RELATIONSHIP BETWEEN MHC II EXPRESSION ON T-LYMPHOCYTES AND REJECTION

A MODEL OF HETEROTOPIC HEART TRANSPLANTATION

RADEK CTIRAD KADERABEK

M. D., Charles University, Prague, Czech Republic, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF SURGERY

We accept this thesis as conforming to the required standard

.....

.....

.....

.....

The University of British Columbia
November, 2004

Abstract

Relationship between MHCII expression on T-lymphocytes and rejection (a model of Heterotopic Heart Transplantation)

Introduction: The method of choice for monitoring post-transplant rejection status for heart transplant patients is the evaluation of multiple tissue biopsies. The diagnostic technique is time consuming, costly and invasive with possible life threatening complications. The major histocompatibility complex is the most widely distributed and significant determinant for transplantation.

We hypothesized that the MHC II expression on peripheral T-lymphocytes is reflective of rejection status in allotransplanted hearts.

The objective of this project is to determine if MHC II expression on peripheral T-lymphocytes correlates with histological findings of rejection after transplantation.

Materials and methods: The heterotopic heart transplant was performed in 24 female domestic swine, weighing 27-30 Kg. Animals were divided into two groups. Group A (6 donors + 6 recipients) was not treated with immunosuppressive therapy. In group B (6 donors + 6 recipients) immunosuppressive therapy (Cyclosporine 6mg/Kg/BID, Methylpednisolone 1.8mg/Kg/day and Azathioprine 3mg/Kg/day) was used. Cyclosporine levels in blood were maintained at 400 ng/mL. Donor animals were anaesthetisized and the heart was harvested through a median sternotomy. The hearts were antegradely perfused with isoosmolar Tyers's solution and were then preserved in a hypothermic storage for two hours at 4°C. In the postoperative period animals were monitored by ECG, heart-rate, body temperature, blood samples for MHC II expression,

cyclosporine levels, CBC plus differential and creatinine daily . Animals in Group A, survived for a period of five days and animals in Group B, where immunosuppressive therapy was applied, survived for five days also. The endomyocardial biopsies (EMB) were taken from the graft's free wall, apex and septum on day one and five for both groups A and B. The evaluation of #120 EMB (biopsies) was done using modified International Society Heart and Lung Transplant Grading Scale.

Results: Results of this experimental endeavour demonstrated that MHC II expression on T-lymphocytes significantly correlates with biopsy findings from apex ($P<0.01$; $R^2 = 0.83$) for Group A (non-immunosuppressed animals) on day five. The combined data for both Groups A and B (non-immunosuppressed and immunosuppressed animals) at this time were also correlated significantly ($P<0.01$; $R^2 = 0.63$) for free wall and ($P=0.01$; $R^2 =0.7$) for apex despite a significant difference between non-immunosuppressed and immunosuppressed animals.

Conclusions: Results of this experimental protocol suggested that there is a significant correlation between MHC II expression on peripheral T-lymphocytes and Grade of rejection assessed by endomyocardial biopsy on day five after surgery. Further studies will be required to establish the long term relationship of MHC II expression on peripheral T-lymphocytes and histological findings, particularly in animals with immunosuppressive therapy.

Table of Content:

List of Tables

List of Figures

Abbreviations:

APC	**Antigen Presenting Cell**
C	**Complement**
CP	**Calcineurin phosphatase**
CTDR	**Cardiac Transplant Research Database**
CR	**Chronic Rejection**
DNA	**Deoxyribonucleotic Acid**
ETWA	**Evoked T-wave Amplitude**
FDA	**Food and Drug's Administration**
FITC	**Fluorescein Isothyocyanate**
FK 506	**Tacrolimus**
FKBP	**Tacrolimus Binding Protein**
HA	**Hyaluronan**
HLA	**Human Leukocyte Antigen**
IFN	**Interferon**
IL	**Interleukin**
ISHLT	**International Society of Heart and Lung Transplant**
IVRP	**Isovolumetric Relaxation Period**
IMDH	**Inosine Monophosphate Dehydrogenase**
Ig	**Immunoglobulin**
KD	**Kilo Dalton**
LV	**Left Ventricle**
MHC	**Major Histocompatibility Complex**

MP	**Mercaptopurine**
MMF	**Mycophenolate Mofetil (MMF)**
MRI	**Magnetic Resonace Imaging**
MZ	**Mizorbine**
PE	**Phycoerythrin**
PET	**Positron Emission Tomography**
RNA	**Ribonucleotic Acid**
S – BQR	**Sodium Brequinar**
SMC	**Smooth Muscle Cell**
TCR	**T-cell Receptor**
TFN	**Tumor Necrotizing Factor**
TVD	**Transplant Vascular Disease**
VTI	**Velocity Time Integral**

Acknowledgment

I would like to express my appreciation to my supervisor, Dr. Karim Qayumi for his guidance, mentoring and support during my Masters program. I also want to say to other friends and colleagues for their help mentoring and support, especially to Dr. Charles Slonecker, Dr. York Hsiang and Dr. John English.

My special thank is being addressed to my best friend and wife, Prof. Nancy J. Hermiston for her love, support, and patience.

I. INTRODUCTION

A major challenge in cardiac transplantation is the prevention of acute graft rejection [1]. Surveillance of the transplanted heart must be performed in order to maintain the recipient at an appropriate level of immunosuppression to protect the allograft from the alloimmune response. Surveillance must also be performed to detect early signs of acute rejection in order to institute immediate changes in case management. Histological grading of the rejection process via examination of endomyocardial biopsy specimens is the current gold standard for diagnosis of rejection and to monitor efficacy of antirejection therapy .[2,3]

Many centers perform routine surveillance biopsies up to eighteen times in the first post-transplant year and annually thereafter. Additional procedures are performed during periods of clinically suspected rejection .[4] Methods that provide immediate, accurate results with limited invasiveness, reduced risk, and are less inconvenient to the patient, are attractive alternatives to numerous endomyocardial biopsies.[5,6]

To date, surface and intracardiac ECGs ,[7,8] echocardiographic parameters, including 2D and Doppler measures of systolic and diastolic function, [9,10,11,12] and radionuclide imaging including ventriculography and antimyosin imaging, [13,14,15] and MRI [16] have demonstrated limited predictive accuracy or practical usage for monitoring and detection of transplant rejection. [4] The method of choice for monitoring post-transplant rejection status for heart transplant patients is the evaluation of multiple tissue biopsies. This diagnostic technique is time consuming, costly and invasive with possible life-threatening complications. Matches between donor and recipient at the MHC locus is the most widely distributed and significant determinant of transplantation success.

We hypothesized that *de novo* MHC class II expression by recipient peripheral T cells, which is a marker of activation, reflects the rejection status of allotransplanted hearts and may

beused as an accurate descriptor of the rejection process. The objective of this project was to determine if MHC class II expression on recipient peripheral T cells correlates with histological findings of rejection after cardiac transplantation.

We found that there is a significant correlation between the MHC II expression on T-lymphocytes in peripheral blood and Grade of rejection in the allotransplanted hearts in the porcine model.

1. HISTORICAL REVIEW

1.1 Transplantation Surgery

Until the 20th century, transplantation efforts were limited to the grafting of tissues, not organs, because no techniques were available to control bleeding during organ resection or to re-establish circulation after transplantation.

Organ transplantation became possible with the development of surgical techniques that permitted blood flow to be interrupted and re-established in the recipient.[17] Dr. Yahoo Carrel, M.D. in France and Father Ditsie Guthrie, M.D. in the USA created the method of anastomosis, joining two blood vessels together to allow blood circulation to "achieve" anastomosis (from Gk, *anastomosis:* the surgical union of parts, esp. of hollow tubular parts).[18] They developed anarterial clamp to prevent excessive blood loss, as well as techniques to hold arteries in place.The first cadaveric kidney transplant in a human was performed in 1936 in an attempt to treat renal failure caused by mercury poisoning, The patient died two days later [19]. Five subsequent attempts during the next decade were surgically successful, but lack of

immunosuppression resulted in organ failure due to rejection [20]. In the 1950s, at least eight attempts at renal transplantation between ABO-compatible subjects in France were unsuccessful.[21]. In 1967, Christian Barnard became fascinated by work with heart transplantation in dogs in the USA. He went to Cape Town, South Africa, and performed the first successful heart transplant in human history. This first recipient died within three weeks due to complications from surgery, but Barnard's second patient lived for seventeen months [22]. At that time, other centres began to perform the procedure. After one hundred and sixty-six heart transplantations had been performed, only 23 recipients were still alive, for an overall mortality rate of greater than 85%, due to complications of rejection[23]. Although practical aspects of organ transplantation improved as surgical expertise developed, it was clear that the ability to achieve long term organ function would depend on the development of methods to control rejection [24].

