THYMIC EPITHELIAL CELL TRANSPLANTATION TO THE HOST THYMUS: STUDIES IN IMMUNOMODULATION AND TOLERANCE INDUCTION

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This work is dedicated to:

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Dr. Thomas Wesley Allen,

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and my dad.

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ALS	antilymphocyte serum
APC	antigen presenting cell
BM	bone marrow
BMC(s)	bone marrow cell(s)
CTLA	cytotoxic T lymphocyte associated antigen
DA	Dark Agouti inbred rat strain (MHC haplotype Rt1a)
DM	dexamethasone
DMSO	dimethyl sulfoxide
FCS	fetal calf serum
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
ES	equine serum
ETOH	ethanol
GWAJC	WAJC media from GibcoBRL
HBSS	Hank's Balanced Salt Solution
IFs	intermediate Filaments
IL	interleukin
IFN-γ	Interferon-gamma
IP	intraperitoneal
IT	intrathymic
LDA	1st filial generation of Lewis crossed with Dark Agouti rat
Lew	Lewis inbred rat strain (MHC haplotype Rt1l)
LFA	leukocyte function associated antigen
n	number
PBS	phosphate buffered saline
PSF	penicillin/streptomycin/fungizone
RPM	revolutions per minute
RSC	renal subcapsular space
SCS	supplemental calf serum
TEA3A1	TEC line of Lewis/DA strain from Dr. Hayashi
TECs	thymic epithelial cells
TEM	transmission electron microscopy
TNF	tumor necrosis factor
VLA	very late antigen
WAJC	W. Alton Jones Cell Research Center media

ABSTRACT

The most significant determining factor in successful allotransplantation is the ability to avert graft rejection. Innovative transplantation techniques have evolved to include presurgical and postsurgical immunosuppressive regimens, some of which continue to pose serious short- and long-term health risks. The intrathymic (IT) inoculation of certain donor cell types into an adolescent recipient thymus gland in the circumstance of short-term immune ablation with a single intraperitoneal injection of antilymphocyte serum has been shown to induce tolerance to a variety of subsequent donor-specific grafts in many animal models of allogeneic transplantation. Using this technique of immune re-programming, the resultant peripheral immune population has shown long-lasting efficacy in tolerance induction even after the withdrawal of non-donor-specific, systemically acting immunosuppressive drug regimens. Cellular inoculates for IT tolerance induction have included donor splenocytes, spleen-derived dendritic cells, bone marrow cells, bone marrow-derived dendritic cells (included in the class of cells known as antigen presenting cells), renal glomeruli, and pancreatic islets of Langerhans. To date, the potential of primary culture-immortalized thymic epithelial cells (TECs) to modify the host immune system using a similar experimental regimen has not been investigated in a rat model of allogeneic

transplantation. This may be due to the difficulty in deriving and maintaining these cells as a readily available source for extensive experimentation.

Although the mechanism by which IT inoculation of antigenic cells induces tolerance has not been fully elucidated, numerous studies have demonstrated the efficacy of this technique in inducing specific unresponsiveness in a host. These studies have confirmed a significant role for the thymus in immune maturation, antigen recognition, and induction of specific unresponsiveness (central tolerance). It is believed that cell/tissue grafts within the thymus allow presentation of foreign antigens to immature host T cells, resulting in reeducation of the host immune system through clonal deletion of target specific cells by apoptosis. The proven efficacy of IT bone marrow transplantation in tolerance induction studies has served as background for the use of bone marrow transplantation in several of today's pre-transplant conditioning regimens for human tissue transplantation. Some of the techniques used for total myeloablation prior to allogeneic bone marrow reconstitution in human pre-transplantation conditioning regimens are associated with significant health risks related to a severely immunocompromised state. Hematopoietic cell transplantation is not trivial as bone marrow is also limited in supply. Additionally, bone marrow lacks cellular uniformity, sometimes harbors viral contaminants, and current technologies aimed at primary bone marrow culture are limited. TECs were chosen for this investigation based on their thymic origin, epithelial nature, cellular uniformity, and ability to be maintained and manipulated

in vitro. Bone marrow cannot offer these same benefits. In light of the ability of certain cells to induce intrathymic tolerance, it was initially hypothesized that a combination of gene therapy on TECs and intrathymic transplantation of the genetically altered TECs might form a new paradigm for the prevention or treatment of transplant rejection. Before embarking on this project, the effects of unmanipulated TECs transplanted to the intrathymic environment had to be investigated. It was hypothesized that peripheral reactive lymphocyte depletion with antilymphocyte serum followed by TEC transplantation to the thymic environment would result in repopulation of the immune repertoire with cells recognizing TEC antigens as self. In such a paradigm, the immunogenic proteins expressed by TECs within the host thymus would function as tolerogens, resulting in the modification of the host immune repertoire. This approach would allow the host immune system to be specifically modified to accept grafted tissue or cells without impinging on its ability to effectively respond to other foreign immunogens.

The early studies outlined in this project sought to identify techniques of establishing primary cultures of TECs from a variety of donor rat strains. TEC primary cultures were characterized in varying growth medium conditions using several parameters, including morphological changes, proliferative capacity, junction formation, cell contact, and terminal differentiation. The best suitable culture conditions were defined for the establishment of the primary cultures as a cell line. These TECs were then used in transplantation experiments to

determine their ability to induce allogeneic tolerance to subsequent donor matched grafts. The experimental design in this project used a rat model of allogeneic tissue transplantation. Graft rejection was based on rejection scores determined by a histological analysis of donor matched and mismatched transplanted tissue. The tolerogenic effects of TECs upon intrathymic transplantation were compared to the tolerogenic effects of bone marrow cells upon intrathymic transplantation. The comparison evaluated the ability of the intrathymic treatments to modify the natural history of graft rejection as quantified by the extent of lymphocytic infiltration, general inflammation, and preservation of structural architecture. On a scale of 0 to 4, with a score of 4 indicative of the most aggressive graft rejection processes, intrathymic transplantation experiments revealed a reduction in mean graft lymphocytic infiltration from 1.78 ± 0.47 in the negative control groups (intrathymic saline, IP saline; n=10) and 1.50 ± 0.60 (intrathymic saline, IP ALS; n=8) to 0.25 ± 0.25 ($p < 0.01$) in the positive control group (intrathymic bone marrow + ALS; n=8). A reduction in graft mean lymphocytic infiltration to 1.00 ± 0.31 in the experimental group (intrathymic TEC, IP ALS; n=16) occurred, but the p value did not reach statistical significance. Overall, the results revealed that IT inoculation of TECs results in some immunomodulation, although not reaching the same effectiveness as bone marrow. This might be related to previous reports of a dual role for the TEC in both positive and negative selection. Overall, it appears that intrathymic inoculation of TECs can be used to reeducate the host immune system following reactive peripheral lymphocyte depletion and may provide an opportunity for

future research using *in vitro* cellular manipulation techniques to further investigate pre-transplant conditioning regimens employing tolerance induction.

CHAPTER 1.

TRANSPLANTATION, THE IMMUNE SYSTEM, AND THYMIC EPITHELIAL CELLS FOR TOLERANCE INDUCTION

1.1. INTRODUCTION

Transplantation offers a means of replacing absent or dysfunctional tissue with viable cells, tissues, or organs to meet the physiologic demands of the body. Autotransplantation involves transplantation within one individual; homogeneic or syngeneic transplantation involves transplantation between two histocompatibly matched individuals; and allogeneic transplantation (allotransplantation) involves transplantation between two histocompatibly mismatched individuals. Xenogeneic transplantation involves transplantation between individuals from separate species. Although there are documented reports of attempts to successfully transplant animal organs or tissues to humans dating as early as ancient Egypt and China circa 8,000 B.C. [1, 2], allotransplantation has been the mainstay of current investigations into successful human transplantation. Great strides have been achieved in the successful replacement of organs, tissues, and cells. Advances in surgical techniques and graft treatments prior to transplantation have led to technical success in the placement of grafted organs and tissues.

Pharmacological discoveries have led to the development of immunosuppressive agents that are almost universally administered to prevent acute and chronic graft rejection. New treatment regimens can even offer the possibility of graft survival in the transplantation of a completely genetically mismatched organ. Nevertheless, pharmacological agents used to prevent graft rejection are associated with significant complications, indicating a need for an alternative treatment which is as effective in reducing immune rejection process.

This chapter will summarize the history of transplantation through the 21st century, explain normal immune system development and mechanisms of immune function, describe categories of graft rejection and ways of preventing it, and introduce tolerance induction mechanisms as a method of bypassing toxic immunosuppressive regimens and inducing antigen-specific immune unresponsiveness.

1.2. THE HISTORY OF TRANSPLANTATION

Blood transfusions and dental transplantation have been documented as far back as the 15th century. Prior to the late 18th century, transplantation focused on replacement of limbs and teeth, and transferring skin and other body parts from one animal to another. By the late 19th and early 20th centuries, a number of unsuccessful animal transplants were attempted using animal organs, including

kidney transplants between dogs. The first reliable report of a human transplant surgery was a skin graft performed by Dr. Carl Bunger in 1823, although it is not reported whether this surgery was successful. It was not until early in the 20th century that successful transplants between humans were reported, offering new avenues of renewed health. The concerted efforts of researchers and physiologists helped to develop the surgical techniques needed for performing successful transplants, but rejection still remained a problem.

Through the late 19th and early 20th centuries, corneal transplants, skin allografts, joint and vascular reconstruction, and blood transfusions became commonplace. In the 1920's and 1930's, the concept of "self versus non-self" was starting to become recognized and the field of transplant immunology was born. Dr. Emile Holman, a young Boston surgeon, suggested from his results in skin transplantation that host immunity increased after a second grafting from the initial donor but not from a third party. [1] In 1937, Dr. J. B. Brown transplanted skin between identical twins and made a comparison between the indefinite survival of these grafts and the gradual "absorption" of maternal skin on burned patients. In 1942, Oxford zoologist, Dr. Peter Medawar, was able to demonstrate the impracticality of allografts compared to autografts and reported hastened destruction of second allografts, concluding that accelerated rejection involved the "destruction of foreign epidermis…brought about by a mechanism of active immunization." These ingenious perceptions led to the induction of Sir Medawar

as “the father of transplantation biology”. [3] A more detailed outline of historical events in transplantation can be found in tabular form at Table A.

“The replacement of diseased organs by healthy ones has been a milestone of medicine in the mid-twentieth century.” [1] However, organ transplant success is quite limited due to a number of factors, including availability of donor organs, immune graft rejection, and the side effects of immunosuppression. One way to eliminate graft rejection is to use the recipient’s own tissue when possible (blood, skin, bone marrow, blood vessels). Although successful experiments in renal transplantation in animals can be documented as early as 1902, it was 50 years later before the first successful living-related human kidney transplant. [4] This was followed by venous allografts, kidney transplants between genetically mismatched siblings, cadaveric kidney, liver, lung, kidney-pancreas, heart, and pancreas transplantation.

The accomplishments of early transplant surgeons and the growing popularity of organ transplantation led to many practical and ethical issues related to donor organ availability. The establishment of brain death criteria in 1968 served to increase the supply of viable donor tissue for transplantation. [5] However, most of today’s graft recipients still must remain on a waiting list. (In 2004, according to the United Network for Organ Sharing [UNOS], there were approximately 14,000 donors available for nearly 90,000 recipients on the transplant waiting list

in the United States alone.) The establishment of UNOS in the 1980's supported organ procurement organizations and outlawed the purchase and sale of organs.

Cryopreservation and biologist Dr. Jean-Francois Borel's discovery of the immunosuppressant cyclosporine in 1972 (not clinically used until 1982) have been pivotal in the development of modern transplantation techniques. The development of organ preservation solutions (e.g. Viaspan or University of Wisconsin [UW] solution) for improved organ preservation during cold storage and transport has led to significant improvements and greater success rates in transplantation by reducing the incidence of primary graft non-function (heat shock, reperfusion injury, ischemia). [6] Simultaneous multiple organ transplantation has also led to increased success. As success in transplantation has progressed, the demand for donors has grown. In order to supply increasing demands, artificial tissue has been used to replace missing or dysfunctional tissue. Through the 1980's, artificial heart, single-lung, heart-liver, double-lung, and liver-small intestine transplantation techniques and protocols were established.

Still, the increasing demand for viable transplantable tissues has led researchers to explore other avenues, such as advanced immunotherapies that would improve transplantation efficacy and xenotransplantation techniques that would allow extrahuman expansion of the donor pool.

1.2.1. Immunology and Transplantation

In 1863, it became well documented that tissues transplanted from one person to another resulted in graft rejection. Forty years later, this graft rejection was classified as immunologic in nature. Irradiation by X-rays became the first available method of immunosuppression in the 1950's, and after the 1958 discovery of the histocompatibility gene complex and the development of tissue typing and matching, transplantation underwent a transformation from a technical science to an immunological science. Namely, transplant surgeons had previously focused on technical aspects of transplantation, but the limited success of transplants impeded by the immunologic process of graft rejection led to a focus on preventing immune mechanisms that would destroy a technically well-placed graft. Minimizing histocompatibility differences, along with early anti-rejection medications like prednisone, azathioprine (Imuran), and anti-lymphocyte serum, offered some limited protection from rejection, but greatly increased the risk of postoperative infection.

Lymphocytes were functionally distinguished and divided into T and B cell subsets in the late 1960's, and not until the 1970's were subsets of T cells defined using antibodies developed with recently discovered monoclonal antibody technology. [1] Overall improvements in graft survival coincided with the conceptualization by Dr. Alfred Singer of subpopulations of T helper/inducer cells and the T cell receptor-MHC antigen interaction. Since the FDA's approval

of cyclosporine, there has been significantly reduced graft rejection and longer graft survival times with improved quality of life in many transplant recipients. The Nobel Prize for Medicine was shared between Dr. Joseph Murray in 1990 for his work in kidney transplantation and Dr. George Hitchings for his work in chemical immunosuppression. Dr. E. Donnall Thomas was also awarded the Nobel Prize for bone marrow transplantation as a practical therapy in the treatment of leukemia. Transplantation techniques have increased in complexity even since the 1990's, employing newer, more sophisticated immunosuppressive agents whose mechanisms of action can be defined at the cellular level, and in some cases, the molecular level. These agents continue to offer improved graft survival with fewer side effects. The later part of the 20th century (1980-1999) also brought advances in immunology, tissue typing, and surgical techniques; as well as advances in organ donation, distribution, and preservation. In addition, the passage of several laws has provided additional support and direction to organ donation and transplantation in the United States. The Uniform Anatomical Gift Act of 1968 and the National Organ Transplant Act of 1984 began the process of establishing the organ donation and transplantation system that we know today. [7-9] Over the years, other federal polices and laws have provided for Medicare coverage and medication funding for some qualified transplant recipients.

Because of the disparity between donor supply and recipient demand, transplant waiting lists are rapidly growing. A combination approach has been developed to

curtail this disparity: the expansion of the donor pool and the advancement of immunosuppressive therapy, despite its side effects. Many transplant centers have come to accept the use of living, unrelated donors in kidney allotransplantation. The immunosuppressive regimens of today have allowed biologically unrelated donors the option of transplantation.

The 21st century holds the promise of many new and exciting advances in the field of transplantation which include artificial organs and support devices, stem cells as sources of functional transplantable tissue, xenotransplantation (the use of animal cells and organs), advances in living and cadaveric organ donation, splitting of organs to help multiple recipients, and development of better anti-rejection medications. At the forefront of transplantation science, future studies may allow a better understanding of the immune system and specific applications of immunomodulatory treatments in the prevention of graft rejection.

1.3. THE EVOLUTION OF 21ST CENTURY TRANSPLANTATION

1.3.1. Blood Transfusion

The most common form of transplantation performed in modern medicine is the blood transfusion. The first successful blood transfusion was performed in 1818 by Dr. James Blundell. Transfusions became more widespread in World War I

following the discovery of blood group antigens by Dr. Karl Landsteiner, and the first use of an effective anticoagulant for stored blood, sodium citrate, in 1914. [10, 11] Early blood transfusions were associated with transfusion reactions and infections, but over the past 90 years, advances in immunology and chemistry have transformed blood transfusions from harrowing experiences to a routine and relatively safe form of treatment. The allogeneic blood supply is screened for viral antibodies, antigens, and nucleic acids, elevated enzyme levels, and evidence of syphilis. The ability to screen for these particular elements, and to remove contaminated blood from the blood supply, has greatly increased the success of transfusion. In terms of transplantation immunology, transfusion may elicit the formation of antibodies reactive to antigens on the donor red blood cells, leukocytes, or platelets. Many transfusion recipients will become transiently febrile. Significant transfusion reactions are generally limited to blood group antigen disparities, mostly commonly due to human error.

1.3.2. Vascularized Transplantation

An organ is a fully differentiated unit of tissues with specific cellular architecture that is specialized to work in concert to perform specific functions. Each organ's survival and function is dependent upon the provision of an adequate blood supply, and transplanted organs require the maintenance of patent vascularity following transplantation.

Among the most frequently transplanted single organs are the kidney, heart, liver, pancreas and lung. An alternative to multiple single organ transplantation is the option of combined organ transplantation. Combined organ transplants are designed to share a common, extensive vascular supply, and are therefore more likely to survive. Frequently combined transplants include heart-lung. Bowel/cluster transplants or multivisceral transplants can be used to replace the function of multiple organs including the small bowel. Additionally, large flaps of tissue (full thickness skin, muscle) that require an extensive blood supply can be successfully transplanted with vascular anastomoses. While graft rejection following vascularized transplantation is still a common complication, the major cause of hospitalization during the first post-transplant year is infection [12], with graft versus host disease a significant complication in cluster transplantation.

Immunosuppressive drug regimens that include multiple agents, each working through a different mechanism of action, have evolved to effectively reduce graft rejection and support survival of transplanted organs. Although conventional immunosuppression includes triple therapy with sirolimus, cyclosporine, and prednisone, the specifics of which will be discussed in following sections, individual drug-related toxicity, recipient-related risk factors, and donor organ characteristics are all taken into account when deciding upon an immunosuppressive treatment regimen [13]. Most protocols combine a primary immunosuppressant (e.g. cyclosporine or tacrolimus) with one or two adjunctive agents (e.g. azathioprine, mycophenolate mofetil, sirolimus, corticosteroids). These improvements in

immunosuppression have made allograft rejection “the exception rather than the rule” [14], reducing acute cellular rejections to about 10-20%. [15] The reduced incidence of acute and chronic renal graft rejection has allowed renal transplantation to replace hemodialysis as the preferred hemofiltration option in end-stage renal disease. [14]

Heart, lung, and combined heart-lung transplants have been clinical options for resuming adequate circulation and gaseous exchange in congenital heart disease patients and those patients with end-stage heart and lung disease, and have been a standard part of the treatment paradigm for selected patients with these diseases over the past 35 years. Emphysema and chronic obstructive pulmonary disease account for the pathology associated with the majority of single lung transplants, while the most frequently reported indication for heart transplantation in the United States is coronary artery disease. The recipient’s medical condition is the most important factor in patient survival following lung transplantation, while the recipient’s age, gender, and race are the significant determining factors for successful heart transplant outcomes. [12]

The indications for liver transplantation have expanded greatly, and techniques have been simplified over the years. As the waiting list for liver transplantation continues to grow, and numbers of cadaveric donors remains static, the availability of transplantable livers cannot keep up with need. The proliferative capacity of hepatocytes has made the use of live donors and the procurement of

split-liver grafts for two recipients a scientifically and medically sound approach towards reducing the shortage of donor organs. [16] For unknown reasons, the liver is known to be associated with a downregulated immune response and excellent graft survival. Although it has been reported that up to 70% of patients develop acute liver rejection, this often spontaneously resolves and chronic rejection is rarely seen. [17] Because of the relatively forgiving nature of liver transplantation, cadaveric donors, living-unrelated related donors, and older donors with co-morbidities and moderate elevations in liver function tests can be added to the relatively small pool of living related donors to address the increasing demands for donors. [16] Exploring beyond the realm of human transplantation, xenotransplantation (transplantation of tissue between disparate species) has been attempted to expand the donor pool for heart, liver, and kidney transplantation. Attempts at xenotransplantation from chimpanzees and baboons to humans have been limited by hyperacute rejection due to preformed antibodies. Molecular genetic techniques offer the option of knocking out genes that encode cell surface antigens prior to xenotransplantation. Large offspring numbers and appropriate organ size for human transplantation make porcine to human xenotransplantation a realistic option for meeting increased demands for donors. There is still concern about the threat of latent viruses in animal tissue.

In diabetic patients, pancreas transplantation is routinely performed along with the scheduled kidney transplantation (often due to renal failure secondary to diabetes) and dramatically improves the quality of life of the patient. The probability of

success of pancreas transplantation has reached that of other organ transplants. However, the inherent cotransplantation of surrounding exocrine pancreatic tissue along with the endocrine pancreatic islets is unnecessary and associated with complications such as enzymatic tissue damage. Isolated islet transplantation, as will be described below, offers a more specific means of resupplying the recipient with needed insulin producing beta cells.

1.3.3. Nonvascularized Transplantation

Tissues are composed of uniform cells which are organized to perform a specific function. Unlike an organ, a tissue's viability does not depend on an organized vascular supply. When faced with removal from its source of vascular perfusion, a tissue, if placed in a nurturing environment, will be infiltrated and perfused by surrounding blood vessels through the process of neovascularization. Commonly transplanted tissues include skin, bones, ligaments/tendons, corneas, and heart valves.

Skin transplantation is usually used to replace epidermal regions damaged by trauma (e.g. burns) or by disease (e.g. cancer). Split thickness skin grafting involves a skin harvest which is processed to form a mesh, allowing expansion of the graft for greater surface area coverage compared to a full thickness skin graft. The prognosis for autologous skin engraftment is usually excellent but allogeneic skin engraftment still presents a challenge.

Bone transplantation may involve the use of live autologous cortical bone harvested from the ilium of the patient. However, the recovery from this procedure is painful and associated with excessive bleeding. Cryopreserved bone matrix (fibular strut) allografts are commonly used as structural support for osteogenesis in spinal fusion surgery. The recipient's blood is used to infiltrate the non-living graft with autologous cells. Minimal graft rejection is associated with this process.

Structural reinforcement can be accomplished with the proper placement of dense connective tissue, such as ligaments or tendons. Most of the time this involves autologous grafting. These tissue allografts are associated with very little complications other than ischemia.

Corneal transplantation is used to replace a damaged cornea. The avascularity of the cornea is the principal feature which determines its immunologic privilege. The trauma of corneal transplantation surgery can compromise this privilege, exposing the graft to the host immune system. Studies over the past 50 years have demonstrated that inflammation and neovascularization predispose a corneal graft to the cell-mediated immune rejection response. Fortunately, the antigenicity of corneal cells is comparatively low. Topically applied immunosuppressive compounds can be used to prevent inflammation and vascularization, thus blocking T cell activation. Blocking antibodies (to CTLA4, for example) and soluble coreceptor blocking agents have led to a considerable reduction of corneal allograft rejection. [18]

Although the procedure is associated with significant mortality risks, replacement of the native aortic valve with a heart valve prosthesis has become one of the most frequently performed procedures in cardiac surgery. [19] Biologic valve prostheses, although limited in durability, offer an alternative to lifelong anticoagulation associated with mechanical prostheses.

1.3.4. Cellular Transplantation

Individual cells or cell clusters can be transplanted to a recipient to replace important physiologic roles lost to disease or damage. When applicable, cellular transplantation offers a safer, less costly alternative to organ transplantation. Cellular transplantation involves the transplantation of such small volumes that the benefits of immunologically privileged site placement can be exploited. Immunologically privileged sites are parts of the body where the immune system is prevented from mounting an effective attack. They include the eye, testis, brain, placenta, and thymus gland, but only the eye's privileged status has so far been exploited (for corneal grafting) in humans. Many factors are involved in immune privilege, such as tight junctions between the cells of the tissue, little expression of MHC Class I molecules, and expression of the apoptotic-inducing ligands on the cell surface (such as Fas).

Although pancreatic islets of Langerhans may be considered as tissues, or even as organs, due to their variety of interacting cell types and independent vascular

supply, they are included in this section. Islet transplantation is a more specific alternative to pancreas transplantation for the replacement of insulin producing beta cells and can significantly reduce surgical morbidity. Unfortunately, reduced beta cell mass occurs with organ dispersal and islet purification. Recent progress in the field of pancreatic islet transplantation, most notably in Edmonton, Canada, has led to recipient insulin independence following single donor islet transplantation. [20] However, current immunosuppressive drug regimens are complicated by the potential of dangerous systemic comorbidities associated with a non-specific mode of action. Many immunosuppressive agents also demonstrate significant islet cell toxicity. (Chaib, 1994) Diabetogenic immunosuppressants should be eliminated in order to allow optimal survival and function of the grafted islets, since lack of glycemic control can influence the survival of newly transplanted islets. [21] Immunosuppressants such as mycophenolate mofetil and rapamycin do not appear to be as toxic as corticosteroids and cyclosporine. [15] Cellular transplantation also offers the possibility of donor-specific tolerance induction.

Other cellular transplants include bone marrow, epithelial cells, neural cells, myoblasts, and hepatocytes. Bone marrow transplantation is performed to replace the hematogenous cell population. This is most commonly secondary to myeloablative diseases. Bone marrow has also been shown to induce donor-specific tolerance in a variety of transplantation models, as will be discussed further in Chapter 4. Neural cell and myoblast replacement procedures are still in

experimental stages. Hepatocyte transplantation through the inferior mesenteric vein is growing in popularity as an alternative to liver transplantation for the correction of acute liver failure, inherited liver based metabolic diseases and coagulopathies, and
chronic liver diseases. [22-24]

1.4. COMMON CHARACTERISTICS OF TRANSPLANTATION

Successful transplantation of organs, tissues, or cells requires adequate tissue perfusion and reduced graft rejection. Tissue perfusion in organ transplantation is dependent upon the infusion technique used to reduce intravascular debris, the skilled surgical anastomosis of the donor and host vessels, and the ability to maintain an unimpeded vascular supply to the graft. Tissue perfusion in non-vascularized transplantation is dependent upon a supportive nutritive environment and an accommodating immune milieu that allows the development of a new vascular supply. Reduction of graft rejection in most cases is achieved by ABO blood type matching between the donor and the recipient and by prevention of immune mechanisms involved in infiltration of the graft by effector cells.

1.5. THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

Antigen presentation in allogeneic transplantation and discussions of the MHC go hand in hand. This section is included to review the MHC complex as it relates to allogeneic transplantation. The MHC is an array of genes on the small segment of chromosome 6 that encodes three structurally, functionally, and distributionally different products: 1) cell surface glycoprotein antigens designated as MHC Class I antigens, 2) cell surface glycoprotein antigens designated as MHC Class II antigens, and 3) soluble protein components of the complement system. The MHC gene is designated Rt1 in the rat and H-2 in the mouse.

The rat MHC was discovered at the turn of the 19th century when the growth of rat tumor cell lines could not be supported across mismatched strains. [25] The genes of the MHC are the most polymorphic known, and it was not until 1999 that the MHC of humans was sequenced.

The principal physiologic function of class I and class II cell surface MHC molecules is to "present" antigenic peptides of foreign proteins to the appropriate antigen-specific T cells of the immune system. T cells can recognize only membrane bound antigens (as opposed to B cells). The MHC antigen is structured to form a small groove which holds a very small antigenic peptide in place on the surface of an antigen presenting cell for signal recognition by a T cell receptor (TcR). [25] The antigenic peptide, usually about 9 amino acids long,

fits into a groove at the surface of either an MHC Class I or MHC Class II molecule. The TcR is not able to recognize these small antigenic peptides without presentation in the context of an MHC because of the many stepwise signals involved in the TcR-MHC binding that are necessary for antigen presentation. These peptides can include fragments of protein antigens derived from intracellular or extracellular parasites (viruses, bacteria, etc.).

1.5.1. Presentation Through MHC Class II

The Class II molecules consist of two transmembrane polypeptides: an alpha chain and a beta chain, and a groove is formed by the interaction of these 2 chains. Class II molecules are not as widely expressed in the body as the class I molecules are, being constitutively expressed primarily on hematogenously derived cells. However, cells where inflammation is occurring may be induced to express class II strongly and provide a powerful stimulus to the immune system.

In general, class II molecules present exogenous antigens (e.g. extracellular microbes, soluble proteins) that are first internalized and processed in the endosomes or lysosomes. Peptides resulting from proteolytic cleavage then associate with class II heterodimers assembled in the endoplasmic reticulum (ER). Finally, the peptide MHC complex is transported to the cell surface, where it can be recognized by CD4+ helper T cells. In this interaction, the CD4 molecule acts as a coreceptor. Because CD4+ T cells can recognize antigens only in the context

of self class II molecules, they are referred to as “class II restricted.” The role of class II antigens in the induction of helper T cells has an important bearing on the genetic regulation of the immune response. The absence of MHC Class II positive APCs reduces islet graft immunogenicity. [26]

1.5.2. Presentation Through MHC Class I

The Class I molecule consists of a transmembrane protein heterodimer that noncovalently binds a polymorphic molecule of beta-2 microglobulin to a polymorphic short alpha peptide chain. A groove is formed in its quaternary structure. Class I molecules are expressed at the surface of platelets and almost all the nucleated cells of the body. They are not expressed in RBCs or the central nervous system.

Biochemical analyses of several different class I alleles have revealed that almost all polymorphic residues line the sides or the base of the peptide binding groove. As a result, different class I alleles bind to different peptide fragments. In general, class I MHC molecules bind to those peptides that are derived from proteins, such as viral antigens, synthesized within the cell. The generation of peptide fragments within cells, their association with MHC molecules, and transport to the cell surface is a complex process. Analogous to CD4+ T cell restriction to the MHC Class II antigen, the CD8 molecule acts as a coreceptor in MHC Class I antigen binding to the T cell receptor.

The human MHC antigen is known as the HLA (Human Leukocyte Antigen) complex. The HLA was initially detected on leukocytes, hence its name. The HLA system is highly polymorphic. Class I HLA antigens (HLA A, B, and Cw) and class II HLA antigens (HLA DR, DQ, & DP) are further subdivided into types. There are 22 different HLA A, 42 different HLA B, 9 different HLA Cw, and 18 different HLA DR antigens. The great diversity of class I and class II HLA alleles, including ones that result in increased resistance to developing certain diseases, has helped to ensure that no single parasite can eliminate the entire human population, but such diversity also has made a perfectly compatible tissue match for transplantation between two humans almost nearly impossible (except in the case of identical twins). The detection of antigens (genetic markers) such as the HLA on the surface of lymphocytes, known as tissue typing, was developed in efforts to overcome barriers in human organ transplantation, and has helped to reduce the incidence of graft rejection prior to the development of more effective immunosuppressive drug regimens. Serologic and DNA-based methods can be used for tissue typing. Serological methods are used most commonly and work to identify tissue cells that are reactive to antibodies specific for known HLA-A, HLA-B, and HLA-DR antigens. DNA typing is being used to improve the sensitivity and specificity of tissue typing, especially with the HLA-D antigens. The United Network for Organ Sharing (UNOS) screens organs using 6 HLA antigens: two each at HLA-A, HLA-B, and HLA-DR.

Even if it were possible to match the donor and recipient at every locus of the MHC, some tissue incompatibility would still remain and immune rejection would occur because of minor histocompatibility antigens. Few of these antigens have been identified, but they include: H-Y, an antigen encoded on the Y chromosome and thus present in male, but not female, tissue, and HA-2, an antigen derived from the molecular motor protein myosin. Therefore, even transplantation of an MHC-matched organ to a non-identical host requires some degree of immunosuppression to avoid graft rejection. This is why many pre-transplantation protocols are ignoring tissue typing mismatches and maintaining their attention on ABO matching and immunosuppression.

1.6. OVERVIEW OF IMMUNE SYSTEM DEVELOPMENT

The process by which circulating hematopoietic stem cells emigrate from the bone marrow and take residence in the thymus gland is known as thymopoiesis. [27, 28] The commitment of these stem cells to the T cell lineage is based upon a response to signals from the cellular microenvironment within the gland. [29] Following commitment to the T cell lineage, these cells will undergo changes induced by close associations with discrete thymic epithelial cell microenvironments or compartments. The specific sequence of events experienced by each T cell will determine its compartmentalization into a particular T cell subset. These processes are surveyed in a series of physiologic

checkpoints prior to release of the T cell into the circulating pool of mature lymphocytes. If the T cell does not meet standards deemed necessary by the thymic epithelium, it will not be rescued from apoptosis; instead, it will be destroyed via apoptosis as part of the process of positive selection. [30-34]

There are different types of thymic epithelial cells (TECs), and their classification is location based. [35] Ultrastructural studies have demonstrated at least four types of TECs: those found at the cortical surface, those found within the cortex, those found in the medulla, and those within Hassall's corpuscles. [36] Those found in the thymic medulla are referred to as medullary TECs. Similarly, those found in the thymic cortex are referred to as cortical TECs. Both the cortical TECs and medullary TECs have been shown to present antigens in the context of the MHC, but the medullary TECs may do so more rapidly. [37] It appears that in terms of MHC class II-restricted CD4 T cell development, the cortical epithelium functions to regulate positive selection, whereas a particular subset of medullary epithelium regulates negative selection. [38]

Specificities of the interactions between T cells and the thymic stroma determine the fate of immature T cells. The thymic stroma includes TECs, interdigitating reticular cells, macrophages, and fibroblasts. It is suggested that the thymic stromal environment is mutually dependent upon the differentiation status of developing T cells, "a phenomenon designated as 'crosstalk'." [39] Namely, thymocyte subsets are believed to influence the development and organization of

the thymic epithelium: the organization of the thymic medulla “depends upon activating signals provided by mature thymocytes to epithelial and dendritic cells.” [40] Disorganization of the corticomedullary thymic epithelial junction and abnormal clonal selection was found to occur as a consequence of lack of these important activating signals even in the presence of normal thymocyte maturation. [40]

Although lymphocyte clonal selection occurs as a consequence of both thymic epithelial and bone marrow cell participation [41], the focus of this review will be immune system development and responsiveness as it relates to the thymus gland and thymic epithelium.

1.6.1. Arrangement of the Thymus Gland

In the neonate, the thymus gland is a bilobed structure that occupies almost the entire mediastinal space, is situated anterior to the great vessels, and is separated laterally from the lungs by mediastinal pleura and from the heart inferiorly by pericardium. Each lobe is characterized by a superior prominence of tissue referred to as a horn, with the right lobe being characteristically larger than the left.

The thymus gland is largest relative to body size at birth. At puberty or during an illness early in life, the human thymus reaches its greatest size, weighing

approximately 40 grams. In the adult, the thymus is seen as a remnant located within the retrosternal fat pad. The atrophy of the gland is attributed to depletion of T cells, primarily by the process of apoptosis.

The name “thymus” originates from the Greek language, meaning “warty-bumpy” and “feel good.” This warty consistency occurs due to a fibrous capsule from which fibrous septa extend, dividing the gland into lobules. The thymic parenchyma is divided into a cortical region and a medullary region, and does not contain lymphoid nodules. The primary vascular supply to the thymus is from the internal thoracic arteries, with venous drainage via the internal thoracic veins. Small arteries follow the septa, branching into arterioles running between the cortex and medulla of the thymus. Capillary loops enter the substance of the cortex. A few small efferent lymphatics flow within the walls of blood vessels and in connective tissue septae.

The thymus gland is derived from embryonic endoderm (TECs or epithelial reticular cells) and mesoderm (lymphocytes). During embryogenesis, the thymus develops from the third and fourth pharyngeal pouches. Connective tissue growth factor (CTGF) is involved in cellular “proliferation, survival, migration, adhesion, and extracellular matrix production” during embryogenesis, with specific detection by day 13.5 in mice. [42] Epidermal growth factor (EGF) also modulates fetal thymocyte growth and differentiation. [43] Non-thymic lymphoid tissue is derived solely from mesoderm.

The thymic cortex (“bark”) is the layer directly beneath the capsule and consists of mostly tightly packed small (resting) lymphocytes and some capillaries surrounded by spaces consisting of a mixture of immune cells surrounded by cuboidal-shaped TECs possessing a single large, ovoid, light staining nucleus which often displays a nucleolus. The space around the endothelium-lined capillaries is bound by a thin layer of collagen (perivascular connective tissue) and a complete covering of TEC processes with their basal laminae. This layered coating prohibits blood from directly contacting cortical T cells. This coating is also known as the blood-thymus barrier and prevents large macromolecules from traversing the vessel wall and entering the cortex. The blood-thymus barrier exists in the cortex only, making it immunologically protected from connective tissue septae and from the thymic medulla. This ensures that antigens escaping from the bloodstream do not reach developing T cells in the thymic cortex. Large numbers of immature T cells (thymocytes) are found in various stages of differentiation within the thymic cortex. The influx of thymocytes into the thymus gland induces the formation and extension of cellular processes from these TECs, which form the thymic stromal network or cytoreticulum that helps to segregate thymocytes from antigens during their maturation. TEC-induced apoptosis of immature thymocytes that have been exposed to antigens involves a unique apoptotic pathway, while acceptable thymocytes, rescued from apoptosis by the TECs, migrate toward the medulla as they mature. [44] Medullary TECs have a particular receptor associated with

apoptosis. Binding of this Fas medullary cell membrane receptor to its ligand results in cellular apoptosis. [45] The majority of thymocytes in the cortex are eventually phagocytosed by macrophages.

