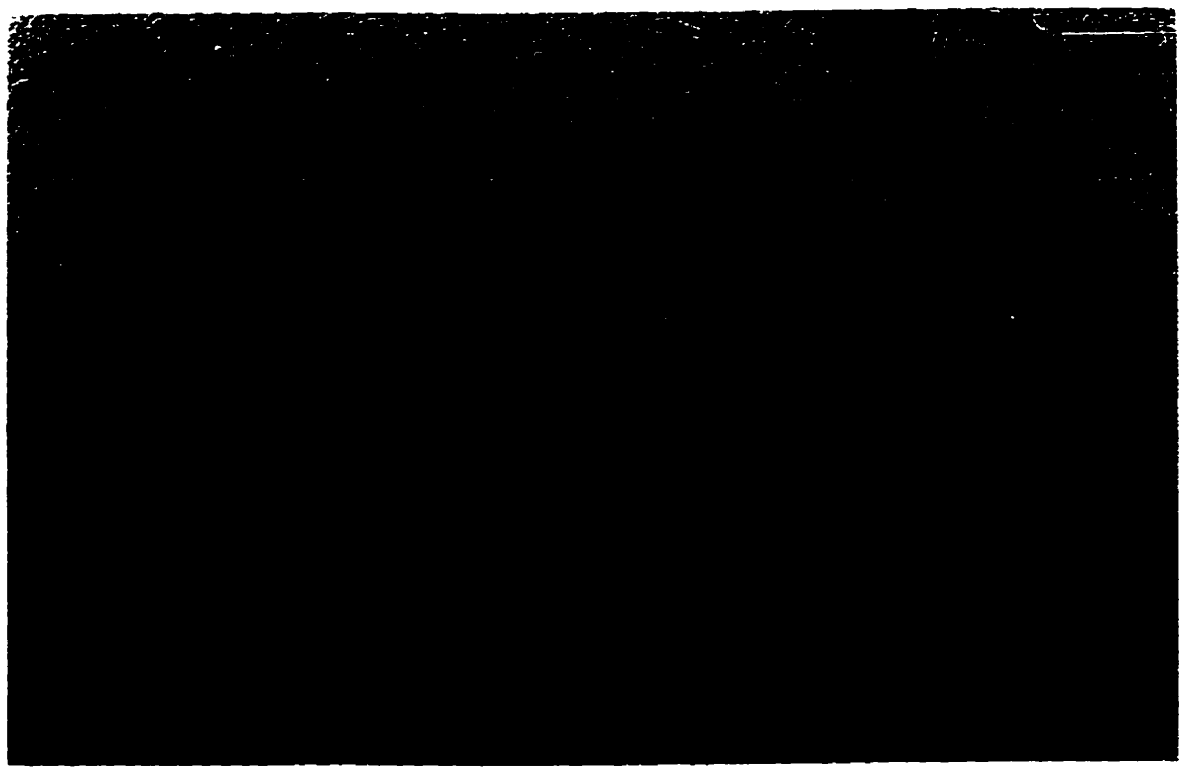


Figure 5:

One week biospy of implanted allogeneic thyroid graft demonstrating loss of follicular epithelial cells and follicular colloid due to rejection reaction. (200x)

Partially collapsed follicle with mononuclear cells in colloid: arrow
Completely collapsed follicles: arrow head

grafts six weeks following transplantation (Figure 6). There was no evidence of graft necrosis in any specimens.

Autopsy specimens of thymus and spleen did not demonstrate any morphologic changes. Functional testing was not done on any tissue specimens.

Figure 6:

Six week biopsy of implanted allogeneic thyroid graft demonstrating late stages of rejection. There is complete follicular collapse and loss of all thyroid tissue organization. The number of basophils is also increased. (100x)

5. DISCUSSION

Many studies (Table 1 and Table 2) have demonstrated that allogeneic graft tolerance can be induced in inbred rats and mice by inoculating the recipient thymus with donor cells/antigen before transplantation. The present study has shown that in outbred rabbits the induction of tolerance using intrathymic inoculation experimental protocols may not be possible.