1.2. The Immunology of Allograft Rejection

Even though the technical/surgical part of transplantation had been mastered, early experiments were obviously doomed to failure because "rejection" was a concept that had not been defined or understood. Histologic analysis performed on some of these early failed allografts demonstrated a pattern of marked inflammation and focal or complete necrosis with occasional thrombosis in rejected tissue, All of these reactions were arising from activation of the host immune response[25].In 1944, Sir Peter Medawar began to describe the rejection process as a reaction of the recipient's immune system to antigens on the donated tissue by a series of experiments on skin transplants in rabbits [26]. Reflection of immune intolerance to foreign tissue has long been visualized using light microscopic techniques. Medawar's work extended these observations to histolopathological descriptions of rejected tissue where a "black band" of lymphocytes was seen infiltrating the dermis of engrafted skin within 1 to 2 weeks of allograft

transplantation. In addition to the formal description of the rejection reaction, Medawar also characterized the decreased time for rejection of a second transplant in an animal sensitized by previous alloantigen sensitization as the "second set response" phenomenon. For these achievements, Sir Peter Medawar has been known as the founder of transplantation immunology[27-8].

Rapaport applied the results of Medawar's work to study the immunology of skin autografts and allografts in humans. [29] These studies provided evidence for the existence of tissue types defined by alloantigens shared between unrelated humans, but on a population basis, differing from individual to individual.

Scientists studying different membrane-bound glycoproteins on groups of leukocytes began collaborating with scientists studying tissue typing. The result of this collaboration was the identification of MHC antigens in humans. In humans MHC molecules are known as Human Leukocyte Antigens (HLA antigens). HLA molecules are the most highly polymorphic group of molecules in humans. To date, application of HLA typing determines donor selection in transplantation. Survival of the transplanted organ depends upon the recipient and donor sharing as many HLA antigens as possible.

In the classic studies of Mann and colleagues, untreated rejection of heterotopic canineallografts was characterized by a prominent cellular infiltrate in the allograft parenchyma.Studies of canine models by Lower and colleagues had indicated that the histopathologic changes of rejection preceded electrocardiographic evidence of voltage loss, and suggested an important role for heart biopsy and rejection surveillance.[26] The most important fact is that to date no indicator of heart allograft rejection in humans has been more sensitive, specific or useful than the endomyocardial biopsy. Rejection, as defined histopathologically, is a very diffuse process. Thus, the likelihood of detecting rejection, when present, has been shown to be

95% when three pieces of bioptome –acquired tissue are evaluated microscopically, whereas the likelihood rises to 98% with four pieces of tissue. [31,32] The sensitivity is greater in the first few months after transplantation when the prevalence of cellular rejection is highest.[3]

1.3. Allograft Rejection

An allograft is an organ or tissue graft from a donor who is of the same species but genetically non-identical to the recipient. An understanding of allograft rejection involves the understanding of the molecular basis of the cells involved in alloantigen recognition and their response to the allograft in an attempt to eliminate it as a foreign invader. In the experiments reported here, the pigs were genetically heterogeneous. The antigens generally capable of eliciting a host rejection response include the HLA, ABO and Lewis blood group antigens, non-HLA monocyte or endothelial cell antigens, and possibly other tissue specific non-HLA antigens[34,35]. The cell surface expression of these different antigenic systems varies among different organs and cells within the same species. The HLA and ABO blood group antigens are the most widely distributed and significant determinants for successful transplantation.[35,36]

There are two major mechanisms and one minor rare mechanism of rejection, each of which involves different immunological effector mechanisms, all of which culminate, in the nonimmunosuppressed host of graft failure.

1.4. The HLA System

The HLA expression is controlled by the highly polymorphic gene loci of the MHC mapped to the sixth chromosome in man. HLA system is divided into two major classes: class I (HLA –A, B and C) and class II(HLA DR, DQ, and DP). It is believed that HLA polymorphism is the result of the evolutionary advantage conferred on the species by a diverse antigen-

processing system capable of interacting with the widest possible range of invading microbes.[35,36] HLA cell surface molecules are important in presenting immunogenic peptides to CD4+ T cells and CD8+ CTL so that foreign cells arising from an allograft can be attacked and eliminated. In allograft rejection, the foreign HLA molecules themselves or HLA bound to processed donor antigens are highly immunogenic and serve as the targets for destruction by host T cells sensitized to these foreign alloantigens.[36,38]

Class I HLA antigens are present on essentially all nucleated cells, although there is some variation in antigen density among different cells and organs. The class I HLA molecules are composed of a 45 kD α heavy chain non-covalently associated on the cell surface with β 2 microglobulin, a 12kD polypeptide which is not coded by HLA genes. Class II HLA antigens under normal circumstances are expressed primarily on antigen presenting cells (APC's) such as dendritic cells, macrophages, and certain specialized epithelial and endothelial cells. These antigens are made up of a 33-36 kD β chain noncovalently linked to a 24-29 kD α-β chain.[3]

The HLA molecules bind foreign peptides for presentation to T-cells (for recognition and activation) in a significantly different way from antibody, which binds much larger conformational antigen epitopes. HLA molecules bind and T-cells recognize and react to antigenic peptides (linear molecules).[40] The cell surface expression of HLA class I and especially class II antigens is affected by cell activation or differentiation. Thus, transplanted cells that may not normally express HLA class II antigens can be induced to increase cell surface expression of these antigens by mediators present at the site of inflammatory responses such as rejection (interferon α, β, γ, TNF-α, TNF-β, and interleukin-4). The increased expression of HLA molecules on the target cell surface probably serves to increase antigen presentation and thus also increases the potential interactions between T cells and immunogenic target antigen.

The HLA molecules themselves are immunogenic as well.[41] Before antigen becomes associated with HLA molecules on the surface of cells, it undergoes processing or degradation

often within the cell that is to express the antigen. There is a suggested difference between antigens which eventually associate either with Class I or Class II surface molecules.[42,43] Endogenous proteins (antigens synthesized within the presenting cell such as viral proteins) areassociated with HLA Class I molecules following intracellular processing.

Immunogenic foreign antigens are not necessarily synthesized within the cell but taken up by the endosomal recycling system and are thought to be mainly HLA class II – dependent proteins.[44] The mature T-cells are represented by two subsets which are phenotypically identified by reaction with different monoclonal antibodies specific for their surface antigens, which are given the cluster designation CD4 and CD8. Both CD4 and CD8 positive T-cells, and are exclusive in terms of their ability to react with class II and class I HLA antigens, a phenomenon known as MHC restriction.[45]

As suggested by many clinical studies, glycoproteins of the donor HLA system play a pivotal role as allogeneic targets in rejection documenting the long term advantages of HLA matching.[46,47] Other studies also suggest that long-term clinical benefits from HLA matching is the reduction of allograft loss during the first five months following transplantation.[48]

1.5. **Immune Recognition and Activation**

Cytokines are a primary signal for the sequential activation and proliferation of T and B cells, both for killing and for antibody production. IL-2 is produced by activated recipient CD4+ Th1 cells after they have recognized the foreign peptide: self HLA complexes expressed on the membranes of professional antigen presenting cells, accompanied by other co-stimulatory events delivered by the same cells. Expression of high-affinity receptors for IL-2 on the membranes of CD8+ CTL's activated by foreign peptide: self MHC complexes are a critical first step in the

proliferation and differentiation of CD8+ cytotoxic T cell clones that will traffic back to the allograft and cause cells in the engrafted tissue to undergo apoptosis.