The medulla is the central portion of the thymus and is where T cells complete their maturation and exit the thymus via venules and efferent lymphatic vessels. The T cells then migrate to non-thymic lymphoid structures. The medulla is continuous throughout the gland and is not separated by lobules. It consists of a variety of cell types, but TECs, small T cells, macrophages, dendritic cells, eosinophils, and plasma cells are among the more commonly observed in this region of the thymus. Hassall's corpuscles are concentrically arranged whorl-like nests of squamous thymic epithelium that increase in number and size with age. They display various stages of keratinization and their specific function, if any, remains unclear. In contrast to vessels within the thymic cortex, there is no barrier around capillaries in the medulla. These medullary thymic capillaries, or sinusoids, allow trafficking to occur freely throughout the medulla. Large numbers of TECs and loosely packed mature T cells cause the medulla to stain differently from the cortex.

1.6.2. Origin of TECs

TECs are endodermally and ectodermally derived from the third pharyngeal pouch and branchial cleft. They are also derived from cranial neural crest cells.

Because vascularization of the primordial thymus gland has not yet occurred, early hematopoietic populants of the thymic rudiment must extravasate through the pharyngeal vessels and home to the thymus through the perithymic mesenchyme. This suggests that the stem cells are "responding to a gradient of…diffusible chemoattractant factors" that are reported to originate from fetal MHC class II+ TECs. "A number of chemokine receptors expressed by thymic precursors and several chemokines, including thymus-expressed chemokine (TECK), are expressed by thymic epithelial cells, resulting in chemotactic activity for isolated thymic precursors." [27, 46]

1.6.3. TEC Function in Immune System Development

The TECs of the thymic cortex are believed to positively select thymocytes, so that only cells with a self MHC-restricted T cell receptor (TcR) are allowed to mature. [47] The bone marrow-derived antigen presenting cells (APCs) found mainly at the corticomedullary junction, are believed to be largely responsible for negative selection, which leads to deletion of T cells with high affinity for self MHC and/or self antigen/self MHC complexes. However, recent studies both *in vitro* using TEC cultures [48], and *in vivo*, using thymic transplantation models in mice, [41, 49] suggest that the thymic epithelium may also be involved in negative selection. Lymphocytes have long been known to reside in close contact with TECs, and have even been found within TEC vacuoles months following initial harvesting of thymic tissue. [50]

1.6.4. Positive and Negative Thymic Selection

The MHC restricted, two-signal model of antigen recognition serves as the framework for understanding cellular immunity. [51-53] The thymus plays a significant role in developing and maturing T cells which exhibit this pattern of specific MHC restriction. [54-56]; [57] Thymocytes develop this specificity by undergoing selective elimination during their maturation and differentiation. [58] In the process of thymocyte development, bone marrow stem cells migrate to the thymus, are retained there by binding of the CD2 molecule, and commit to the T cell lineage under the influence of the thymic microenvironment. [29] The expression of co-receptor molecules CD4 and CD8 characterize the major lineages of T cells thereafter. The earliest precursors are CD4-/CD8- (double negative), which later become CD4+/CD8+ (double positive) cells. Immature CD4+/CD8+ T cells able to interact in a productive manner with self-MHC molecules are retained through the process of positive selection. It is believed that MHC molecules on the surface of the thymic epithelium select which of the double positive cells will mature into CD4+/CD8- or CD8+/CD4- (single positive) cells. [59] [60] Positive selection involves the “positive” recognition by developing T cells of self-MHC proteins expressed on non-lymphoid cells resident within the thymus, particularly TECs. [34, 59, 61, 62]. Bone marrow derived cells or fibroblasts have also been shown to participate in positive selection. [34]

Thymocytes recognizing and targeting self-proteins bound by self-MHC are eliminated by negative selection. Negative selection occurs via the high-affinity recognition by developing T cells of self-peptides bound to MHC proteins expressed on IT APCs, specifically thymic dendritic cells and macrophages. [63]

1.7. OVERVIEW OF HUMORAL AND CELL-MEDIATED IMMUNITY

The lymphatic system consists of several discrete organs (thymus, spleen, tonsils, and lymph nodes), diffuse lymphoid tissue, and cells including T cells, B cells, and macrophages. This system functions to defend the organism by mounting humoral immune responses against foreign substances (antigens) and cell-mediated immune responses against microorganisms, tumor and transplanted cells, and virus-infected cells. An allogeneic immune response involves the coordination of many cell types. There are both general and specific responses to this stimulus. The three main cell types involved in this response are T cells, B cells, and APCs. Other cell types include eosinophils for opsonization of antigens and neutrophils for local inflammation to enhance the overall immune response. Cellular infiltration at the rejection site occurs early in the process of allogeneic graft rejection. The general properties and functions of specific immune cells will be reviewed as background material for the descriptions and evaluations of cellular infiltrates found upon histological evaluations of tissue grafts in this project.

APCs may degrade antigens into small peptides during “processing”. These antigenic peptides are then linked to an MHC molecule expressed within or on the surface of the APC.

In 1961, studies demonstrated that thymectomized neonatal mice never underwent normal lymphoid tissue development, exhibited weak immune responsiveness, and easily developed infection. Because of the B cell response from the intact bone marrow in these animals, antibody responses remained, but the absence of T cells resulted in an immunocompromised state. In 1967, it was shown that antigen-stimulated lymphocyte populations, now known to be T cells, responded by proliferating, but that these cells did not secrete antibodies. The cells of the immune system include exponential numbers of clones of T- and B cells that are able to recognize and respond to a single antigenic determinant (epitope), or a small group of closely related epitopes. Exposure to an antigen and a second signal (one or more cytokines) induces activation of resting T and B cells, leading to their proliferation and differentiation into effector cells. The population of effector cells may also include APCs (e.g. macrophages, lymphoid dendritic cells, Langerhans’ cells, follicular dendritic cells, M cells, and B cells) as well as mast cells and granulocytes. Although B cells can present antigenic epitopes to T cells, and thus are APCs, their role in this function probably is limited to the secondary (anamnestic) immune response rather than the primary immune response.

As described above, T cell maturation occurs in the thymus and involves immunoincompetent progenitor T cells migrating from the bone marrow to the thymus, where they are termed thymocytes. Within the thymic cortex, thymocytes undergo gene rearrangements and begin to express antigen-specific T cell receptors, which are integral membrane proteins. Hence, the cells are now immunocompetent. The cortical thymocytes also begin to express thymus-induced CD (cluster of differentiation) markers, designated CD2, CD3, CD4, and CD8 on their surface. In the thymic medulla, some thymocytes lose CD4 and develop into CD8+ cells; others lose CD8 and develop into CD4+ cells. Medullary thymocytes also develop class I or class II MHC antigens.

T cell subtypes include T helper (Th) and T cytotoxic (Tc) cells. T helper (Th) cells are CD4+ cells which synthesize and release numerous growth factors, known as cytokines (lymphokines), following their activation. The cytokines interleukin 2 (IL-2) and interleukin 4 (IL-4) induce B cells to respond to an antigenic stimulus.

T cytotoxic cells (Tc) are CD8+ cells. They also produce cytokines, and are involved in the effector stage of graft rejection.

Suppressor T cells (Ts) are also CD8+ cells that can modulate the extent of the immune response by suppressing the activity of Th cells. They may serve an important role in preventing autoimmune responses.

T memory cells are long-lived, committed, immunocompetent cells that are formed during proliferation in response to an antigenic challenge. They remain relatively dormant in the circulation or in specific regions of the lymphoid system, and when activated, function to increase the size of the original clone and thereby provide a faster and greater anamnestic response against a future challenge by the same antigen.

B cells originate and mature into immunocompetent cells within the bone marrow. They have surface immunoglobulins (antibodies) attached to the external aspect of their plasma membrane; all the immunoglobulin (Ig) molecules on a given B cell clone recognize and bind to the same antigen determinant, or epitope. They are responsible for the humoral immune response. They proliferate and differentiate following an antigenic challenge to form plasma cells and B memory cells. Plasma cells actively synthesize and secrete antibody specific for the challenging antigen. B memory cells have the same properties as T memory cells.

Null cells, designated Th_0, are part of a small selection of peripheral lymphocytes that lack antigens which would characterize them as T or B cells. Natural killer (NK) cells are one type of null cell, exhibiting nonspecific cytotoxicity against tumor cells and virus-infected cells. The mechanism by which NK cells recognize these target cells is not yet understood. They also can kill specific target cells that have antibodies bound to their surface antigens in a process known as antibody-dependent cell-mediated cytotoxicity (ADCC).

Macrophages function as both APCs and as cytotoxic effector cells in ADCC. They produce IL-1 which helps activate T_H cells, and several other cytokines that influence the immune response or hematopoiesis. They also secrete prostaglandin E2 which decreases certain immune responses. They are activated by interferon gamma which is also known as macrophage activating factor; this activation increases the phagocytic and cytotoxic activity of macrophages. Macrophages recognize foreign cells, cellular debris, foreign material, and apoptotic cells through the process of target recognition. Phagocytosis is triggered by binding of macrophage surface receptors to target ligands, and accomplished by the formation of a coated pit which creates a vesicle known as a phagosome. The phagosome fuses to a lysosome, exposing the engulfed material to hydrolytic enzymes. [64]

The major histocompatibility complex, as described previously, encodes two main classes of antigenic molecules: class I molecules, which are expressed by nearly all nucleated cells, and class II molecules, which are expressed by the various cells that function as APCs. Antigens are molecules that have the capability of inducing an immune response. The word antigen comes from "generating antibody." Immunogens are antigenic molecules that can react with an antibody or a T cell receptor. Some exogenous antigens are endocytosed or phagocytosed by APCs and degraded intracellularly. Involved in this sequence are proteolytic complexes, proteasomes, which digest antigenic proteins into short peptides, and transport proteins, which ferry peptide fragments from the cytoplasm to the ER. Within the ER, antigenic peptides bind to the antigen binding cleft of newly synthesized class I heavy chains, which will associate with beta 2 microglobulin to form a stable trimer. (MHC alloantigens do not require intracellular degradation for antigen presentation.) The epitope-class II HLA complexes are transported to the cell surface for epitope presentation, where they are presented to CD8+ cytotoxic T cells. Endogenous immunogens are produced within host cells (e.g. viral proteins synthesized within virus-infected cells and tumor proteins synthesized within cancerous cells). They are degraded within host cells, yielding antigen peptides that associate with class I HLA molecules. The peptide-class I HLA complexes are transported to the cell surface, where they are displayed (presented) to T cells.

T cells exhibit HLA restriction: T cells of each type (except T memory cells) only recognize epitopes that are associated with either class I or class II HLA molecules as follows. Th cells recognize class II HLA molecules. Tc cells and the majority of Ts cells recognize class I HLA molecules. T memory cells recognize both class I and class II HLA molecules.

1.7.1. Requirement of Antigen Presentation

Before any pro-inflammatory cells infiltrate an area (monocytes, lymphocytes), there must be an immune stimulus. This stimulus is either mismatched MHC antigen or a minor histocompatibility antigen difference strong enough to elicit a response. This antigen must be properly “presented” to the immune system to in the context of MHC and followed by a co-stimulatory ligand binding signal. A strong and organized immune response will not occur without the second signal. APCs may degrade antigen into small peptides during “processing”. These antigens are then linked to an MHC molecule expressed within or on the surface of the APC. Almost simultaneously, release of the second signal of activation by costimulatory ligand binding of the TcR-MHC on the APC elicits an immune response. This second step is crucial. Non-APCs are capable of presenting antigen per se, but they are not capable of eliciting an immune response because the second signal remains absent.

1.7.1.a. Presentation of Alloantigens

During “presentation”, allogeneic MHC on a graft cell can act as an antigen without the intracellular processing and transport back to the cell surface that other antigens must undergo. Generally, the MHC molecules of allograft tissue belong to Class I, but the Class II MHC antigen expressed by enough donor cells carried into the host site within the parenchyma of a graft can elicit a significant rejection response. In terms of non-MHC antigens, they may be bound to either MHC Class I or Class II, but are restricted to whichever one is bound. As a general rule, if the antigen is found within the cell (endogenous), then that antigen will be bound to a Class I MHC molecule, and exogenous antigens are bound to Class II molecules. Dendritic cells are the most potent of the APCs.

Antigen presentation restricted to Class I MHC involves antigen degradation by proteasomes. The antigens are marked for degradation by two mechanisms: 1) natural degradation and 2) labeling by ubiquitin. There are gene-encoded endopeptidases that provide the phagocytic cell with molecules to assist with degradation.

APCs (B cells, macrophages, dendritic cells, and Langerhans cells), together with TECs, endothelial cells, and activated T cells are the only cells in the body that naturally express MHC II proteins on their surface, although almost any cell can be induced to express MHC II molecules by cytokines, namely γ-interferon

[65]. Exogenous antigens, usually recruited by phagocytosis or endocytosis, are bound to MHC Class II molecules within the cell and this complex is transported to the cell surface, where it is made available for interaction with a T cell receptor (TcR). When this MHC II molecule-antigen complex surfaces on the APC, it is recognized by a TcR. This is referred to as the "First Signal." There are other first signal binding complexes than the MHC-TcR. But again, this first signal(s) cannot provide enough of stimulus to activate T cells.

1.7.1.b. The Second Signal or Costimulatory Factor

There are co-stimulatory molecules on the APC that induce activation of T cells. This is referred to as the "Second Signal." The second signal may be a chemical signal, such as interleukin 1, or the binding of B7-1 on the antigen presenting cell with CD28 on the T cell. This binding will lead to the production of IL-2 (also known as T cell growth factor), initially from APCs and later in greater amounts from T cells. The end product of this cascade is the mass proliferation of a particular clone of T cells due to interleukin stimulated activation of transcription factors.

There are other molecules that do not directly participate in antigen presentation, but assist in the process. Two main types of cluster designation (or CD) molecules are present on T cells. CD4 and CD8 (depending on the MHC molecule class) stimulate intercellular events and lead to T cell activation. CD4

molecules help to anchor the bond between MHC Class I molecules and antigen with the TcR and CD8 molecules help to anchor the bond between MHC Class II molecules and antigen with the TcR. These events promote greater affinity in TcR-MHC binding, stimulating T cell activation and proliferation. Other molecules that assist with this binding are LFA-1 (on T cell) with ICAM-1 (on APCs) and LFA-3 (on APCs) with CD2 (found on all T cells).

Both CD8+ cytotoxic and CD4+ helper T cells are needed for complete rejection. Helper T cells express the CD4 molecule and assist in the clonal expansion of more T cells and B cells by the secretion of various interleukins. Helper T cells can either participate in cell-mediate immunity (Th1 cells) or antibody-mediated immunity (Th2 cells). The main chemical mediators involved in cell-mediate immunity are IL-12 and interferon-γ. The main chemical mediators of antibody-mediated immunity are IL-4, IL-5, and IL-10.

Cytotoxic and suppressor T cells express CD8 molecules. Cytotoxic T cells destroy cells which express the antigen that they recognize, and suppressor T cells lower cytotoxic T cell activity. Cellular killing is accomplished in part with the pore-forming lytic protein, perforin.

1.7.2. Cells Capable of Antigen Presentation

1.7.2.a. Cell Types and Their Distribution

Bone marrow derived macrophages, monocytes, B cells, and dendritic cells are characterized by the relevant costimulatory molecules (B7-1 and B7-2) required for antigen presentation to T cells bearing an MHC-restricted TcR. These cells mostly originate from the bone marrow and the thymus gland, and are mostly present in lymphoid organs, the circulating lymph, and the skin. Other MHC Class Ia-positive macrophages are found in the peritoneum, the liver, the lungs, and the blood. "Non-professional" APCs include cells of epithelial origin that are able to present antigens when activated." [66]

1.7.2.b. Origin of APCs

Dendritic cells, included in the class of APCs, were discovered in both skin (termed Langerhans cells) and lymphoid tissues in 1973, and found to be up to 100 times as efficient as B cells in antigen presentation. [67] These cells, also known as passenger leukocytes, are capable of transferring antigens from peripheral tissues back to lymphoid tissues where they present these antigens to naive antigen-specific T cells within lymphoid tissue, initiating an immune response. Dendritic cells are involved in the initiation of autoimmune diseases and are largely responsible for the initiation of graft rejection. [68, 69] Like other APCs, dendritic

cells express MHC Class II antigens. Splenic dendritic cells are believed to derive from a “thymus-independent bone marrow precursor”. [70]

1.7.3. Function of TECs in Antigen Presentation

Immature thymocytes able to express MHC-restricted T cell receptors (TcR) on their cell surfaces are selected to mature. [71-73] Likewise, immature CD4+ and CD8+ T cells must also express appropriate MHC-TcR interactions to continue in the process of thymic maturation and survival following release to the periphery. A sufficient naive thymocyte population is regulated by cytokine signals that induce a baseline level of expansion. Weak TcR-MHC interactions allow constant immune surveillance feedback between circulating T cells and the thymus gland, signifying the expansion of reactive clones when necessary. For T cells to be surveyed on a constant basis, they must remain free for split second interactions with MHC expressing cells. This requires a combination of high TcR-MHC specificity and low affinity. Taking advantage of peripheral immune surveillance mechanisms, investigators have used intravenous infusion of specific T cell populations as a modality to influence T cell subset selection in models of autoimmune disease. [74] The diversity of the mature T cell population can be attributed by the diversity of self-peptides bound to MHC. [75] The long-term maintenance of a peripheral T cell population that remains sensitive to the self-peptide/MHC complex suggests that extrathymic surveillance

continues long after positive selection of mature lymphocytes by TECs has taken place. [76]

The thymus gland, as discussed, is key to the establishment of self-recognition and self-tolerance. [54] [57]. This is evidenced by the fact that neonatal thymectomy has been shown to induce autoimmune disease. [77, 78] In the establishment of tolerance to peripheral tissue-specific antigens, the interaction of the thymus gland with the periphery has not been fully explained, since clonal ignorance, deletion, and anergy can occur independent of thymic selection. The mechanism of thymic dependent active suppression of aggressive responses to autoantigens that are not expressed intrathymically, also known as infectious tolerance, still remains unknown. [79-81] In any case, the thymus gland is important in immune surveillance as evidenced by *in vivo* studies showing that the intravenous administration of various molecular weight antigens results in their rapid transit to the thymus gland for antigen presentation. [82]

1.8. OVERVIEW OF THE IMMUNE RESPONSE TO TRANSPLANTATION

1.8.1. The Normal Physiologic Response to Graft Placement

Anatomical and physiological events following transplantation or other immunological stimulus include massive shifts in lymphocyte migration to host

lymphoid tissues and away from nonlymphoid tissues. Under normal conditions, T cells recirculate continuously between blood, various native tissues, and the lymph system. In the event of an antigenic stimulus, whether via the gut, from a site of skin inflammation such as a delayed hypersensitivity response, or from placement of a vascularized graft, these recirculation patterns change to allow dissemination of the antigenic message as widely and promptly as possible throughout the host immune system. A few lymphocytes will appear in perivascular areas, ready for interaction with APCs. Lymphoid tissues are an important point of interaction between circulating lymphocytes and graft antigen brought by APCs, or with cytokines released into the circulation from the graft site by antigen-activated immune and, potentially, non-immune cells. They also facilitate antigen recognition and activation by naive host immune cells. Also, some of the prompt changes in lymphocyte migration patterns following an antigenic stimulus can be attributed to the activity of adhesion molecules, both on antigenic cell surfaces and on lymphocyte membranes. For instance, upregulated LFA-1 expression causes lymphocyte binding to high endothelial venules, allowing lymphocytic infiltration into lymph nodes by diapedesis. It has been suggested that there are high endothelial venular-like structures in the vessels of organs which allow leukocyte migration at particular sites. Other such molecules on endothelial surfaces, endothelial-leukocyte adhesion molecules, or intracellular adhesion molecules, may also alter migration of recirculating lymphocytes, increasing the immunological message to the entire host. Over the span of two to four days, large numbers of lymphocytes and macrophages enter

the area(s) of graft tissue. As progressive disruption of vessels and other structures associated with tissue necrosis occurs, lymphocytic cells fill recipient lymphoid compartments. A general inflammatory response occurs to the surgical trauma. Tissue damage resulting from the actual transplantation procedure is surveyed by T cells, while pathogen eradication occurs via cell-mediated immunity. Release of proinflammatory factors, like Hageman factor (factor XII), triggers a cascade of events such as the clotting cascade. Fibrinopeptides promote vascular permeability and attract neutrophils and macrophages with subsequent cytokine production. Antigen-antibody complexes target pathogens for phagocytosis and stimulate macrophages to secrete cytokines (tumor necrosis factor β or cachectin, and tumor necrosis factor, interferon γ released locally by infiltrating cells, and interleukin-1), stimulating proinflammatory endothelial responses. Nitric oxide may also be involved in the inflammatory response. T cells activate the classical complement cascade (e.g. C3a and C5a), while mast cell degranulation (histamine, 5-hydroxytryptamine) increases vascular permeability. In the absence of pathogens, macrophages promote repair and structural reinforcement of damaged tissues (regeneration and neovascularization). Endothelial cells, smooth muscle cells, fibroblasts stimulated by fibroblast growth factor, extracellular matrix with its integrin adhesion molecules, interleukin-1, interleukin-6, transforming growth factor, and platelet derived growth factor, all aid in tissue repair.

1.8.2.The Immunologic Process of Graft Rejection

Graft rejection is a cell-mediated response to alloantigens. Based upon its stimulus, severity, and timing, graft rejection can be classified as hyperacute, acute, and chronic. These stages of graft rejection do not necessarily follow a chronological course.

Normally, T cells recognize the foreign peptides presented in the context of a self-MHC molecule. Different individual T cells, or T cell clones, recognize different peptides. After a transplant, the foreign (allogeneic) MHC molecules, with or without peptide ligands, are recognized as non-self-MHC-peptide complexes by many types of T cells. CD8+ and CD4+ cell subsets proliferate in response to the alloantigens on the surface of the foreign cells. The activation of these specific T cell clones triggers the effector stage.

These activated lymphocytes that accumulated by active proliferation in the host lymphoid tissue during early phases of immune responsiveness then migrate to the graft site to act in an effector role toward its destruction. The effector stage involves a large influx of macrophages and T cells to the graft site. Cytolytic T cells lyse cells bearing the specific MHC alloantigen target, in this case the endothelial and parenchymal cells of the graft. Helper T cells, mostly CD4+ cells, activate macrophages and initiate a delayed-type hypersensitivity reaction.

Finally, graft blood vessels are compromised by alloantibodies that bind to the endothelium.

1.8.2.a. Hyperacute Graft Rejection

Hyperacute rejection may occur as early as minutes following transplantation. In general, rejection that occurs up to 24 hours following transplantation is classified as hyperacute. In these cases, the etiology is usually associated with a significant number of preformed antibodies, either generated from memory cells in the case of specific sensitization, or from non-specific cross-reactivity with the graft alloantigens. Once antibodies present in the host encounter donor MHC antigens, particularly on a donor organ's surface endothelial cells, antigen binding triggers the complement system. Neutrophils flood the grafted tissue and platelets aggregate. The graft soon fails as massive inflammation and emboli block the blood flow to the grafted tissue.

The severity of antibody-mediated rejection can vary from case to case. In fact, not all transplant recipients with donor-specific antibodies actually go on to develop hyperacute rejection. In the worst cases, it begins immediately after establishing vascular reperfusion of the allograft. This may be as early as 20 minutes following transplantation. A blood group (ABO) mismatch could cause a hyperacute graft rejection even in the presence of immunosuppressive drug therapy, but such a mistake rarely occurs. [83] The transplanted organ becomes

grossly mottled, cyanotic, and loses its previous function. The organ may enlarge due to marked edema and rupture of the graft may occur. A multisystemic syndrome such as shock may be the first signal that hyperacute graft rejection is occurring.

1.8.2.b. Acute and Delayed Accelerated Acute Graft Rejection

Acute rejection is a T cell mediated response which typically occurs within the first week following transplantation. The most characteristic feature involves the progressive infiltration of the site by host mononuclear cells. Early experiments have shown that this inflammatory immune response, consisting of diffuse lymphocytic infiltration with CD8+ cytotoxic T cells (Tc cells) and CD4+ T helper cells (Th cells), is capable of stimulating acute destruction of an allogeneic graft. Additional supportive evidence includes the fact that neonatal thymectomy results in a blunting of the rejection response to allogeneic skin transplantation, while injection of normal T cells can restore this capacity. Extensive damage occurs to the blood vessels and effector mechanisms cause massive tissue destruction. The two-signal process of lymphocyte activation involves binding of transplantation antigens to the T cell surface receptors (signal 1), which receive, at the same time, a co-stimulatory signal 2 from an antigen-presenting cell (e.g. macrophage, dendritic cell, B cell, etc.). The co-stimulatory signal(s), CD28 and B7 ligand binding, are increased by the adhesion molecules CD2, LFA-1, and VLA-4 and act to stimulate interleukin-2 (IL-2) production. The CTLA4 adhesion

molecule present on activated Th and Tc cells is structurally similar to CD28 and also binds to the B7 ligand. Extracellular matrix basement membrane proteins serve as a substratum for cell adhesion, stimulating lymphocyte migration and exerting co-mitogenic effects upon lymphocyte activation. T cell activation can be increased *in vivo* by the co-mitogenic effects and can be influenced by factors affecting extracellular matrix components. Antigen binding alone without the co-stimulatory signal is not sufficient for activation and differentiation of CD8+ T cells. T cell proliferation follows IL-2 stimulation. T cell subsets are MHC restricted, in that Tc cell precursors are activated by class I MHC antigens and Th cell precursors are activated by class II MHC antigens. IL-1 produced by antigen-activated antigen presenting cells stimulates the proliferation of Th cells. IL-2 produced by the T-helper cells in turn stimulates the differentiation and proliferation of Tc cells. Tc cells recruited secondarily to the site complete the acute rejection process. Cell killing may occur via specific T cell products, granzyme B, a serine esterase protein, and perforin. Alloantigen-stimulated B cells differentiate into antibody-producing plasma cells, secreting both specific and non-specific anti-donor antibodies. Macrophages act as both APCs early in the immune cascade and later as aggressive effector cells contributing to graft destruction. As previously mentioned, products such as IL-1, which activates Th lymphocytes to produce their own cytokines, particularly IL-2, and other macrophage-derived cytokines, upregulating donor MHC antigens on graft cells, leading to even more effective target recognition as the graft response ensues. Pro-inflammatory cytokines may directly injure graft tissue, while IL-4 and IL-10

may counteract these effects. Two myelomonocyte differentiation antigens (MRP8 and MRP14) are expressed during acute rejection and act as calcium-binding proteins with cell activation and differentiation. Natural killer cells, a population of cytotoxic non-T, non-B immune cells, have been found adhering to exposed Fc portions of molecules during antigen-antibody interactions.

Vasculitis results from antibody formation. The T cell mediated response is followed by destruction of the tissue by invading effector cells such as macrophages and lymphocytes. The pathogenesis of antibody-mediated rejection occurring within the first few days of transplantation is believed to be due to preformed antibodies reactive against donor antigens. These antibodies develop during a prior exposure of the recipient to donor-derived antigens in connection with a blood transfusion, pregnancy or abortion. If the titer of these antibodies has dropped, a few days or even weeks may elapse before an amnestic immune response can develop. Antibody mediated rejection in such cases may be somewhat delayed and less severe.

Cytokine production plays an important role in the immune response. In addition to graft parenchymal cells, both donor and host activated immune cells and APCs can participate in the immune response by producing cytokines. Proinflammatory cytokines include Th1 derived IL-1, IL-3, IL-5, IL-6, interferon gamma (IFN-γ), and tumor necrosis factor (TNF). In transplantation, cytokines produced locally by injured vascular endothelium,

particularly IFN-γ and TNF-α, work in many ways to stimulate the immune response. These cytokines work by increasing MHC Class II expression and detection of the T cell receptors on infiltrating host lymphocytes, upregulating both donor (target) and host (recognition) molecules. Adhesion molecules and other mediators expressed or upregulated on vascular endothelium may influence both early and late graft behavior. Selectins are a family of molecules which are associated with the rolling phase of leukocyte adhesion. Intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), LFA-3, and endothelial-leukocyte adhesion molecule-1 (ELAM-1) are all molecules associated with a proinflammatory response and allograft rejection.

The diagnosis and treatment of acute rejection can be extremely difficult at times, as it may even occur in the first 6 to 12 months following transplantation. Rejection is related to levels of adequate immunosuppression, and may be delayed by several weeks if sensitization to donor antigens has occurred in the remote past. For most organs, the only way to show unequivocally that rejection is occurring is by organ biopsy.

1.8.2.c. Chronic Graft Rejection

Chronic rejection has taken the lead in graft rejection since routine preventive and immunosuppressive techniques commonly prevent hyperacute and acute

rejection, respectively. Unfortunately, there are still many undiscovered concepts in the multifactorial process of chronic graft rejection. There may be multiple etiologies resulting in delayed and long-term immune compromise of a graft. The definitive diagnosis of chronic rejection is generally made by organ biopsy. Chronic cardiac graft rejection is often manifest, as in many other grafts, by accelerated graft atherosclerosis caused by smooth muscle cell proliferation. This may be a form of delayed-type hypersensitivity caused by recipient macrophage smooth muscle cell growth factor stimulation. Chronic renal graft rejection is characterized by fibrosis and damage to the microscopic blood vessels in the substance of the kidney. Livers with chronic rejection have a decreased number of bile ducts on biopsy, a condition known as "vanishing bile duct syndrome". Transplanted lungs with chronic rejection are said to have "bronchiolitis obliterans", a type of fibrosis of the lung.

1.8.2.d. Graft vs. Host Disease

When a graft containing enough lymphocytes is placed, donor lymphocyte clonal expansion can occur and result in an immunologic response by donor cells directed against host cells and tissues.

1.9. PREVENTING GRAFT REJECTION

The first method used to lower the risk of graft rejection is cross matching between a particular donor and recipient to decrease the likelihood of acute rejection. Because a perfect match is rarely available, other techniques for preventing graft rejection are included in the transplantation treatment regimen. Despite precautionary measures, chronic rejection can occur weeks or years after the initial graft was accepted. Various immunosuppressive drugs, including mitotic inhibitors such as azathioprine, lymphocyte migration inhibitors such as corticosteroids, and generalized immunosuppressives such as cyclosporine A, FK 506, and rapamycin, can be used to effectively reduce graft rejection. The first successful long-term kidney transplants were between monozygotic twins, biological clones known to inherit the same histocompatibility antigens. Transplants were therefore donor matched. Using a relative and therefore someone with similar inherited histocompatibility antigens as the donor can also reduce graft rejection. Many techniques have been developed to widen the donor pool. One of the most widely used methods of achieving renal allograft success is the use of routine tissue typing on cadaver organs to select matches with the closest histocompatibility. Although retrospective studies comparing 5 year kidney graft survival rates show direct proportionality to lack of HLA mismatches, techniques used to prevent graft rejection have resulted in 80% of all kidneys functioning by the end of the first year, even with mismatches of all 6 HLA antigens. Most transplantation protocols today depend on ABO blood group

matching but have eliminated tissue typing. While reducing the severity of graft rejection, many of these highly potent immunosuppressive drugs are cytotoxic to both the recipient and sometimes to the graft of interest. Even if a recipient can survive potentially lethal side effects due to toxicity, the effects of long-term generalized immunosuppression leave the recipient vulnerable to disease secondary to infectious pathogens and cancer secondary to blunted immunologic surveillance mechanisms. From 1% to 5% or more of transplant recipients will develop cancer within a few years of receiving their allograft. This represents a 100-fold increase in risk compared to the general population. Allograft recipients are particularly prone to developing B cell lymphomas. Their risk is 350 times higher than that of the general population. In most cases, stopping the immunosuppression leads to regression of the cancer, but often rejection of the transplant as well. More precise methods of immunosuppression would prevent rejection without the dangerous side effects of infection and cancer. Immunosuppressive drugs are also responsible for a significant financial and psychological burden to the recipient. Patient compliance is another significant factor.

Solid organ transplantation success continues to improve, allowing for longer periods of graft survival. Unfortunately, the longer the graft survives in the host, the greater the chances for adverse events and comorbidities. [84] Most protocols combine a primary immunosuppressant (cyclosporine or tacrolimus) with one or two adjunctive agents (azathioprine, mycophenolate mofetil,

sirolimus, corticosteroids). While the benefits of today's immunosuppressive regimens essentially guarantee graft survival, and are necessary and vital to the successful outcome of the transplant, immunosuppressive agents taken over long periods may be associated with the onset of hypertension, hyperlipidemia, osteoporosis, nephrotoxicity, islet cell toxicity, neoplasia, and may possibly contribute to other comorbid conditions. Immunosuppressive treatments may also cause cosmetic changes, such as gingival hyperplasia, hirsuitism, alopecia, and weight gain, leading to patient noncompliance. [84] When toxic levels are reached, both cyclosporine and tacrolimus may cause neurological symptoms such as major speech or language abnormalities, delirium, seizures, tremors, or even coma. Vasogenic white matter edema (as seen on MRI) may lead to apoptosis and cytotoxic edema. Enhanced nitric oxide production may cause blood-brain barrier dysfunction and may inhibit drug clearing from the central nervous system. Drug interactions are common and the source of significant complications/comorbidities. These drug side effects have traditionally been so significant that ethical arguments against their usage have sometimes outweighed arguments supporting transplantation in non-life threatening diseases.

1.9.1. Immunosuppression

Immunosuppressive agents are used to inhibit the recipient's immunological response against the transplanted antigens. The application of generalized

immunosuppressive drugs in transplantation has dated since the commencement of azathioprine and steroid use in the early 1950's, and has continued with the development of antilymphocyte globulin in 1960, and the first specific immunosuppressive agent, cyclosporine, discovered in 1970. [85] Over recent years, "improved knowledge of the immune mechanisms underlying transplantation rejection has resulted in the development of new immunosuppressive agents capable of selectively blocking various steps of the immune response." [86] Specifically acting immunosuppressive agents are associated with fewer side effects, maintaining the necessary balance between immunosuppression and immunocompetence (preservation of general immune protective mechanisms) in order to maintain the general health of the transplant recipient. A regimen which is effective, easy to follow, less costly, and has fewer side effects can dramatically improve patient compliance and morale.

Acute graft rejection is the most significant factor in short-term graft outcome. Real-time ultrasound-guided allograft biopsy and routine histological evaluation using Banff criteria has permitted close monitoring of each graft. [14] The use of erythropoietin to treat anemia allows patients to avoid transfusions, thereby reducing potential immune sensitization which could trigger acute and/or hyperacute rejection episodes. Sirolimus and everolimus have antiproliferative effects on smooth muscle that retard vascular remodeling that is characteristic of chronic allograft nephropathy. Avoiding drug-drug interactions, especially in

pregnancy, intravenous dosing, and caring for minority patients [87], is of extreme importance.

Triple immunosuppressive therapy is a common strategy used to reduce the incidence and severity of graft rejection, and includes the use of a calcineurin inhibitor such as cyclosporine A (CsA, trade name Sandimmune, Neoral), Tacrolimus (FK-506, trade name Prograf), or Sirolimus (Rapamycin, trade name Rapamune); an antimetabolite such as mycophenolate mofetil (MMF, trade name CellCept) or azathioprine (trade name Imuran); and a corticosteroid, such as prednisone, methylprednisolone, or hydroxycorticosone.

Calcineurin inhibitors are natural products isolated from microbial fungal cultures of *Streptomyces tsukubaensis* (CsA and FK-506) and actinomycetes found in the fermented soil of Rapa Nui (Rapamycin). These macrolide immunosuppressants inhibit the signaling pathway used by T cells to turn on their genes for activation, e.g. IL-2 secretion. They work by forming complexes with intracellular receptors (cyclophilins or FK-506 binding proteins [FKBPs]). These complexes block the calcium/calmodulin dependent serine-threonine protein phosphatase, or calcineurin (CaN), lymphocyte activation pathway, preventing activation of specific T cell transcription factors involved in cytokine (IL-2) production and thus signal transduction. [86]

Antimetabolite purine analogs are potent, selective, non-competitive, reversible inhibitors of inosine monophosphate dehydrogenase (IMPDH), inhibiting DNA *de novo* synthesis, and thus lymphocyte proliferation, without incorporation. Like cancer drugs, purine analogs may induce side effects associated with rapidly dividing cells (e.g., lining of the intestine, hair follicles) and myelosuppression. The active metabolite of MMF, mycophenolic acid (MPA), "selectively targets activated lymphocytes" and works to augment the actions of standard immunosuppressive agents without adding to toxicity. It works by inhibiting purine synthesis and thus B and T cell proliferation. The addition of MMF will allow a dose reduction of cyclosporine, tacrolimus, or corticosteroids while still avoiding acute graft rejection. MMF is also used in the definitive and prophylactic treatment of graft versus host disease (GVHD). [88]

Prior to the development of the latest pharmaceuticals, corticosteroid immunosuppression was a mainstay in triple therapy treatment of graft rejection. Corticosteroids are still sometimes administered at high intravenous doses intraoperatively, and at moderately high intravenous doses 1 to 2 days postoperatively. Higher doses initially induce lymphocyte migration to the extravascular space through effects on adhesion molecules and impedance of leukocyte rolling. Later, there is non-specific blockage of antigen recognition by the reduction of IL-1, TNF, and IL-6 through transcriptional inhibition of macrophage mRNA, and modulation of T cell, B cell, and monocyte activity by glucocorticoid receptor effects. High doses can result in lymphocyte death with

resultant shrinkage of lymphoid tissues, decreased cytokine production, decreased antibody synthesis, and inhibition of the complement pathway due to increased cell membrane lipid accumulation. Corticosteroids are tapered to moderate oral doses over the postoperative week, and further tapered over the following two to three months. Lower doses are actually associated with elevated antibody synthesis, possibly accounting for rebound graft rejection following discontinuation of steroids. The final dose is usually 0.2 to 0.3 mg/kg/day, which is still associated with significant clinical comorbidities.