5.1 ALS Treatment

Almost all successful tolerance induction experiments in rats and mice have required the use of ALS. The theoretical basis is that the ALS depletes the peripheral T-cell pool. This in turn signals the bone marrow to release additional stem cells for re-establishment of circulating T-cells. During this process, the stem cells must pass through the thymus, now inoculated with donor antigen. In this way a new population of recipient T-cells, tolerant to donor antigen may be produced. In the current experiment, ALS was given the evening before thymus inoculation with donor lymphocytes. Our results demonstrate a 66% reduction in the levels of peripheral lymphocytes. The antiserum is specific for membrane antigen on T-cells and reacts with cells in the thymus (96%), spleen (40%), lymph nodes (80%), appendix (30%), and bone marrow (15%). Specific lymphocyte subset numbers were not measured, however B-cell's typically represent 20% of peripheral lymphocytes. This would then translate to a minimum of a 83% reduction in total T-cell count. It is

possible that since the rabbits had subclinical Pasteurellosis the B-cell percent would be higher than 20%. The relative reduction in T-cells is similar to that obtained in successful rat models (45). The rabbits appear to quickly develop anaphylactoid sensitization to the ALS, as two animals given a follow-up dose of ALS within 24 h died within minutes of respiratory and circulatory failure.

The timing of ALS with respect to thymic inoculation of T-cells may be important since the ALS will also react with donor lymphocytes in the thymus. Thymic inoculation was performed 18-20 hours after injection of the ALS. It is not known how quickly the anti-rabbit ALS is deactivated and cleared from the circulation. However, studies on OKT-3, the anti-human lymphocyte equivalent, indicates the monoclonal anitbody is completely bound to T-cells within minutes of intravenous injection and the globulin is then quickly cleared from circulation (40).

5.2 Intrathymic Inoculation of Donor Lymphocytes

The majority of previous studies in the rat have utilized splenocytes as the innoculant (see Table 1). The spleen provides a simple matrix from which lymphoreticular cells can be collected. Spleen tissue is simply ground, coarsely filtered, and then treated with ammonium chloride to lyse the erythrocytes. This would provide a mixture of cells that include T and B lymphocytes as well as macrophages, providing both MHC Class I and II antigens. Other groups have inoculated with T-cells (65), leukocytes (51), glomeruli (52),

bone marrow cells (67), and islets (46) with successful induction of tolerance. In addition, both native and synthetic T-cell and splenocyte cell surface antigens (Class I or Class II) have also been used successfully (Table 2). In the present study, the processing of whole blood with Histopaque-1077, leads to the collection of T and B lymphocytes and monocytes with a reported viability of >98% and full functionality for MHC testing.

The number of lymphocytes injected into the thymus was 5×10^7, chosen empirically after review of the range (10^6 - 10^8 cells) of other published successful experiments in rats. Oluwole (77) defined a specific weight of donor antigen for inoculation that resulted in tolerance induction, however the results were never described in terms of cell counts or a dose per given thymic weight. In our experimental design, in order to insure donor viability, the maximum single blood withdrawal for lymphocyte harvesting was 10 ml, considering that blood was also required for MLC assay. These two tests resulted in a withdrawal of about 15% total body blood volume, the maximum single withdrawal reasonable within a chronic survival experiment. It is possible that the larger volume of the rabbit thymus, compared to the rat, did not expose new pre-T-cells to a sufficient concentration of donor antigen. If it is assumed that rats and rabbits have the same concentration of thymocytes per unit volume of thymic tissue and that the thymocytes are spheroid and have similar volumes, a ten-fold difference in thymic volume would require an identical increase in inoculated cell number to provide the same exposure to the pre-T-cells. Our inoculated count, compared to other successful experiments, is well within this range. It is not known how long donor innoculant remains in the thymus.