Antigen presenting cells (APCs) and both helper and cytotoxic T cells play the leading role in the immune recognition or afferent phase of allograft rejection. The normal rate and pattern of lymphocyte migration is disrupted by transplantation, resulting in an increase in the influx rate of white blood cells into the graft.[49] The APCs, a group that includes dendritic cells, macrophages, B cells, endothelial and some parenchymal cells, process antigen so that a peptide of eight to ten amino acids (MHC Class I) or ten to twenty-two amino acids (MHC Class II) HLA molecule for presentation to potentially reactive naïve, mature T cells.[50,51]

The T-cell receptor, the associated signal transduction molecular complex CD3 and the CD4 or CD8 co-receptors are all essential in graft rejection reactions. In nearly all T cells the TCR (T-cell receptor) is composed of two polypeptides, α and β, connected to each other by a disulfide bond and closely associated in the cell membrane with another protein group, the CD3 complex, which is made up from at least 4 distinct signal transduction peptides (ε, γ, δ, and ζ). Each molecule in the CD3 complex contains at least one motif, known as an ITAM, which contains tyrosine residues. A number of additional T cell surface molecules such as LFA –1, CD2, and in particular CD28 serve signaling and adhesion functions vital to both the afferent priming phase and the effector phase of T cell activation and clonal expansion.[52]

1.6. Class II HLA Antigens

The HLA class II antigens are noncovalently bound heterodimeric glycoproteins but they differ in their function, biochemistry, and genetics. MHC molecules and other molecules encoded in a tightly linked complex of genes involved in the processing and presentation of antigens are encoded within the major histocompatibility locus of the pig both α (heavy) and β

(light) chains and are encoded within the major histocompatibility complex.The class II antigens are constitutively expressed on only a limited number of cells in humans, including B cells, monocytes, macrophages, vascular endothelial cells and, dendritic cells. They could be expressed on lymphoid or parenchymal cells during certain inflammatory reactions, especially and typically mediated by γ –interferon.[53] The class II genes occur in clusters of alpha and beta genes in which the number of each can vary.

For the HLA –DR cluster, one invariant α gene is present with several genes. The DRβ1 gene possesses that account for most of the HLA-DR specificities (DR β-1 chain determining specificities DR1, DR2, DR3, DR4, DR5). Other DR β genes are less variable and encode theDRw52 and DRw53 broad specificities. These antigens are analogous to Bw4 and Bw6 in that they are associated with different groups of HLA-DR specificities, but different in that they are not encoded by the same genes as the individual alleles in its cluster. The other major class II loci include DP and DQ, each of which consists of two α and two β genes, not all of which are expressed. Both the α and β genes of DP and DQ express allelic polymorphism.[54] Immune recognition requires that the nominal antigen (peptide) be co-recognized with MHC molecules on the cell surface. It is known that MHC heavy and light chains in conjunction with MHC binding peptides are involved in the conformation, trafficking and recognition of the MHC molecules. In allorecognition T cell clones were able to distinguish MHC/peptide complexes arising from different cell types. Both T and B lymphocytes are involved in generating an immune response to alloantigens. Subsequently, antibodies and /or effector T lymphocytes can generate an immune response to allo-MHC antigens resulting in a cascade of events that ultimately leads to destruction or inactivation of the target.[55]

Recruitment of activated T cells into the graft is used as a diagnostic tool for rejection. In fact, even activated T cells circulating in the host periphery after interacting with the graft are shown to be the best markers of acute renal rejection[56].*Reinke et al* have demonstrated that MHC II antigens are upregulated on the surface of activated circulating cells during rejection episode and propose that determination of circulating MHC II-DR^+ T cells complements biopsy and is very important for clinicians if histology is equivocal[56]. Although the precise role of MHC II on T cells is not well understood, it has been shown to be involved in enhancement of cell to cell interaction between T cells themselves and other inflammatory cells. It also enables to present antigens and enhances the magnitude of the inflammatory cascade.[57]

2. Prevention and Treatment of Allograft Rejection: The Drugs Used in this Study, Their Mechanisms of Action and Toxicology

Immunosuppressive drugs that inhibit recipient T lymphocytes or kill T lymphocytestraveling in the graft constitute the principal treatment regimens for graft rejection. Our current success in immunosuppression results largely from our ability to inhibit and control the activation and differentiation of allograft-specific recipient CD4+ and CD8+ T cell clones with TCR's specific for epitopes from the graft following transplantation.

Initial attempts at immunosuppression were very nonspecific and involved total body irradiation. These experiments revealed that the immune system could be destroyed and thereby made to accept the transplanted organ. Although each agent has its specific actions, all suppress humoral or cell-mediated immunity or both. Patients undergoing immunosuppression are particularly vulnerable to opportunistic infections such as cytomegalovirus, *Pneumocystis carinii*, *Candida albicans*, and *Aspergillus*, and may rapidly succumb to sepsis.[58] This is the downside of immunosuppression.

2.1. Azathioprine

In 1959, Schwartzand and Damashek, while searching for a less toxic substitute to whole body irradiation, discovered that 6-mercaptopurine (6-MP) suppressed the immune response of antigen-primed rabbits when they received subsequent challenges with the priming antigen.[59] Azathioprine, as 6-MP is now called, was first used in clinical organ transplantation in 1963. The compound is a purine anti-metabolite that is converted by the cytochrome oxidase system or hepatic Cytochrome c in the liver to 6-MP.

It is a strong but non-specific immunosuppressive agent which inhibits both DNA and RNA synthesis, blocks *de novo* purine nucleotide synthesis, and causes breaks in chromosomal DNA.[60] Azathioprine, therefore, inhibits clonal expansion of lymphocytes activated by antigens expressed by and presented on engrafted cells. The efficacy of azathioprine as an immunosuppressive agent is due to inhibition of lymphocyte proliferation.[61]

The primary toxicity of azathioprine is evident in the bone marrow, where it causes leukopenia and thrombocytopenia. Other major adverse effects are on the gastrointestinal system, where it produces nausea, vomiting, and symptoms of pancreatitis and hepatitis.[62] Periodic monitoring of blood cells and platelet counts, as well as serum chemistries for pancreatic enzymes and liver function, are recommended for timely detection of toxicity.[63]

2.2. Corticosteroids

Corticosteroids are used in the organ transplant regimen at low doses as part of allograft maintenance protocols in combination with other drugs, and at high doses for induction of immunosuppression in the engrafted recipient.

Corticosteroids cross the cell membranes, combine with specific binding proteins, and are then transported across the nuclear membrane to sites near cytokine genes. The steroid/protein complex inhibits the transcription of the genes and the production of cytokines.[63] Treated macrophages cannot release IL-1[64] and IL I also provides a signal for activated T-cells to express the high affinity form of the IL-2 receptor. IL 6 may supply a necessary coordinating signal. Without a high-affinity IL-2 receptor, an antigen-binding T cell will not clonally expand and differentiate. IL-2-activated CD4+ Th1 cells also secrete interferon γ (IFN-γ), which induces macrophages to synthesize more IL-1 and IL-6, so corticosteroids also immunosuppress via feedback inhibition.[65]

Corticosteroids also inhibit macrophage release of the inflammatory mediators leukotriene B4, thromboxanes and chemokines, which are chemotaxic for neutrophils and effector CD4+ Th1 T cells. Cytotoxic responses directed at engrafted cells in a tissue are thus suppressed.[66]

After an antigen-specific immune response has been initiated, the acute inflammation that produces allograft damage is maintained by non-specific release of cytokines, complement activation, and the activation of the clotting cascade. Therefore, the anti-inflammatory effects of corticosteroids remain crucial even when rejection is ongoing.[65]

2.3. Cyclosporine

Cyclosporine A was introduced for clinical use in 1978. By the mid-1980s' most immunosuppression protocols were cyclosporine-based.[62] Derived from a fungus *olypocladium inflatum,* cyclosporine is a cyclic decapeptide.[63] Both cyclosporine A and tacrolimus block T-cell proliferation by inhibiting the phosphatase activity of a Ca^{2+} dependent enzyme called calcineurin at nanomolar concentrations. Calcineurin has a major role in transmitting signals

from the T-cell receptor to the nucleus. Both drugs reduce the transcription of several cytokine genes that are normally induced on T-cell activation, including interleukin-2 (IL-2).