Table 1.1. Side Effects of Corticosteroid Therapy. Multisystemic side effects can occur in transplant patients with long-term steroid treatment.

Side Effect	Features
Cushing's syndrome	(excess glucocorticoids stimulate lipogenesis in face, neck, and trunk)
Osteoporosis	(increased osteoclast:osteoblast activity; reduced gastrointestinal calcium absorption)
Myopathy	(fluorinated steroids)
Cataracts	(toxicity)
Peptic ulcers	(mucosal irritation)
Skin fragility	(atrophy)
Adrenal suppression	
Hypertension	(mineralocorticoids)
Insulin resistance/Diabetes	

Despite improved renal allograft survival over the past 30 years, drug-related nephrotoxicity and chronic rejection remain obstacles in successful kidney transplantation in a significant number of cases. Development of laboratory assays focused on identifying patients with donor antigen-specific hyporeactivity

may help to customize the immunosuppressive drug regimen in stable renal allograft patients. [14]

Antilymphocyte serum (ALS) is antibody-rich serum derived from an animal that has been immunized against lymphocytes. It is used as a potent, non-specifically-acting immunosuppressive agent that destroys circulating lymphocytes. Because of its lot-to-lot variability in potency, it is generally used for animal research. The gamma globulin component of ALS is antithymocyte globulin (ATG, or thymoglobulin, trade name Atgam). Antithymocyte globulin has been used clinically to modulate the immune response. However, due to its relatively non-specific mechanism of action, it has been replaced by more targeted immunosuppressive agents.

New drugs available offer the potential to achieve optimal immunosuppression while reducing toxicity by combining lower doses of toxic drugs or replacing them. [85, 86, 89] Newest to the armamentarium against graft rejection are the monoclonal antibodies directed against specific molecules key to the 2-signal model of T cell activation. Immunosuppressants as well as treatments that recruit leukocytes to allografts are some of the strategies used to study allograft tolerance [17], and many of these studies have led to the development of new monoclonal antibodies for the inhibition of T cell signaling in graft rejection.
Modern immunosuppressants, such as basilixumab (trade name Simulect) and daclizumab (trade name Zenepax), are monoclonal antibodies that target specific

receptors ligands in the immune response (such as IL-2), and other soluble receptor hybrid molecules may serve to reduce required doses of toxic chemical immunosuppressants and provide more specific immune suppression. [14, 17, 86] Moromonab-CD3 (OKT3, trade name Orthoclone®CD3) targets the CD3 component of T cell receptors, inhibiting the Signal 1 of T cell activation. [90] Daclizumab targets the IL-2 receptor and thus inhibits only activated T cells. Efalizumab (trade name Raptiva) is a humanized monoclonal antibody that targets the T cell lymphocyte function-associated antigen-1 (LFA-1) receptor through the CD11a side chain. [91] Monoclonal antibodies that inhibit B7-1 and CD28 ligand binding are being developed to inhibit Signal 2 of T cell activation. [92]

1.9.2. Immunomodulation and Donor-Specific Immune Tolerance

Immunosuppressive agents are invariably required for all allografts as of today, although immunologic tolerance induction techniques may reduce or perhaps someday eliminate the need for these agents. With continued improvements in transplantation outcomes, the drawbacks associated with conventional immunosuppression regimens become increasingly apparent. If allograft tolerance could be established, immunosuppression and its accompanying risks could be withdrawn. Further identification and development of novel agents that target specific components of the allograft response will help achieve greater target-specific immunosuppression, and will aid in the development of true donor-specific

tolerance, “the Holy Grail of solid organ transplantation,” as described by Luke and Jordan.

Generally, the introduction of an immunogen at a period following the completion of immune development in the presence of some inflammatory stimulus will sensitize the recipient to subsequent exposures of that immunogen. However, IT inoculation of antigenic tissue with concomitant depletion of the extent of the peripheral lymphocyte population has been shown to specifically modify recipient immunity, although the mechanism by which immune reeducation occurs has not been fully elucidated. Numerous studies have demonstrated that the introduction of specific polypeptide sequences to the immunologically privileged IT site with depletion of circulating and noncirculating peripheral lymphocytes will induce tolerance.

The ability to induce donor-specific tolerance in an allogeneic host raises the possibility of introducing specific antigens into the thymus to study the ability of these known antigens to act as tolerogens, and induce antigen specific unresponsiveness. [93-96] By altering conditions such as the timing and route of administration of antigenic peptides or the genes encoding them, it may be possible to induce specific tolerance to subsequent exposure to that same antigen.

1.9.2.a. Intrathymic (IT) Transplantation and Central Tolerance Induction Through Thymic Reeducation

Selection processes that occur within the thymus, prior to T cell differentiation, are referred to as “central” selection processes, while post-differentiation T cell regulatory mechanisms on the effective immunological repertoire of lymphocytes are referred to as “peripheral” selection processes. It has been concluded that “only recent thymic emigrants, but not peripheral resident mature T cells are susceptible to this process of functional education, which also requires exposure to specific antigens and occurs entirely in the periphery.” [97] Based upon the cellular type, timing, and route of administration, cellular inoculation treatments can be used to harness natural central or peripheral tolerance induction mechanisms. [47] The thymus is of great importance in T cell development and self-recognition, but its function was unknown until the current century. [54] [57]. It plays an essential role in the establishment of self-tolerance to antigens expressed on its stroma by clonal elimination or functional inactivation of self-reactive T cells. [98] Cortical TECs are believed to positively select self-MHC-restricted thymocytes for maturation. [47] The bone marrow-derived APCs found mainly at the corticomedullary junction are believed to be largely responsible for negative selection [99], which leads to deletion of T cells with high affinity for self MHC and/or self antigen/self MHC complexes. This hypothesis is supported by

recent studies demonstrating that TECs can regulate the functional maturation of CD4+ thymocytes. [100, 101]

1.9.2.a.1. Maintenance of the T Cell Repertoire

Models of autoimmune disease serve to elucidate mechanisms of immune function. In these models, autoantigens serve as naturally occurring foreign antigen representatives, and are classified as either intrathymic or extrathymic. [74] The binding of immature autoreactive T cells signals a process that results in apoptosis and thus clonal deletion [98]. In comparing the autoimmune response to the normal cell-mediated immune response, antigen presentation studies have been used to detect abnormalities in the normal 2-signal model of antigen recognition. Cells bearing autoantigens are thought to be presented to T cell receptors by APCs that are missing key costimulatory molecules. [102] [66] Activation of signal 1 without coactive signal 2 renders autoreactive T cells either anergic or indifferent. [103-105]

This is how passive self-tolerance is said to occur. However, self-tolerance to autoantigens may also require active suppression mechanisms to address the autoreactive potential of cells that somehow are neither clonally deleted nor rendered anergic. [106, 107] Likewise, it has been shown that IT selection of the T cell repertoire is dependent upon peripheral fallback mechanisms regulated by

circulating mature suppressor T cells, answering the question of how tolerance to peripheral self-antigens is achieved. [74]

In terms of tolerance induction, if deletion (anergic) mechanisms were responsible for unresponsiveness, the persistence of thymic tissue unexposed to donor alloantigen would permit normal maturation of alloreactive T cells, preventing donor-specific tolerance. Donor alloantigen introduced into one thymic lobe would result in clonal reeducation in that part of the thymus, but the remainder of the thymus gland would remain naive to the donor alloantigen. This would indicate why it would be important to inoculate both lobes with antigenic tissue for successful tolerance induction. If a suppression mechanism were responsible unresponsiveness, then the presence of alloantigen-naive thymic tissue would not affect the IT induction of tolerance. IT tolerance induction studies support the clonal deletion mechanism. It was shown that "inoculation of bone marrow into only one lobe of the native thymus and/or ectopic thymus did not promote consistent survival of subsequent…cardiac allografts." [108] Tolerant hosts actually had reduced numbers of precursor cytotoxic lymphocytes targeted against donor alloantigens. "Adoptive transfer of spleen cells from tolerant WF hosts harboring longstanding cardiac allografts led to permanent survival of LEW cardiac allografts in secondary recipients, indicating that the unresponsive state after IT inoculation of bone marrow cells is primarily mediated by deletion and/or inactivation of donor-specific T cell precursors maturing in a chimeric thymus." [108] These findings reflect that persistence of donor

alloantigen supplied by cells in the allograft is key to the maintenance of an unresponsive state. “Adoptive transfer of spleen cells from tolerant WF hosts treated with LEW bone marrow but not transplanted with cardiac allografts did not lead to permanent survival of LEW cardiac allografts in naive secondary recipients.” [108] Immune mechanisms are never quite so straightforward, however.

The intravenous infusion of specific T cell populations to induce tolerance or to prevent autoimmune disease takes advantage of the immune surveillance capacity of these cells in the peripheral immune system. It has been shown that as few as 600,000 CD4+/CD8- cells collected from the donor thoracic duct and injected intravenously into lymphopenic rats can protect approximately half of the animals from diabetes. [74] The mechanisms of immunomodulation associated with peripheral administration of lymphocyte populations may not fall under the category of tolerance induction per se, as T cell-mediated suppression of immunity may be the cause of perceived donor-specific unresponsiveness.

Transplantation biology remains a very complicated science. The very same immunosuppressive methods used to treat one of the major obstacles in the success of transplantation, graft rejection, may also lead to graft failure and other intolerable side effects. Familiarity with current techniques in transplantation combined with an intricate understanding of the immune response will guide the development of modern strategies to reduce graft rejection, and at the forefront of

this field, to induce transplantation tolerance. It is hypothesized that more specifically targeted approaches towards reducing immune graft rejection that avoid chronic therapy would eliminate both the threat of graft rejection and the secondary complications of long-term immunosuppression.

1.9.2.b. Intrathymic Inoculation of Soluble Proteins and Non-Viable Cells and Tolerance Induction

In the establishment of tolerance to peripheral tissue-specific antigens, the role of the thymus is much less clear, since recessive mechanisms of peripheral tolerance (ignorance, deletion, anergy, immunodeviation) do not require thymic selection. The mechanism of thymic dependent specific suppression of aggressive responses to autoantigens that are not expressed intrathymically still remains unknown. [79-81] It is known that the expression of autoantigen mRNA and protein in the thymus correlates with resistance to autoimmune disease, supporting the idea that introduction of antigens into the thymus could be used to establish central tolerance. [109] Allogeneic and xenogeneic murine thymic tissue transplantation has been shown to induce donor-specific tolerance. Similarly, allogeneic thymic tissue transplantation in a euthymic miniature swine model supported host thymopoiesis. These studies suggested that thymic transplantation could be adapted into a donor-specific xenogeneic tolerance induction regimen. [110] The specific mechanism by which tolerance induction occurs has not been characterized, but it has been proposed that TECs are

primarily responsible for imprinting MHC-restricted specificity, and bone marrow derived macrophages or dendritic cells are responsible for the elimination of high affinity bound self-reactive T cells.

IT inoculation of soluble alloantigens, including MHC, has been shown to induce donor-specific tolerance to murine cardiac allograft, [93, 111-114] and IT inoculation of sonicated cells has also been shown to induce donor-specific allograft tolerance via the oral route in a mouse model of corneal allotransplantation. [115] Allograft tissue presented intrathymically in this fashion is not recognized as foreign and rejection processes are avoided. The exact mechanisms behind IT soluble alloantigen inoculation induced tolerance induction are not well explained, and the IT inoculation of specifically functioning cells bearing antigens may be a more targeted approach towards tolerance induction.

Further studies have demonstrated that this thymic reeducation is also efficacious in an autoimmune setting. In some models of autoimmune disease, such as experimental autoimmune uveoretinitis, a correlation has been shown between “constitutive expression of autoantigens in the thymus (mRNA and protein)” and resistance to disease. [109] This naturally occurring central tolerance to the “relevant autoantigen” could explain why certain individuals are more susceptible to autoimmune diseases than others. [109, 122]

1.9.2.c. Intrathymic Inoculation of Viable Cells and Tolerance Induction

1.9.2.c.1. Tolerance Induction with Islets of Langerhans

IT islet transplantation in prediabetic BB/Wor rats was shown in several studies to inhibit the progression towards the diabetic state. [116-121] These historical studies are further discussed in Chapter 4.

In many of the studies involving IT cell transplantation for tolerance induction, it is difficult to determine whether the reduced immunogenicity is related to the type of cells transplanted or simply due the presence of allogeneic antigens in the transplantation milieu. It is hypothesized that genetically altering and then transplanting a specific cell type would further specify the mechanism by which tolerance induction occurs.

1.9.2.c.2. Tolerance Induction with Renal Glomeruli

An IT glomerular inoculation study was published just after a similar study showing that IT inoculation of donor islet cells induced tolerance to subsequent donor matched islet grafts, as described above. [123] The IT inoculation of isolated renal glomeruli into the thymus of MHC incompatible rats pretreated with cyclosporine was shown to induce long-term donor-specific tolerance to subsequently transplanted kidney grafts while medium only injected rats rejected their grafts

within 7 to 9 days. [124] This study expanded upon the specificity of tolerance induction because it demonstrated that the tolerance induction was not only MHC-specific, but it was tissue-specific.

1.9.2.c.3. Tolerance Induction with Bone Marrow

Donor-matched tolerance to a variety of tissues, including skin, has been achieved following the IT injection of donor-matched bone marrow cells into each lobe of the thymus coadministered with a single 1 mL intraperitoneal injection of ALS. [125-128] Studies supporting this tolerance induction strategy are discussed in greater detail in Chapter 4.

1.9.2.c.4. Tolerance Induction with Spleen Cells

IT inoculation of MHC mismatched splenocytes with a concomitant single intraperitoneal dose of ALS led to donor-specific unresponsiveness in a model of rat cardiac allotransplantation. Graft survival was dependent upon the route of inoculation (intravenous splenocytes did not induce tolerance) and the present of ALS coadministration. [129]

1.9.2.d. Use of TECs in Thymic Reeducation

TECs were chosen for this investigation based on their thymic origin, epithelial nature, and their ability to be maintained and manipulated *in vitro*. It is hypothesized that TEC expression and presentation of an antigenic gene product in the thymic environment will result in the recognition of the antigen as self, preventing immune targeting of this antigen. In such a paradigm the immunogenic proteins within the host thymus, whether native products such as allogeneic MHC, or transgenic products from novel transferred genes, would function as tolerogens, resulting in the modification of the host's immune repertoire. It is hypothesized that this approach would allow the host immune system to be specifically modified to accept transplanted tissues or cells without impinging on its ability to effectively respond to other foreign immunogens.

1.9.2.d.1. Primary Culture of Mammalian TECs

1.9.2.d.1.a. The Demand for Interest in Primary Keratinocyte Cell Lines and Application to TEC Cultures

It is hypothesized that deriving and culturing TECs would allow for studies on the role of TECs in thymic education and tolerance induction. The development of new and intriguing *in vitro* manipulation techniques which function to isolate and effectively maintain cells in prolonged culture have allowed more efficient

individual and throughput research protocols to materialize. Harvesting and culturing primary cells is a tedious process associated with inconsistency, inconvenience, and expense. Consistency depends upon the reliability of a harvesting source, sensitivity of specific processing techniques, timing of crucial steps in the harvesting process, and technical skill of the tissue harvesting team. Tissue and cell harvesting can be labor intensive, sometimes requiring repeated mid-process sampling to ensure adequate product yields. To maintain genetic consistency between harvest sources, littermates of the same gender are often chosen. Therefore, many other factors, including the reproductive capacity of an animal colony and the availability of appropriately sexed offspring can be technically and financially limiting. Even when the protocols for high yield harvesting from multiple animals have been established, some technologies are not suited for tissues or cells from multiple animals. For example, the gene therapeutic techniques that might potentially be used on cells require that they originate from a single animal source and that the cells are “carried for multiple passages”. The ability to cryopreserve the cells would also be advantageous for repeating experiments and comparing results among primary cell lines. [130] To obtain sufficient numbers of cells for experimental manipulation and evaluation, the development of a method allowing continuous culture of thymus-derived epithelial cells becomes necessary [131-133]. Typically, culture protocols for epithelial-derived cells require a high density of cells to sustain viability in primary culture.

Epidermal keratinocytes are an epithelial-derived cell type which have been extensively studied in *in vitro* systems, and when plated at a high density, demonstrate proliferation and improved overall viability compared to cells cultured at low density [134]. Studies also demonstrate increased plating efficiency for human epidermal keratinocytes derived from newborn as compared to adult donors, signifying the advantage of using partially undifferentiated or immature cells for establishing cell lines. It is reported that even though some keratinocyte cultures can be established and subcultured, there is a limited lifetime in culture ranging from 20 to 50 cell generations. [135]

While the culture and subculture of human keratinocytes from a single biopsy have been well documented, culture techniques for murine keratinocytes have required plating at high density for seeding of sufficient numbers of cells, and poor division capacity has necessitated pooling from several animals. [135] [136] [137] These limitations in murine keratinocyte culture highlight the demand for an interest in primary keratinocyte cell lines.

General aspects of primary keratinocyte culture can be applied to TEC culture, as TECs may be included in the category of keratinocytes. TECs express keratin in the form of keratin intermediate filament bundles, or tonofilaments, and desmosomes, defining them as epithelial in nature. [36] Additionally TECs express an endocrine function, secreting thymosin, serum thymic factor, and thymopoietin in cytoplasmic secretory granules. These hormones all function in

the transformation of immature T cells into immunocompetent T cells, though they may have other functions. Thymosin has been connected to functions in reproductive physiology, wound repair, angiogenesis, and tumorigenesis.

The thymic microenvironment controls IT T cell differentiation via influence by prolactin (PRL) and growth hormone (GH). Both of these hormones, along with thyroid stimulating hormone, stimulate thymulin secretion by TECs. [138] Thymulin, also a hormone, has been shown to regulate GH secretion [139] while inducing T cell differentiation [140], and is controlled by TEC negative feedback mechanisms. [141] Insulin-like growth factor 1 (IGF-1) works synergistically with GH to upregulate thymulin secretion. Prolactin and GH receptors have been located on both TECs and thymocytes. [142] Established TEC lines secrete thymic hormones and promote thymocyte proliferation *in vitro*. One group refers to these secretory products as thymic hormonal factors (THFs). [143]

Minced human thymic tissue has been cultured with and without trypsinization. Cuboidal shaped epithelial cells can appear in a layer within the first 24 to 48 hours, and the *in vitro* thymic epithelial cells were able to influence thymocyte proliferation for at least two weeks. [144]

The differentiation of TECs toward a “neural-oriented cell fate” occurs under the influence of epidermal growth factor (EGF). [145] Neural-oriented differentiation

also appears to be calcium dependent. [146] This could be related to TEC derivation from cranial neural crest cells.

1.9.2.d.1.b. Previous Reports of TEC Isolation

The isolation and culture of TECs in humans, mice, and rats has been described previously. Many of the advances in TEC isolation and culture have resulted from studies aimed at the characterization of monoclonal antibodies for immunology research. These studies are discussed further in Chapter 2.

1.9.2.d.1.c. Culture Conditions and Media Supplements

Many techniques for isolation and culture of TECs have been described, mostly employing conventional primary tissue culture techniques combined with hormonal supplementation and intermittent chelation therapy to reduce fibroblast overgrowth. Other manipulations used to enhance specific growth of this cell type include the deletion of serum from the culture medium, supplementation of the medium with D-valine [147-149], growth of cells on extracellular matrix-coated surfaces [150], growth of cells over “irradiated fibroblasts as filler cells” [135, 149], and culture of cells in low calcium medium. [146, 151-155] Using this background knowledge, it is hypothesized that TEC culture isolation can be developed to supply the need for proliferative TEC lines (Chapter 2).

TECs may be distinguished from other thymic cells based on characteristics specific to epithelial-derived cells. The presence of cytoplasmic keratin intermediate filaments (IFs) or tonofilaments interacting with desmosomal junctions are the primary morphological features allowing identification and characterization of TECs. [156, 157]

Thymic epithelial cell cultures are highly susceptible to overgrowth by fibroblasts. The substitution of horse serum for fetal calf serum has been shown to reduce fibroblast overgrowth. [158] Various serum-free methods of primary keratinocyte culture have proven effective in establishing and maintaining cells through several passages. Types of keratinocytes studied have included but are not limited to epidermal keratinocytes and prostatic epithelial cells. Supplements used in prostatic epithelial cultures include transferrin (1 microgram/mL), EGF (10 ng/mL) and insulin (3.7 micrograms/mL or 0.1 IU/mL). Glucocorticoids like dexamethasone and retinoids like retinyl acetate have also been added as supplements to regulate epithelial proliferation by dose dependent synergism with the effects of insulin and EGF. [159, 160] It is suggested that the TECs exhibit paracrine glucocorticoid activity on thymocytes [161] and that reduction of glucocorticoid production by TECs with age may be the cause of thymic involution. [162] It has been shown that hydrocortisone treatment of glucocorticoid expressing TECs augments their adhesion to thymocytes, thymic hormone secretion, cytokeratin expression, and extracellular matrix production. These cells have been shown to express glucocorticoid receptors and are

sensitive to hydrocortisone in terms of thymic hormone secretion, cytokeratin expression, and ECM production. Additionally, dexamethasone treatment *in vitro* has been shown to induce keratin distribution changes *in vitro* in TECs. [163] It is not known how these effects correlate with *in vitro* primary culture per se, although several growth factors have been used to maintain cultured keratinocytes. [164]; [146, 165] [166] Insulin-like growth factor-1, GH, and PRL have been shown to enhance *in vivo* expression of high molecular weight cytokeratins and to stimulate *in vitro* TEC proliferation. [142].

Already employing serum-free methods of primary keratinocyte culture, one group investigated the effects of glucose and electrolytes. Titration experiments showed that optimal glucose concentration is 0.8 mM and sodium chloride concentration is 100 mM. There appeared to be an abnormal lipid profile in keratinocytes in primary culture, but the lipid profile normalized with the adjustment of glucose and sodium chloride levels. [167] The accumulation of lipid granules within TECs might indicate a need for culture medium glucose and electrolyte optimization.

A common denominator in the formulation of culture medium for the development of primary murine cell lines has been the maintenance of cells in medium containing low levels of calcium. Several studies report on the impact of reduced medium calcium concentration on the proliferation and effortless passaging of epidermal keratinocytes [146, 151-155]. Media containing calcium concentrations less than 0.09mM supported continued growth and proliferation of

keratinocytes, while higher calcium levels initiated terminal differentiation and decreased cell proliferation. [165] Further, the expression of biochemical markers indicative of late stage epidermal differentiation occurred following culture of cells in media depleted of growth factors and containing calcium at a high concentration (0.15mM) [168]. Keratinocytes from newborn mice were grown on dishes coated with collagen IV and maintained in fibroblast-conditioned medium containing 0.06 mM calcium and added growth factors. By removing growth factors and increasing the medium calcium level to 0.15 mM, keratinocytes were induced to produce mouse keratin I. However, this expression was lost by the 15th passage. [130] Other groups also reported that higher calcium levels initiated terminal differentiation and decreased cellular proliferation. [146] [169] The calcium-induced response of TECs is discussed in Chapter 3.

Another supportive investigation showed that monolayers of human prostatic epithelial cells have been grown to the 4th passage using collagenase digestion of prostatic acini, low calcium concentrations, supplementation with “a growth factor that is concentrated in bovine neural tissue” and subculturing before confluence reaches a certain threshold. [170] Trypsinization with EDTA may not be as effective in passaging cells due to their tight adherence to growth surfaces and to each other. Gentle collagenase IV digestion for 5 minutes at 37° C, then rinsing with PBS and 0.02% EDTA, followed by pelleting at 600 to 800 rpm and replating can allow passaging of especially adherent cells.

Limited dilution cloning is a technique that has been used to isolate and expand small numbers of epithelial cells. (Figure 2.1.) [171] Additionally, the reduction of incubation temperatures from the usual 37° C to 31° C has been shown to increase epidermal cell proliferation. [164]

1.9.2.d.2. Immortalization (Transformation vs. Nontransformation)

"Subculturable cell lines" can be established with the cellular application of carcinogen treatments. [164] TEC lines derived from thymomas and malignant thymomas were compared to those derived from normal rat thymus tissue and shown to exhibit differential expression of proto-oncogenes and tumor suppressor genes *in vitro*. [172] These thymoma-derived cell lines produced cells of a larger size than cells derived from normal thymic culture, and were "stabilized" to a fully differentiated TEC phenotype with the addition of 1μm dexamethasone. [163, 173] Alternately, TEC lines have been transformed using oncogenic viral vectors, such as SV40, an oncogenic simian polyoma virus. [174-176] Because of the differences between TEC lines derived by induced transformation vs. spontaneous transformation, a spontaneously transformed cell line might be preferable. It was hypothesized that altering culture conditions would allow the cultivation of spontaneously transformed primary TEC lines for further use. The isolation and culture of several TEC lines, one of which spontaneously transformed, is covered in Chapter 2.

1.9.2.e. Tolerance Induction and Gene Therapy

Statistical analyses of the number of transplantation recipients suffering from significant systemic side effects of routine immunosuppressive agents would lead one to conclude that an alternative to current strategies should be closely investigated. Induction of specific immunologic tolerance to subsequent grafts would offer a safe alternative to long-term immunosuppression and combat donor organ shortage. Tolerance induction would be an ideal way to treat autoimmune diseases. The possibility of imparting specific immunologic tolerance to autoimmune diseases such as diabetes mellitus, autoimmune thyroiditis, or system lupus erythematosus serves as a great impetus for working towards a new treatment paradigm that would simply and effectively prevent some of the world's most common diseases. [177] Gene therapy has been and continues to be examined as a means to introduce novel genes into the cells of a graft in an effort to modify graft immunogenicity, introduce localized/microlocalized immunosuppression, modify the graft environment to improve engraftment, and/or modify the function of transplanted cells/tissues. One method of introducing a protein antigen within the thymic microenvironment is via the insertion of genetic material encoding that antigen using gene transfer techniques. [178]

"Gene therapy is currently being used in attempts to induce immune tolerance to a variety of immune mediated diseases. In transplantation, gene therapy

strategies to prolong graft survival involve gene transfer and expression of immunomodulatory or graft-protecting molecules." [179] Induction of immunologic tolerance to alloantigens is a major goal in the field of transplantation. The recent addition of stable gene transfer vectors that can be expressed in a controlled manner has allowed focus towards clinical applications of gene therapy in transplantation of various organs, including the heart. [179]

While many studies support the IT route of administration for inoculation of genetically modified cells for tolerance induction, one showed that neither intraperitoneal nor intrathymic inoculation of genetically modified fibroblasts induced recipient tolerance to the protein encoded by the transgene without the use of immunosupression (cyclosporine A). In fact, when later compared to the intraperitoneal/cyclosporine A treated control group, recipients of intrathymic cells mounted a heightened antibody response to the protein, suggesting sensitization rather than tolerance induction. [180]

It is hypothesized that the combination of gene therapy and tolerance induction strategies, resulting in the production and expression of specific novel proteins and their expression as tolerogens within the thymus, offers a highly attractive paradigm for developing novel therapeutic approaches to transplantation immunology, autoimmune disease(s), and potentially other immune-related issues. [182] The desire to use genetic manipulation necessitates a method allowing continuous TEC culture. [135-137]

CHAPTER 2.

ESTABLISHMENT AND MAINTAINANCE OF A RAT PRIMARY THYMIC EPITHELIAL CELL LINE

2.1. INTRODUCTION

Induction of specific unresponsiveness has been demonstrated following IT transplantation of whole cells, cell clusters (i.e. islets), and IT inoculation of soluble proteins and non-viable cells. It was hypothesized that the IT injection of donor MHC-mismatched TECs, native residents of the thymic environment, would demonstrate improved transplantability and survivability at the transplant site, and, as demonstrated for other cell types, would induce donor-specific tolerance. The successful establishment of a proliferating population of TECs would be the first step in demonstrating the feasibility of this hypothesis. The desire to utilize large numbers of TECs for *in vitro* manipulation and characterization, transplantation experiments, and for other future studies including genetic manipulation in a rat model necessitated the establishment of at least one primary TEC line. The best suitable conditions for isolation of TECs and establishment of a TEC line were investigated by modifying previously published techniques, and by varying culture and medium conditions.

Previously, studies focusing on ontogeny of the thymus gland and defining the *in vitro* effects of TECs on T cell maturation in normal and diseased animal and human models required the ability to establish and maintain TECs in primary culture. [183-188] Some groups applied the results of research on thymomas to normal TEC responses. [189-194] Other studies focused on raising monoclonal hybridoma antibodies against TECs for studies characterizing the *in situ* architecture of the thymic cortex as it related to the blood-thymus barrier and clonal selection. [195-209] Murine-derived TECs and other cells which express keratin intermediate filaments are reported as difficult to establish and maintain, possibly explaining why a primary rat TEC line has not been made commercially available. [135] [136] [137] Like primary TEC cultures, primary keratinocyte cultures are often complicated by fibroblast overgrowth. [158] Additionally, primary keratinocyte cultures have been established using serum free and low calcium growth conditions [146, 151-155]. These varied conditions of primary culture, common to keratin-expressing cells, were applied to the primary TEC cultures derived from four different rat strains, the Lewis, Dark-Agouti, LDA (Lewis x Dark Agouti), and Wistar Furth strains, outlined below.

2.2. MATERIALS AND METHODS

2.2.1. Animals

Three to fourteen day old Wistar Furth (WF, $RT1^{u}$), Lewis (Lew, $RT^{1}l$), Dark Agouti (DA, $RT1^{a}$), and Lewis-Dark Agouti (LDA, $RT1^{a,l}$, F1 offspring of Lew x DA) rat perinates were used as tissue donors.

2.2.2. Neonatal Thymus Gland Excision

Perinates were sacrificed using craniocervical dislocation, the neck and thoracic regions were prepared with 70% ethanol, and thymic dissection was performed in an aseptic environment. Using aseptic technique, a midline vertical skin incision was made beginning in the mid epigastric region and extending towards the neck. The skin was retracted laterally. Forceps were used to lift the epigastric musculature and scissors were used to puncture the musculature of the diaphragm, and an incision was made through both the musculature and the ribs, just lateral to the sternum, extending cranially all the way through the clavicle. The ribs were retracted laterally, and the gray, bilobed thymus gland was identified overlying the great vessels. Microforceps were used to separate the thymus tissue from the great vessels. To minimize cellular autolytic changes, the fresh thymic tissue was placed in 15 to 20 mL of cold (0°C) Ca++/Mg++ free Hank's balanced salt solution in a glass Petri dish over ice.

2.2.3. Preparation of Thymic Cell Suspension – Enzymatic *In Vitro* TEC Isolation

Freshly harvested neonatal thymi were placed in a sterile glass microconcavity slide with a small volume of Ca++/Mg++ free HBSS. (Fisher Scientific, St. Louis, MO) Sharp dissection with microforceps and a microscalpel under a dissecting microscope, was used to remove any visible capsular and pericapsular connective tissue, fat, coagulum, blood vessels, and debris. Under a positive pressure laminar flow hood, the tissue was minced in the sterile microconcavity slide using curved 9 cm surgical scissors, producing approximately 2-3 mm^2 particles in a viscous cellular mix. Occasionally rinsing the scissors with a small volume of Ca++/Mg++ free HBSS helped to clean it of adherent tissue. The minced particles were pipetted into a 50 mL centrifuge tube containing a sterile-filtered collagenase (Boehringer Mannheim, Mannheim, Germany) solution (15 mL, 1 mL solution/perinate, 1 mg/mL collagenase in Ca++/Mg++ free HBSS) and incubated for 30 minutes at 37°C in a water bath with vigorous agitation every 10 minutes. The addition of cold Ca++/Mg++ free HBSS was used to arrest enzymatic activity, and the digest was centrifuged at 1500 rpm for 5 minutes. Following centrifugation, the supernatant was removed and the digested tissue washed and centrifuged with Ca++/Mg++ free HBSS two additional times. A pipette was used to remove the supernatant from the pellet of minced thymic tissue. (Depending on the donor strain, cells were treated with 0.05% EDTA in Ca++/Mg++ free HBSS.) The pellet was resuspended to a total of 7 mL in growth medium (type varied with strain of rat), and plated to T25 tissue culture

flasks (approximately 3 thymi per flask). (Falcon, Fisher Scientific, St. Louis, MO) Plated thymic explants were maintained in a water-jacketed incubator at 37°C with an atmosphere of 5% CO_2 in air. On day 7 of culture, the tissue was refed by removing and replenishing approximately half the media volume. Depending on the donor strain, 0.05% EDTA in Ca++/Mg++ free HBSS or PBS was used to chelate calcium from the culture medium, inhibiting fibroblast overgrowth. Unattached thymic cells and tissue explants removed with the media change were pelleted by centrifugation for 5 minutes at 1500 RPM, the supernatant discarded, and the pellet resuspended and replated to a new flask in fresh media.

All flasks were refed and EDTA-treated every 3 days thereafter. The thymic cells attached to the culture dish surface and cellular outgrowth occurred, eventually forming a monolayer. Culture supernatants were collected daily for one week and used for subculture to conserve any unattached cells, providing them further opportunity to settle down, attach, and thrive. The cultures were observed daily under an inverted tissue culture microscope. Unattached non-epithelial thymic cells such as thymocytes were discarded with the supernatant and attached fibroblasts were eliminated over a period of approximately 3 weeks by exclusion of supplements required for their growth and by differential trypsinization of cultures.

2.2.4. Pure Thymic Epithelial Cell Culture

Initial culture techniques included collagenase and EDTA treatment followed by plating of minced tissue for two hours, re-plating of collected centrifuged supernatant mixture (500 rpm), and refeeding with fresh medium. The effects of collagenase and EDTA treatment of the thymic suspension and the supernatant were evaluated. The medium used for the initial plating was adjusted over time to optimize primary TEC culture. (Table 2.1) Evaluation of early cell populations revealed a majority of cells with a fibroblast-like phenotype, with a minority of epithelial-appearing cells. Sterile poly-L-lysine was used to treat the growth surface of tissue culture flasks in attempts to increase cellular attachment. Incubation settings were also optimized. Re-evaluation of the culture techniques eventually led to the following modifications in the protocol: The collagenase and EDTA-treated minced tissue was plated into T25 culture flasks containing the medium best suited for each rat strain and maintained at incubation settings of 37°C, 5% CO_2. The strain-specific optimization of media was empirically determined using a variety of media and media combinations (Table 2.1.). Depending on the rat strain, the tissue was again EDTA treated on day 7 and every 3 days thereafter until the epithelial nature of the monolayered cells was confirmed using light microscopy, immunocytochemical staining, and electron microscopy.

Table 2.1. Media Formulations Assayed for Applications in Primary TEC Culture. Various formulations with differing concentrations of medium components. GWAJC supplements = cholera toxin, EGF, insulin, transferrin, and DM.

Base medium	2° medium (conc.)	serum (conc.)	Buffer (conc.)	antibiotic (conc.) 1%	Supplements (conc.)
DMEM	N/A	equine (15%)	HEPES (1%)	PSF	NaPy (1%)
DMEM	Ham's F12 (50%)	N/A	$NaHCO_3$	PSF	N/A
WISH		fetal calf (15%)	$NaHCO_3$	PSF	L-glutamine (1%)
GWAJC	DMEM (7.5%)	fetal calf (7.5%)	HEPES and $NaHCO_3$	PSF	GWAJC
GWAJC	DMEM (2.5%)	supplemental calf (2%)	HEPES and $NaHCO_3$	PSF	GWAJC
GWAJC	DMEM (7.5%)	supplemental calf (2%)	HEPES and $NaHCO_3$	PSF	GWAJC
GWAJC	DMEM (2.5%)	supplemental calf (7.5)	HEPES and $NaHCO_3$	PSF	GWAJC
DMEM	N/A	N/A	$NaHCO_3$	PSF	GWAJC
DMEM	N/A	supplemental calf (2%)	$NaHCO_3$	PSF	GWAJC
DMEM	N/A	supplemental calf (7.5%)	$NaHCO_3$	PSF	GWAJC
DMEM	N/A	supplemental calf (10%)	$NaHCO_3$	PSF	GWAJC
DMEM	N/A	fetal calf (10%)	$NaHCO_3$	PSF	GWAJC
Ham's F12	N/A	supplemental calf (2%)	$NaHCO_3$	PSF	GWAJC
Ham's F12	N/A	fetal calf (7.5%)	$NaHCO_3$	PSF	GWAJC
Ham's F12	N/A	fetal calf (10%)	$NaHCO_3$	PSF	GWAJC
Ham's F12	N/A	supplemental calf (10%)	$NaHCO_3$	PSF	GWAJC
GWAJC	DMEM (50%)	supplemental calf (5%)	HEPES and $NaHCO_3$	PSF	GWAJC
GWAJC	DMEM (50%)	supplemental calf (7.5%)	HEPES and $NaHCO_3$	PSF	GWAJC
GWAJC	DMEM (50%)	supplemental calf (10%)	HEPES and $NaHCO_3$	PSF	GWAJC
Promocell	N/A	N/A	N/A	PSF	N/A
GWAJC	N/A	N/A	HEPES and $NaHCO_3$	PSF	Promocell
Promocell	N/A	N/A	N/A	PSF	GWAJC
Promocell	N/A	supplemental calf (2%)	N/A	PSF	Promocell
Promocell	N/A	supplemental calf (5%)	N/A	PSF	Promocell
Promocell	N/A	supplemental calf (7.5%)	N/A	PSF	Promocell
Promocell	N/A	supplemental calf (7.5%)	N/A	PSF	Promocell + D-valine
Promocell	N/A	supplemental calf (2%)	N/A	PSF	GWAJC
Promocell	N/A	supplemental calf (5%)	N/A	PSF	GWAJC
Promocell	N/A	supplemental calf (7.5%)	N/A	PSF	GWAJC

A culture isolation technique using sterile vacuum grease and cloning cylinders was used to isolate and expand cuboidal, epithelial appearing cells or cell clusters. (Figure 2.1.) When a small cluster of adherent cells was found in early stages of primary culture, the area was marked, medium volume was reduced, sterile vacuum grease was applied to one end of the cloning cylinder, and under sterile conditions, the cylinder was applied with the cylinder lumen surrounding the cell cluster. The isolated cells were lightly trypsinized, and they were passed to a new growth surface, thereby increasing their purity and relative concentration. This technique was used to increase cell-to-cell contact and maintain a pure population of cells.