Timing of intrathymic inoculation with graft implant is also highly variable in published reports. Successful tolerance induction has been seen with IT injection over a range of seven days post grafting to 13 weeks pregrafting (26). Our experimental protocol resulted in grafting of the thyroid tissue 19 weeks after thymic inoculation. It is possible that although a new T-cell population, non-reactive to donor tissues, was created within the first few weeks following inoculation, enough of this peripheral population did not survive until the time of grafting. It has been shown that non-activated T-cells remain in the peripheral circulation for months to years while activated T-cells and memory T-cells have a much shorter lifespan. It is possible that T-cell activation rates were higher in this rabbit model due to the underlying Pasteurellosis especially following ALS treatment resulting in early destruction of the tolerant clone population. It is known that in mice activated T-cells can return to the thymus and appear to remain there for about 30 days (18). It is also possible that the higher rate of return likely in these rabbits resulted in alterations of the thymic environment and prevented tolerance induction.

5.3 MLC Assay

The MLC assays demonstrated no change from preinoculation through to the period of thyroid graft placement. Although the MLC stimulation values for experimental animals appear to decrease, there are no statistical differences. Reporting MLC as stimulation index provides a means to compare MLC values between individuals but implies that there is a relationship between the proliferation of the stimulated cells (a result of contact

with foreign antigen) and the background proliferation, which is not entirely valid. The data can also be presented as a simple subtraction of control proliferation values from activated values. Using this method there were no differences between the controls and experimental group, or from one sampling period to the next. The ideal method for monitoring changes in MLC would be to use cryogenically maintained pooled blood obtained from a wide selection of donor rabbits. This pooled sample could then be used as a standard to which individual donor-recipient pairs could be compared.

5.4 Rabbits

The rabbits used in this study were all outbred strains of New Zealand Whites and Californians. It is not known what specific genetic immune difference exists between them. However, out of the nine grafts performed, not a single one survived even beyond the first week after grafting. When donor and recipient incompatibility in both major and minor MHC existed (from detailed genetic testing), there was no extended survival of grafts (60,64,66). Similar results were found in the xenograft (95,96) and outbred canine models (94), where one would expect significant MHC differences. Similar differences would be found in non-related live or cadaveric human donor-recipient combinations. It would appear that in rats, the thymic inoculations may provide a form of tolerance that resists only minor differences in MHC and that is possibly overwhelmed in situations of major antigen differences.

The rabbits used in these experiments were not specific pathogen free or maintained in pathogen free environment. All rabbits received treatment with enrofloxacin for one week at the time of receipt, as a preventative measure against Pasteurellosis. However, after treatment with ALS, the recipients often demonstrated symptoms of Pasteurellosis. In some cases this consisted of occasional sneezing and mucous discharge while other cases had severe respiratory symptoms, requiring extended treatment with enrofloxacin. It has been noted in mice that infections (acute parasitic (127) or chronic viral (128,129) can result in the loss of tolerance to specific antigens. One study by Ohashi showed that after chronic in utero infection with virus, the neonatal thymus has the ability to remove the viral antigen and restore normal non-tolerance to the virus, in spite of viral antigen being available peripherally (129).

Many medications and biological stressors have been found to cause changes in thymus size and activity (9). Drugs used in the current study experiment included anaesthetic agents (ketamine, xylazine, and isoflurane), analgesics (torbugesic and buprenorphine), and antibiotics (enrofloxacin). Buprenorphine has been studied in relation to its chronic effects on the immune system of young rats. Thymic and lymph node weights increased during a four week high dose administration of buprenorphine, which might indicate some immunotoxicity (130). It is not known what effects if any would be seen with acute use in rabbits. Chronic cephalosporin administration in mice causes increases in IgM levels but does not appear to affect host resistance or cell-mediated immunity (131). Enrofloxacin however is a quinilone carboxylic acid producing its antibacterial effects

through inhibition of bacteria gyrase (DNA synthesis promoter) This drug does not appear to have any deleterious effects on the hematologic system even at doses 30 times normal. (Package insert) There is no data on immune functional changes with the acute use of the other drugs from this study.