Cyclophilin, a specific cyclosporine binding protein, has been identified in both the cytoplasm and nucleus of T-cells.[65] Cyclophilin is one of the peptidyl-prolyl *cis –trans* isomerases, a family of cytoplasmic enzymes which plays an important role in normal protein folding during protein synthesis.[64,67] The interaction between the drug-binding protein complex and the relevant genes results in the inhibition of IL-2 transcription.[68,69] As a result of cyclosporine binding cyclophilin loses its activity. Thus, cyclosporine inhibits a cytoplasmic signal required to initiate normal cytokine gene transcription in the nucleus. Cyclosporine is a potent inhibitor of almost all the known cytokines, including IFN-γ and IL-2 through IL -4.[65]

Although cyclosporine does not directly block macrophage production of IL-1, it may reduce the production of this cytokine by inhibiting T cells through the production of interferon gamma.[70] Thus, cyclosporine, while targeting a different cell and cytokine, produces feedback inhibition similar to that of corticosteroids.[65] Therefore, cyclosporine and corticosteroids synergistically inhibit recipient immune responses. This provides a rationale for their combined use in clinical transplantation.

Cytokines are a primary signal for the sequential activation and proliferation of T and B cells, both for killing and for antibody production. IL-2 is produced by activated CD4+ Th1 and Th2 cells after they have been activated by foreign peptide: self HLA complexes expressed on the membranes of professional antigen presenting cells and other co-stimulatory events. Expression of high-affinity receptors for IL-2 on the membranes of CD8+ CTL's activated by the same peptides on recipient MHC molecules is a critical first step in the proliferation and differentiation of CD8+ cytotoxic T cell clones that will traffic back to the allograft and cause cells in the engrafted tissue to undergo apoptosis (programmed cell death).

The most common adverse effects of cyclosporine on organ systems of the body are hyperkalemia, hypertension, hyperuricemia, nephrotoxicity and a variety of infections. Other problems with less incidence (less than 20%) include hepatotoxicity, tremors, hirsuitism, gingival hypertrophy, anorexia, gout, paresthesia, and hypomagnesemia.[58,62]

2.4. Other Immunosuppressive Agents

Immunosuppressive regimens based on cyclosporine, azathioprin, and corticosteroids are associated with significant drug toxicity, resistant acute rejection and opportunistic infections. Relatively nontoxic alternatives to the cytotoxic class of drugs can now be used for immunosuppression in transplant patients. Fewer complications are associated with tacrolimus, sodium brequinar (S-BQR), rapamycin, mycophenolate mofetil (MMF), and mizoribine.

2.4.1. Tacrolimus (FK 506)

Discovered in 1984, FK 506 received FDA approval in 1994 for use in the USA. FK 506 is a macrolide lactone, a metabolite extract from the filamentous bacterium *Streptomyces tsukubaensis,* found in Japan. It inhibits the proliferation and differentiation (priming) of CD4+ Th1 and Th2 T cells and CD8+ CTL's (cytotoxic T lymphocytes). The inhibited lymphocytes include those which are specific for foreign epitopes on the allograft. Tacrolimus penetrates the cell and it specifically inhibits synthesis of interleukins (IL-2 ,-3 and -4), colony stimulating factors, and IFN-γ.[71] FK 506 binds to a cellular protein. The FK 506 binding protein (FKBP), and the bound complex interferes with the activity of calcineurin phosphatase (CP). CP is an enzyme that is part of the biochemical cascade that transduces activation signals from the surface of a cell to its nucleus, causing the transcription of genes for cytokines. By interfering with CP,

FKBP effectively reduces expression of IL –2 and other cytokines, thereby blocking T-cell activation. This is a very selective action, because only the T-cell dependent immune responses are blocked, while the T cell-independent B-cell responses are preserved. T cell stimulation through alternative pathways (CD28) is also not affected.[74,76] The adverse effects profile of FK 506 is similar to that of cyclosporine, including nephrotoxicity, neurotoxicity, and new-onset diabetes.

Neurotoxicity extends from mild symptoms of tremor, headaches, and insomnia to syndromes of dysarthria, seizures and coma. Like cyclosporine, there are three clinical settings in which the nephrotoxicity of FK 506 commonly occurs: (1) in the immediate post-surgical period where graft ischemia associated with the vasoconstrictive effect of FK506 may occur; (2) with concurrent administration of other nephrotoxic agents; and (3) with chronic renal insufficiency due to progressive interstitial fibrosis.[71]

2.4.2. Sodium Brequinar (S-BQR)

S- BQR was originally developed as an antimalignancy drug, and was later discovered to have immunosuppressive properties. It has been extremely effective in synergistic combination with cyclosporine in experimental conditions, and whether the synergism exists in clinical transplant situations remains to be evaluated.[72] S-BQR inhibits the action of dihydro-oronate dehydrogenase, an enzyme involved in the *de novo* biosynthetic pathway of pyrimidines involved in the synthesis of DNA and RNA. Inhibition of the *de novo* synthesis of uridine also blocks the salvage pathway and decreases the function of adhesion molecules, because glycosylation of these molecules requires the pyrimidine nucleotides.[73]

Adverse effects of S-BQR include thrombocytopenia, desquamative maculopapular dermatitis, mucositis, and gastrointestinal side effects.[71]

2.4.2. Rapamycin (Sirolimus)

Rapamycin, also known as sirolimus, was isolated from another *Streptomyces hygroscopius,* an actinomycete called "Rapa Nui" by the Easter Island natives.[60] Rapamycin has a structural similarity to FK 506, being a macrolide, but it does not appear to interfere with cyclosporine A activity. Rapamycin affects both T and B cells directly by preventing cytokines from activating these cells, an action that is uniquely different from that of cyclosporine.[74] Rapamycin and FK 506 are reciprocal antagonists of IL-2 secretion and should not be used in combination. There is no evidence to suggest that rapamycin works in synergy with cyclosporine in animal models.[71]

The full spectrum of activity of rapamycin is not known. Rapamycin can inhibit mitogenic responses in cultured fibroblasts, hepatocytes, endothelial cells, and smooth muscle cells as well as lymphocytes. Because the results of human trials are still pending, we must look at results of trials in experimental animals for a toxicity profile. Dogs are unusually sensitive to the toxicity of rapamycin, whereas in other animals the toxicity has been restricted to weight loss, testicular atrophy, and lethargy. Rapamycin is known to be diabetogenic in rodents, but not in nonhuman primates.[75]

2.4.4. Mycophenolate Mofetil (MMF)

MMF, previously identified as RS 61443, is a semisynthetic derivative of the active agent mycophenolic acid (the active agent), isolated from the mold *Penicillium glaucum.*[71] MMF has been used for more than two decades in the treatment of psoriasis and has been proven to be safe. MMF suppresses immunologic rejection responses by inhibiting the enzymes involved in the purine salvage pathway, thereby reducing the availability of purines for DNA synthesis.

Lymphocytes activated by engrafted antigens have a high proliferative index and this drug inhibits their proliferation and differentiation into effector cells.[71] MMF *in vivo* is phosphorylated to become mycophenolic acid, which noncompetitively inhibits the enzyme inosine monophosphate dehydrogenase (IMDH). IMDH is involved in the *de novo* synthesis of guanine.[76] The drug can be administered orally, with high bio-availability, and it is eliminated in the bile.[71] However, because the drug is very effectively inactivated, it has a low potency, and it binds with low affinity to IMDH.[76] For effectiveness, it is important for patients to maintain immunosuppressive levels of the drug in the blood. Furthermore, inadequate doses could lead to T- and B- cell proliferation because they have been held in check at a late stage of activation.[76]

The majority of side effects are minor gastrointestinal ones. Upper respiratory tract infections and herpes viruses (HHV's) infections also have been reported.[71] Animal studies reveal that side effects include anemia in rats; leukopenia, diarrhea and anorexia in monkeys; and gastrointestinal hemorrhage in dogs.[70]

2.4.5. Mizoribine (MZ)

Mizoribine, also known as Bredinin, has been used in Japan, but not widely. Like MMF (mycophenolate mofetil), mizoribine is a pro-drug that needs to be phosphorylated to be active. Mizorbine, like MMF, competitively inhibits IMDH. So far no studies have proved any kind of advantages of mizoribine over drugs in the current therapeutic armamentarium. Toxicity of mizoribine is especially evident in dogs, who developed hemorrhagic enteritis and erosive mucosal lesions and, when on high doses, endotoxemia.[76]

2.4.6. Other Therapeutic Options

Those include prostaglandin E, lipoxygenase inhibitors, and the older, but still effective, antithymocyte globulins. Still another therapeutic option available is leflunomide, which suppresses lymphocyte activation by inhibiting Lck, Fyn and ZAP-70, all tyrosine kinases involved in signal transduction after phosphorylation of ITAM's and signal transduction which results in the transcriptional activation of the IL-2 gene and the α chain of the high-affinity IL-2 receptor.