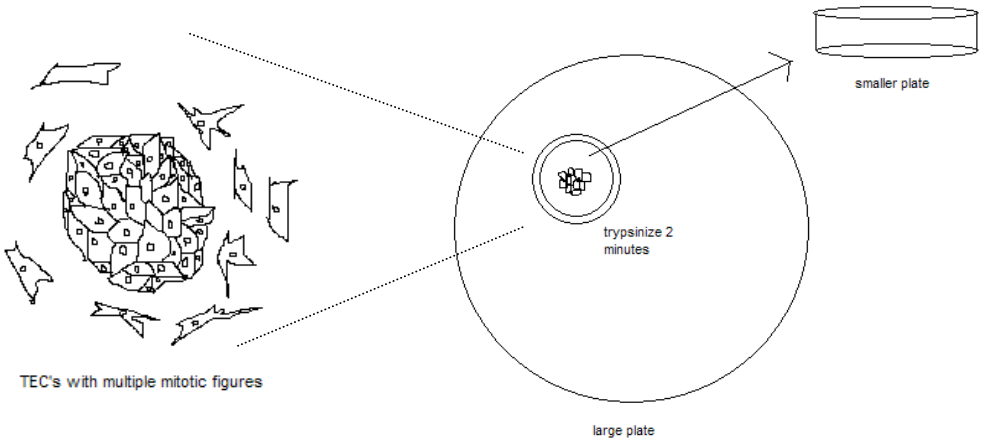


Figure 2.1. Isolation of TECs with Cloning Cylinders. Schematic diagram showing isolation technique used to concentrate a small TEC cluster within a large culture plate using a cloning cylinder (aka cloning ring).

The primary cell line and cultures from the different rat strains that were developed and maintained in this project were refed every 3 days with the

medium that supported their continuous growth. Cells were maintained in T25 tissue culture flasks at 37° C in 5% CO_2. Doubling times were approximately 48 hours long. A completely confluent flask of TECs usually contained 1 x 10^6 TECs, as shown by cytometric analysis. During the *in vitro* experiments, the isolated cells from thymic suspensions were regularly investigated for the presence of cytoskeletal cytokeratin.

2.2.4.a. Passaging of Thymic Epithelial Cells

Adherent cells were passaged when they approached confluence, with replating at a density of approximately 2 x 10^5 cells per T25 flask. For trypsinization, media was removed and cells were rinsed with room temperature calcium free PBS. Trypsin was prepared by adding 20 mL of calcium free PBS to 5 mL aliquots of thawed 0.25% stock solution. Cells were immersed in this 0.05% trypsin solution for 10 minutes at 37° C. Cold calcium free PBS was used to inactivate the trypsin. Cells were agitated off of the flask by jet pipetting, then collected and centrifuged in 50 mL centrifuge tubes at 1500 RPM for 5 minutes. The supernatant was removed from the pellet and the cells were suspended in 25 cc flasks in fresh supplemented GWAJC medium. LDA cells were trypsinized and reseeded at a 1:2 or 1:3 concentration, while WF, Lewis, and DA cells were reseeded at a 1:1 concentration.

2.2.4.b. Cryopreservation and Cryogenic Storage of Thymic Epithelial Cells

Cells from the continuous cell line were stored at a density of 1 x 10^6 TECs in GWAJC medium supplemented with 10% cryoprotectant, DMSO. Nearly confluent LDA TECs grown in antibiotic free media for 3 passages were trypsinized, triple washed in calcium free saline solution, and 1 x 10^6 cells were frozen in a vial containing 1 cc of GWAJC media containing 10% DMSO, and stored at –80° C. For reconstitution, the vial was thawed at room temperature, cells and media were transferred to a T25 flask, and 6 mL of GWAJC medium added to the flask for incubation at 25° C, 5% CO_2. Cells were refed every 3 to 4 days with fresh GWAJC medium.

2.2.5. Morphological Analysis of Thymic Epithelial Cells by Light Microscopy

2.2.5.a. Immunocytochemical Labeling of Thymic Epithelial Cells for Keratin

The immunocytochemical labeling of keratin within epithelial-appearing cells derived from the rat thymus was used to determine whether the neonatal rat thymic tissue cultures included TECs. Like other endodermally-derived epithelial cells, TECs should express cytoplasmic keratin IFs. A modified avidin-biotin-peroxidase immunocytochemical technique was used. [210, 211] Early attempts at staining had been performed using a mouse anti-human anti-keratin monoclonal antibody. (DAKO N Series Mouse anti-human cytokeratin, MNF

1166, class IgG, kappa, Carpenteria, CA); however, experimental results and negative controls (monoclonal mouse antibody, isotype IgG1, kappa, clone DAK-GO1, toward Aspergillus niger glucose oxidase, code no X 0931, Lot 047, 100 mg/L) were poorly differentiated when using an avidin biotin complex-diaminobenzidine (ABC-DAB) staining technique with the DAKO LSAB 2 kit (Carpenteria, CA). Therefore, the cytokeratin antigen was labeled with a rabbit anti-bovine polyclonal anti-cytokeratin antibody (keratin wide spectrum screening 20622, DAKO, Carpenteria, CA).

Immunocytochemical staining was successfully performed using a modified avidin-biotin-horse radish peroxidase technique with the polyclonal anti-cytokeratin primary antibody described above to characterize adherent primary cultured thymic cells.

Thymic epithelial cells were cultured in filter-top culture flasks (Fisher Scientific, St. Louis, MO) over a period of 5 to 6 weeks. Attempts to lift the cells by trypsinization and subsequently passage them into chamber slides did not initially yield adequate numbers of adherent cells for staining. Thus, following the establishment of a confluent monolayer of cells, staining was performed within the culture flasks. Cells on the flask's growth surface were not allowed to dry at any time. All reagents were equilibrated to room temperature prior to usage, except as specified.

Monolayered cells adhering to the bottom of the plates were washed 3 times with calcium-free phosphate buffered saline (PBS) (Fisher Scientific, St. Louis, MO). Cells were fixed by the addition of absolute methanol (2 mL per flask) at –4°C for 5 to 6 minutes, then washed with PBS, and treated with peroxide (0.1 mL of 3% H_20_2 in 10 mL of methanol at room temperature for 5 minutes) to quench endogenous peroxidase activity, after which cells were again washed with PBS. Whole goat serum (diluted 1:50 with PBS, 2 mL per flask) was applied to fixed cells for 15 minutes at 37° C to block non-specific binding by the secondary antibody, followed immediately by application of primary antibody diluted 1:250 in PBS for one hour at 37°C. PBS was used in place of the primary antibody to control for non-specific binding of the secondary antibody or the avidin-HRP complex. After removal of the primary antibody solution, cells were washed in PBS for 10 minutes, followed by application of a goat anti-rabbit biotinylated secondary link antibody (2 mL per flask) for 1 hour at 37° C, after which cells were washed again with PBS for 10 minutes. Avidin (streptavidin conjugated to horseradish peroxidase [HRP]) (2 mL per flask) was then applied to the cells for 15 minutes at 37°C to allow binding to the biotinylated secondary antibody. Cells were again washed and then immersed in PBS before the staining procedure.

2.2.5.b. Immunocytochemical Labeling of Thymic Epithelial Cells for Vimentin

The same modified avidin-biotin-peroxidase immunocytochemical technique was used for vimentin staining of TECs. A monoclonal anti-mouse vimentin antibody

(DAKO V9 vimentin, MO 725) diluted 1:10 in PBS was applied using the same ABC-DAB technique as described above. Equine serum was used to block non-specific secondary antibody staining.

2.2.5.c. Staining Procedure using Diaminobenzidine as Chromogen

3,3-Diaminobenzidine (DAB, DAKO, code k3465, Carpenteria, CA) was used as the chromogenic substrate to react with HRP. [210, 212, 213] For TEC staining, DAB was diluted (80 microliters of DAB in 3.92 mL of buffer) approximately 30 minutes prior to use. Excess solution was removed from the cells and 2 mL of the DAB solution were placed in each flask to react with the avidin-conjugated HRP at room temperature. Flasks were observed under the microscope for deposition of the DAB reaction product. Development was continued until optimum staining was achieved, usually in approximately 5 minutes. Gentle rinsing with distilled water helped to wash away unbound excess reaction product in each flask. For cells stained in flasks, initial viewing was accomplished with an inverted light microscope. For counterstaining and coverslipping of thymic epithelial cells stained in flasks, the growth surface of the flask that contained DAB-stained cells was removed, a hematoxylin counterstain was applied for 2 minutes and then rinsed. The flask surface was glued face up over a glass slide with melted gelatin (28-30°C) and a coverslip was applied over the cells with a few drops of fructose mounting media (Fisher Sci., prepared by heating a 3:1 solution of fructose in distilled water, to 55°C and then chilled) containing thymol

(J.T. Baker) as an antifungal preservative. Subsequently, optimization of culture techniques and conditions allowed the successful adherence and growth of TECs on chamber slides, which greatly simplified the immunocytochemical processing of culture TECs, and allowed coverslipping with Permount mounting medium.

2.2.6. Morphological Analysis of Thymic Epithelial Cells by Phase Contrast Microscopy

The growth surface of flasks containing living media fed cell cultures was visualized using an inverted microscope using Hoffman phase contrast illumination. Cells were photographed using a digital camera.

2.2.7. Morphological Analysis of Thymic Epithelial Cells by Transmission Electron Microscopy

Transmission electron microscopy of TECs allows detection of cytoplasmic keratin IFs and a desmosomal type of intercellular junction between cells. Cultured TECs were evaluated as either cell monolayers grown on coverslips, or as a pelleted cell mass.

For cells grown on coverslips, approximately $1x10^6$ cells suspended in 0.5mL low calcium medium (WAJC with standard supplements) were plated onto Thermanox coverslips in 30mm tissue culture plates and incubated for 2hr (37°C, 5% CO_2).

This allowed for a concentrated number of TECs to seed to the coverslip surface. After the 2hr incubation, an additional 1.5mL of WAJC medium was added to each plate, and cells were incubated an additional 24hr, after which time cells were fixed and embedded for transmission electron microscopy. Coverslips were released, fragmented, glued to Polybed812 bullets, sectioned at 70nm, and stained with uranyl acetate and lead citrate.

Alternately, Bouin's fixative was extracted from previously fixed cells using 2 washes of 70% ETOH, followed by rehydration in graded solutions of 50% ETOH, 25% ETOH, and buffer. These fixed cells were then pelleted into a microfuge tube by centrifugation, and infiltrated with 10% neutral buffer formaldehyde (NBF), rinsed with buffer, submerged in gelatin at 37° C for 1-2 hours, and cooled in ice. The hardened gelatin was overlaid with toluene saturated with Polybed embedding medium. After baking, the cell button was cut out of the microfuge tube for sectioning.

2.3. RESULTS

Thymic cells, derived from neonatal WF, Lew, DA, and/or LDA rats were isolated and primary cultures were established under reduced calcium conditions. Cells derived from Lew, WF, and DA strains were maintained up to the 5th passage, while cells derived from LDA rats were maintained through over 65 passages.

Cultures were refed at varying intervals and subcultured at varying levels of confluence, as empirically determined to be appropriate by experimentation.

2.3.1. Establishing Primary Cultures of WF Thymic Epithelial Cells

Initial attempts toward TEC isolation and culture in the WF strain employed the use of thymic fragmentation, *in vitro* enzymatic dissociation, and plating in either Nutrient Mixture (Ham's) F12 medium or Dulbecco's Modified Eagle Medium. (Figure 2.2.) These high calcium medium formulations resulted in the growth of a variety of cell types with limited proliferative capacity. Some cells appeared cuboidal, while others appeared more fusiform (fibroblast-like), with the majority of cells appearing as anuclear, membrane bound ghostlike structures adherent to the flask growth surfaces. (Figure 2.3.) After cells grew to a higher confluence, trypsinization and replating resulted in a morphologic change of cells from epithelioid to a more fibroblast-like appearance. Plating into glass chamber slides resulted in minimal to no adhesion. Likewise, replating into plastic chamber slides resulted in insufficient cell adhesion to allow processing of the cells for microscopy. It was concluded that greater numbers of adherent cells were required in the initial phases of culture establishment, and that once cultures had been established, expansion of small cell clones was not a feasible approach.

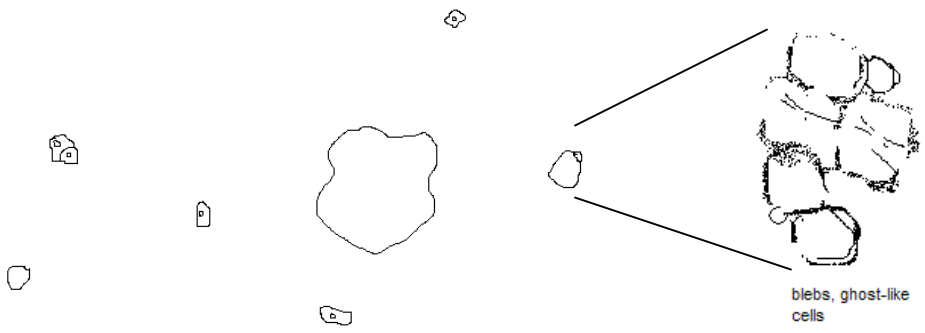


Figure 2.2. Initial Appearance of Thymic Cells from the WF Strain. In this drawing depicting the initial primary culture of explants derived from the WF thymus, very few cuboidal-shaped nucleated cells were seen. To the far right, an enlarged view shows pyknotic cellular remnants and blebbing of cellular membranes in cultures maintained in Ham's F-12 Nutrient Mixture.

Later attempts at TEC isolation and culture in the WF strain employed the use of media containing 50% Ham's F12 with L-glutamine (GibcoBRL, Gaithersburg, MD) and 50% enriched high glucose DMEM (GibcoBRL, Gaithersburg, MD) supplemented with 1% PSF (GibcoBRL, Grand Island, NY), 1% sodium pyruvate (GibcoBRL, Gaithersburg, MD), 1% HEPES buffer (GibcoBRL, Gaithersburg, MD), prolactin (PRL, Sigma, St. Louis, MO), ES (Hyclone, Logan, UT), and insulin-like growth factor 1 (IGF-1, Sigma, St. Louis, MO). Supplementation with equine serum resulted in fibroblast overgrowth and reduced TEC proliferation.

The medium formulation that was most effective for TEC establishment in the WF strain was DMEM with 10% supplemental calf serum (SCS), cholera toxin (20 ng/mL), dexamethasone (10 nM), epidermal growth factor (10 ng/mL), insulin (10 μg/mL) and transferrin (10 mg/mL). A heterogeneous population of cells could be visualized with ghostlike cells that demonstrated a ring-like structure

surrounding a small yet intact nucleus, rounded cells with a central nucleus, and oblong, spindle shaped cells. (Figure 2.3.) Many cells appeared vacuolated, and some appeared to have a frothy cytoplasm. Other cells appeared to be anuclear.

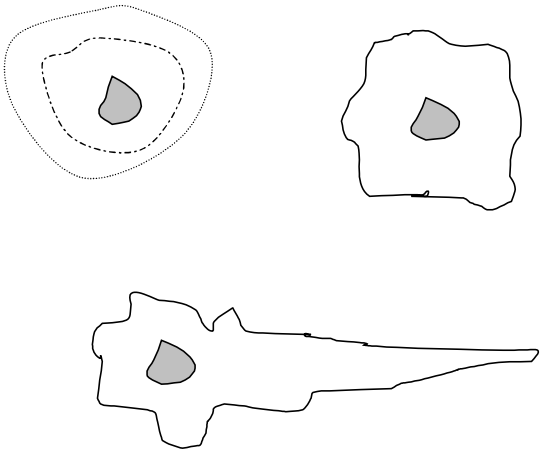


Figure 2.3. Variety of Cell Types in Primary Thymic Culture. Drawing demonstrating the 3 general types of cells seen in early stages of primary culture using thymic tissue fragments.

In spite of improvements in the initial yields of WF TECs from primary cultures, the proliferative capacity of these cells remained limited. However, following a switch in culture medium within a week of plating from the DMEM with 10% SCS and supplements, used for the initial plating process, to WAJC medium, a low calcium formulation from the W. Alton Jones Cell Research Center, the cells demonstrated a continued proliferation, producing sufficient numbers of cells for passaging without dilution. This represented a considerable improvement in the establishment of primary cultures of WF TECs compared to initial results.

2.3.1.a. Maintaining Primary Cultures of WF Thymic Epithelial Cells

Established cells were maintained in WAJC media. Cells were refed by aspiration of spent medium and replacement with fresh medium. Due to the relatively limited proliferative capacity of TECs of the WF strain, completely confluent TECs were lightly trypsinized and reseeded without dilution. Cell numbers diminished over the span of approximately 5 passages, with eventual loss of cellular colonies due to lack of proliferation and terminal differentiation. Because thinning of cell numbers resulted in loss of cellular colonies, populations of cells grown in several flasks were consolidated, centrifuged, and subcultured into a single new flask. This failure of the cells to thrive in conditions of low cell density seemed to indicate that a certain amount of intercellular contact was necessary to induce proliferation, and proliferative rates of this cell population were never sufficient enough to allow the spontaneous transformation of the WF TECs into a cell line.

2.3.2. Establishing Primary Cultures of LDA Thymic Epithelial Cells

LDA TECs were isolated by the *in vitro* enzymatic tissue digestion technique with tissue plating in specialized low calcium medium (WAJC) supplemented with 2% Supplemental Calf Serum (SCS, Hyclone, Logan, UT), cholera toxin, dexamethasone, insulin, transferrin, and EGF. WAJC medium has previously been used for the isolation and maintenance of the TEA3A1 primary cell line

derived from LDA rats. [132] Both the TEA3A1 cell line and WAJC medium were donated as generous gifts by Dr. Jun Hayashi at the University of Maryland to aid in the derivation and characterization of new primary TEC lines. This medium formulation was later custom ordered through GibcoBRL. Gibco WAJC (GWAJC) supplemented with 2% SCS, 2.5% DMEM, cholera toxin 20 ng/mL, dexamethasone 10 nM, insulin 10 μg/mL, transferrin 10 mg/mL, and EGF 10 ng/mL was used to establish a primary LDA TEC line for this project. (Figure 2.4.)

The initial protocol for establishing LDA TEC primary cultures involved surgical excision and enzymatic digestion of the thymus, and culture in WAJC media (low calcium, 0.098mM), supplemented as described above. Later, primary cultures of LDA TECs were maintained in GWAJC (GibcoBRL, Gaithersburg, MD) supplemented with 2% SCS. Each T25 flask was able to accommodate thymic fragments for 3 neonatal rats. Contact inhibition was noted with tissue densities which exceeded this level.

The above techniques resulted in establishment and proliferation of partially differentiated epithelial cells with concomitant depletion of lymphocytes and other non-epithelial cells. Cells were refed after the formation of a sufficient cell monolayer and maintained proliferative capacity indefinitely. Upon microscopic examination, cells cultured in low calcium medium displayed round to spindle shaped morphology and failed to establish physical contact between cells even

at confluence. In passaging cells, single cell suspensions were easily obtained by light trypsinization, further indicating a paucity of intercellular contact. Isolated LDA TECs maintained under reduced calcium conditions (using WAJC media supplemented with growth factors) retained a high *in vitro* mitotic frequency and spontaneously transformed into a cell line, designated OKTE4-01.

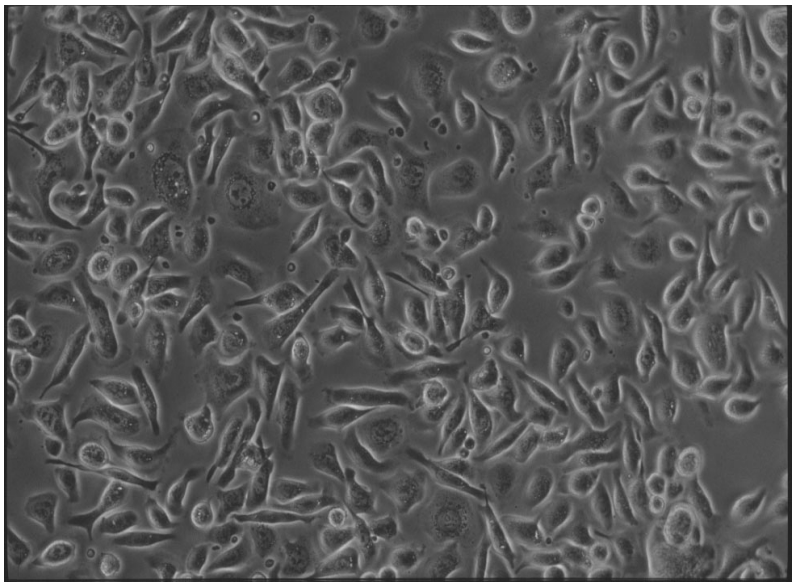


Figure 2.4. Monolayer of Proliferative TECs from the LDA Hybrid Strain. Thymus-derived cells displayed the above morphology in primary culture at high confluence. These cells remained proliferative when maintained in GWAJC medium supplemented with 2% SCS and growth factors. 135 X.

2.3.2.a. Maintaining Primary Cultures of LDA Thymic Epithelial Cells

Once LDA TEC primary culture was established, cells were maintained in T25 flasks containing 7 cc's of medium until subculture became necessary. Passaging of the cells was imperative before they became overly confluent. Even though these cells normally appeared to have increased intercellular

spacing, a morphological change resulted from excessive confluence, and the cells began to exhibit cobblestoning and occasionally some cellular inclusions, typical of TECs from other strains which had lost proliferative capacity. The cause of this was unknown, although it was speculated that release of calcium from intracellular stores in cultures of overpopulated cells might have been the causative factor. Thus, cells were gently trypsinized at approximately 80% confluence (i.e. approximately 80% of the growth surface was occupied by cells).

Cells were cryopreserved for storage and future use at multiple passages. Frozen cells were easily reconstituted, and appeared to be relatively unaffected by the cryogenic procedure. No changes in morphology or proliferative capacity were observed following cryopreservation.

2.3.2.b. Effects of Cholera Toxin Concentration

Cholera toxin levels were increased to determine their effects on TEC morphology. The original concentration of cholera toxin in the WAJC solution was 20 ng/mL. No significant changes in TEC morphology were seen at cholera toxin concentrations of 40, 80, and 200 ng/mL. However, primary culture of TECs was dependent on maintaining minimal cholera toxin concentrations of at least 20 ng/mL in the growth medium. Exclusion of this supplement from the medium used for initial plating resulted in the inability to establish TEC primary culture. Once TECs were established in primary culture, the cholera toxin could

be excluded from the medium without any apparent deleterious effects on TEC morphology or proliferative capacity.

2.3.3. Establishing Primary Cultures of Lewis Thymic Epithelial Cells

Using a calcium free thymic tissue fragmentation and collagenase digestion method, numerous media types were tested for their capacity to allow establishment of Lewis TEC colonization and proliferation. Each media type was supplemented with cholera toxin, EGF, insulin, dexamethasone, and transferrin. GWAJC and Promocell media, despite supplementation with 2%, 5%, 7.5%, and 10% serum, were the least permissive medium for initial Lewis TEC survival. The use of equine serum resulted in fibroblast overgrowth and limited TEC proliferation. Cells initially collagenase-digested and then chelated with EDTA appeared to retain a greater proliferative capacity and could be maintained for longer periods than cells initially treated with collagenase but never exposed to EDTA treatment.

The EDTA chelation of the medium is believed to have reduced available calcium, thereby preventing cell/tissue exposure to calcium levels high enough to induce irreversible terminal differentiation. Serum free WAJC resulted in minimal to no growth while supplementation with 7.5% SCS allowed the formation of minimal cell colonies. Serum free DMEM with hormonal supplements initially allowed small numbers of scattered cells and cellular remnants to attach, but

within a week, there was differentiation of some cells and almost complete loss of attached cellular material. Interestingly, use of calcium-free medias did not improve initial culture success as much as EDTA chelation of calcium from higher calcium medias.

Primary culture of Lewis TECs was possible following comparative studies using DMEM with 2% to 10% SCS and Ham's F12 with 2% to 10% SCS. DMEM with 2% SCS and supplements allowed the formation of small nests of TECs that expanded over the first week of culture. DMEM with 7.5% SCS and supplements had similar results. DMEM with 10% SCS and supplements allowed the initial formation of even larger nests of TECs, but these cells became less well organized over time and were eventually overgrown by fibroblasts. Similarly, use of Ham's F12 and WAJC supplements with 2% SCS resulted in some scattered nests of cells, and with 10% SCS exhibited larger nests containing mitotic figures despite increased fibroblast contamination compared to other media types. Both DMEM and Ham's F12 with 10% SCS and supplements allowed the formation of larger, better organized TEC colonies. This might have been due to the higher serum concentrations in the media, although this would not explain why GWAJC with 10% serum did not support Lewis TEC colonization. Unfortunately, over time, even the most robust TEC colonies dwindled in number due to loss of proliferative capacity and terminal differentiation. As time passed, some cells appeared to display effects of toxicity, as reflected by accumulation of lipid droplets. Cells cultured in Ham's F12 transformed into dendritic appearing cells.

Eventually, mitotic cells disappeared and all of the cells appeared to be senescent.

DMEM was mixed 50/50 with GWAJC to take advantage of the positive effects of both types of media; however no beneficial effect as evidenced by TEC growth was observed with serum supplementation of 5%, 7.5%, or 10% SCS. The application of extracellular matrix (ECM) or poly-L-lysine (PLL) to the cellular surface prior to thymic tissue seeding did not improve cellular attachment over control flasks without ECM or PLL.

Finally, it was hypothesized that an initial establishment of TEC colonies using DMEM with high serum followed by a switch to GWAJC media containing only 2% SCS at a critical stage in the establishment of primary TEC culture would prevent or minimize loss of proliferative capacity. Lewis TEC primary cultures were established by initially plating the tissue in DMEM with 10% SCS. After a period of approximately 7 days when colonies of cuboidal cells appeared, the medium was switched to GWAJC, the low calcium medium used to establish LDA TECs. This switch allowed the establishment of TEC colonies in high calcium and the subsequent proliferation of TECs in low calcium medium. Once Lewis TEC culture was established, TECs were maintained by refeeding with GWAJC every 3 to 4 days. (Figure 2.5.) However, Lewis TECs did not spontaneously transform into a cell line.

2.3.3.a. Maintaining Primary Cultures of Lewis Thymic Epithelial Cells

After colonies of TECs were established with DMEM containing 10% SCS and supplements, switching the media to CGWAJC prolonged cell survival of these primary TECs to the 7th passage. The ability to establish colonies in one type of medium and maintain them in another type of medium suggested that this medium switching technique could be applied to the establishment of TEC culture from other rat strains. It also suggested that application of this strategy might make feasible the establishment of primary cultures of other keratinocyte types not previously shown to be amenable to primary culture.

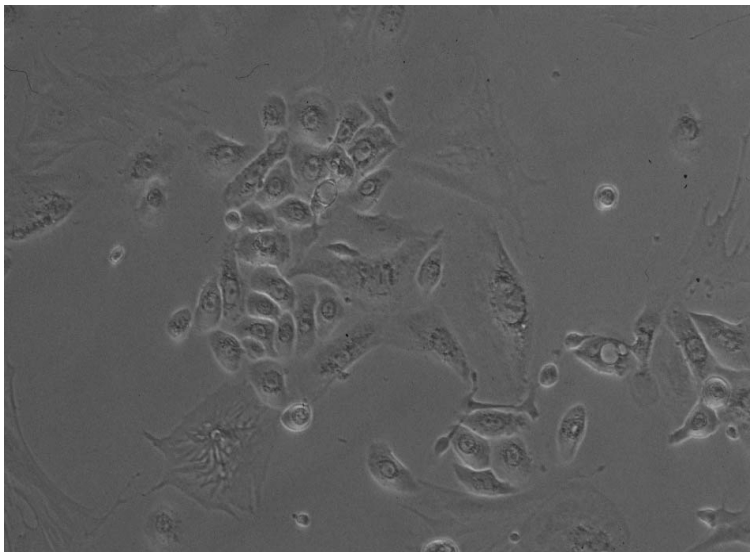


Figure 2.5. Lewis TECs in Primary Culture. Cells derived from Lewis thymic are polygonal in shape and are moderately flattened. 135 X.

2.3.4. Establishing Primary Cultures of DA Thymic Epithelial Cells

Primary culture of TECs from the DA strain initially demonstrated similar responses, as did primary cultures of Lewis and WF TECs. Like TECs derived

from WF and Lewis strains, TECs derived from the DA strain did not demonstrate growth and proliferation in response to initial culture in either high calcium or low calcium medium. After the first passaging, cells terminally differentiated into fibroblast-like cells. Some cells had a dendritic appearance. Employing the medium switch strategy in which initial plating was accomplished using high calcium medium (DMEM with 10% SCS and supplements) and then after establishment of TEC colonies, switched to low calcium medium (GWAJC) within the first week of tissue culture, the cells maintained an epithelial cell morphology, retained some proliferative capacity, and could be passaged without dilution. This represented a significant improvement in DA TEC primary culture over initial attempts, allowing the establishment of monolayers of adherent DA TECs, although the cultures did not spontaneously transform into a primary TEC line.

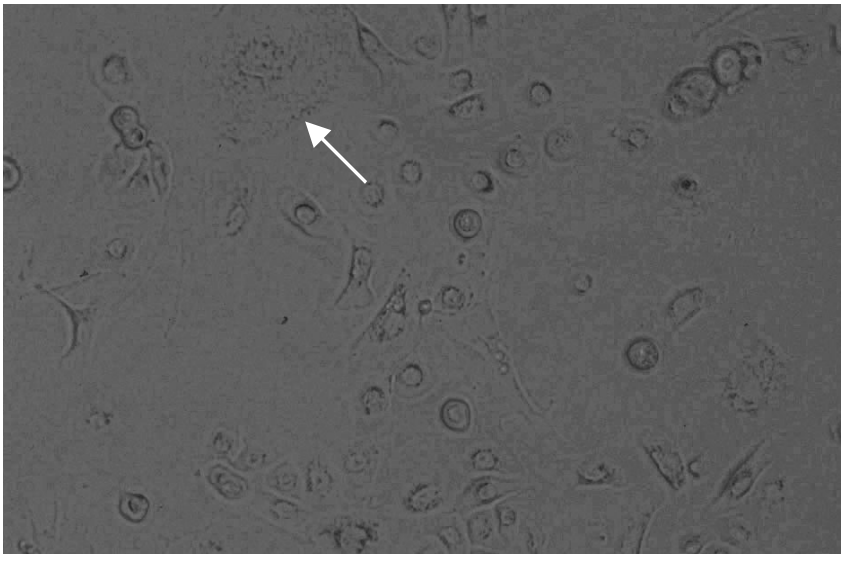


Figure 2.6. DA TECs at Moderate Density. A pyknotic cellular outline (arrow), with blebbing at the upper right hand corner, and scattered cuboidal cells at a late passage of TEC culture derived from the DA strain. 135 X.

2.3.4.a. Maintaining Primary Cultures of DA Thymic Epithelial Cells

As with TECs from other rat strains, once TEC culture from the DA strain was established, cells were refed every 3 days with low calcium GWAJC medium with supplements and 2% SCS.

2.3.5. Immunocytochemistry and Verification of TECs

The purpose of immunocytochemical evaluation of established TEC colonies was to demonstrate definitive epithelial cell characteristics in the isolated cells obtained from neonatal rat thymic tissue harvests, thereby validating that these cells were indeed TECs. The definitive identification of cultured thymic cells as epithelial was of interest in related studies that would take advantage of both the thymic origin and the epithelial nature of these cells. Keratin IFs are known to exist within epithelial or epithelial-derived cells of ectodermal origin in the form of intracytoplasmic bundles and are also known to insert into desmosomes. Other thymus-derived cells would express IFs of a different type, uncharacteristic of endodermally derived epithelial cells, and would not be characterized by desmosomes. Therefore, the expression of cytokeratin within these cells, as visualized by light microscopic immunostaining, was used as the defining characteristic to identify these thymus-derived cells as epithelial. Because TECs contain the antigen cytokeratin, this demonstration was accomplished by staining with anti-cytokeratin antibodies. Positive specific staining would definitely

establish the thymus-derived cells to be epithelial in nature, and thus TECs.

(Figure 2.7.)

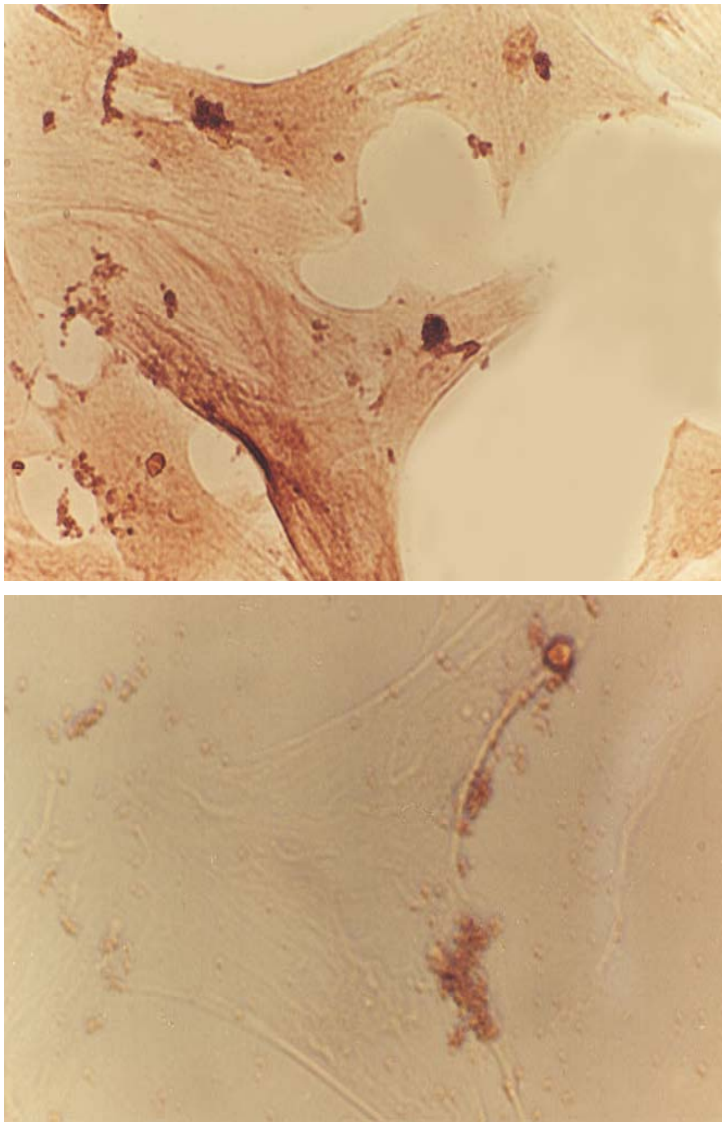


Figure 2.7. Immunocytochemistry on TECs with an Anti-cytokeratin Primary Antibody. Cytokeratin staining was performed on the experimental flask (top), with DAB-labeled TECs throughout with prominent cytoplasmic uptake in a cytoskeletal pattern and no uptake in the nucleus which was highlighted by the surrounding stain. The negative control flask (bottom) demonstrates no specific DAB staining. 425 X.

Not shown, immunocytochemistry on TECs with an anti-vimentin primary

antibody was also negative.

2.3.6. Electron Microscopy and Verification of TECs

Electron microscopy of cultured thymic cells was also used to discriminate thymus-derived epithelial cells from other thymic cell types, including non-ectodermally derived epithelial (e.g. endothelial) cells. Since these cells were isolated from the thymus gland, demonstration of characteristics unique to epithelial cells would define the cells as TECs. In previous studies examining thymic epithelium [149, 214, 215], both *in situ* and *in vitro*, characteristics of TECs have been described. Common to many epithelial-derived cells, these cells demonstrate keratin IFs and desmosomes (as described above) and display undulating cell membranes that possess microvilli. Further, TECs have been noted to possess significant numbers of intracytoplasmic lipid droplets, lysosomes, and residual bodies.

The presence of individual keratin intermediate filaments and filament bundles indicates the epithelial derivation of these cells. [157] Endothelial cells, fibroblasts, and other connective tissue cells do not demonstrate these characteristic filament bundles. [31, 35, 156, 158] Rather, they express vimentin as individual IFs, the appearance of which tend to be more curved or spiral in EM examination than keratin IFs. The EM demonstration of desmosomes also indicates the epithelial derivation of these cells. These desmosomes, or macular adherens junctions, are associated with keratin IFs which insert into components of the junction.

All of these characteristics, distinctive for epithelial cells, and specifically for TECs, have been observed within both the TEA-3A1 and OKTE4-01 cell lines, confirming these thymus-derived cells as TECs. Standard TEM was performed using methanol fixed, osmium/lead stained cell clusters to localize ultrastructural characteristics definitive of epithelial cells. (Figures 2.8. and 2.9.) The definitive identification of cultured cells as TECs was important in establishing the ability to isolate and maintain a thymic specific cell.