5.5 Thyroid Transplant

Thyroid tissue was chosen for transplantation in our model because of the technical simplicity of grafting. It was imperative that there be no mortality associated with the surgical placement of the grafts since these donor-recipient pairs had been studied extensively over a 19 week period. Loss of graft recipient at the time of graft placement would require each pair to be restarted. As seen in a number of human studies, thyroid grafts can be placed without the necessity of providing direct vascular micro anastomoses. The two week survival of autografts in all animals was evident on histological examination. It is not known whether these rabbit grafts would respond appropriately to TSH. In the human studies the heterotopic autografts did demonstrate at least partial functionality although all demonstrate follicular hyperplasia at the implant sites.

In two studies in rats using intrathymic inoculations of either antigen (78) or splenocytes (62) and ALS, survival of one tissue type was contrasted by rejection of another although both groups received the identical tolerance developing protocol. In the study by

Nakafusa (78), mismatched (RT) rats demonstrated long term acceptance of heterotopic heart grafts, yet the same protocol did not provide acceptance of renal or skin grafts. They felt that the higher number of passenger lymphocytes in the latter two tissues might have caused an enhanced reaction to the tissues. Goss and Nakafusa (62) conducted a similar experiment LEW donors and Buffalo rat recipients. Again, heterotopic heart allografts demonstrated extended survival while small bowel grafts suffered from acute rejection. Once again, it was felt that the high concentration of donor lymphocytes in the donor bowel (Payer's patches) may have caused enhanced immune response from the host. In other rat strain combinations, kidney, skin, and bowel do demonstrate extended survival with splenocyte or antigen inoculation of the thymus (64,65,68). Biopsy of normal thyroid tissue does not demonstrate an abundance of lymphocytes (Figure 2). The thyroid does have an extensive capillary blood supply associated with the basement membranes.
It is possible that normal thyroid possesses potential antigens or autoantigens that would not be expressed by the donor lymphocytes inoculated into the rabbit thymus. In this case, T-cell clones reactive to any thyroid specific antigen would not have been deleted within the thymus resulting in immune reaction at the graft site.

Hashimoto's thyroiditis is most commonly seen in middle aged women. It is associated with the production of autoantibodies to thyroglobulin and thyroid peroxidase, and often coexists with other autoimmune diseases including rheumatoid arthritis and systemic lupus erythematosis (132). The affected glands demonstrate a massive infiltration of plasma cell, lymphocytes, and macrophages. These cells almost completely destroy the

normal structure of the gland leading to increased gland size (goitre) and hypothyroidism (due to the reduced number of follicles). The thyroid peroxidase is present on the apical surface of microvilli as well as in the cytoplasm and microsomes. Genetic differences in outbred rabbits could result in recognition of similar autoantigens on the thyroid implants not compensated for by any tolerance induced by intrathymic inoculation. Such split tolerance has been described in a model of myasthenia gravis (133).

6. CONCLUSION

In contrast to a significant body of published research with the rat model, inoculation of donor lymphocytes with concomitant treatment by anti-lymphocytic serum does not result in the induction of tolerance to allogeneic thyroid grafts in outbred rabbits. This study is in agreement with other studies in outbred dogs and in intraspecies grafting. The reasons for failure of tolerance induction in this model remain unclear but may be due to insufficient donor lymphocyte inoculation, immune peculiarities of the thyroid, insufficient recipient induction lymphopenia, or large donor-recipient MHC incompatibilties that could not be overcome with simple thymic inoculation treatment.

Further studies focusing on evaluating the dose of donor antigen per mass of recipient thymus, the relationship of recipient infections, and the survival of other graft types need to be performed in order to determine the feasibility of this method for use in humans.

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