2.5. Nonpharmacological Therapies

Among the non-pharmacological options available are techniques such as total lymphoid irradiation, photo-chemotherapy, thoracic duct lymphocyte depletion and pre-treatment of the graft with a monoclonal antibody (like OKT3) specific for the extra-cellular aspects of the CD3 complex followed by complement.

3. Pathology of Heart Allograft Rejection

The immune intolerance and its reflections had been visualized for many years with the light microscope. Later on, with interest in biopsies of the human heart in the 1950's, and the invention of the transvenous bioptome in Japan, the possibility of monitoring heart allograft rejection became feasible in the 1970s'.

Among the many pioneering contributions of the Stanford Heart Transplant Program, the most important was the modification of the transvenous bioptome by Philip Caves and its application to human heart allograft rejection.[77] Secondly, the biopsy became the Gold standard in the management of heart allograft recipients, and is still being used today. Thanks to Dr. Margaret Billingham,[78] the classification for the heart allograft rejection status was established

and is being used to guide clinical management of heart allograft recipients. The high sensitivity of the endomyocardial biopsy (generally 85%-100% for three to five pieces of endomyocardium) in detection of cellular rejection relates to the universality of alloantigens in every region of the endomyocardium. The sensitivity is greater in the first few months after transplantation when the prevalence of cellular rejection is highest.[77]

3.1. Hyperacute Graft Rejection

Hyperacute rejection is characterized by thrombotic occlusion of the graft vasculature that begins within minutes to hours after host blood vessels are anastomosed to graft vessels. This reaction is mediated by pre-existing antibodies in the host's circulation that bind to the donor's endothelial antigens.

Hyperacute rejection is very rare. Vascularized organs (especially heart and kidney) are at risk for hyper-acute rejection if the patient has in his blood preformed donor-specific alloantibodies. This humoral presensitization (or alloimmunization) may be caused by a previous transplant, blood transfusion or pregnancy. The primary target of the donor–specific antibodies is the vascular endothelium of the transplanted organ. The most common target antigens are HLA class I, ABO system, but other less well defined antigens have also been considered. HLA class II antigens seem less relevant in hyper-acute rejection because they are not strongly expressed on the vascular endothelium. Antibodies mediating hyper-acute rejection are almost always complement-fixing and they could be IgG or IgM type or both. Alloreactive T lymphocytes are occasionally involved.

A critical step in alloantibody-mediated rejection is the initiation of the complement cascade. This involves the binding to and activation of C1q, which requires at least two complement-fixing sites on the antigen/antibody complex. This requires a close proximity

between antigens and no complement activation will occur if the antigen density is too low on the cell surface. Activation of the complement cascade leads to the release of various inflammatory mediators and the initiation of the coagulation and fibrinolytic systems. Hyper-acute rejection is manifested by rapid vascular constriction, edema and thrombotic occlusion. Endothelium stimulation and exposure of subendothelial basement membrane proteins activate platelets which then adhere and aggregate in the vasculature. Soon afterward, polymorphonuclear leukocytes (PMN) adhere to the vascular endothelium.

3.2. Acute Rejection

Acute rejection is a process of vascular and parenchymal injury mediated by T cells and antibodies that usually begins after the first week of transplantation. Acute allograft rejection remains one of the leading causes of death and morbidity in cardiac transplantation.[78,79] The incidence of rejection reported in a cohort of seventeen hundred patients transplanted between 1992 and 1994 in the Cardiac Transplant Research Database (CTDR), was 1.4 - 1.5 rejections per patient in one year. Forty-two percent of the patients were free of rejection, 31 % had only one rejection episode, and 27 % had more than one rejection at one year. The incidence of hemodynamic compromise was 8%. Twelve percent of patients received cytolytic antibody therapy for the treatment of rejection.

Significantly, there is no simple biochemical marker of cardiac rejection, and over 90% of episodes of acute cellular rejection are not accompanied by any cardiac symptoms or measurable change in graft function. This is, in part because biopsies are performed on a routine protocol basis in an attempt to detect rejection before it induces cardiac dysfunction. The entireapproach of using protocol biopsies to diagnose rejection in cardiac transplantation is based on the very high mortality associated with the development of hemodynamic compromise or

evidence of significant graft dysfunction in heart transplant recipients[80]. Data from a review of three thousand three hundred and thirty-seven patients transplanted between1990-1994 showed that patients with hemodynamic compromise had a 40% mortality in the six months following such an episode and up to 50% by one year. Ten percent of patients treated with cytolytic therapy for hemodynamic compromise in this series had no histologic evidence of rejection on the biopsy[81]. Acute rejection is mediated by lymphocytes that have become alloactivated against donor transplantation antigens.

In vivo stimulation of alloreactive T cells takes place primarily in the peripheral lymphoid tissues of the recipient although intragraft sensitization may occur. The strongest stimulus is provided by donor dendritic cells (also referred to as passenger leukocytes) present in the allograft which will enter the circulation and end up in the lymphoid tissues of the recipient. These dendritic cells function as antigen presenting cells and provide a strong stimulus to MHC class II-restricted CD4+ cells, which can stimulate the growth and differentiation of HLA class I reactive CD8+ cytolytic lymphocytes.

Alloreactive lymphocytes enter the circulation and react with antigens on allograft vascular endothelium, the primary target of the initial stage of cellular rejection. The lymphocyte- endothelial interactions depend on the expression of appropriate target antigens on the endothelium. HLA class I antigens are expressed at the cell surface whereas HLA class II antigens expression must be induced by various cytokins, especially gamma interferon. Furthermore, adhesion molecules (such as integrins, selectins) play an important role in the cell surface interactions between lymphocytes and endothelium cells, and in their activation and release of cytokines and inflammatory mediators. These processes lead to the migration (extravasation) of lymphocytes through the vascular wall.

Graft-infiltrating lymphocytes mediate various effector mechanisms of allograft immunity. Besides having a direct cytotoxic effect on graft parenchymal cells, lymphocytes may mediate a delayed type hypersensitivity mechanism of graft rejection. The latter involves therecruitment of macrophages and NK cells. Varying proportions of CD4+ and CD8+ lymphocytes are found in cellular infiltrates of rejecting allografts. Many of them express T cell activation markers, like IL-2 receptors and through release of gamma –interferon and other cytokines; there is an increased expression of HLA antigens (especially class II) on the vascular endothelium and other target cells in the graft parenchyma.

Consideration must be given to additional immunological mechanisms such as graft injury secondary to infection, especially those caused by viruses. This means that other types of lymphocytes may infiltrate the graft and could mediate immune effector responses against virus-infected cells within the graft. Another possibility is that graft-infiltrating lymphocytes mediate the recurrent autoimmune disease process.

Severity of rejection is defined histopathologically by the number of infiltrating inflammatory foci associated with the increasing amount of parenchymal, vascular, and myocyte damage. In very severe forms of rejection there are found phenomena such as edema, necrosis and hemorrhage. The classification of acute rejection was originally proposed by Dr. Billingham in the 1970's and later simplified and unified by ISHLT. Besides the usual development of a rejection, defined as acute rejection, there could also develop conditions which are not as well understood called hyper-acute rejection and chronic rejection.

When rejection is due to preformed alloantibody (presensitization), it can result in very rapid graft loss in what has been called hyper-acute rejection.[82] The role and importance of alloantibodies directed primarily against MHC class I has been studied using animal models and in clinical studies as well.[83,84] Antibodies reactive with MHC II class antigens have been found in transplant recipients and have been shown to cause acute graft loss.[85] Both CD4 (MHC class II

reactive and CD8 (MHC class I reactive) T cells are important to rejection and each have over-lapping or exclusive effector roles.