Figure 2.8. Electron Microscopy of TEC Intermediate Filaments. Cells cultured in high calcium WAJC media contained individual and bundled cytoplasmic keratin IFs coursing through the cytoplasm (arrow). 42,500 X.

Cells grown in high calcium medium were adjoined by desmosomes, further confirming their TEC nature. (Figure 2.9.)

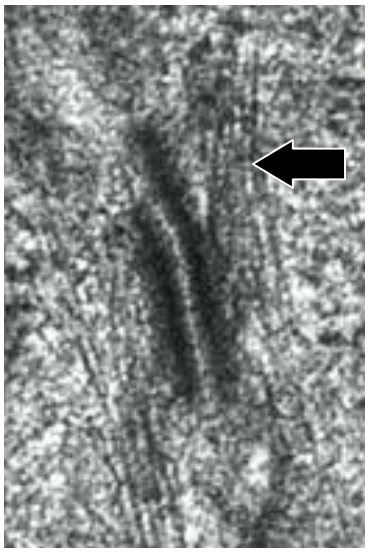


Figure 2.9. Electron Microscopy of TEC Desmosomes. A typical desmosome with keratin IFs (arrow) attaching into dense plaques. 80,000 X.

Maintenance of TECs in low calcium medium was not associated with the typical arrangement of keratin IFs and formation desmosome structures. Cytosolic keratin IF bundles were considerably less numerous although individual IFs remained visible and were scattered throughout the cytoplasm.

An incidental finding noted in the evaluation of electron micrographs of TECs grown in low calcium media was the presence of IFs arranged in whorls, complete and incomplete concentric rings, and other curvilinear bundles. Some of these structures demonstrated a dense laminar structure, while others appeared to possess more diffuse laminae. These unique IF arrangements were observed in regions containing linear keratin filaments, present as either individual filaments or as filament bundles. (Figure 2.10.)

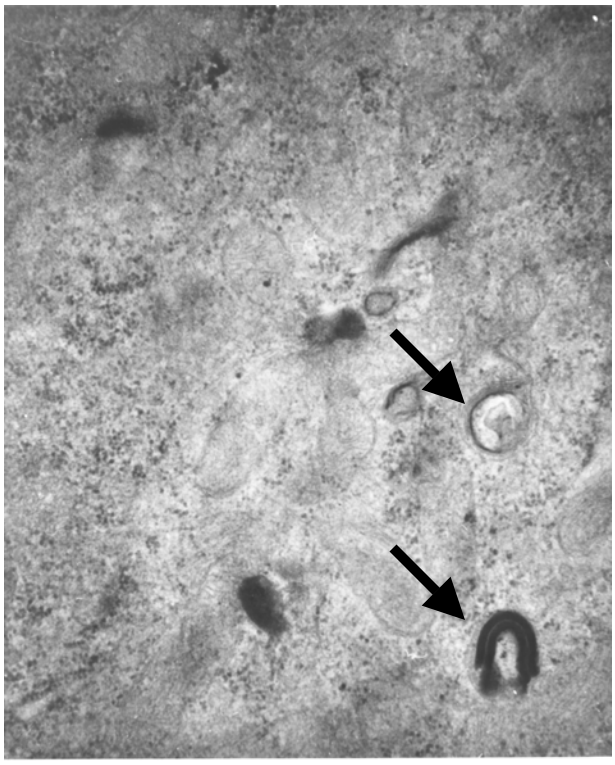


Figure 2.10. TECs Maintained in Low Calcium Medium. Concentric whorls and curvilinear structures (arrows) are visible throughout the cytoplasm of TECs maintained in medium containing low calcium levels. 37,500 X.

2.4. DISCUSSION

As noted by authors of previous reports, TECs are difficult to obtain and maintain in culture. While LDA TECs spontaneously transformed into a cell line when plated and maintained in low calcium medium, WF, Lewis, and DA TECs required plating in high calcium medium (DMEM with 10% SCS) and switching to low calcium medium (WAJC) for maintenance. Though not previously reported, it appears that rat TEC primary culture is dependent upon the specific strain from which the thymic tissue is derived. These calcium-dependent characteristics of

rat TECs are further evaluated by morphological and functional studies in Chapter 3.

Because the ability to obtain sufficient TECs was limited to the LDA rat strain and its MHC expression, further studies using an allogeneic model of rat transplantation tolerance induction were directed towards respectively MHC mismatched donors and recipients (Chapter 4). Additionally, preliminary studies on *in vitro* manipulation and genetic transfection of TECs (discussed briefly in Chapter 5) were also limited to LDA TECs.

It is hypothesized that it may be of value to employ a medium change like the one used to expand upon the proliferative capacity of WF, Lewis, and DA TECs during the crucial period of cellular attachment in primary cultures of other cell types that could not previously be established using other techniques.

CHAPTER 3.

EVALUATION OF INDUCED MORPHOLOGICAL CHANGES IN THYMIC EPITHELIAL CELLS

3.1. INTRODUCTION

The common denominator which led to the successful culture of TECs was knowledge of the TEC response to medium calcium levels. To establish and maintain cell populations proportionate to the demands of this project, the calcium-related morphological and proliferative responses of TECs became a primary focus. These responses of TECs to varying medium calcium concentrations were evaluated in great detail following the establishment of their primary culture.

Attempts at primary culture of TECs derived from WF, LDA, Lewis, and DA rat strains revealed that there were strain dependent differences in responses to cell culture conditions, and only the LDA derived TECs spontaneously transformed into a cell line. It is known that there is a causal relationship between medium calcium levels and desmosome formation. Desmosomes, or macular adherens, are structures composed of protein plaques with attaching keratin IFs, and they

function in maintaining structural enforcement between keratin-expressing cells. "The IF bundle system in epidermal cells appears to be involved in shape formation, shape maintenance, the establishment of desmosomes, nuclear centration, and cell-cell contact." [216] A relationship between desmosome formation and calcium exposure has been demonstrated in many cell types [217-219], but this relationship has not been defined in TECs. It was hypothesized that TEC proliferative capacity was dependent upon desmosome formation. The morphological studies that ensued were aimed at interrelating the effects of medium calcium levels, TEC proliferative capacity, and desmosome formation.

3.2. MATERIALS AND METHODS

3.2.1. Phase Contrast Microscopy of TECs

The growth surface of a flask containing living media fed cell cultures was visualized using an inverted Hoffman phase contrast microscope. Cells were photographed using a digital camera.

3.2.2. Light Microscopy and Immunocytochemistry

The immunostaining protocol using a modified ABC-DAB technique was previously described in Chapter 5, and was used on TECs maintained in varying

culture conditions. Cells were maintained and treated in chamber slides. Primary cultured LDA TECs maintained in WAJC medium with 2% DMEM were compared to LDA TECs which had originally been maintained in WAJC medium with 2% DMEM and then switched to a higher calcium medium (WAJC with 7.5% DMEM). During the immunocytochemical labeling process, cells were not allowed to dry at any time. All reagents were equilibrated to room temperature prior to usage, except as specified. Immunocytochemical staining was performed using an avidin-biotin-horse radish peroxidase technique with polyclonal rabbit anti-bovine anti-cytokeratin primary antibody (keratin wide spectrum screening 20622, DAKO, Carpenteria, CA) and DAB as chromogen to characterize adherent primary cultured thymic cells. Cells in chamber slides were fixed in absolute methanol at –4°C for 5 to 6 minutes and treated with peroxide (0.1 mL of 3% H_20_2 in 10 mL of methanol at room temperature for 5 minutes) to quench endogenous peroxidase activity, after which cells were again washed with PBS. Fixed cells were treated with blocking serum diluted 1:50 with PBS for 15 minutes, incubated with rabbit anti-bovine cytokeratin polyclonal primary antibody diluted 1:250 in PBS for one hour at 37°C, washed again, incubated with goat anti-rabbit biotinylated secondary antibody diluted 1:250 in PBS, washed again, and incubated with avidin HRP solution for 15 minutes at 37°C. DAB was used as the chromogenic substrate, and hematoxylin counterstaining was also applied.

3.2.2.a. Staining Procedure using Diaminobenzidine as Chromogen

The same DAB staining technique described in Chapter 5 was used to stain TECs exposed to varied medium calcium concentrations. Briefly, DAB was diluted (80 microliters of DAB in 3.92 mL of buffer) approximately 30 minutes prior to use. Excess solution was removed from the cells and 2 mL of the DAB solution were placed in each flask. Flasks were observed under the microscope for deposition of the DAB reaction product. Gentle rinsing with distilled water was used to wash away excess reaction product. Cells were counterstained for 2 minutes with hematoxylin and then rinsed, and a coverslip was applied with Permount mounting medium.

3.2.3. Transmission Electron Microscopy

As described in Chapter 5, TEM of TECs allowed detection of a desmosomal type of junction between cells and cytoplasmic IFs. Quantitative analysis of these calcium-dependent morphologic structures in TECs could therefore be compared in TECs exposed to varying calcium concentrations with a TEM study on cells grown on coverslips.

Approximately $1x10^6$ cells suspended in 0.5mL low calcium medium (GWAJC with standard supplements) were plated onto Thermanox coverslips in 30mm tissue culture plates and incubated for 2hr (37°C, 5% CO_2). This allowed for a

concentrated number of TECs to seed to the Thermanox coverslip. After the 2hr incubation, an additional 1.5mL of GWAJC medium was added to each plate. Cells were allowed to adhere for 18hr, at which time medium calcium levels were adjusted, as appropriate, to 0.0925 mM for the low calcium condition by maintaining cells in GWAJC with 2% SCS and 2.5% DMEM, and to 0.188mM for the high calcium condition by maintaining cells in GWJAC with 2% SCS and 7.5% DMEM. Cells were incubated an additional 24hr, after which time cells were fixed and coverslips were embedded for transmission electron microscopy. Embedded coverslips were released, fragmented, glued to Polybed812 bullets, sectioned at 70nm, and stained with uranyl acetate and lead citrate.

3.2.3.a. Quantification of Adherent Junctions and Apposed Membranes

To characterize and compare TEC morphological changes, cells were systematically scanned from low magnification (135 X) to high magnification (between 3,600 and 7,200 X). Fields of cells were visualized with TEM and photographed. The numbers of cells per field were counted, apposed membranes between cells traced and measured, desmosomes between cells counted, and length of desmosomes measured in electron micrographs which were analyzed (measured to scale) using MetaMorph cell analysis computer software (Universal Imaging Corporation, Nikon Instruments, Lewisville, TX). Data variables examined included number of cells per field, number of juxtaposed cell membranes,

juxtaposed cell membrane length, number of desmosomes, and length of desmosomes. Data was analyzed using chi squared analysis and student's T test.

3.3. RESULTS

TECs maintained in low calcium medium displayed different morphological characteristics from TECs maintained in high calcium medium, and these morphological characteristics could be induced by elevating medium calcium levels.

A minimum medium calcium concentration was required for the establishment and maintenance of TEC cultures. When culture conditions were modified from low to high calcium concentrations, thymic cell morphology was dramatically altered. In high calcium (0.188mM) medium, cultured cells were more densely populated, forming confluent monolayers lacking intercellular spacing, and were resistant to trypsinization (using 0.05% trypsin/EDTA). This morphological change from a partially differentiated spindle-like monolayer to a fully differentiated cobblestone-like monolayer was freely inducible in all thymic cell cultures maintained in WAJC media by increasing the media calcium content. Culture conditions were altered from low calcium media to high calcium media by incrementally increasing DMEM supplementation to assess morphological changes. (Figures 3.5. and 3.6.) Trypsinization at concentrations sufficient to lift

cells (0.05%) resulted in detachment of cells in sheets, and induced cellular damage (blebbing and cell lysis). Further, thymic cells maintained in high calcium media became quiescent and no longer exhibited logarithmic growth. Immortalized TECs established in low calcium media, once exposed to higher calcium levels, converted to a morphology similar to primary TECs grown in high calcium media. This differentiated state appeared to be permanent and irreversible, as returning cells to the low calcium culture condition after an 18 hour period in the high calcium condition failed to restore the cellular morphology and spacing observed in cells never exposed to high calcium culture conditions.

3.3.1. Light Microscopy: Morphological Analysis of Calcium-Induced Thymic Epithelial Cell Changes

As mentioned above, culture conditions were altered from low calcium media to high calcium media by incrementally increasing DMEM supplementation. Histological characteristics of TECs cultured at both low calcium and high calcium concentrations were evaluated by immunocytochemistry for keratin distribution and light and electron microscopy to determine the effects of media calcium levels on cellular structure and ultrastructure. These morphological analyses were also used to confirm that immortalized TECs retain the ability to fully differentiate following exposure to high calcium media.

3.3.1.a. Phase Contrast Morphological Analysis of Thymic Epithelial Cell Changes To Compare Effects of Differing Culture Conditions on TEC Morphology

High calcium GWAJC medium (7.5% DMEM supplemented) has a calcium concentration of 0.188 mM. To further investigate the effects of medium calcium levels on TEC morphology, medium calcium levels were incrementally increased from 0.098 mM to 0.280 mM in increments of 0.012 mM by adding DMEM. After 24 hours, cells were visualized using Hoffman phase contrast microscopy. As opposed to technology behind a normal phase contrast microscope, which allows visualization of cells using the interference between a central light path and a halo of light around a cell caused by an annular ring, Hoffman phase contrast microscopy employs technology that modulates the phase contrast light path with a slit lamp effect to produce a three-dimensional image of cells. The percentage of cells transformed from an appearance consistent with cellular proliferation (greater intercellular spacing, more three dimensional texture) to an appearance consistent with cellular differentiation and decreased proliferation (reduced intercellular spacing, more two dimensional texture) was recorded.

A three dimensional appearance was seen in TECs maintained in low calcium while a two dimensional appearance was seen in TECs maintained in high calcium, reflecting the flat morphology of cells grown in high calcium medium. (Figure 3.1. and 3.2.)

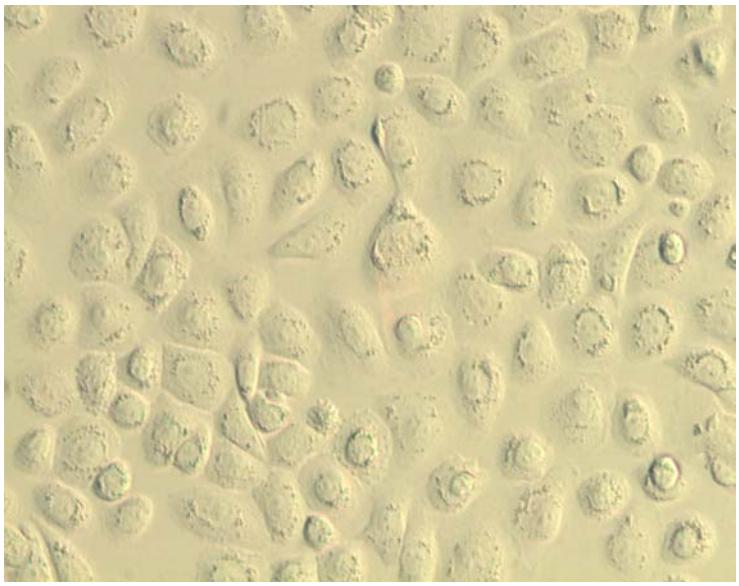


Figure 3.1. Hoffman Phase Microscopy of TECs Maintained in Low Calcium Medium. Cells have more intercellular spacing and a three dimensional appearance than those maintained in high calcium medium. 135 X.

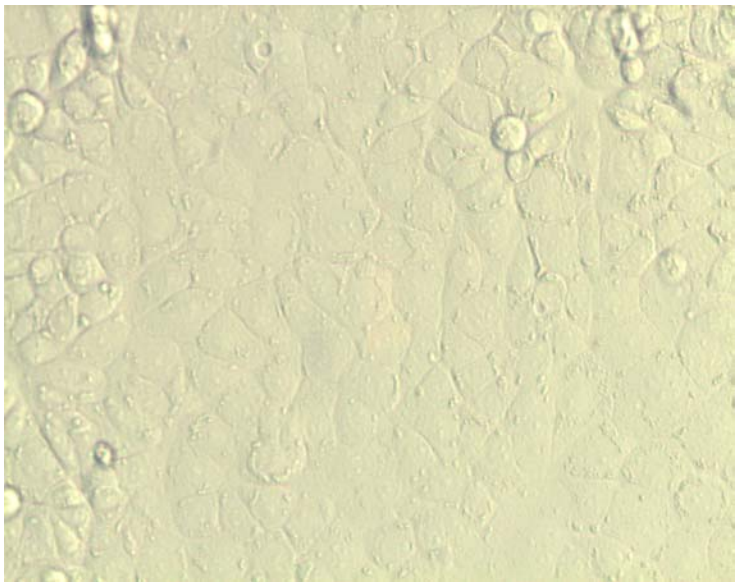
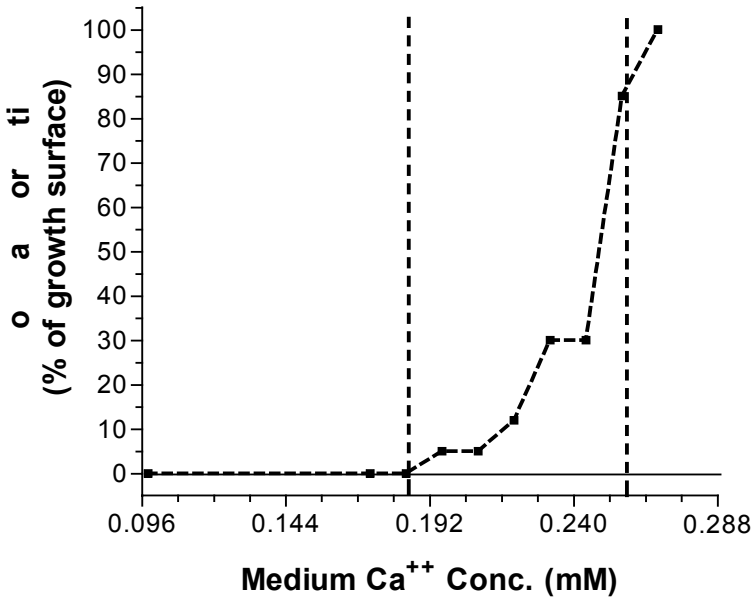


Figure 3.2. Hoffman Phase Microscopy of TECs Maintained in High Calcium Medium. Cells have decreased intercellular spacing and a 2 dimensional appearance, indicating their squamous, adherent nature. 135 X.

Incrementally increasing the medium calcium concentration resulted in an alteration of TECs from the proliferative morphology to the non-proliferative

morphology. These results correlate with the earlier immunocytochemical results demonstrating that the GWAJC medium containing a high calcium level (0.188 mM) induces a morphological change in TECs. (Table 3.1.)

Table 3.1. Effects of Medium Calcium Concentration on TEC Morphology. Areas of altered cells appeared when medium calcium concentrations were between 0.184 and 0.196 mM.



Calcium (mM)	% alteration
.098	0
.172	0
.184	0
.196	5
.208	5
.220	15
.232	30
.244	30
.256	85
.268	100
.280	100

3.3.1.b. Immunocytochemistry To Compare Effects of Differing Culture Conditions on TEC Morphology

Two populations of cells were employed in this investigation. TEA-3A1 cells (LDA TECs) were previously derived, and have been established as an immortal line of TECs [132] by Dr. Hayashi at the University of Maryland. Primary cultures of TECs (designated OKTE4-01) were derived in our laboratory using the

techniques described above. Both TEA-3A1 and OKTE4-01 cells were maintained in low calcium medium, as described above. For this study, culture conditions for some cells were altered from low calcium medium to high calcium medium by increasing the concentration of supplemented Dulbecco's Modified Eagle's Medium (DMEM) from 2.5% to 7.5%. (TECs did not thrive in DMEM concentrations below 2.5%.) Because DMEM was originally added to Ca++ free WAJC medium (GibcoBRL, Gaithersburg, MD) to introduce a minimal calcium level necessary for cell viability (creating low calcium WAJC404A), we used DMEM accordingly to further increase calcium levels to make high calcium WAJC404A. DMEM was chosen over serum or other supplements for increasing medium calcium levels because of the number of unknown components and sometimes intangible effects of serum on the cultured cells. These four cell populations (TEA-3A1, low Ca^{++}; TEA-3A1, high Ca^{++}; OKTE4-01, low Ca^{++}; OKTE4-01, high Ca^{++}) were evaluated by light and electron microscopy to determine the effects of medium calcium concentration on histological and ultrastructural characteristics. Quantitative analysis of macula adherens density was performed on the OKTE4-01 cells.

Immunocytochemistry was used to visualize the keratin IF distribution pattern of TECs exposed to low calcium WAJC medium (with 2.5% DMEM) (Figures 3.3. and 3.4.) and TECs exposed to high calcium WAJC medium (with 7.5% DMEM) (Figures 3.5. and 3.6.). Evaluation of stained cells demonstrated differences in

distribution of keratin IFs between the cells cultured in low vs. high calcium media.

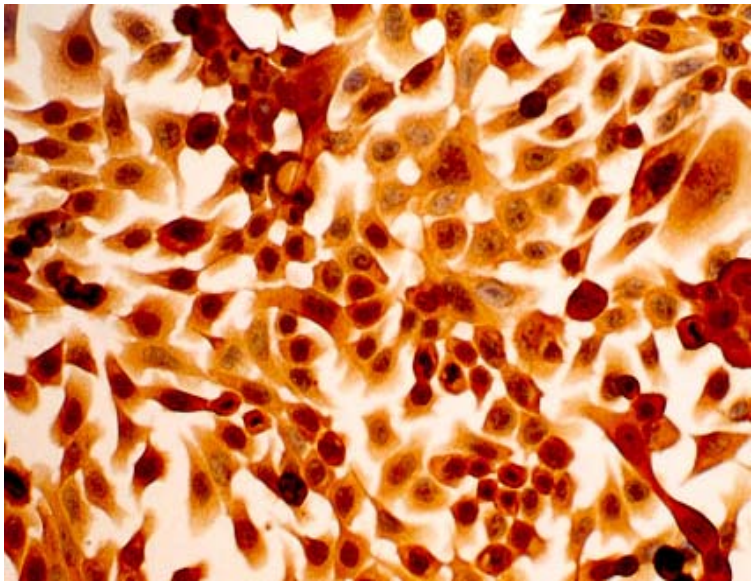


Figure 3.3. Low Magnification View of TECs in Low Calcium WAJC Medium. Immunocytochemical keratin staining demonstrated a diffuse distribution of keratin IFs with increased intercellular spacing and spindle-like morphology even at confluence. 135 X.

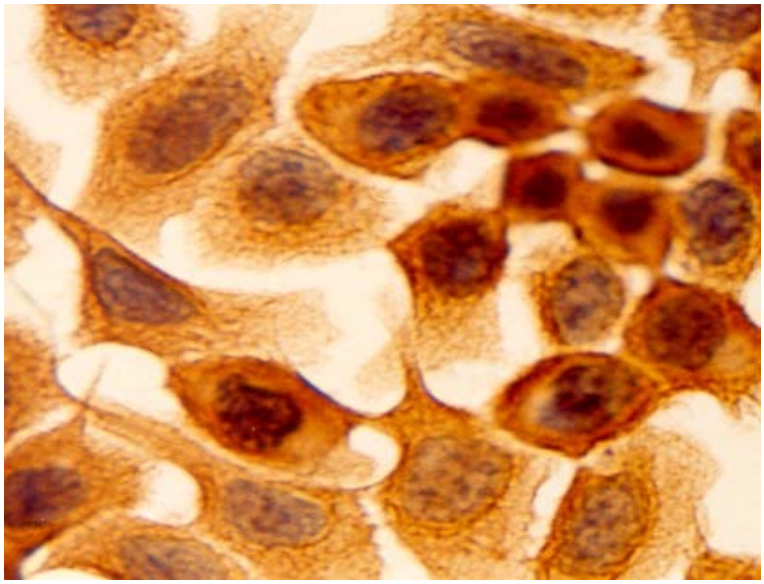


Figure 3.4. Higher Magnification View of TECs in Low Calcium WAJC Medium. Immunocytochemical keratin staining demonstrated keratin IFs arranged in a reticular pattern through the cytoplasm without visible condensation of filaments in any particular area. Spherical cells were common and in these a darker staining pattern was apparent. 640 X.

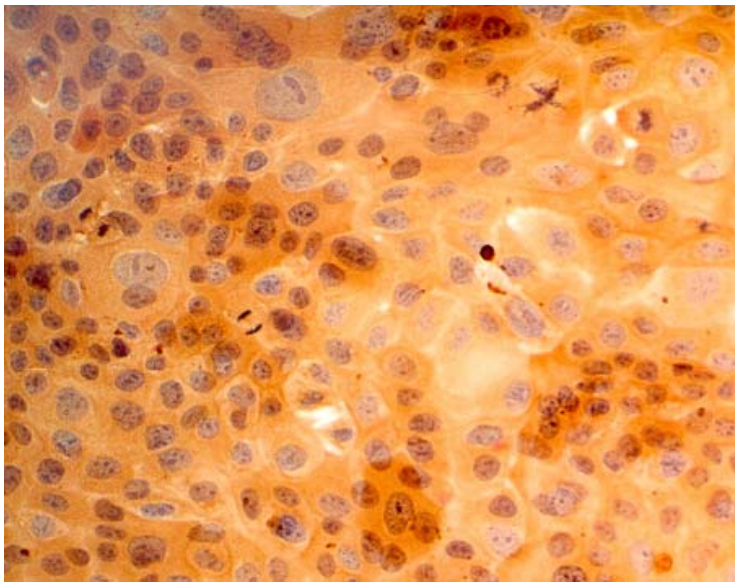


Figure 3.5. Low Magnification View of TECs in High Calcium WAJC Medium. Immunocytochemical keratin staining demonstrated decreased intercellular spacing with a cobblestoned appearance at confluence. 135 X.

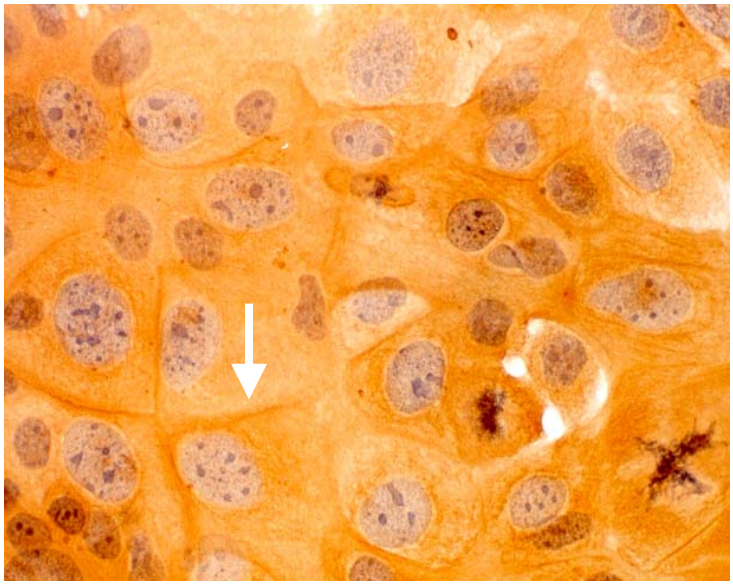


Figure 3.6. Higher Magnification View of TECs in High Calcium WAJC Medium. Immunocytochemical keratin staining demonstrated increased specific staining with linear profiles of keratin IFs evident near cell borders. (arrow) 640 X.

3.3.2. Transmission Electron Microscopy To Compare Effects of Differing Culture Conditions on TEC Morphology

Transmission electron microscopy was used to further characterize and compare the nature of TECs cultured in low calcium WAJC media, and TECs cultured in low calcium WAJC media and subsequently transferred to high calcium WAJC media (supplemented with 7.5% DMEM). Any steps in the EM processing protocol that might expose TECs maintained in low calcium to elevated calcium levels were performed following fixation to avoid inadvertent induction of morphological changes. The two cell populations, TECs exposed to high calcium and TECs exposed to low calcium, were analyzed by counting cell number, number and length of areas of apposed membranes (examples given in Figure 3.7.), number of desmosomes between cells, and length of each desmosome.

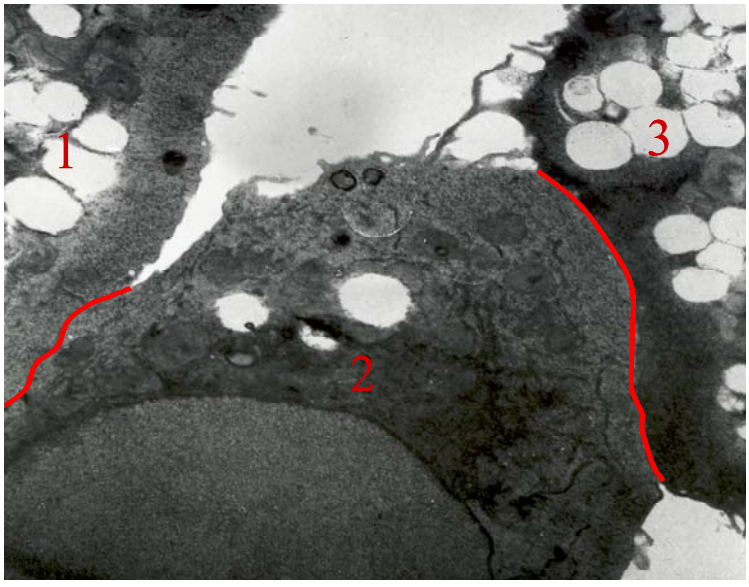


Figure 3.7. Transmission Electron Microscopy of TECs. Three cells (1-3) and apposed membranes (highlighted by drawn in lines) were counted in a typical field of TECs exposed to low calcium.

In total, examination of 87 distinct TEM fields identified 222 cells from high calcium cultures (total apposed membrane length of 1489.3μm in 153 discrete areas), and assessment of 48 distinct TEM fields identified 124 cells from low calcium cultures (total apposed membrane length of 627.3μm in 48 discrete areas).

3.3.2.a. Length of Apposed Membranes

Statistics were calculated for 87 fields of high calcium cells and 48 fields of low calcium cells. The average number of cells observed per field was not significantly different (2.55±0.10 cells/field, high calcium; 2.58±0.12 cells/field, low calcium; $p>0.05$); however the length of apposed membranes between cells differed significantly between the high and low calcium cultured TECs (6.59±0.49μm/cell, high calcium; 4.88±0.41μm/cell, low calcium; $p<0.02$). These results support the subjective finding that TECs maintained in low calcium medium appear to retain greater intercellular spacing than TECs maintained in high calcium medium. (Table 3.2.)

Table 3.2. Analysis of TECs for Juxtaposed Membrane Length. Number of cells per field and length of apposed membranes between cells. Analysis revealed no difference between cells per field but there was a significant difference between lengths of apposed membranes between the two cell populations.

	High Ca++	**Low Ca++**	**p***
n	87	48	
cells/field	2.55 ± 0.10	2.58 ± 0.12	NS
apposed membrane length μm	6.59±0.49	4.88 ± 0.41	p < 0.02

3.3.2.b. Number of Desmosomes

Morphological changes were characterized by a quantitative analysis of desmosome formation. In the 222 high calcium cultured cells, a total of 239 desmosomes were identified, while in the 124 low calcium cultured cells, only a single desmosome was observed in all of the juxtaposed cells evaluated. To assess desmosomal density (desmosomes/μm of apposed membrane), the number of desmosomes per micrometer of membrane length was calculated for each observed region of apposed membrane, and averaged with values derived from other regions. These extrapolations revealed one desmosome per 6.23μm of cell membrane (total apposed membrane length/total number of desmosomes) in TECs maintained in high calcium medium (GWAJC/7.5%DMEM/2%serum). For statistical evaluation, the number of desmosomes per unit membrane length (μm) was calculated for each region of apposed membrane examined and averaged to provide a mean value of desmosomal density biased by desmosomal distribution within the observed membrane regions. This calculation yielded a value of 0.263±0.035 desmosomes/μm of apposed membrane (one desmosome per 3.8μm) for TECs cultured in high calcium medium vs. 0.001±0.001 desmosomes/μm of apposed membrane (one desmosome per 690.7μm) for TECs cultured in low calcium medium. This represented a significant difference between the distribution-biased densities of desmosomes in TECs cultured in high vs. low calcium medium ($p<0.001$). (Table 3.3.)

Table 3.3. Analysis of Desmosome/Membrane Relationship Between TECs. TECs exposed to high calcium levels had a significant number of desmosomes while TECs exposed to low calcium levels had essentially no desmosomes.

	High Ca++	Low Ca++	p*
n	87	48	
cells/field	2.55 ± 0.10	2.58 ± 0.12	NS
desmosomes/μm membrane	2.63±0.35	.001 ± .001	p<0.001

3.3.2.c. Length of Desmosomes

Average desmosome length was approximately 416±19 nm for the high calcium TECs, and was 201 nm for the single desmosome observed in low calcium TECs. A valid statistical comparison of desmosome length between the cells grown in high calcium and low calcium was not feasible due to the small sample size (n=1) of desmosomes in the low calcium condition. Nevertheless, the single desmosome's 201 nm length was within size ranges documented for normal desmosomes. [220] (Table 3.4.)

Table 3.4. Analysis of Desmosome Length in TECs. There was no significant difference between the length of desmosomes in TECs exposed to high calcium levels and TECs exposed to low calcium levels.

	High Ca++	Low Ca++	p*
n	240	1	
desmosome length	0.416 + 0.019	0.201	NS

3.4. DISCUSSION

The differences in growth and proliferative characteristics of TECs isolated and maintained in medium with varying calcium concentrations appeared to be related to irreversible changes in their morphology. These relationships appeared to be especially well demonstrated by the steep rise in morphological alteration in the phase contrast microscopy study. (Table 3.1.). Also, the almost all-or-none phenomenon of desmosome formation as it related to medium calcium levels would indicate that desmosome formation is an important signature of decreased cellular proliferation in TECs. While hemidesmosome formation has been demonstrated within 5 minutes of exposure to elevated calcium levels in other cell types [221], the relationship of desmosome formation and TEC exposure to calcium is unique to this project.

CHAPTER 4.

IMMUNOMODULATION WITH THYMIC EPITHELIAL CELLS

4.1. INTRODUCTION

The introduction of "foreign" antigens into the thymus has been shown to reeducate the immune system by selectively restricting the T cell repertoire such that the "foreign" antigen may be considered "self." This finding has great implications for the potential therapeutic strategies in the prevention of organ graft rejection and in autoimmune diseases, particularly Type I diabetes. Although the mechanism by which IT inoculation of antigen or antigenic tissue has not been fully elucidated, numerous studies have demonstrated the efficacy of this technique in inducing specific unresponsiveness in a host, and have resulted in decreased numbers of target-specific effector T cells. [222] These studies also confirm a significant role for the thymus in immune maturation, antigen recognition, and induction of specific unresponsiveness (central tolerance). This has led to the development of the hypothesis that cell/tissue grafts within the thymus allow presentation of foreign antigens to immature host T cells, resulting in depletion of cells bearing TcR specific for the reeducation of the foreign antigen. The overall effect is reeducation of the host immune system leading to specific

unresponsiveness. Recent experimental work in an animal model has shown that preconditioning of a transplant recipient by IT injection of donor antigen can induce donor-specific unresponsiveness, eliminating the necessity for subsequent long-term immunosuppression. This pioneering work described the transplantation of allogeneic pancreatic islet tissue into the thymus of streptozotocin (STZ)-induced diabetic recipient rats, and reported the induction of unresponsiveness to a subsequent, donor-matched, extrathymic islet graft placed at the renal subcapsular site. [223] It was hypothesized that IT placement of allogeneic adult islets prevented the rejection of the subsequent islet graft, and the recurrence of the recipient's STZ-induced diabetes, due to tolerogenic presentation of the donor-specific MHC antigen within the host thymus.

Some studies have compared the effects of intrathymic inoculation to extrathymic inoculation. It has been confirmed that the IT site is specific for tolerance induction, whereas neither the renal subcapsular nor the intravenous route of inoculation can induce tolerance. [126, 224] Authors proposed that IT inoculation of cells exposes T cell precursors to B cell-specific autoantigen(s), resulting in clonally selective deletion.

In these studies, antilymphocyte serum (ALS) was used to transiently deplete peripheral T cells. The generalized immunosuppressive effect of ALS abates in 14 to 21 days and permits T cell repopulation through IT maturation of lymphocytes. It has been suggested that alloreactive maturing thymocyte clones

encountering their specific target molecule in the host thymus undergo central deletion while peripheral alloreactive lymphocytes are eliminated by ALS administration. It is proposed that IT inoculation with a large amount of donor-specific cells/antigen allows for a more complete central deletion, reducing the number of peripherally reactive lymphocytes while the effect of ALS is still present. In a skin model of allograft transplantation using a similar tolerance induction mechanism with bone marrow, 21 days passed before a challenge skin graft was placed and graft survival was prolonged from 8 days to 24 days with treatment. [128] Similarly, donor-specific unresponsiveness to murine cardiac allografts has been induced induced by IT administration of soluble alloantigens. [111] Allograft tissue presented intrathymically in this fashion is not recognized as foreign and rejection processes are avoided.

This central tolerance induction hypothesis has now been tested in a number of rodent models of allogeneic transplantation. Studies have been conducted using pancreatic islet [223, 225], renal [226], cardiac [227-229], skin [230], liver [231, 232], and small bowel [233] allotransplantation. Induction of specific unresponsiveness has been demonstrated following IT inoculation with whole cells, cell clusters (i.e. islets), or soluble cell proteins.

Several studies have shown that the IT inoculation of genetically mismatched donor BMCs has been shown to induce long-lasting allogeneic tolerance to subsequent grafts. [128, 234] Injection of donor-matched bone marrow cells into

each lobe of the thymus coadministered with a single intraperitoneal injection of 1 mL of ALS has been shown to induce tolerance to a variety of tissues [125-127], including the "gold standard of transplantation tolerance," skin. [128] (Table 4.1.)

Table 4.1. Bone Marrow Transplantation and IT Tolerance Induction. Donor-specific tolerance induction to allograft tissue induced by intrathymic bone marrow inoculation with single concomitant immunosuppression.

Year	Author	Intrathymic donor cells	Donor Tissue
1992	Posselt, et al.	BM + ALS	islets
1992	Odorico, et al.	BM + ALS	cardiac
1993	Matsuura, et al.	BM + ALS	cardiac
1993	Campos, et al.	BM + ALS	liver
1994	Hara, et al.	BM + ALS	cardiac
1995	Alfrey, et al.	BM + ALS	liver
1998	Li, et al.	BM + tacrolimus	lung
1999	Cober, et al.	BM + ALS	skin
2000	Wekerle, et al.	BM + anti-CD154 + anti-CTLA4 Ig	skin

Based upon the results of these previous studies, it was hypothesized that the IT administration of genetically mismatched TECs might result in specifically tailored immune modification. To assess this possibility, IT preparations of equivalent numbers of TECs or bone marrow cells (BMCs) were used as treatments prior to challenges with donor-specific tissue grafts. Animals received either IT TECs,

BMCs, or saline accompanied by an IP injection of either saline or ALS. After a designated *in situ* period, challenge grafts were removed and analyzed for significant differences in rejection responses using a graft rejection scoring system.

To establish a baseline to assess immune unresponsiveness in study animals, a series of renal subcapsular allografts to recipient rats, biopsied at sequential time points, was performed. This made possible a visual comparison of the histological changes between the various study groups. Those grafts in the treatment groups whose histological features appeared to resemble less mature stages of acute graft rejection in the control groups could be characterized as coming from recipients which experienced some level of immune modification.

4.2. MATERIALS AND METHODS

4.2.1. Animals

Both Lewis ($Rt1^{l}$) and DA rats ($Rt1^{a}$) were used as donors and recipients (DA to Lewis, Lewis to DA challenges) for transplantation studies. Donors were over 60 days old and recipients were males 4 to 6 weeks of age. For baseline graft rejection studies, 6 week old DA males received thyroid and pancreatic challenge grafts from adult male Lewis donors without concomitant immunosuppression.

Grafts were harvested at 2, 4, 6, 8, 10, 12, 14, and 21 days following transplantation.

The MHC mismatched TEC inoculum was limited to the spontaneously transformed LDA (F1 cross between Lewis and Dark Agouti, $Rt1^{a,l}$) cell line established in the first arm of the project. Therefore, for parallel studies using bone marrow instead of TECs, adult LDA rats were used as bone marrow donors. It was hypothesized that the $Rt1^{a}$ haplotype from IT inoculation of LDA ($Rt1^{l/a}$) TECs into Lewis ($Rt1^{l}$) recipients would induce donor-specific tolerance to DA ($Rt1^{a}$) allografts. Likewise, it was hypothesized that the $Rt1^{a}$ haplotype from IT inoculation of LDA ($Rt1^{l/a}$) TECs into DA ($Rt1^{a}$) recipients would induce donor-specific tolerance to Lewis ($Rt1^{l}$) allografts. Also, it was hypothesized that this low responder donor-recipient combination of Lewis to DA strains might have a less robust alloresponse than the high responder donor-recipient combination of DA to Lewis strains. [235] Fully allogeneic IT Lewis bone marrow with ALS was used as an overall positive control.

4.2.2. Intraperitoneal Injection of Antilymphocyte Serum

Sterile frozen 1 mL ALS (rabbit-anti-rat ALS, Accurate Chemical, Westbury, NY) aliquots were thawed at room temperature, aspirated into tuberculin syringes, and slowly injected intraperitoneally (IP) into 4 to 6 week old recipients approximately 4 to 5 hours prior to IT transplantation. [236] If a group of younger animals appeared

to experience a violent reaction to the injection, two separate 0.5 mL injections were administered approximately 15 minutes apart. [237, 238] Sham controls used IP saline as treatments.

4.2.3. Bone Marrow Harvest

Bone marrow donors were anesthetized with isofluorane via general inhalation and the lower extremities shaven and washed with 70% ethanol. An incision was made just over the hip joint and extended toward the knee. Sharp calipers were clamped through the tibial plateau to stabilize the proximal tibia. With downward traction on the leg, the acetabulum was disjointed and the femur was removed by sharp dissection of knee ligaments. Muscular tissue was cleaned from the femur and both ends were sharply removed with a sterile splicing instrument. A 12-gauge needle on a syringe of cold HBSS was used to flush bone marrow cells from the bone marrow cavity into a centrifuge tube. [239]

4.2.4. Intrathymic Transplantation

4.2.4.a. Cell Preparation and Concentration of TECs

As mentioned previously, each confluent T25 flask yielded 1 x 10^6 TECs. Cells were trypsinized and triple washed in Ca++/Mg++ free HBSS, and concentrated numbers were disbursed into microfuge tubes for centrifugation

(1500 RPM, 5 minutes) and volume adjustment to make 50 microliter aliquots containing 5 x 10^5 TECs per microfuge tube.

4.2.4.b. Cell Preparation and Concentration of BMCs

Bone marrow harvests were consolidated into one centrifuge tube and spun at 1500 RPM to form a pellet. The pellet was resuspended in 10 mL of sterile, endotoxin free water (Hyclone, Logan, UT) for 30 seconds and then centrifuged in 50 mL of HBSS; the supernatant was decanted. To gauge experiments, cell counts were evaluated by cytometric analysis. Following erythrocyte lysis, the number of cells derived from two rat femurs was reduced from between 1.3 and 1.5 x 10^6 to 7 x 10^5 cells. Pellets were resuspended in enough HBSS to make 50 μl aliquots containing 5 x 10^5 BMCs per tube.

The same number of TECs and BMCs were prepared for each recipient. Cellular inoculates were concentrated via centrifugation to 5 x 10^5 cells in 50 microliters of HBSS/1 mL microfuge tube for inoculation of each thymic lobe (2 tubes per recipient). [240] [241] [74] [234]

4.2.4.c. Intrathymic Inoculation Procedure

Donor cells (either BMCs or TECs) were prepared and concentrated as described. Recipient rats, aged 4 to 6 weeks, were anesthetized with isofluorane via inhalation technique and prepared for surgery. The neck and

upper thorax were shaven and washed with 70% ethanol, and the animal was placed supine with the neck extended by placement of a towel roll beneath the scapulae. Forceps were used to lift the skin overlying the trachea and a midline incision was created using straight scissors. Similar to documented techniques [242], sharp dissection techniques were used to retract the skin laterally, to expose the sternohyoid muscle overlying the trachea, and to incise the muscle. Rongeurs were used to remove the manubrium down to the first sternebra. The two halves of the sternohyoid muscle were further separated down to the cut end of the manubrium, revealing the cephalad portion of the thymus gland. A cotton-tipped applicator was used to further delineate the edges of the thymus gland. With a 27-gauge butterfly syringe, the cell suspension was gently aspirated and expelled to disperse any settled cells at the bottom of the microfuge tube. The 50 microliter solution containing cells for inoculation was finally aspirated into the syringe. Any liquid at the tip of the needle was removed and the solution carefully injected into one lobe of the thymus. As the needle was removed, a sterile cotton tipped applicator was used to cover the puncture in the thymus and absorb any leakage. The process was then repeated on the other lobe of the thymus. Grossly visible leakage was recorded as a technical difficulty and considered upon statistical analysis. The feasibility and accuracy of IT injection was initially tested by injecting ink into the thymus gland (Figure 4.16.) using the above described technique.

4.2.4.c.1. Intrathymic Inoculation of TECs

Primary cultured LDA ($Rt1^{l/a}$ hybrid) TECs were transplanted intrathymically into 4-6 week old Lewis males ($Rt1^{l}$). 5×10^5 cells were injected into each lobe of the thymus, resulting in a total of 1×10^6 IT TECs. Concomitantly, recipients received either IP saline (1mL, sham control) or anti-lymphocyte serum (1mL, rabbit anti-rat ALS, Accurate Chemical, Westbury, NY) for peripheral lymphocyte depletion.

Freeze-thawed TECs were prepared by exposing the cells to 4 freeze-thaw cycles between liquid nitrogen and a 65°C heating block to supply dead TECs (FT-TECs). (Figure 4.28.) Cell death was confirmed by a cellular replating and incubation technique in supplemented GWAJC medium at 37°C, 5% CO_2.

4.2.4.c.2. Intrathymic Inoculation of BMCs

LDA or Lewis BMCs were transplanted intrathymically into 4-6 week old DA or Lewis recipients. 5×10^5 cells were injected into each lobe of the thymus, resulting in a total of 1×10^6 IT BMCs.

4.2.5. Challenge Grafts—Harvest and Preparation

After a period following IT TEC or BMC inoculation with or without concomitant IP ALS, recipients received a challenge graft placed in the renal subcapsular

space, and the graft site carefully documented. (DA recipients received Lewis challenge grafts.) After an *in situ* period, the graft was recovered, fixed in Bouin's solution, prepared for routine paraffin histology (5μm sections, H&E staining) and analyzed histologically for graft rejection.

The harvesting techniques for pancreatic and thyroid tissue challenges were similar. A sterile Petri dish was filled with 25 mL of HBSS (GibcoBRL, Gaithersburg, MD) and placed over ice. Lewis, DA, or LDA rats of sexual maturity were used as the source of tissue. Animals were anesthetized with isoflurane via inhalation technique.

4.2.5.a. Challenge Grafts - Pancreas Harvest and Preparation

The abdominal area was shaven and washed with 70% alcohol. Using aseptic technique, blunt forceps were used to lift the skin of the right upper quadrant. Straight scissors were used to extend a skin incision across to the left upper abdomen. Blunt dissection was used to separate the skin from the underlying connective tissue, and the skin was retracted. The underlying muscular layer was lifted with forceps and incised from right to left in a similar fashion. The peritoneum was incised and separated using blunt dissecting techniques. The spleen was lifted with clean blunt forceps and clean scissors were used to dissect the tail of the pancreas from the spleen and the greater curvature of the stomach. A fragment of pancreatic tissue was sharply excised and placed in the

Hank's solution over ice. The animal was either prepared for thyroid tissue excision or sacrificed via carbon dioxide asphyxiation. The pancreatic tissue was cleaned of connective tissue, fat, and blood vessels under a dissecting stereomicroscope (Hitschfel SZX-ZB9, St. Louis, MO), and then dissected into 2 mm fragments for immediate transplantation to the renal subcapsule. The unused tissue was discarded.

4.2.5.b. Challenge Grafts - Thyroid Harvest and Preparation

The neck was shaven, and the surgical field was washed with 70% alcohol. Using aseptic technique, blunt forceps were used to lift the skin overlying the trachea. Straight scissors were used to extend a skin incision cephalad from the jugular notch cranially to the submandibular region. The scissors were spread to separate the skin from the underlying connective tissue, and the skin was carefully retracted. The connective tissue surrounding the each lobe of the thyroid gland was separated using a scissors opening technique and blunt forceps were used to stabilize the thyroid gland while a fresh pair of curved scissors was used to harvest a large fragment of tissue. The parathyroid glands and unwanted material were carefully dissected away and the cleaned thyroid tissue was minced to 2 mm fragments and placed in Hank's solution over ice for immediate transplantation to the renal subcapsule.

4.2.5.c. Placement of Challenge Grafts

Recipient animals were labeled and identified by metal numbered ear tags (National Brand & Tag Company, Newport, KY) to eliminate confusion or misidentification, and the recipient animal's ID number was recorded prior to beginning the procedure. The kidney subcapsule (Serie *et al* 1983, Hegre *et al* 1984) provided a convenient site for easy graft placement. The animal was anesthetized using ether or isofluorane and one or both flanks were shaven with an electric shaver. The area was washed with 70% ethanol, and the animal was placed in a lateral decubitus position.

The costal margin was located and used as an anatomical landmark. The skin was opened with scissors at a point slightly inferior to the costal margin. Blunt dissection was used to separate the adipose and loose connective tissue underlying the dermis from the underlying lateral muscular wall of the abdominal cavity prior to incision of the muscular wall. This layered dissection was used to minimize the development of adhesions. Irrigation was performed with HBSS.

The kidney was isolated and delivered out of the wound. A mosquito hemostat was applied to the fat pad associated with the renal capsule, stabilizing the kidney during placement of the graft tissue. A #11 blade scalpel was used to make a 1-2 mm incision in the renal capsule. One arm of a blunt forceps, such as a #5 eye-dressing forceps, was inserted and angled approximately

30 degrees both clockwise and counterclockwise to create a pocket for tissue placement, taking care not to injure the underlying renal cortex. The blunt forceps were used to hold the capsule open while other forceps were used to advance an appropriately sized intact tissue fragment under the capsule about 1 cm. Donor tissue fragments were prepared immediately prior to transplantation as described above and stored in cold HBSS over ice. The capsule was gently replaced, and the fragment manipulated through the capsule by gentle pressure to prevent the transplanted tissue fragment from slipping out of the subcapsular space. The mosquito hemostats for kidney stabilization were unclamped, and the kidney was returned to the body cavity. Sterile HBSS was used for wound irrigation and to replenish lost fluids. The wound was closed by first reapproximating the two edges of the muscle wall incision, and suturing with 4-0 Vicryl suture (Ethicon, Inc., Somerville, NJ) using simple running suture. Subsequently, the skin edges were brought together and stapled with sterile wound clips or sutured with nonabsorbable suture in a simple interrupted or uninterrupted fashion. Once the closure of the skin layers was complete, generic triple antibiotic ointment was topically applied.

The animal was returned to normal caging after recovery from anesthesia, where it was carefully monitored for complications during a one hour recovery period. All procedures were documented, and any irregularities noted.

4.2.6. Excision and Processing of Grafts

4.2.6.a. Macroscopic Evaluation of Grafted Tissue

At harvest, transplanted tissue was examined macroscopically as part of the protocol to assess the host's immune response. The graft was evaluated in terms of general appearance, visible necrosis, neovascularity, purulence, coagulation, and scarring. The overall assessment was rated as either accepted, or partially to completely rejected. Macroscopic evaluation was included for correlation with subsequent histological evaluation, but had no bearing on the actual immune scores assessed by histologic evaluation.

4.2.6.b. Renal Subcapsular Graft Excision

Animals were placed under general anesthesia, the surgical site shaven and prepped with ethanol, and the skin incised at the same location as the original incision. Blunt and sharp dissection were used to open the layers of the wound, separating subcutaneous fat and connective tissue from the underlying muscular layer, and noting any excessive adhesion formation, hematoma, or abscess. The previous incision was reopened with scissors, mosquito hemostats were applied, and the kidney was delivered through the wound and stabilized. The graft was located based on previous documentation, and macroscopically evaluated as described above. After recording macroscopic observations, the graft area was excised using sharp curved scissors to fashion an oval shaped wedge including

3 mm of kidney cortex with the overlying graft. An alternative to the scissors technique was the use of a #11 scalpel blade (Becton Dickinson and Co., Lincoln Park, NJ) to incise the kidney cortex circumferentially around the graft site, although the renal capsule tended to slip and distort the graft placement with this technique. The specimen was retrieved with blunt forceps, rinsed in saline solution, and placed in Bouin's fixative solution.

4.2.6.c. Fixation, Embedding, Sectioning and Mounting

Harvested graft tissue was bathed in 5 mL Bouin's fixative solution for approximately 18 to 24 hours. The Bouin's solution was then decanted off and the tissue was immersed in approximately 25 mL of 70% ethanol. Ethanol was replaced daily for 4 to 5 days to ensure more complete clearing of Bouin's solution from the tissue. Porous paper embedding bags were labeled appropriately, and samples were placed within each bag. Samples were then rinsed twice more, 3 minutes each time, in fresh 70% ethanol, and air bubbles were eliminated by piercing the bags with a needle. A silk tie was stapled to the bags which were then hung into a fleaker of fresh 70% ethanol, with continuous agitation of the solution by a magnetic stir bar for one hour. Bathing solutions were replaced hourly thereafter with 2 changes of 95% ethanol and 3 changes of 100% ethanol, resulting in dehydration of the tissue sample. The sample was placed in toluene (3 changes, each an hour long) until tissue translucence was confirmed with a flashlight.

Paraffin (Surgipath, Richmond, IL) embedding was accomplished with the aid of a Sakura Finetech Tissue Tek II tissue processing station. Tissue samples were immersed in three successive paraffin baths, followed by placement of the tissue within a mold filled with molten paraffin with the aid of a Leica EG1160 histological embedding station. The paraffin block was allowed to cool and separated from the mold. The block was placed in a Leica RM2155 microtome and sectioned rapidly until the tissue was encountered. Chemically etched single frosted glass slides (Anapath, Statlab, Lewisville, TX) were labeled with pencil or etched with a diamond pen using a numbering system corresponding to treatment groups. Serial tissue sections of 5μm thickness were obtained and mounted to the labeled slides using a water bath floatation technique. The entire tissue sample was serially sectioned to ensure that the entire graft could be evaluated. Blocks were stored for later sectioning and referral.

4.2.6.d. Staining for Histological Examination

Harris' Hematoxylin was prepared as follows. One hundred grams of aluminum potassium sulfate (Sigma, St. Louis, MO) were dissolved in a 2 liter flask containing 1 liter of water. In a 250 mL beaker, 25 mL propylene glycol, 25 mL 70% ETOH, and 5 gm Hematoxylin (Kodak, C.I. No. 75290, Rochester, NY) were combined and stirred at 50-60°C until dissolved. The two formulations were combined at room temperature. In place of mercuric oxide, 0.5 gm potassium iodate was added.

For deparaffinization and staining, slides with sections were baked at 60° C and washed twice in xylene to clear them of paraffin, and then rehydrated in graded ethanol solutions (two washes each of 100% ethanol; then 95% ethanol; then 85% ethanol).

Rehydrated slides were rinsed in tap water and stained for 15 minutes in filtered Harris' Hematoxylin. This was followed by a 5 minute water rinse, differentiation in acid alcohol, and refining of stain color in ammonia water. After a final water rinse of 10 minutes, slides were dehydrated to 85% ETOH and counterstained to the desired intensity with Eosin (Polysciences, Warrington, PA) which had been prepared within a week by mixing equal stock solutions of 0.178% eosin Y (C.I. No. 45380) and 0.026% phloxine B (C.I. No. 45410), each dissolved in 83.3% ETOH. The working stain was acidified with 0.7 mL glacial acetic acid (EM Science, Cherry Hill, NJ) per 150 mL of the mixture. The dehydration process was continued on stained slides through 2 changes each of 95% and 100% ethanol and back through 2 washes with xylene before coverslipping with Permount mounting medium.

4.2.7. Histological Examination and Immune Scoring of Grafts

Grafts were evaluated using a graft rejection scoring system reflecting: (1) allograft viability based upon morphological integrity of the graft, and (2) a graft rejection scoring system based upon the mononuclear cell infiltration into the

region of the graft. Scores ranged from 0 to 4, where 0 signified the least altered graft viability or the lowest number of mononuclear cells infiltrating the graft; and 4 signified the most significant reduction in graft viability to the formation of a scar and the most significant mononuclear cell destruction of the graft. (Figure 4.1.) The graft scoring system used in the histological analysis of the challenge tissue and surrounding graft site has been used in other transplantation study protocols, and makes an objective measure of the immunologic response feasible. (Tables 4.2. and 4.3.) As lymphocytic infiltration occurs in focused concentrations, accurate cell counts are difficult to obtain based upon 5 μm section samplings through grafts measuring between 1 and 2 mm. The quantification and sizing of lymphocytic foci, along with the application of the graft grading system, was therefore used to assign graft scores above zero. Graft fibrosis (Figure 4.2.), necrosis (Figure 4.3.), polymorphonuclear cell infiltration (Figure 4.4.), macrophage infiltration (Figure 4.5.), and vascularity were also evaluated.

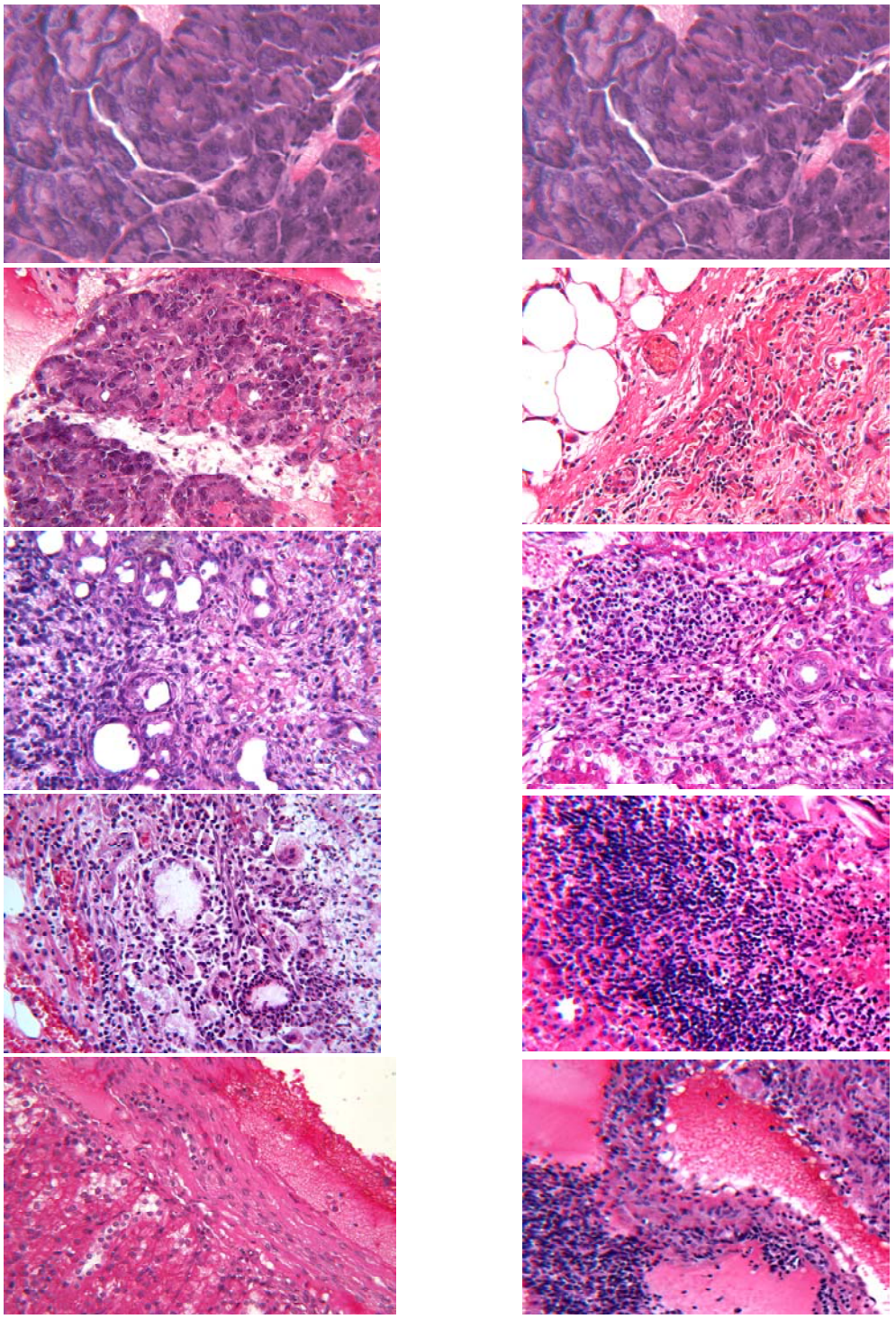


Figure 4.1. Graft Rejection and Lymphocytic Infiltration Scoring Systems.
Overall graft rejection (left column) is scored 0, 1, 2, 3, and 4 from top to bottom; graft lymphocytic infiltration (right column) is scored 0, 1, 2, 3, and 4 from top to bottom. Score descriptions can be found in Tables 4.2. and 4.3. Each image = 100 X.

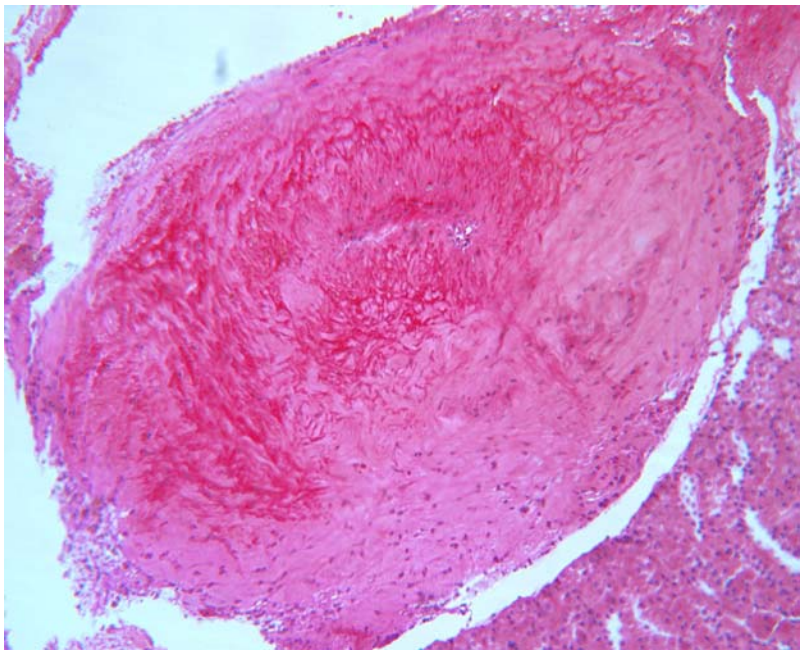


Figure 4.2. Graft Fibrosis. Eosinophilic collagen fibers replaced viable graft tissue as the rejection process ensued 90 X.

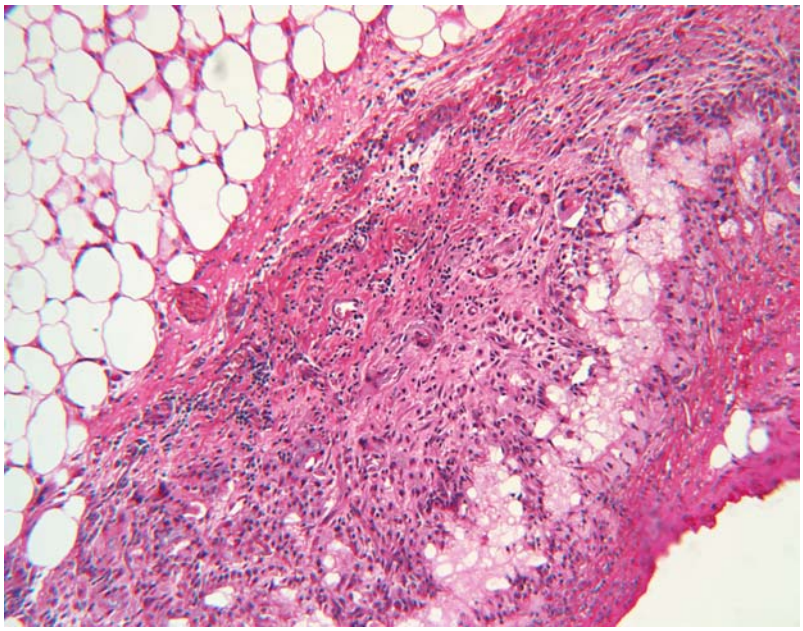


Figure 4.3. Graft Necrosis. Involution of graft structure exceeded the level of lymphocytic infiltration expected to cause this extent of graft destruction. 100 X.

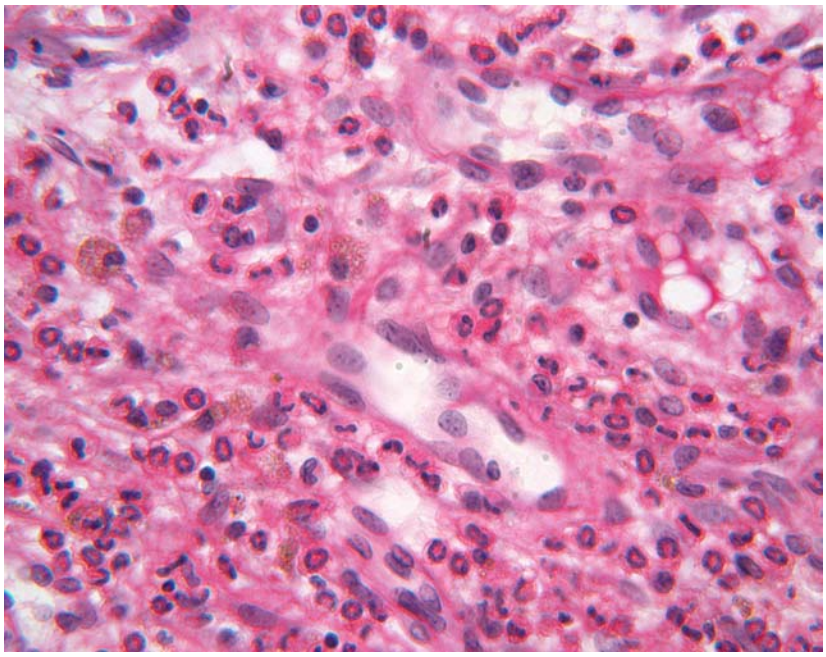


Figure 4.4. Graft Neutrophilic Infiltration. Neutrophilic bands are concentrated between nucleated cells in acute stages of graft inflammation. 135 X.

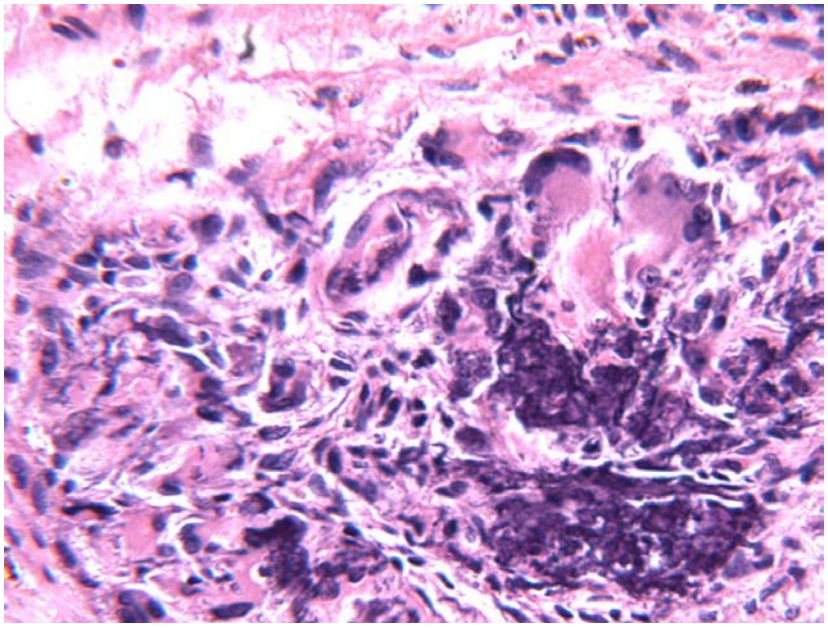


Figure 4.5. Graft Macrophage Infiltration. Macrophages and multinucleated giant cells phagocytose graft tissue remnants. 240 X.

Grafts sometimes contained foreign bodies including fur. (Figure 4.6.) When fur was seen, it was usually associated with a histological graft appearance consistent with late stages of graft rejection, suggesting that its presence heightened the immune response by elevating proinflammatory cytokine levels. This is not an unexpected finding, as rejected third party grafts can also induce rejection of previously accepted donor-matched allografts by localized alteration of cytokine milieu. [243]

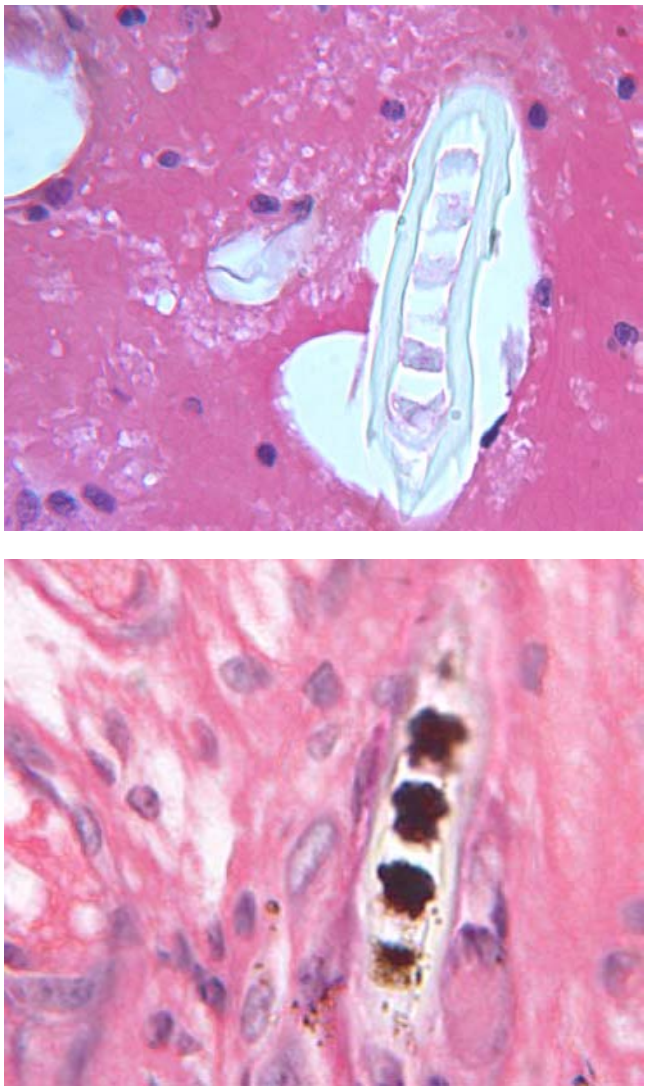


Figure 4.6. Graft Foreign Bodies. White fur from a Lewis rat (top) and brown fur from a DA rat (bottom) are seen within challenge grafts. 240 X.

Lymphocytic infiltration and graft viability (preservation of histological architecture) were evaluated, as described above, using separate grading systems based on criteria for allograft evaluation developed by Hegre and Ketchum. (Hegre *et al* 1984) (Figure 4.1.)

Grafts of pancreatic and thyroid fragments were histologically examined and assigned graft scores based upon lymphocytic infiltration of the graft, and relative immune-related destruction of graft tissue by the following sets of criteria:

Table 4.2. Lymphocytic Infiltration Scoring System. Scores of 0 to 4 were assigned based upon presence and size of lymphocytic foci.

Lymphocytic infiltration Score	Description
0	Graft site free of lymphocytic proliferation, allowing for a few scattered lymphocytes.
1	Graft site with one or more small distinct lymphocytic infiltrates containing between 10 and 30 cells.
2	Graft site with one or more distinct lymphocytic infiltrates containing 40 or more cells.
3	Graft site with several large lymphocytic infiltrates with some overlap or extensive infiltration throughout.
4	Graft without almost complete, indistinct lymphocytic infiltration obscuring normal graft tissue.

Table 4.3. Allograft Viability Scoring System. Scores of 0 to 4 were assigned based upon histological architecture.

Allograft Viability Score	Description
0	Viable graft, no evidence of immune involvement
1	Viable graft, structurally intact tissue with limited mononuclear infiltrate
2	Immune response, with a largely intact graft with evidence of mononuclear infiltrate and minimal evidence of graft destruction
3	Immune response, with recognizable tissue with significant mononuclear infiltrate and evident significant destruction of graft tissue
4	Rejection, remnant scar tissue only, with complete resolution of mononuclear infiltrate

4.2.7.a. Statistical Analysis

Fisher's Exact Probability Test was initially used to identify statistically significant overall differences in outcome among smaller treatment groups. The chi squared test was also used to determine the statistical significance of the qualitative preliminary data between preliminary treatment groups. The student's T test was then used to evaluate differences between the graft scores of individual treatment groups, with a one-way ANOVA for comparisons between numbers of groups. [244] Graphpad Prism 4 and GraphPad InStat (GraphPad Software, San Diego, CA) computer software programs were used for statistical outputs.

4.3. RESULTS

4.3.1. The Natural History of Allograft Rejection: Temporal Studies in the Course of Immune Rejection

As described in Chapter 1, the immunologic response to graft rejection differs from the natural response to graft placement, i.e. inflammatory response associated with unavoidable surgical trauma, such as in the case of an autograft. These graft rejection events are dependent upon factors such as the extent of the surgical wound, immunocompetence of the recipient, histocompatibility

between the donor and recipient, cellular composition of the graft, antibody formation following engraftment, and immunosuppressive regimens.

Almost no immune response to allogeneic transplantation occurs 2 days following graft placement. (Figure 4.7.) There is retention of pancreatic acinar structure and minimal lymphocytic infiltration.