Our current success at immunosuppression results largely from our ability to inhibit and control the changes within rejection reaction effector cells following immune stimulation. The initial attempts were very non-specific and involved total body irradiation. Those experimentsrevealed that the immune system could be destroyed and thereby made to accept the transplanted organ; however, the recipient remained defenseless to infection and rapidly succumbed to sepsis.[86] Current methods to prevent graft rejection primarily involve the induction of energy in potentially alloreactive T cells through specific immunosuppressive agents.[61]

3.3. Chronic Rejection

Transplant vascular disease (TVD) is one of the major causes of morbidity and mortality in long-term survivors of heart transplantation. TVD is the most serious clinical manifestation of chronic rejection process. Chronic rejection (CR), a process involving progressive developmentof fibrosis and vascular stenosis, is responsible for the majority of late allograft failures. The pathogenesis of CR is believed to be a cycle of recurrent immunologic injury followed by an injury repair process. During the injury repair process, vascular smooth muscle cells (SMCs) proliferate, migrate to form a neo-intima. The generation of myofibroblasts and synthesis of matrix lead to a scar, which, after repeated cycles of injury and repair, enlarges and compromises vascular flow. These SMCs preferentially express a number of genes that cause the secretion of extracellular matrix proteins. Some of these regulate cell motility. One of these is the hyaluronan (HA) receptor RHAMM. Interference with RHAMM:HA interactions after vascular injury may limit SMC proliferation and migration, thereby reducing the vascular narrowing associated with chronic rejection.

3.4.1. Histopathology of Hyperacute Graft Rejection

The histopathology of hyperacute rejection is manifested by the following findings:

1. Thrombosis and edematous changes in the rejected organ
2. No pronounced infiltration of mono-nuclear cells
3. Deposits of immunoglobulin, complement C3 and fibrinogen (immunostaining)

Antibody –mediated rejection may also occur without thrombotic occlusion. In this case, the histological pattern is a vasculitis which may lead to necrosis of individual cells of graft blood vessels. Because lymphocytes may be also involved, this process is referred to as acute vascular rejection.

3.4.2. Histopathology of Acute Rejection

Histopathology of acute cardiac rejection is fully described and presented by histopathologic criteria summarized in ISHLT grading table (Grade 0 – Grade 4). Table No.1

3.4. Histopathology of Chronic Rejection

The histopathology of chronic rejection is characterized by following findings:

1. Progressive fibrosis of endocardium, myocardium, epicardium, and epicardial coronary intima.
2. Increasing frequency of B-cells and plasma cells in endocardium, epicardium, and epi-cardial coronary intima.
3. Myopathic myocyte enlargment, myocyte nuclear prominence and hyperchromatism, and myocyte atrophy with interstitial and replacement fibrosis.

4. The Diagnosis of Cardiac Allograft Rejection

Until 1978, the diagnosis of cardiac allograft rejection was made largely by summated R-wave voltage on the electrocardiogram.[78] The presumption was that rejection was associated with the development of myocardial edema causing a loss of voltage. The introduction of transvenous biopsy of the heart by Caves in the mid –1970s, significantly increased the sensitivity and specificity of making the diagnosis of rejection, and has become the Gold standard for establishing the diagnosis of rejection in cardiac transplant recipients.[87,88]

On the other hand, there have been a number of non-invasive markers of rejection that have been suggested over time for non-invasive diagnosis of rejection.[78] This includes placement of catheters into the heart at the time of transplantation to directly record intramyocardial electrograms which can be read by telemetry while the patient is asleep to measure any decrease in voltage in the heart.[89-91] This approach has been particularly applicable in children where a biopsy may be technically difficult, and has been shown to have high sensitivity and specificity in the limited trials to date.[91]

The second area of significant investigation has been the use of echocardiography to measure a number of parameters of systolic and diastolic function[92]. This approach has not been broadly adapted due to the impact of changes in preload and heart-rate on these measurements. Echocardiography is a primary method of following children, where alterations in posterior wall thickening are usually the primary indication to warrant invasive biopsy.

A number of nuclear techniques have also been evaluated, including radio labeled antimyosin antibodies, PET scanning, and MRI imaging to assess myocardial water edema[93] . These tests have also not had significant clinical utility. Most recently, the rise in plasma concentration of Troponin T, a cardiac-specific isoform used to diagnose acute myocardial infarction, has been shown to have some correlations with cardiac rejection.[94]

The problem with all of these immunologic markers is that they were highly sensitive and specific when correlated with biopsy evidence of rejection only within the first three months post-transplant, limiting the utility and diagnostic accuracy of these tests[80].

4.1. Cardiac Catheterization

Cardiac catheterization is the general name for a group of procedures in which long, thin tubes (catheters) are placed in the heart and its adjacent blood vessels. The tubes may be hollow or solid. The hollow tubes allow measurements of pressures, injection of fluids such as X-ray dyes, and or withdrawal of blood. The solid tubes contain wires for recording electrical activity and or for pacing the heart. Specialized instruments, such as bioptomes, permits one to obtain a tiny piece of heart muscle for laboratory study.

4.2. Endomyocardial Biopsy

Endomyocardial biopsy involves the use of a flexible bioptome to obtain tissue samples from the right (and occasionally left) ventricle of the heart. The heart is approached via a transarterial or transvenous heart catheterization. Endomyocardial biopsy results in a small tissue sample (average size 1 to 2 mm); multiple samples (usually 4 to 6) are required because pronounced topographic variations may be found within the myocardium. Endomyocardial biopsiesare typically performed for the monitoring of cardiac allograft rejection and anthracycline cardiotoxicity, and in detecting infiltrative disorders of the myocardium (i.e. myocarditis).

4.3. Classification of Allograft Rejection by Histopathologic Criteria

The International Society of Heart and Lung Transplantation has established a biopsy grading classification, that is based on the amount of cellular (lymphocytic) infiltration and the presence or absence of myocyte necrosis.This classification divides biopsies into mild [(1a and b) Grade 1], focal (Grade 2), diffuse moderate[(3a)Grade 3] or diffuse severe rejection [(3B and 4) Grade 4].

In essence, rejection severity is measured histopathologically by increasing numbers of infiltrating inflammatory cells and increasing number of inflammatory foci, associated with an increasing amount of parenchymal and vascular damage and, in most severe forms, often accompanied by an appearance of edema and evidence of hemorrhage. As such, the histopathologic severity of inflammation in acute rejection follows a logical geographical algorithm, progressing from perivascular and interstitial aggregates and, ultimately, to widespread mixed cellular infiltrates.

4.4. Artefacts

The standard range of bioptome and biopsy-related artefacts apply to the study of specimens from heart allografts. Recollection of the normal constituents of the endomyocardium is essential to the process of evaluating biopsy specimens from allografts. These normal features include the endothelial lining, stromal fibrous proteins, like collagen and elastin, smooth muscle cells, fibroblasts, and pericytes, Thebesian venous channels, small arteries, arteriols and capillaries,myocardial fibres including Purkinje fibres, and resident must cells, lymphocytes and monocytes.

Biopsy sites–the histopathology of biopsy site reflects the acute injury of the bioptome followed by healing and repair. Fresh microthrombus may be found overlying areas of acute

myocyte necrosis, focal hemorrhage and leukocytic infiltration when a repeated biopsy is performed within a few days of the previous biopsy.

Endocardial fibrous tissue-may ensue from several healing processes including acute rejection, biopsy sites and mural thrombus.

Adipocytes–are associated with the myocytes and connective tissue of the endomyocardium. The adipose tissue may be extensive at times and may masquerade as a site of biopsy-induced epicardial perforation. The extent of endomyocardial adipose tissue is determined in part by the use of corticosteroids in immunosuppressive therapeutic regimens, in part by a tendency to cardiac adiposity in some people, and in part by the process of healing upon repeated injury.

Myocardial calcification may be found in biopsy specimens, or on autopsy examination of heart allografts. The significance of such calcification is unclear, although global myocardial ischemia at the time of harvest or later during hemodynamic instability may be a factor.