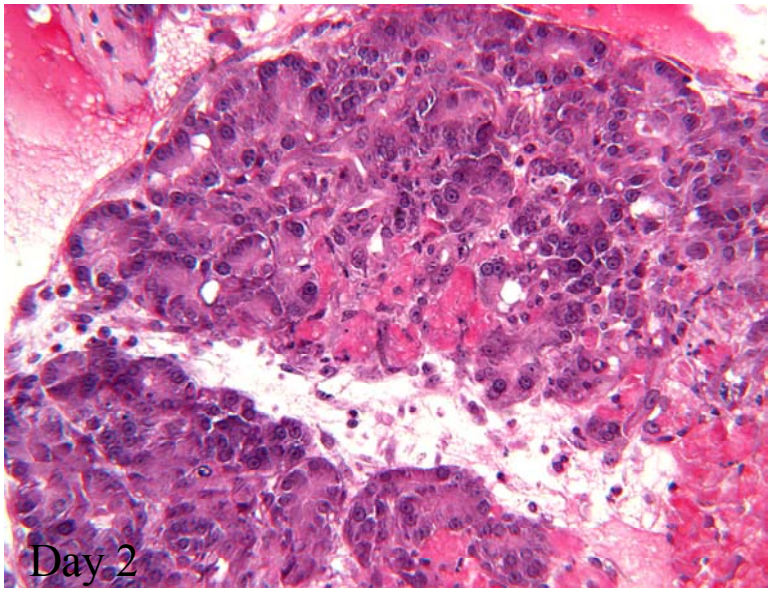


Figure 4.7. Pancreatic Graft 2 Days Following Allogeneic Transplantation.
Tissue demonstrates normal pancreatic acinar structure and a minimal number of infiltrating mononuclear cells. 120 X.

By Day 4 following allogeneic transplantation, there is a significant change in graft morphology with loss of basophilic staining pancreatic acini. (Figure 4.8.) Ductal elements are still supported by an intact, eosinophilic staining basal lamina, making it still evident that this is glandular tissue. Compared to the day 2 graft with minimal lymphocytic infiltration, there is some mild perivascular

lymphocytic infiltration. There is no evidence of scarring or deposition of fibrotic tissue.

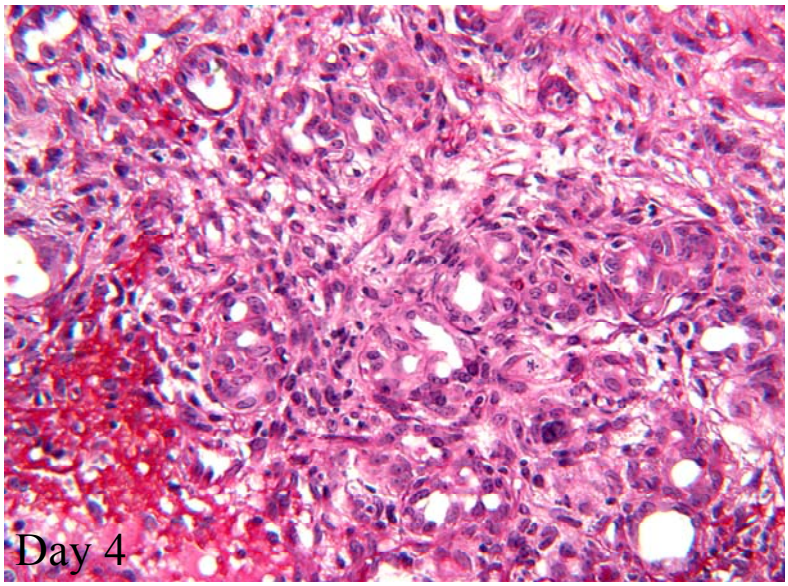


Figure 4.8. Pancreatic Graft 4 days Following Allogeneic Transplantation. Normal acinar appearance is lost but ductal elements remain supported by an eosinophilic basal lamina. Mild mononuclear cell infiltration. 120 X.

Similar findings to the 4 day pancreatic graft are seen in this thyroid graft at 7 days. (Figure 4.9.) Follicular elements are seen, and there is increased lymphocytic infiltration compared to the 2 day graft. Loss of tissue density between the follicular elements and replacement with lightly eosinophilic fibers signify early fibrotic changes.

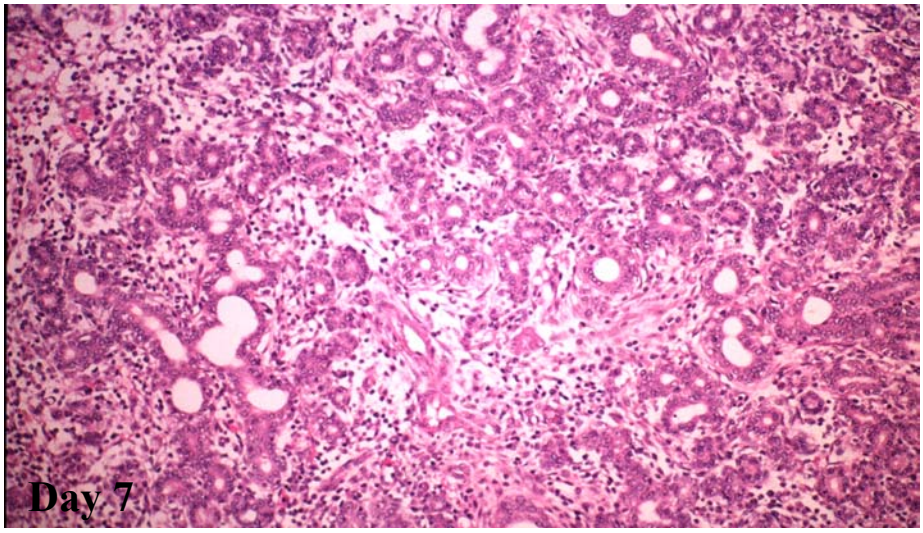


Figure 4.9. Thyroid Graft 7 Days Following Allogeneic Transplantation. Follicular elements of thyroid glandular tissue remain while lymphocytic infiltration ensues along fibrotic areas of the graft. 115 X.

By day 10 following allotransplantation, pancreatic ductal elements are losing structural integrity, suggested by loss of the smooth appearance to the ductal lumen. (Figure 4.10.) This may be due to lack of support from the disintegrating basal lamina. There is increased lymphocytic infiltration around the ductal elements compared to the 4 and 7 day grafts, occupying loose fibrotic areas.

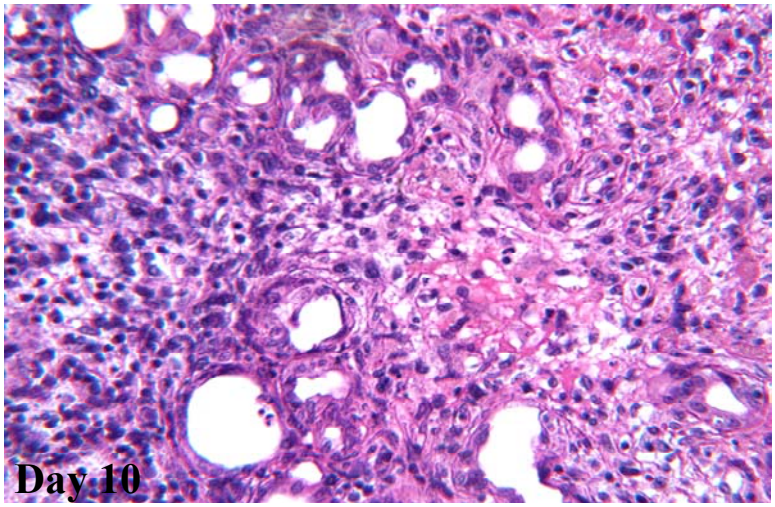


Figure 4.10. Pancreatic Graft 10 Days Following Allogeneic Transplantation. Ductal lumina appear less well preserved and lymphocytic infiltration continues to destroy the graft. 125 X.

Tissue destruction continues until basal laminae are no longer evident after 12 days. (Figure 4.11.) Lymphocytes and macrophages predominate. Neovascularization is also noted. This tissue is no longer identifiable as glandular in structure. Much of the tissue has been replaced by lightly eosinophilic staining collagen fibers.

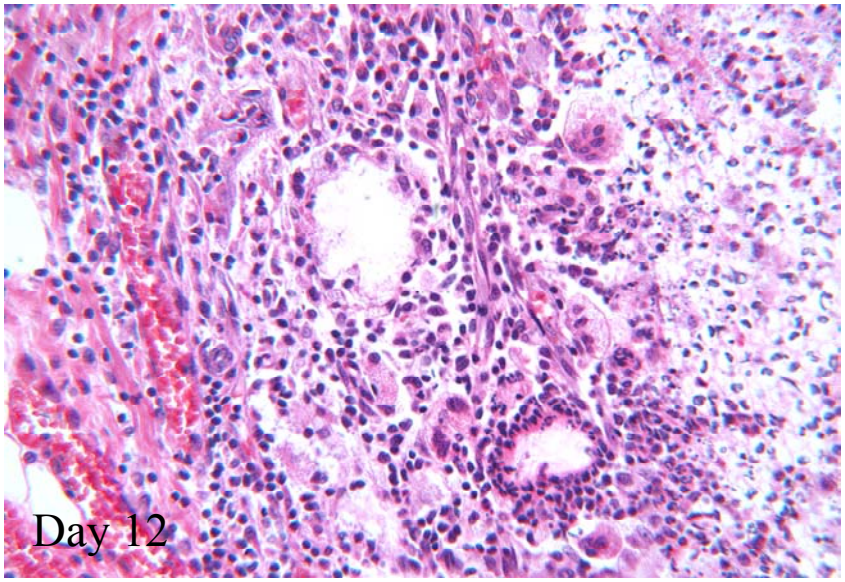


Figure 4.11. Pancreatic Graft 12 days Following Allogeneic Transplantation. Ductal elements are replaced by eosinophilic collagen fibers while lymphocytic infiltration destroys graft viability. 125 X.

At 14 days, the majority of the graft takes on a lightly eosinophilic, fibrotic appearance but there is still a moderate lymphocytic infiltrate. (4.12.)

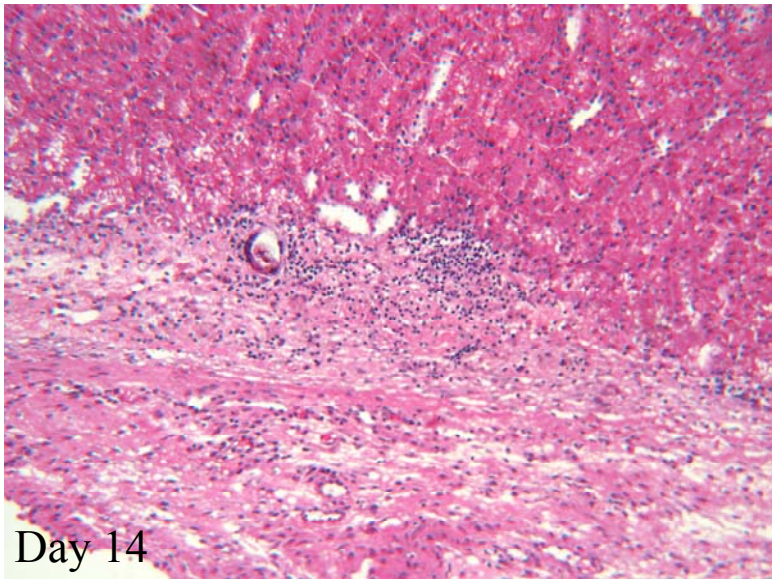


Figure 4.12. Pancreatic Graft 14 Days Following Allogeneic Transplantation. A few lymphocytic foci remain at the junction between the graft and the renal Subcapsule. Eosinophilic collagen fibers account for the majority of the graft. 100 X.

Finally, by day 21, all that remains of the graft is a scar devoid of nuclei. (Figure 4.13.) Paucity of lymphocytes in the area indicate that the immunologic process of rejection has resolved.

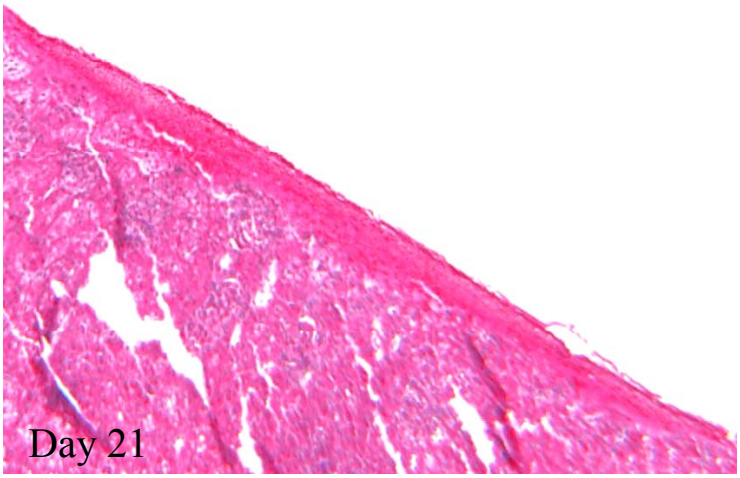


Figure 4.13. Pancreatic Graft 21 Days Following Allogeneic Transplantation. A scar of eosinophilic collagen is all that remains of the tissue graft which overlies normal kidney tissue. Lymphocytic infiltration has resolved. 100 X.

Graft score categories of overall graft rejection and lymphocytic infiltration were plotted, and this reflected the expected natural history of the immunologic response to allotransplantation. (Figure 4.14.)

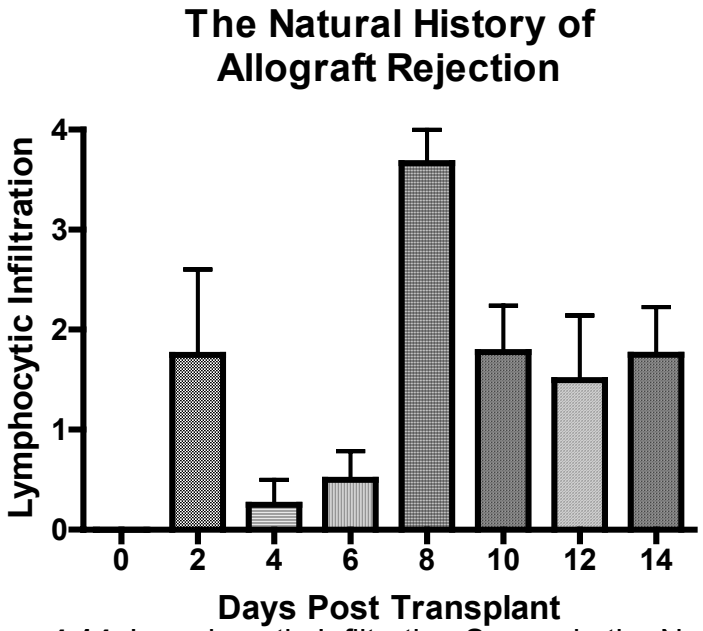


Figure 4.14. Lymphocytic Infiltration Scores in the Natural History of Graft Rejection. Lymphocytic infiltration was elevated 2 days post transplant, and peaked at 8 days post transplant.

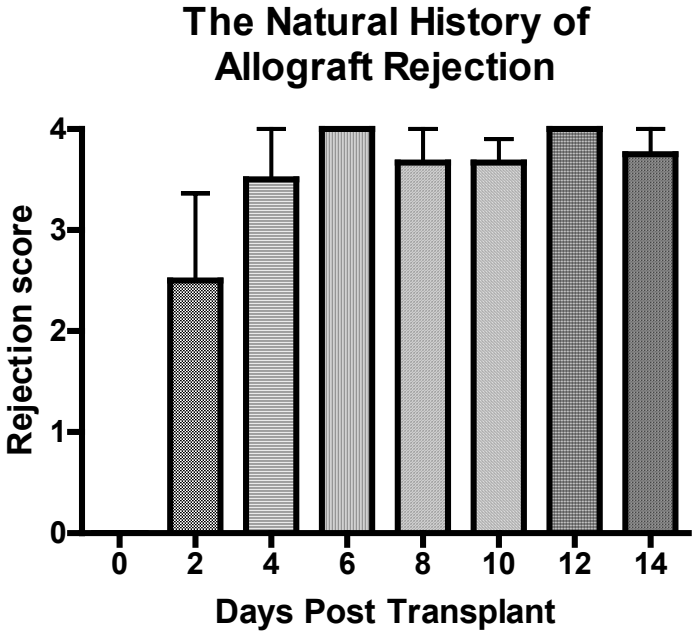


Figure 4.15. Graft Rejection Scores in the Natural History of Graft Rejection. Graft rejection rose and became relatively steady past day 4.

Rejection scores were similar from days 4 through 14, and therefore not as useful for comparing graft scores in treatment groups. Averages of combined rejection and lymphocytic infiltration scores were not as physiologically relevant but fit a more bell shaped distribution (not shown) with a peak between 8 and 10 days. The findings in the natural history of graft rejection helped to outline the time frames chosen for challenge graft harvesting to determine a treatment-related shift in graft immune response from the normal response.

4.3.2. Allogeneic Transplantation from DA to Lewis Rats

As mentioned in the Materials and Methods, 4 to 6 week old recipients received a total IT inoculation of 1 x 10^6 LDA BMCs or TECs, or 50 μL saline, with a concomitant intraperitoneal injection of 1 cc ALS or saline.

Successful IT injection with retention of inoculum was easily established and documented using India ink, as described in p. 142.

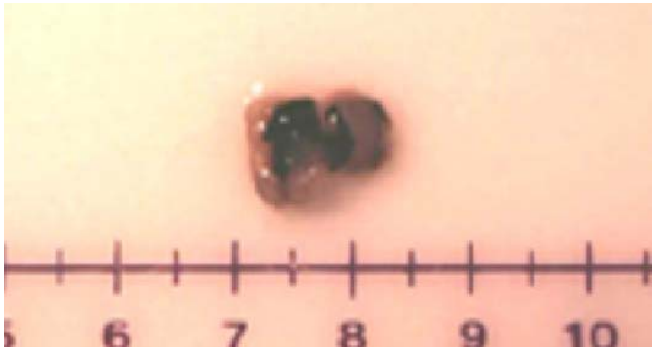


Figure 4.16. Adolescent Rat Thymus Following India Ink Inoculation. The intrathymic injection technique was tested for accuracy using an ink injection.

After a treatment to challenge period of 10 days, DA challenge grafts of thyroid and pancreatic tissue fragments were placed in the renal subcapsule. DA Grafts were harvested for processing and analysis after 21 days *in situ*. Any technical difficulties were recorded. This was referred to as the "10/21" time frame.

To determine whether the *in situ* period had an effect on graft recovery studies, the treatment to challenge frame was extended to 21 days and the *in situ* challenge time was reduced to 10 days. This was referred to as the "21/10" time frame.

To control for sensitization, syngeneic Lewis graft responses were evaluated following IT TEC and IP ALS or saline on the 21/10 time frame.

The DA to Lewis transplantation experiments are summarized in Table 4.4.

Table 4.4. DA to Lewis Challenge Grafts Following Varied IT Treatments. Lewis recipients received either IP ALS or saline and either IT cells or saline for tolerance induction studies.

Group	Donor	Recipient	IT inoculum	ALS	Challenge (Days post Rx)	Harvest (days post-challenge)
1	DA	Lew	saline	N	10	21
2	DA	Lew	saline	Y	10	21
3	DA	Lew	LDA BMC	Y	10	21
4	DA	Lew	LDA BMC	N	10	21
5	DA	Lew	LDA TEC	Y	10	21
6	DA	Lew	LDA TEC	N	10	21
7	DA	Lew	LDA TEC	Y	21	10

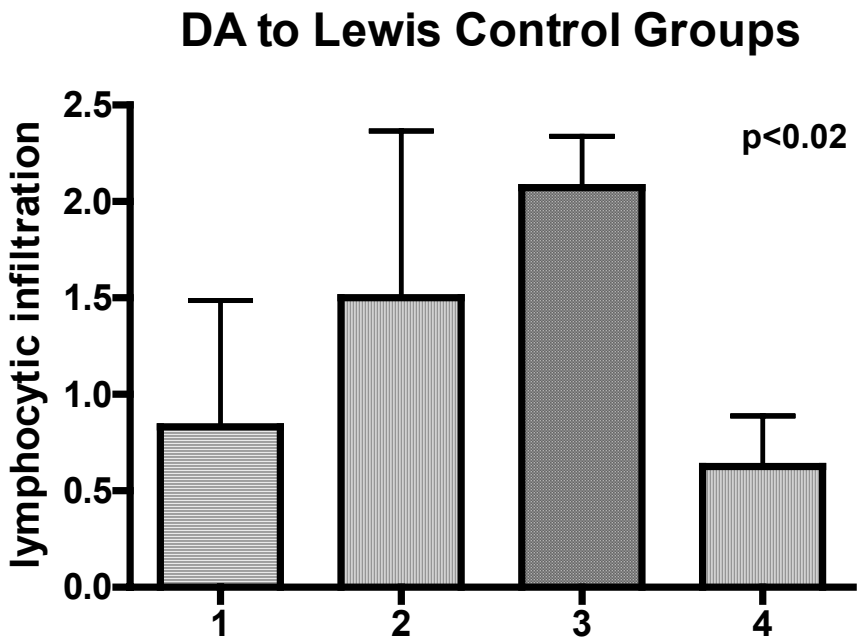


Figure 4.17. DA to Lewis Control Groups. There was a statistically significant elevation (ANOVA, $p<0.02$) in lymphocytic infiltration in the ALS/ LDA BMC (positive control, group 3) compared to negative controls (groups 1, 2 and 4). There were no statistically significant differences among the negative control groups (1, 2 and 4).

A one-way ANOVA of the control groups revealed significant overall differences between the groups with a $p<0.02$. A Dunn's multiple comparison post-test revealed no significant differences between the negative control groups, indicating that ALS alone or BMCs alone did not exert statistically significant immunomodulatory effects compared to saline. There were significant differences ($p<0.05$) between group 3 (positive control, ALS + BMCs, mean lymphocytic infiltration 2.1 ± 0.27, $n=13$) and negative controls, including group 1 (saline + saline, mean lymphocytic infiltration 0.83 ± 0.65, $n=6$, $p<0.05$) and group 4 (saline + BMCs, mean lymphocytic infiltration 0.63 ± 0.26, $n=8$, $p=.002$). The immunological effects of intrathymic LDA BMCs (group 4) were significantly enhanced by the addition of IP ALS to the BMC protocol. Of interest, there was

no significant difference between group 3 and group 2 (ALS + saline, mean lymphocytic infiltration 1.5±0.87, n=4, p NS) in a 10/21 time frame. The ALS only group was also used to control for the effects of IT BMCs. The brief IT reeducation period and lingering effects of ALS during the *in situ* challenge period might have been the cause of these results, and the impetus behind the protocol switch to the 21/10 time frame.

The saline negative control and the IP ALS/IT LDA BMC positive control were used as direct comparisons to the treatment group of IT LDA TECs with concomitant IP ALS. (Figure 4.18) As mentioned previously, the period from ALS administration and IT inoculation to challenge graft placement was either 21 or 10 days, and the *in situ* graft period was either 10 or 21 days, respectively. These time periods were evaluated for their significance and to highlight any outcomes between the treatment groups as related to the period of thymic reeducation.

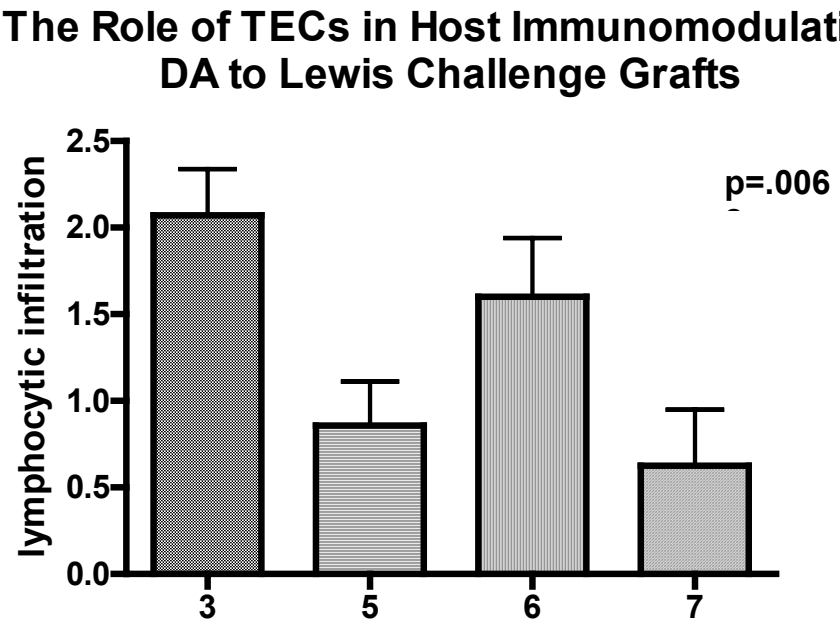


Figure 4.18. DA to Lewis Experimental Groups. The degree of immunomodulation was significantly higher (ANOVA, p=0.006) in the positive control group (3, ALS+BMCs) compared to the IP ALS/IT TEC treatment groups (5 and 7). Group 6 (saline+TECs) was associated with immunomodulation not significantly different from group 3, suggesting a physiological role for TECs.

A one-way ANOVA including group 3 (positive control) and experimental TEC treatment groups revealed statistically significant differences between the groups (p=.006). A Dunn's multiple comparison post-test revealed significantly higher mean graft lymphocytic infiltration scores between group 3 (ALS+BMCs) and the treatment groups 5 (ALS+TECs, 10/21 time frame, mean lymphocytic infiltration 0.86±0.25, n=12, $p<0.05$) and 7 (ALS+TECs, 21/10 time frame, mean lymphocytic infiltration 0.63±0.32, n=4, $p<0.05$). There were no significant differences among any of the TEC treatment groups despite differences in time frames, and despite the addition of ALS to the TEC treatment protocol. This was different from the effect of adding ALS to the BMC treatment protocol, which resulted in a synergistic immunomodulatory effect, even though the ALS came from the same source. Of interest, there was not a statistically significant

difference between group 3 and group 6 (saline+TECs, mean lymphocytic infiltration 1.6+0.34, n=17, $p<0.05$), suggesting that IT TECs have significant immunomodulatory effects when administered without ALS. However, group 6 was not significantly different from group 1 (saline control) in host immunomodulation (not shown, p NS). This was confirmed by a t-test between the two groups. Therefore, the potential clinical significance of IT TEC inoculation remains limited due to the lack of statistical significance when comparing the TEC group with the saline negative control.

Overall, when compared directly, it appeared that TECs alone were as effective as BMCs with concurrent ALS administration, suggesting a physiologic role for IT injected TECs. The determine the safety of IT TEC inoculation, syngeneic Lewis recipients were treated with IT LDA TECs without concomitant ALS. (Figure 4.19.)

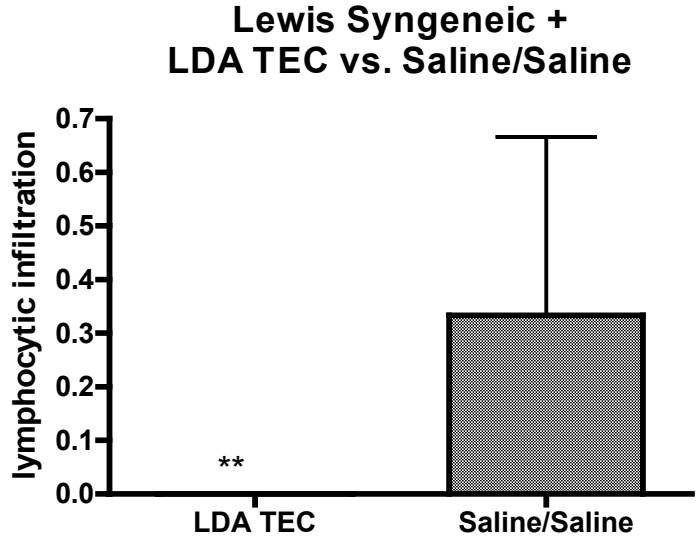


Figure 4.19. The Effect of Allogeneic IT TECs on Syngeneic Grafts. IT inoculation of LDA TECs resulted in reduced graft lymphocytic infiltration (average score 0), indicating no deleterious effects in syngeneic recipients.

Cases in which technical difficulties led to leakage of the TEC inoculum into the mediastinal cavity were recorded. Not shown, a comparison between the IT TEC inoculations without technical difficulties and those with technical difficulties ("leaks") revealed a statistically significant ($p<0.05$) loss of graft viability. The relevance of these results appears dimmed by the lack of significant change in graft lymphocytic infiltration or rejection scores between the groups.

4.3.3. Allogeneic Transplantation from Lewis to DA Rats

Strain-dependent differences have been reported to affect the success of allograft survival following IT inoculation of donor BMCs with concomitant ALS. [245] The $Rt1^a$ haplotype of DA rats has been reported to induce a stronger response to allograft placement compared to the $Rt1^l$ haplotype of Lewis. A combination of rat strains which results in such a response is referred to as a "high responder" combination. [235] Therefore, the DA to Lewis transplantation direction was reversed to create a "low responder" combination. This was done to highlight any observed modulations in the immune response following the hypothesis that TECs might induce donor-specific tolerance.

Recipients aged 4 to 6 weeks received an IT inoculation of 1×10^6 LDA BMCs or TECs, or 50 μL saline, with a concomitant IP injection of 1 cc ALS or saline. The negative control group was treated with IP and IT saline. As a control for IT cellular immunomodulation, animals received IP ALS and saline as a treatment.

After a treatment to challenge period of 21 days, Lewis challenge grafts of thyroid and pancreatic tissue fragments were placed in the renal subcapsule. Lewis grafts were harvested for processing and analysis after 10 days *in situ*. Again, any technical difficulties were recorded. The Lewis to DA experiments are summarized in Table 4.5.

Table 4.5. Lewis to DA Challenge Grafts Following Varied IT Treatments. DA recipients received either IP ALS or saline and either IT cells or saline for tolerance induction studies.

Group	Donor	Recipient	IT inoculum	ALS	Challenge (Days post Rx)	Harvest (days post-challenge)
1	Lew	DA	saline	N	21	10
2	Lew	DA	saline	Y	21	10
3	Lew	DA	Lew BMC	Y	21	10
4	Lew	DA	LDA BMC	Y	21	10
5	Lew	DA	LDA TEC	Y	21	10
6	Lew	DA	LDA TEC	N	21	10
7	Lew	DA	LDA FT-TEC	Y	21	10

To control for the tolerance inducing capacity of the partially mismatched Rt1 haplotype, Lewis BMCs ($Rt1^{l}$) (ALS/Lew BM) were substituted for LDA BMCs ($Rt1^{l/a}$) for the IT treatment with IP ALS in a 21/10 time frame (ALS/LDA BM).

The target experimental group was given ALS and TECs (ALS/TEC). To control for a physiological role for TECs as opposed to a purely antigenic role, freeze-thawed LDA TECs were injected intrathymically with IP ALS in a 21/10 time frame. (Figure 4.20.)

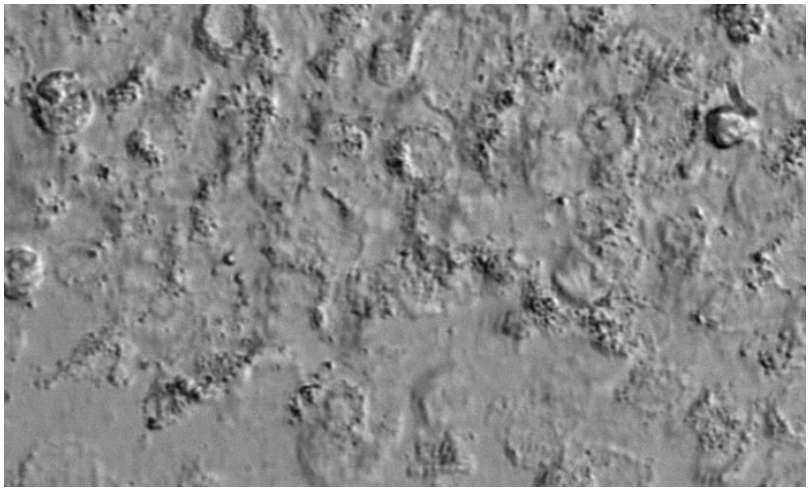


Figure 4.20. Hoffman Phase Microscopy of Freeze-Thawed TECs. TECs immediately following 4 freeze-thaw cycles; cells were injected intrathymically as a negative control to confirm a physiologic role for live TECs. 260 X.

To control for an allogeneic Lewis to DA response and for graft necrosis, syngeneic DA controls were included in a 21/10 time frame, as were syngeneic Lewis controls.

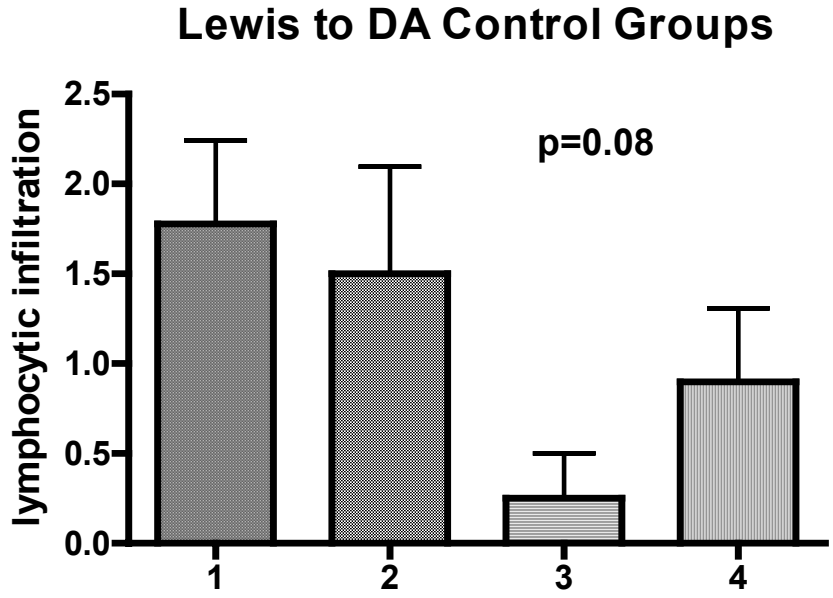


Figure 4.21. Lewis to DA Control Groups. As expected, compared to the saline control (group 1), IP ALS without an IT cellular treatment (group 2) did not significantly modulate the host immune system. Compared to negative control groups (1 and 2), group 3 (ALS+Lewis BMCs) and group 4 (ALS+LDA BMCs) resulted in reductions in mean lymphocytic infiltration of the grafts (ANOVA, p=0.08).

As a positive control, allogeneic BMCs were injected intrathymically with a concomitant IP injection of ALS. A one-way ANOVA of the Lewis to DA control groups revealed not quite statistically significant overall differences between the groups (p=0.08). However, a selected Dunns analysis revealed the mean lymphocytic infiltration for group 1 (saline control, mean lymphocytic infiltration 1.78+0.47, n=10) to be statistically different from group 3 (ALS+Lewis BMCs, n=6, p=0.01), indicating that Lewis BMCs resulted in significant modulation of the host immune system. The reversed direction of graft scores from high to low in the positive control was felt to reflect the 21/10 time frame as it related to the natural history of graft rejection. Though appearing to reduce lymphocytic infiltration to some degree, the lack of statistically significant immunomodulation by group 4 (ALS+LDA BMCs, mean lymphocytic infiltration 0.9+0.41, n=6) compared to group 1 (p NS) may have been related to strain specific differences in the allograft response, or it may have been a function of the new 21/10 time frame. In this specific strain combination, the inclusion if a Lewis-Dark Agouti hybrid bone marrow plus ALS treatment group was more experimental when compared to the use of fully allogeneic Lewis bone marrow plus ALS as a positive control treatment group, although there was no statistically significant difference in the mean lymphocytic infilration scores between group 4 to group 3. The comparison of group 4 to group 2 (ALS+saline, mean lymphocytic infiltration 1.5+0.6, n=8, p NS) was included to control for the effects of IT LDA bone marrow, suggesting that the addition of ALS to the IT BMC treatment regimen was not as effective in immunomodulating the host immune system in this low

responder strain combination, compared to the DA to Lewis strain combination. The difference in the mean lymphocytic infiltration scores between group 2 and group 3 approached statistical significance (p=0.07), suggesting a physiologic role for IT Lewis BMCs in DA recipients and a synergistic role for ALS in host immunomodulation when combined with fully allogeneic Lewis BMCs.

The immunomodulatory effects of IT LDA TECs were compared to those of IT Lewis and LDA BMCs.

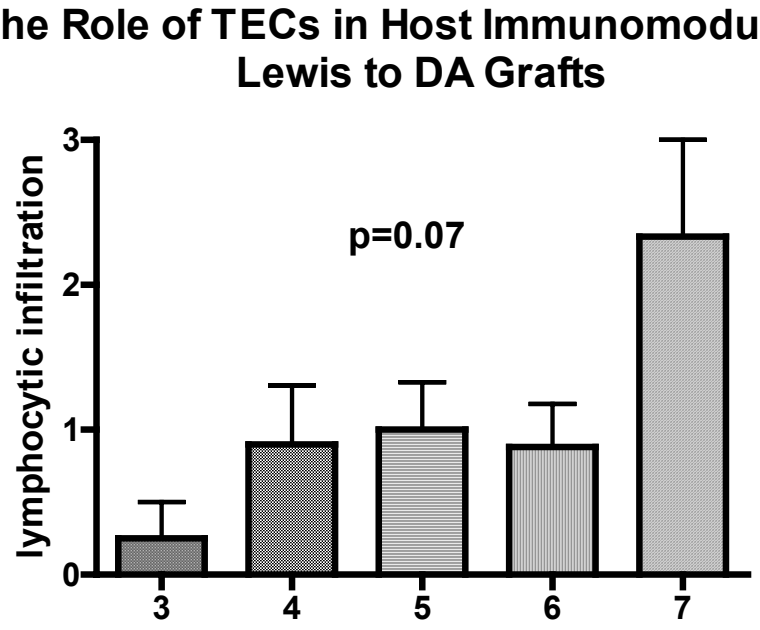


Figure 4.22. Lewis to DA Experimental Groups. Immunomodulation as demonstrated by reduced lymphocytic infiltration scores was higher (ANOVA, p=0.07) in the positive control group (3, ALS+Lewis BMCs) compared to the IP ALS/IT TEC treatment groups (5 and 6). Treatment groups 5 and 6 were not significantly different from group 4 (ALS+LDA BMCs). Group 7 (ALS+FT-TECs) showed the least immunomodulation compared to saline controls (not shown).

A one-way ANOVA of the Lewis to DA experimental groups comparing the immunomodulatory effects of groups 5 and 6 (IT TEC treatment groups with ALS, mean lymphocytic infiltration 1.0±0.33, n=14, or without ALS, mean lymphocytic

infiltration 0.88±0.3, n=11) to those of groups 3 (ALS+Lewis BMCs) and 4 (ALS+LDA BMCs) revealed statistically not quite significant overall differences ($p=0.07$). Using Dunn's multiple comparison test to compare groups 4, 5, and 6 to group 3, there was no significant difference between the use of ALS and live LDA TECs or the use of LDA BMCs with concomitant ALS administration in the Lewis to DA strain combination. There was a statistically significant difference between group 3 and group 7 (ALS+IT freeze-thawed TECs, mean lymphocytic infiltration 2.33±0.67, n=6, $p<0.01$), reflecting the difference between physiologically active BMCs and dead cells such as TECs. The lack of significant differences between the immunomodulatory response to freeze-thawed TECs with ALS vs. the saline control group further suggests a physiological role for both live TECs and BMCs in this experimental model. Although a comparison between recipients receiving live TECs and ALS vs. recipients receiving freeze-thawed TECs and ALS did not quite reach statistical significance ($p=0.7$), these findings are suggestive of a physiological role for the TECs as opposed to a simply antigenic role.

As with the majority of experiments, graft rejection score inconsistencies within the treatment groups made those scores unreliable indicators of host immunomodulation. Therefore, mean lymphocytic scores were used. Overall, the Lewis to DA allotransplantation studies revealed the lowest mean lymphocytic infiltration scores in the positive control group (ALS+Lew BMCs), similar results between experimental groups (ALS+LDA TECs vs. ALS+LDA

BMCs), and similar results between negative controls (saline control vs. ALS + freeze-thawed TECs). These results were exactly opposite the results of DA to Lewis allotransplantation studies, where highest mean lymphocytic infiltration scores occurred in the positive control group (ALS+LDA BMCs), and the lowest scores occurred in the negative control groups. A striking similarity between the groups, however, was the placement of the TEC treatment groups somewhere in the middle between positive and negative controls. Intrathymic TECs with concomitant intraperitoneal ALS immunomodulated graft lymphocytic infiltration scores when compared to saline controls, but not with any statistical significance in either strain direction. Additionally, a comparison between treatment with IT TECs and ALS vs. ALS alone failed to demonstrate a statistically significant physiologic role for TECs in the Lewis to DA strain combination. Perhaps this reflects the relatively reduced synergistic effect of ALS with IT cellular treatments in the Lewis to DA transplants.

The results of the above transplantation studies may be meaningful from an educational standpoint, but they may not become clinically relevant if immunomodulatory responses are strain dependent. Therefore, it was hypothesized that the differences in treatment responses were related to strain combinations.

4.3.3.a. DA Syngeneic and Lewis Syngeneic Graft Controls

Syngeneic and allogeneic transplant results were compared. (Figure 4.23)

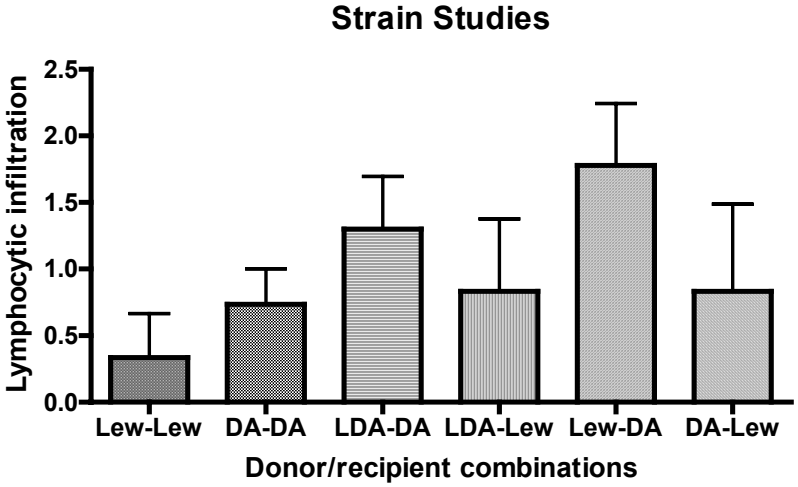


Figure 4.23. Untreated Groups in Various Strain Combinations. LDA to DA transplants and Lewis to DA transplants resulted in stronger alloresponses, providing a plausible explanation for differences between the immunomodulatory responses using reversed strain combinations.

Syngeneic grafts were included as controls for host immunomodulation. Both graft rejection scores and lymphocytic infiltration scores were significantly lower in the control groups as compared to the Lewis to DA allograft group. (Figure 4.24 and 4.25) Not shown, the graft rejection score was 2.83±0.44 for the Lewis syngeneic group and 3.33±0.25 for the DA syngeneic group, significantly lower than the Lewis to DA allograft group at 3.67±0.22, $p<0.05$.

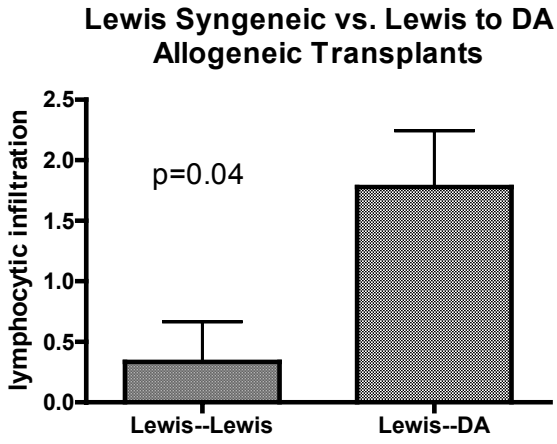


Figure 4.24. Lewis to Lewis Syngeneic vs. Lewis to DA Allogeneic Transplants. Syngeneic Lewis challenge grafts had statistically significant reductions in graft lymphocytic infiltration compared to allogeneic challenge grafts.

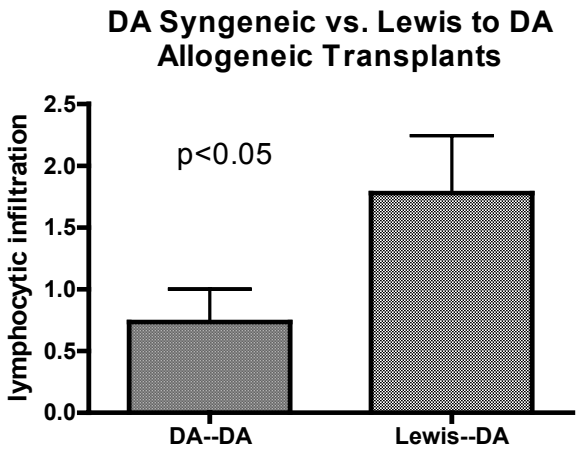


Figure 4.25. DA to DA Syngeneic vs. Lewis to DA Allogeneic Transplants. Syngeneic DA challenge grafts had statistically significant reductions in graft lymphocytic infiltration compared to allogeneic challenge grafts.

To further analyze the findings, the strain direction was directly compared to determine whether a "high responder" strain combination was the cause of these differences in allogeneic response. There was found to be no significant difference between the DA to Lewis compared to the Lewis to DA TEC/ALS treatment group in a 21/10 time frame.

4.4. DISCUSSION

The transplantation studies used to plot the natural history of allograft rejection served to put in perspective the results of the experimental transplantation studies. The original 10/21 time frame, where animals received IP ALS and IT inoculation with a 10 day time period before challenge graft placement to the RSC that remained *in situ* for a period of 21 days, was chosen because of its

expected success. The results indicating that TECs were not as effective as hypothesized led to a shift in the time frame to the 21/10 day protocol. Although some of the results were suggestive of immunomodulation by TECs, the null hypothesis was disproven. One reason might have been the effect of prolonged *in vitro* culture of TECs prior to their use. Repeated trypsinization at cellular passaging could have resulted in down regulation of surface antigen molecule expression, particularly MHC antigens, making the cells less tolerogenic. Since BMCs had neither been cultured for a prolonged period *in vitro* nor exposed to trypsinization, down regulation of surface antigenicity would not have been likely. The trypsinization of BMCs would have been an educational control, but would not have been clinically applicable since BMCs in current pre-transplantation tolerance induction protocols are not exposed in such a way. Another reason for the lack of robust tolerance inducing capacity in TECs might have been their proposed dual role in both positive and negative T cell selection. [246] Foreign TECs might be effective in eliminating MHC mismatched clones in the host via negative selection while also recruiting donor-matched clones via positive selection. The end result would have been void due to this dual role. The results of these transplantation studies are further discussed in Chapter 5.

CHAPTER 5.

DISCUSSION AND OVERVIEW

Successful organ, tissue, or cell transplantation is dependent on two primary factors: 1) achieving technical success, which maximizes tissue/organ engraftment and survival, and 2) avoiding immune rejection of the graft. Previous transplant protocols have focused on minimizing deleterious immune responsiveness, and allograft rejection, by either minimizing graft immunogenicity or by modifying the host immune environment by systemically suppressing immunoreactivity.

Interest in the thymus, and thymic induction of central tolerance, has increased over the last several years as a consequence of studies demonstrating the induction of immunological tolerance following the placement of donor antigen within a host thymus with concomitant depletion of the peripheral lymphocyte population. In the initial landmark report, isolated pancreatic islets of Langerhans, transplanted to the thymus of adolescent rats with a concomitant single dose of anti-lymphocyte serum, were challenged after 200 days of graft residence by a second graft of donor-matched pancreatic islets placed in the renal subcapsular space [247]. Apparent immunological tolerance was

observed, as indicated by the failure of the host to reject the challenge islet graft. This tolerance was proven to be donor-specific because of the rejection of third party control grafts. Subsequently, numerous other studies have reported similar outcomes using a variety of cell types and/or antigen sources as the IT inoculum [121, 124, 126, 127, 129, 248-251]. It was hypothesized from these studies that the IT administration of novel antigens resulted in immune reeducation and specific tolerance to the introduced antigens. These reports stimulated significant interest in the thymus and the thymic cells involved in this process, including the TEC. The epithelial nature of TECs would suggest that these cells would be highly amenable to culture and to *in vitro* manipulation. To show the efficacy of this paradigm, the feasibility of TEC isolation and proliferative culture had to be demonstrated and established. TECs were isolated and cultured from neonatal thymi of several rat strains. The cells were manipulated for extensive characterization in differing culture conditions. Valuable information was gathered regarding the establishment, maintenance, and manipulation of primary keratinocyte cultures.

5.1. THYMIC EPITHELIAL CELL CHARACTERISTICS

A continuous cell line of TECs was necessary for disproving the null hypothesis that IT inoculation of TECs with concomitant ALS would induce donor-specific tolerance. It was soon discovered that these cells could not be easily established

in primary culture, and this led to investigations on these limitations. Initial attempts using mammalian cell culturing techniques in commonly used media formulations for epithelial culture led to the outgrowth of scattered, slow proliferating cells that mostly resembled fibroblasts. Literature review on the primary culture establishment of specialized epithelial cells (keratinocytes) pointed towards the supplementation of medium with various growth factors (insulin-like growth factor, bovine pituitary extract) and the reduction/removal of serum from the medium. The combined focus of previous experience with primary culture of other cell types and further literature searches finally pointed to references [252] which alluded to the low calcium culture methods that eventually led to successful establishment of a rat TEC line.

Calculations of the calcium levels in the medium varieties that were initially used for this project showed them to be significantly higher than the calcium levels maintained in reported keratinocyte permissive media formulations. In retrospect, the inability to easily trypsinize those higher calcium cultured cells that were able to attach to the growth surface, the limited proliferative capacity of the cells, and the early differentiation of the cells into a dendritic phenotype suggested an interrelated cause--calcium levels were suspected.

Histologic techniques used to identify the cells as TECs demonstrated the presence of structures and proteins (desmosomes and keratin IFs) characteristic

of epithelial-derived keratinocytes. This led to the question of why these particular keratinocytes would not proliferate in culture.

Following a low calcium, serum free method for establishing primary TECs in culture proved difficult despite correspondence with authors who reported ease of culture. Attempts to recapitulate experiments using the reported components (approximately 70 additives) were technically difficult due to the trace concentrations necessary. To assist the learning process, Dr. Jun Hayashi at the University of Maryland generously donated a sample of the custom formulation, WAJC, to the laboratory. The TEA-3A1 cell line was derived from neonates of the LDA strain of rat (Lewis/Dark Agouti cross) [132] and is of the same genetic background as the new line of TECs derived in this study. Not coincidentally, the use of WAJC medium in the initial establishment of primary cultures of rat TECs was limited to the LDA strain. As presented in the results section on primary culture isolation of TECs (Chapter 2), thymic tissue isolated from the other rat strains did not respond as favorably as thymic tissue from LDA rats. The LDA rat cell line for this project, designated OKTE4-01, was compared to the TEA-3A1 cell line by examining for cytokeratin content and structural elements. The presence of desmosomes and tonofilament bundles is typical and characteristic of epithelial cells, and the ultrastructural visualization of these structures allowed these thymus-derived cells to be characterized as TECs. These cells appeared identical in morphology and cellular proliferation to the LDA TEC cell line established for this project. While the OKTE4-01 cell cultures spontaneously

transformed, it was a struggle to obtain a monolayer of cells from the other strain. It was hypothesized that the ability to establish rat TEC primary culture might be strain specific. Further, it was hypothesized that the morphological nature and proliferative capacity of TECs was calcium related.

Indeed, it was demonstrated that TECs maintained in medium containing low calcium levels expressed significant differences from TECs maintained in medium containing high calcium. Specifically, proliferative capacity, morphologic appearance, intercellular spacing, and macula adherens density were found to be dependent upon calcium levels in the media. (Chapter 3)

The information gained from these findings was applied to primary culture techniques. It was hypothesized that the failure of DA TECs to attach early in the process of the *in vitro* isolation and culture using the low-calcium GWAJC medium could be due to lack of calcium-dependent cellular attachment. When the medium calcium concentrations were increased, DA thymic tissue responded as expected—there was adequate tissue attachment to the tissue growth surface. Unfortunately, when medium with higher calcium concentrations was used, the attached DA tissue would adhere so tightly to the surface that trypsinization was nearly impossible due to lifting of cells in sheets. Within a short time frame, approximately 7-10 days, the DA cells consistently assumed a terminally differentiated, dendritic appearance. After many attempts to grow the cells in different types of media with different

supplements, the idea to establish attachment to the growth surface early in the primary culture establishment process with higher calcium medium followed by the withdrawal and replacement of medium with lower calcium medium resulted in the successful culture isolation of DA rat TECs. This technique of early high calcium culture followed by a switch to low calcium culture was applied to the other strains of thymic tissue and resulted in successful culture establishment of primary TECs. As part of this project, primary rat TEC cultures were derived from several different strains. It is hypothesized that the application of this new method of high to low calcium medium switch technique could lead to the successful primary culture establishment of many other types of keratinocytes in varying strains and species.

Thymic epithelial cell lines, both OKTE4-01 and TEA-3A1, grown in WAJC medium supplemented with 2.5% DMEM were highly proliferative (with doubling times of ≈48hrs), and were readily passaged via light trypsinization (0.05%). Increasing medium calcium concentration induced a distinct change in cell morphology, including the approximation of cell boundaries, and the formation of increased areas of cell contact. Upon examination with electron microscopy, cells maintained in high calcium medium appeared more closely spaced, with confluent TECs closely apposed to adjacent cells and spread evenly across the growth surface. Cells maintained in low calcium medium appeared more widely

spaced, with visible gaps between adjacent cells and fewer regions of closely apposed TECs.

As stated above, the *in vitro* maintenance of the new OKTE4-01 cell line, as well as the TEA-3A1 line, required a significantly reduced medium calcium concentration to 0.098mM, which was achieved using WAJC404A medium supplemented with 2.5% DMEM. (DMEM alone has a calcium concentration of 1.815 mM.) Previous studies have reported using Nutrient Mixture (Ham's) F12, whose calcium levels are already extremely low (0.299mM calcium) in comparison to other media, to achieve the low calcium levels required for successful TEC culture [149, 157]. However, culture of both OKTE4-01 and TEA-3A1 in Ham's F12 resulted in conversion of the cultured cells to a non-proliferative state, displaying closely approximated adherent cell boundaries and increased adherence to the growth surface similar to that observed in our TEC populations cultured in high calcium WAJC404A medium.

These observations indicate that increased desmosome formation results from increased medium calcium concentrations, and would explain the strong adherence characteristics, both between cells and to the growth surface, demonstrated by cells maintained in high calcium conditions. This is the first instance in which the calcium-dependent morphological changes in TECs have been quantified and statistically analyzed. These calcium-dependent adherence characteristics may not be limited to thymic epithelium only.

Desmosome formation in mouse epidermal keratinocytes was documented ultrastructurally just one to two hours following elevations in medium calcium levels. [221] An unexpected finding upon TEM examination of TECs maintained in low calcium medium was the presence of laminar, whorled and curvilinear cytoplasmic densities. These structures were found near keratin IFs and were thought to be immature keratin protein structures. Their significance was not determined, although future studies using labeling techniques in combination with electron microscopy might be useful in determining their composition.

Numerous studies demonstrate induction of desmosome formation and cell differentiation by increasing extracellular calcium levels [253] In fact, a “dramatic reorganization” was shown to occur concurrently between microtubules, microfilaments, and keratin IFs only hours after cell culture medium calcium changes in human epidermal keratinocytes [254]. Many studies attempt to demonstrate that calcium induced cell differentiation mechanisms are not all-or-none phenomena [255]. While the observations presented here indicate that once exposed to higher levels of calcium, cells form adherent junctions with other cells that cannot be reversed, in some epithelial cell cultures it may be of value to reduce medium calcium concentrations with the intent of decreasing intercellular adhesion to allow for increased cell proliferation.

Serendipitously, the LDA rat thymic tissue culture isolation and maintenance in GWAJC as described in the techniques section resulted in spontaneous transformation into the OKT4-01 cell line, allowing a constant supply of TECs for the remainder of this project. Additionally, the OKT4-01 cell line allowed for the focused studies on calcium-dependent cellular changes and served as a control cell for comparison with the TECs from other rat strains. The evaluation of the effects of calcium on TECs revealed that TECs maintained in high calcium media became highly differentiated and did not exhibit logarithmic growth, while TECs maintained in low calcium media supplemented with specific growth factors could be used to maintain these partially differentiated cells at high mitotic frequency *in vitro.* It was also an important finding that a minimum calcium level was necessary for TEC establishment and maintenance.

Based on the irreversible nature of changes induced by high calcium exposure, we have concluded that our ability to passage these cells was based primarily on initial culture conditions, strain of donor tissue, and lack of exposure to calcium, rather than on the passaging protocol. This could explain why a limited number of thymus-derived epithelial cell lines have been documented among humans, mice, and rats. [214, 256-261].

5.2. GENERATION OF IMMUNOLOGIC UNRESPONSIVENESS TO INTRATHYMIC TRANSPLANTATION OF ALLOGENEIC TISSUE

Due to their organ of origin and their hypothesized role in the process of immune education, modified TECs were considered excellent candidate cells for induction of immune tolerance in models of autoimmunity and/or transplantation. Statistical analysis of the graft scores in allograft recipients pretreated with IT TECs and ALS comparing negative controls (sham experiments) and positive controls (IT BMCs and ALS) reflected some reduction in lymphocytic infiltration, effectively shifting the time course of graft rejection to the left (reducing the expected level of rejection at a given time point). However, numerical values did not reach statistical significance to support the hypothesis that TECs and ALS would be better candidates than BMCs and ALS for inducing donor-specific tolerance, despite changing the treatment to challenge time frame, changing the *in situ* challenge graft time frame, altering the type of donor tissue to avoid autolysis, switching from a high responder to a low responder strain combination, and increasing treatment group sizes. The comparison between positive and negative control groups did reach statistical significance with p values < 0.3, indicating that the IT inoculation of cells previously proven to induce donor-specific tolerance was effective in this particular animal model of allograft rejection. A comparison between the donor-specific tolerance inducing capacity of living and non-living TECs with ALS also did not reach statistical significance, despite the fact that

non-living TECs were also not significantly more effective than saline in inducing donor-specific tolerance. In many of the transplantation experiments, p values reached near statistical significance, suggesting a dual role for TECs that might have confounded the results. If both positive and negative tolerance induction mechanisms were in effect, one would have expected equivalent results. Following peripheral lymphocyte depletion, allogeneic TECs injected intrathymically might be retaining immature T cell clones specific for the allogeneic MHC, thus actually sensitizing the host. Simultaneously, they might be inducing tolerance in the host by eliminating T cells that are reactive to the new "self" antigens, thus buffering the sensitization, producing an overall neutral effect.

Evidence of sensitization, as demonstrated by accelerated graft rejection, was not seen in the transplant recipients. This could be related to the dual role of foreign IT TECs, or the immunoprivileged graft site. The relatively placid effect of TECs compared to BMCs might actually be of benefit when considering the use of TECs as a platform cell for gene therapeutic techniques. The effects of genetically altered TECs on the host immune system have yet to be evaluated.

Terminal differentiation of cells exposed to high calcium levels *in vivo* might also affect the results of TEC transplantation to the host thymus. Specifically, inoculating TECs that were originally maintained in low calcium *in vitro* conditions into relatively high calcium *in vivo* conditions might have resulted in terminal differentiation of these proposed immunomodulatory cells, inhibiting a

physiologic role. In view of the sensitivity of TECs to calcium levels, gene therapeutic protocols employing higher calcium levels might have negative effects on the physiologic role of TECs.

Another possible explanation for the less than statistically significant results was the use of TECs derived from an F1 hybrid rat strain due to limitations in cell supply. Future studies might involve a different animal model with the unlimited availability of fully allogeneic TECs.

5.3. GENETIC TRANSFECTION OF THYMIC EPITHELIAL CELLS

Integrating gene therapy techniques with IT transplantation of TECs could provide a potential transgene platform for transfection of novel antigens and subsequent IT transplantation of genetically altered TECs to modify host immunity. Sufficient numbers of TECs were made available by early experiments to pave the way for future gene transfer studies. As a sideline to transplantation studies, the feasibility of TECs to serve as such a vehicle for the delivery of novel genes was investigated using various gene therapeutic techniques. TECs were transfected with green and red fluorescent protein reporter genes using liposome techniques and electroporation, and with humanized green fluorescent protein using adenoviral-associated vectors with transient expression encoded proteins with all gene therapy technique applications.

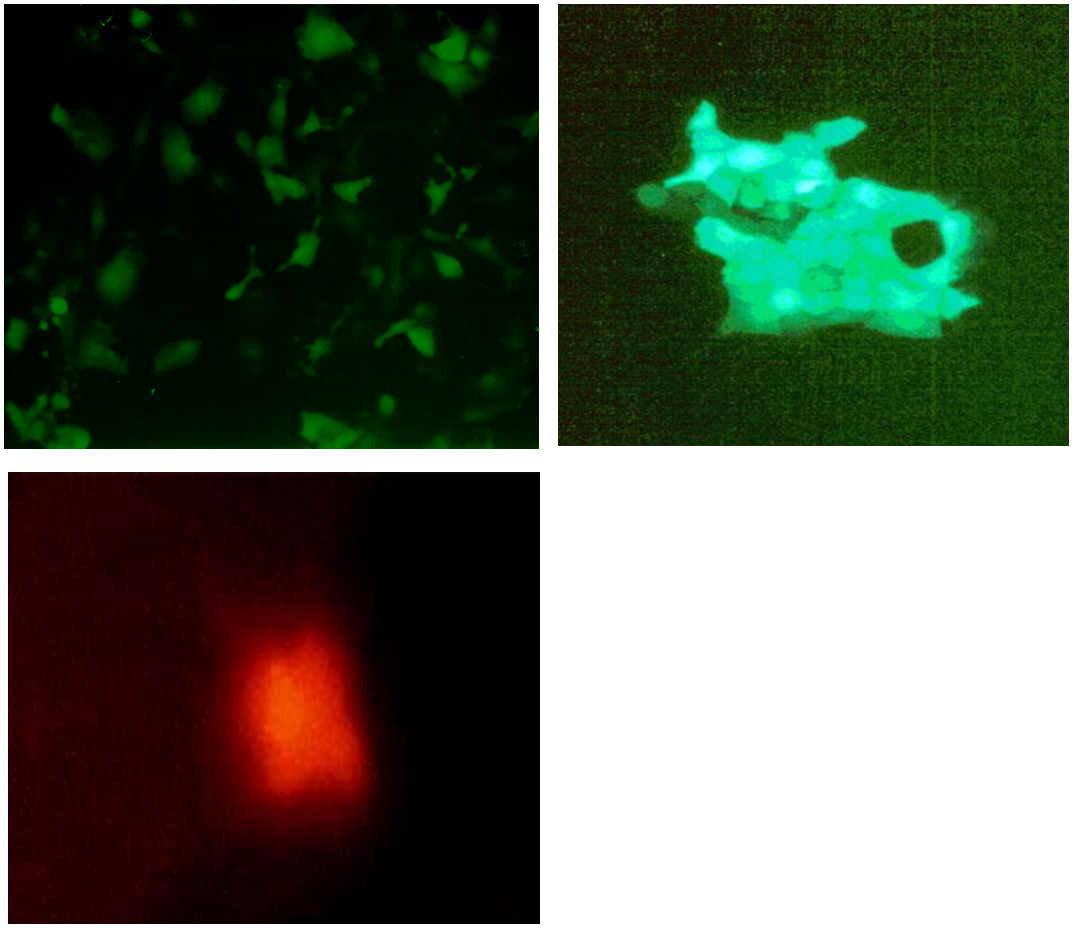


Figure 5.1. Light Microscopy of TECs Transfected Using Varied Techniques. TECs with GFP using liposomes (top left and right). TECs transfected with RFP using an adeno-associated viral vector (left, 75 X) and with liposomes (right, 125 X). TEC transfected with RFP using liposomes (bottom, 135 X).

A transfection efficiency of only 3%, for example, in any assay system would seem to require further optimization, but some authors will report such preliminary results. [262] As mentioned previously, the transfection studies performed at the time of this particular dissertation project were preliminary and were not the primary focus of this research, but the best transfection efficiency was found to be almost 30% employing the AaV technique.

5.4. ROLE OF TECs FOLLOWED BY A SECOND GRAFT

TECs are normal thymic residents, representing a significant portion of the cells which constitute the stroma of the thymus. Although their precise function has not been definitively elucidated, it is known that TECs are involved in the process of T cell development and immune education. Experimental evidence indicates that TECs are involved in the process of positive thymic selection, which selects a population of immature T cells for retention within the thymus based on the ability of their TcR to interact with self-recognizing MHC proteins expressed on TECs [62, 263, 264]. Other reports suggest an ability of TECs to act as an APC within the thymus, and to present antigens to maturing thymocytes in the process of negative selection, in which T cell subsets with a high affinity for self-MHC antigen are selected and deleted, thereby excluding cells capable of recognizing and responding to autologous antigens from the peripheral immune repertoire. [62, 73, 265, 266]. Some reports go so far as to ascribe both positive and negative selection functions to TECs, with functional effects determined by the cellular location within the thymus [99, 267], or by the degree of expression of MHC antigens [268]. Almost all nucleated cells, TECs included, express MHC Class I antigen, and as APCs, TECs also express MHC Class II molecules. The objective of this project was to employ *in vitro* TECs most closely mimicking an *in vivo* functional role. Hence, the effects of donor-MHC-specific tolerance induction studies were expected to potentially reveal new information about

how TECs function in the immune response and clonal selection. While it has been shown that interferon gamma (IFN-γ) can induce the upregulation of MHC Class I and II molecules on TECs [269], this was felt to be less reflective of usual *in vivo* conditions. Additionally, a direct comparison between the effects of unaltered IT allogeneic bone marrow with concomitant immunosuppression and the effects of unaltered IT allogeneic TECs with concomitant immunosuppression is a novel approach, and altering the TECs with IFN-γ might be interesting as a focus for future experiments.

As with any treatment, significant potential complications may occur in performing IT cellular transplants for tolerance induction. One complication considered was accelerated graft rejection. In one background study, it was shown that IT injection of purified liver and spleen derived allogeneic dendritic cells (DC) "…plus a single dose of ALS did not prolong allogeneic islet graft function but rather induced accelerated rejection of islet allografts" administered intraportally. [240]

Intrathymic inoculation of LDA TECs prior syngeneic Lewis challenges were used to control for accelerated graft rejection, and no such response was found.

This project took advantage of the high mitotic frequency and proliferative capacity of culture immortalized TECs for a continuous supply of cells to use in transplantation experiments investigating the ability of TECs to induce donor-

MHC specific tolerance to subsequent allografts, a novel approach towards to tolerance induction because of the inherent nature of the TECs. Successful transplantation and gene transfer experiments using TECs derived from primary culture were performed once cells were established. These studies appeared to demonstrate the modulation of host immunity, with reduced allograft rejection following IT TEC transplantation. Despite indications of modified immunity, success, as gauged by complete protection of the challenge graft from immune destruction, was not achieved. Initial essays at transfection of a novel gene into these cells demonstrated definite expression of the reporter gene in primary cultures of TECs. However, stable transfer of novel genes to these sensitive primary cell lines will require further optimization. The immunomodulatory capacity of transgenic TECs upon IT transplantation will be the focus of a future project.

The above works, while unique in revealing potentially widespread applications of conditions associated with primary epithelial culture, TECs from many different strains, and the donor-specific tolerance inducing capacity of TECs, served as precursors towards the development of a treatment paradigm combining IT transplantation with gene therapy to allow introduction and prolonged expression of antigens within the thymus. The application of gene therapy to TECs destined for IT inoculation for tolerance induction is also a novel approach.

The use of cultured-isolated MHC-mismatched TECs for host IT inoculation as a model to induce donor-specific tolerance is a unique approach. Since the inception of this project, a research group at the Tohoku University School of Medicine in Japan chose to investigate the effects of IT thymic stromal cell transplantation in a mouse model of allogeneic skin transplantation, demonstrating prolongation of graft survival. [269]

Combining gene therapy with IT transplantation using a unique cell type, the TEC, is hypothesized to serve as a means to introduce immunogenic proteins into the thymus of a host. Once successful TEC transfection protocols can be established, follow-up studies would include IT transplantation of TECs transfected with genes encoding specific novel antigens and investigations into the successful expression of these genes within the thymic environment. An indirect measure of gene expression would be an assessment of modification of host immunity following IT transplantation of genetically modified TECs. Further investigations would be necessary to optimize the preliminary TEC transfection results before going forward with such an ambitious project.

“Donor/recipient MHC class II matching permits survival of experimental allografts without permanent immunosuppression, but is not clinically applicable due to the extensive polymorphism of this locus.” [95] The transplantation tolerance induction arm of this project using MHC mismatched

TECs served as a precursor towards the use of IT transplantation of genetically modified TECs expressing novel donor MHC molecules for induction of graft specific tolerance in virtually any population of transplantation recipients. Since TECs are amenable to culture techniques, they were hypothesized to provide an appropriate transgene platform for the transfection of novel antigens and subsequent transplantation of altered cells. Based on the finding that the majority of transplant rejection is due to an MHC Class I mismatch between the donor and the recipient, using donor-matched TECs transfected with a mismatched MHC Class I specific gene sequence for IT inoculation might provide insight into transplantation tolerance induction mechanisms. Follow-up studies would investigate the ability of TECs transfected with a gene sequence encoding a specific antigen and inoculated into the thymus to induce antigen specific tolerance. In these experiments, genetically modified syngeneic TECs transplanted to the thymic microenvironment would be assessed regarding their ability to present a foreign antigen to the developing immune system of a young recipient, resulting in permanent modification of host immunity. Host specific TECs would be transfected with the gene encoding a specific antigen and injected IT following a single IP injection of ALS. Finally, the immune response to subsequent peripheral administration of this antigen would be viewed as a reflection of central tolerance induction.

The most successful donor-specific tolerance induction strategies would benefit graft recipients suffering from various comorbidities associated with non-donor-specific immunosuppressive drug regimens. The clinical relevance and practicality of this approach in the prevention and treatment of immune diseases and hypersensitivity states is yet to be determined, but as researchers continue to discover and genetically sequence the autoantigens responsible for the development of specific autoimmune diseases, the feasibility of using such an approach becomes more realistic. For example, IT transgenic TEC expression of autoantigens targeted by the immune system in type I diabetes mellitus could induce tolerance to these autoantigens, resulting in the prevention of the immune mediated destruction of insulin producing pancreatic beta cells. Most ambitious, due to the risk of inadvertently exposing a healthy recipient to a serious lifelong autoimmune disease, is the idea of applying a combination of IT tolerance induction strategies combined with gene therapy to potentially be used as a cellular vaccine in children to prevent the early onset of autoimmune diseases such as autoimmune thyroiditis or diabetes.

Procedure or Event	Date	Surgeon or Scientist
Limb and tooth tr.	Late 1700's	John Hunter
Xenogeneic skin tr.	Late 1700's	G. Baronio
First skin autograft	1823	Carl Bunger
Allograft tr.	1863	Paul Bert
Skin allografts	1880's	Astley Cooper
Corneal tr.	Late 1800's	Edward Zim
Surgical tr. techniques	Early 1900's	Alexis Carrel, Charles Guthrie
Renal tr. (dogs/goats)	1902	
Skin allograft	1908	Jacques Louis Reverdin
Cadaveric knee joint replacement	1908	Eric Lexer
First blood transfusions	1918	
United States Navy Tissue Bank	1949	
x-radiation	1950's	Main and Prehn
Living-related human kidney tr. between identical twins	1954	Joseph E. Murray, David Hume, Harrison, Merrill
Histocompatibility system	1958	Jean Dausset
Non-twin kidney tr.	1959	Joseph E. Murray
Heart valve tr.	1962	
Cadaveric kidney tr.	1962	David Hume
Liver tr.	1963	Thomas Starzl
Lung tr.	1963	James Hardy
Kidney-pancreas tr.	1966	
Human heart tr.	1967	Christian Bernard
Pancreas tr.	1966	Richard C. Lillehei, William D. Kelly
Cryopreservation	1970's	
Living related pancreas tr.	1979	
Heart-lung tr.	1981	Norman Shumway, Bruce Reitz
Cyclosporine	1982	Clinical use
Artificial heart tr.	1982	
Single-lung tr.	1983	
Cyclosporine	1983	FDA approval
Heart-liver tr.	1984	Thomas Starzl
Baboon to human heart tr.	1984	
Establishment of UNOS	1984	
Double lung tr.	1987	
Partial lung tr.	1991	
Small intestine tr.	1991	
Split-liver tr.	1996	
Islet tr.	1999	James Shapiro, Edmonton protocol

Table A. Historical Events in Transplantation. List of major events from late 18th century through the 21st century. tr. = transplantation

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VITA

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Institution: Center for Health Sciences, Oklahoma State University
Location: Tulsa, Oklahoma

Title of Study: THYMIC EPITHELIAL CELL TRANSPLANTATION TO THE HOST THYMUS: STUDIES IN IMMUNOMODULATION AND TOLERANCE INDUCTION

Pages in Study: 225 Candidate for the Degree of Doctor of Philosophy

Major Field: Biomedical Sciences

Scope and Method of Study: This study's purpose was to determine whether thymic epithelial cells (TECs) would work in a pre-transplantation intrathymic tolerance induction regimen, necessitating investigations on the establishment of a primary TEC line, characterization of the response of TECs to varying medium conditions, and investigation of immunologic effects of intrathymic TEC transplantation on the natural history of graft rejection in an allogeneic rat transplantation model. The efficacy of bone marrow has been established in a regimen of pre-transplant tolerance induction. It was hypothesized that isolated TECs might be better candidates. Primary tissue culture techniques were used to establish TECs from different rat strains. Light immunocytochemistry and transmission electron microscopy were used to evaluate the effects of varying medium conditions on cellular morphology and differentiation. Allotransplantation following intrathymic cellular inoculation with histological analysis of challenge grafts examined the tolerance inducing capacity of TECs. Multiple techniques tested the feasibility of gene therapeutic techniques on TECs

Findings and conclusions: Rat TECs were established from 4 different strains of inbred rats, one of which became a cell line. Light immunocytochemistry revealed cytokeratin intermediate filament expression, and electron microscopy revealed both cytokeratin intermediate filaments and desmosomes. Quantification of morphological changes revealed 1 desmosome per 6.24 μm of cell membrane in high calcium grown cells, and virtually no desmosomes in low calcium grown cells ($p < 0.03$). Intrathymic transplantation experiments revealed a reduction in graft mean lymphocytic infiltration from 1.78 ± 0.47 in the negative control groups (intrathymic saline, IP saline; n=10) and 1.50 ± 0.60 (intrathymic saline, IP ALS; n=8) to $0.25 \pm .25$ ($p < 0.01$) in the positive control group (intrathymic bone marrow + ALS; n=8), and a reduction in graft mean lymphocytic infiltration to 1.00 ± 0.31 in the experimental group (intrathymic TEC, IP ALS; n=16), p value > 0.05. Overall, the results revealed that IT inoculation of TECs results in some immunomodulation, although not reaching the same effectiveness as bone marrow. This project served as the predecessor to a future project using genetically modified intrathymic TECs in a new paradigm for allogeneic tolerance induction. Initial transfection of TECs resulted in positive expression of reporter genes.

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Advisor's Approval: _____