Opportunistic infections - they may"masquerade"as alloreactive inflammatory process. Bacterial, fungal, viral, and protozoal infections may involve more than 50% of the transplant population. Systemic infections derived from respiratory and urinary tracts or skin are most common. Bacterial and fungal infections typically elicit a mixed inflammatory infiltrate pre-dominated by polymorphonuclear leukocytes. The infiltrate associated with viral or protozoal infections may be quite reminiscent of rejection.

Quilty effect- the prominent, nodular, endocardial infiltrates described by Margaret Billingham are well known in cyclosporine treated patients[95]. These infiltrates are primarily mononuclear (B-lymphocytes) with occasional prominent p-lasma cells. Typically, there are well vascularized with blood vessels lined by prominent endothelium. The size of infiltrates varies

from tiny to very large and the border between the endocardial infiltrate and the underlying myocardium may be smooth and distinct or may suggest an "invasive" **endocardial infiltrate.**

II. HYPOTHESIS

We **hypothesize** that MHC II expression on T-lymphocytes in peripheral blood is an indicator of rejection status in the allotransplanted heart

III. OBJECTIVE

The objective of this study was to investigate if MHC II DR – beta expression on peripheral T lymphocytes correlates with rejection status in a porcine heterotopic allotransplanted heart model.

IV. SPECIFIC AIMS

1. To design an experimental model for heterotopic heart transplantation and to identify 2 groups of animals (A – non-immunosuppressed; B- immunosuppressed) in this experiment.
2. To identify an accredible method for quantitative evaluation of cardiac biopsies.
3. To quantify the expression of MHC II DR – beta lymphocytes in peripheral blood.
4. To develop less invasive diagnostic method for early determination of rejection status in organs after the transplantation.
5. To identify the relationship between the grade of rejection and MHC II DR – beta lymphocytes in peripheral blood.

V. EXPERIMENTAL DESIGN

Twenty-four 3-months old female domestic swine *Sus scrofa domesticus,L.*, each weighing approximately 27 to 30 kg's, were used in this study. The animals were randomly subdivided into two experimental groups. The aim of the first experiment (group A)was to establish what correlation, if any, exists between acute allograft rejection and the upregulation of the expression of the MHC Class II histocompatibility molecule DR –beta on peripheral T lymphocytes in recipient animals that had not received immunosuppressive treatment. This control group (A) consisted of 6 age–matched donors and 6 age-matched recipients and did not receive immunosuppressive drugs prior to or post-transplantation.The aim of the second experiment (group B) was to determine what correlation, if any, exists between graft rejection and the expression of the MHC Class II molecule DR-beta on peripheral T lymphocytes in recipient animals that had received immunosuppressive treatment prior to and during the post-transplantation period until the time of sacrifice.

Pilot study

The heterotopic heart transplantation was performed in both immunosuppressed and non-immunosuppressed groups with *ex vivo* preservation times of the donor hearts between one and half to two hours at 4^0 C in hypothermic storage. Although surgical procedures are routinely performed in our laboratories, our project was proceded by a pilot study using eight experimental animals, four donors and four recipients. During the pilot study we had been developing the most reliable transplant procedure examinig following options: First approach. Upper –middle laparotomy the graft was implanted into the recipient's retroperional space, with the donor ascending aorta anastomosed end to side with the recipient's abdominal aorta and the donor pulmonary artery anastomosed end to side with the recipient's inferior vena cava. Second approach. The incision was made in the right flank of the abdomen between the iliocostal muscle

and external oblique abdominal muscle in order to prepare a „pocket“ between the external and internal abdominal muscles for the graft. The donor-ascending aorta was anastomosed end to side with the recipient's abdominal aorta and the donor pulmonary artery was anastomosed end to side with the recipient's inferior vena cava.

The second approach was chosen as a standard surgical procedure for our experiment.

The reasons were:

1. Less invasive and therefore less risk for the postoperative complications for the animal.
2. Better conditions for postoperative care and monitoring of the animal.

Assessment parameters included biopsies, blood sampling and monitoring of vital and physiological signs. All animals were maintained in accordance with the guidelines of the Canadian Council of Animal Care under the supervision of the Animal Care Committee of the University of British Columbia.

VI. RATIONALE

The rationale for the use of a large animal model were:

1. This was considered as a preclinical trial; hemodynamic and functional measurements are easier in large animals;

2. The amount of tissue and blood required for biochemical and pathophysiological analysis of this project can not be acquired from a small animal;

3. Only in the large animal, according to this type of project, is the evaluation and correlation of functional, biochemical, and morphological analyses possible.

The rationale for using swine included:

Swine have enzymatic similarities, such as the presence of tissue xanthine oxidase, amount of red and white blood cells, blood volume, to humans.

1. It was simple to control sex, age, type, and size for optimal modeling.
2. Our laboratory have had extensive experience with the use of swine for our other experimental models.

The induced expression of MHC II –DR-beta molecules in peripheral T lymphocytes was used as a measure of T cell activation because in our previous studies, using the swine model of single lung transplantation, we observed that MHC II DR –beta molecules increase their surface expression in the host peripheral T-lymphocytes[97]. Other studies proved that increased expression of MHC class II antigen occurs during cardiac and lung allograft rejection.[96,98] The expression of this antigen in T –cells influences diverse aspects of the immune response system including stimulation of the mixed lymphocyte reaction[99], interaction of T cells – T cells and T cells-monocytes[100-1], and the ability of T cells to present antigen in alloantigen-specific immune response[136] . MHC II antigen has been shown to be upregulated on the surface of activated

circulating T cells during the rejection episode[102] . Determination of circulating MHC II – DR^+ T cells are proposed to complement biopsy in renal transplantation and is shown to be very important for clinicians if histology is equivocal.[102]

In solid organ transplantation the histological analysis of allograft biopsies remains the "gold standard" for diagnosing rejection.[103] Therefore we have chosen the endomyocardial biopsy evaluation as a tool for the assessment of grade of rejection.

VII. MATERIALS AND METHODS

1. Surgical Techniques

In the donor procedure using the midline longitudinal mediastinal sternotomy, the heart is harvested with large vessels including the aortic arch, pulmonary arteries up to the entrance to the lungs (hilum), pulmonary veins and their branches, inferior and superior vena cava, and azygos and hemiazygos veins had been ligated.

After the vessels were mobilized, the place for aorta cross-clamping was prepared. Heparine sodium (300 IU/kg) was administered intravenously. Following the procedure of anterograde crystalloid cardioplegia, the cannula was inserted into the ascending aorta and supplied with cardioplegic Tyer's solution. After cross-clamping the aorta, IVC/SVC were ligated immediately and 1000 mL of cold Tyer's solution was administered to arrest the heart.All the vessels were ligated. The heart was removed from the chest and placed in 1000 ml Tyer's solution maintained at 4^o C.

In the recipient procedure the incision was made in the right flank of the abdomen between the iliocostal and external oblique abdominal muscle in order to prepare a "pocket" between the external and internal oblique abdominal muscle, for the graft. Through the retro-peritoneal approach, IVC and the descending (abdominal) aorta were mobilized. After the

donor's heart was prepared, all the branches of the aorta and pulmonary artery were ligated, leaving the main stump of the aorta. The animal was heparinized (300 IU/Kg) and the descending aorta of recipient was partially clamped and anastomosed to the ascending aorta of the donor heart. The sidewall clamp was removed and air was evacuated from the hole in theascending aorta of the donor heart. The hole was ligated. The same procedure was repeated with the anastomosing pulmonary stump to the IVC. The cross clamp was removed, then the heart was reperfused and subsequently defibrillated. A 6 mm diameter Hortex prosthesis was anastomosed to the right atrium of the donor heart and to the subcutaneous tissue of the posterolateral abdominal wall area. It served as a "tunnel" for the introduction of the bioptome into the right side of the engrafted heart. The wound is drained through the contra-aperture underneath. The neck approach was taken in the external jugular vein introducing the 2-3 lumen catheter for blood sampling and administration of drugs. The tip of the catheter was tunneled just under the skin to the posterior triangle of the neck near the ear where it was externalized.

2. Positioning of the Bioptome for Endomyocardial Biopsy

This procedure consists of the following steps. The bioptome is inserted via the external jugular vein, the ima vein, and the superior vena cava into the right atrium with the tip pointed toward the lateral wall of the right atrium. At the level of the mid-right atrium, the bioptome is rotated anteriorly approximately 180^0 and advanced through the tricuspid valve apparatus toward the right ventricle. The bioptome is advanced to the interventricular septum with the jaws opened.

3. Technical Considerations

Proper handling of endomyocardial biopsy specimens obtained by standard bioptome catheters from the right ventricular septum is imperative in efforts to issue accurate diagnoses. Three to six samples of endomyocardium, each with a minimum dimension of 1.5 to 2 mm, should be obtained and fixed in 10% neutral buffered formalin. Tissue is processed for paraffin embedding and serially sectioned at 4 microns thickness throughout the block. Five slides with three to four levels each are stained with H&E; with two complementary slides stained with Masson's trichrome. This is the minimum recommendation for adequate assessment of rejection. Fibrotic samples, fatty samples, and clots can be readily distinguished from endocardium with considerable myocardial tissue. Electron microscopy and immunohistochemistry are not necessary for the pathologic diagnosis of rejection, although they may be included in certain research protocols. The nature of the specimens available for diagnostic pathologic examination will change considerably over time in a given patient. It will include many chronic changes. Such changes may indicate that the pathologist will stay in constant contact with transplant physicians regarding each patient's clinical progress.

3. Parameters of Assessment

The investigational methods included blood sampling, endomycocardial biopsy collections and physiological monitoring. The peripheral blood samples were centrifuged to separate white blood cells for the further measurements which were done by two-color flow-cytometry analysis obtaining MHC II DR-beta intensity on peripheral T-Lymphocytes. This was done to assess cyclosporine levels in blood, using fluorescence polarization, and competitive binding radioimmunoassay, to evaluate CBC and differential using cell counter, and to assess creatinine levels in peripheral blood by calorimetric analysis using picric acid. Endomyocardial

biopsies were taken from the free wall, apex and septum in the right ventricle. Physiological monitoring included ECG, heart rate, respiratory rate, temperature, behavior, food and water intake. Both blood sampling and physiological monitoring were performed on daily basis and EMBs were taken on day 1 (day of transplant) and day 5 (last day after transplant).

5. Blood Sampling Protocol

1. The catheter tip was cleaned with an alcohol swab before and after each insertion.
2. Blood was obtained from the distal lumen of the brown-topped catheter.
3. 20 cc and 10 cc syringe with an 18-gauge needle were used. In the event of a problem, i.e.,an agitated pig, a short extension catheter (butterfly) was used for safe blood sampling to avoid personal injury or the injury to the pig.
4. Approximately 15 cc of heparinized saline (Heparine and 0.9% NaCl physiological solution) was drawn and used to flush the catheter.
5. A 20 cc syringe was used for withdrawing blood, once inserted into the catheter. Clear the syringe with one or two withdrawals and reinsertion of blood. Fill three 7 cc's yellow-topped ACD tubes for MHC II –T-lymphocyte monitoring.
6. Six to eight cc of blood was withdrawn. Approximately 2 cc of this blood was placed into two EDTA 3cc purple-topped tubes. These blood samples were used for determining CBCD (differential) and cyclosporin A levels.
7. The remaining blood was placed into 10 cc SST tube (golden-topped). This sample was used for the determination of urea, bilirubin, ALT, AST, and creatinine (for liver and kidney function) content..
8. After the blood was drawn, catheter was flushed with a bolus of heparinized saline under positive pressure, using the 20 cc syringe.
9. 2 cc of pure heparin was used as a heparin "lock" to prevent occlusion of the catheter from thrombi or coagulation. After harvest, all peripheral blood samples were centrifuged through a Ficoll Hypaque gradient in order to crudely separate white blood cells for further measurements.

6. Intravenous Drug Administration

1. The catheter tip was cleaned with an alcohol swab after each use.
2. The proximal (white topped) lumen of the catheter was flushed with 5 to 10 mL heparinized saline after each drug administration.
3. A heparin "lock" was placed after the last drug administration (2 to 3 mL of pure heparin in the 5cc syringe).

7. Immunosuppression / Drug Regimen

7.1. Prophylaxis

Commencing both donor and recipient , Ceftin (Cefuroxime acetyl) 500 mg, BID, p.o. was administered 2 to 3 days before surgery as a prophylactic treatment.

7.1.1. Day 1

On day 1 (day of surgery) antibiotics such as Ancef, Flagyl and Gentacidin had been administered in the following order: Ancef (Cefazolin sodium) 15 mg /Kg, BID,I.V. First dose was administered after induction of anesthesia. The second dose of the identical amount, I.V., was administered at 11:00 p.m. Optionally, this dose was administered post-surgically via I.M. route. Flagyl (Metronidazole) 20 mg/Kg - commencing on the day of surgery. Gentacidin (Gentamycin sulfate) 2-4 mg/Kg (I.M. or I.V). This antibiotic was used as an adjunct only if infection was suspect/occured.

7.1.2. Immunosuppressive Drugs

Imuran (Azathioprine) 3 mg/Kg, p.o. was administered prior to induction of anesthesia. Infusion time for administration of CS-A (6 mg/Kg) had been over 2 to 4 hours. Blood sample was taken 30 minutes after completion of CS-A administration to determine peak level of CS-A in peripheral blood.Solumedrol (Methylprednisolone): **TD** = 14.3 mg/Kg x Mr (TD = total dose; Mr = the mass of the recipient animal) had been administered according to following regimen on the day 1 (day of surgery):

- ½ TD administred I.V. at induction of anaesthesia
- ½ TD administred I.V. at reperfussion of the implanted organ
- ½ TD administred I.V. 8 hours after surgery; approximately 11:00 p.m.

7.2. Day 2

07:00 a.m. the following drugs had been administered:

- Cefazolin sodium, 15 mg/Kg, I.V.
- Flagyl, 20 mg/Kg, p.o.
- CS-A, 6 mg/Kg, I.V. (infusion time over one hour; glass bottles)
- Methylprednisolone, 1/8 TD, I.V.
- Azathioprine, 3 mg/Kg, p.o.

03:00 p.m.

- Methylprednisolone, 1/8 TD, I.V.

07:00 p.m.

- CS-A, 6 mg/Kg,I.V. (infusion over an hour, glass bottle)
- Cefazolin sodium, 15 mg/Kg, I.V.

7.3. Day 3

07:00 a.m. the following drugs had been administered:

- CS-A : 5.5 mg/Kg; I.V.
- Azathioprine : 3 mg/Kg; P.O.
- Methylprednisolone : 1 mg/ Kg; I.V.
- Metronidazole : 20 mg/Kg, P.O.
- Cefazoloin sodium : 15 mg/Kg; I.V.

07:00 p.m. Cyclosporine in infusion over an hour and Cefazoline sodium had been administered:

- CS-A : 5.5 mg/ Kg, I.V.
- Cefazolin sodium : 15 mg/Kg; I.V

7.4. Day 4

On the day 4, the drug regimen protocol was identical with drug regimen for day 3, by which the levels of CS-A were maintained at the concentration of 350 – 450 ng/mL in plasma.

7.5. Day 5

Day of sacrificing the experimental animals (no drug administration was used)

8. Flow Cytometry

8.1. Procedure for Cell Preparation

Blood samples (18 mL) were collected in an acid citrate dextrose (ACD) vacutainers and diluted with equal volume of phosphate buffered saline (PBS). The diluted blood was slowly added to 10 mL of Ficoll-hypaque (Gibco/BRL) in 15 mL sterile tube. Samples were centrifuged for 30 min. at 1500 rpm and washed twice with TC 199 mixed with 2% Fetal calf serum (FCS) for 15 min. at 1500 rpm.

8.2. Procedure for Freezing Cells

Sterile technique was used throughout the procedure. The minimum cell concentration for freezing was $5x10^5$ cells/mL. The maximum was 1x10x8 cells/mL. For our purpose the amount $2x10^6$ of cells/mL was chosen. Cell suspension were frozen in 1ml aliquots. FCS and DMSO were added as 20% and 10% of the total volume.

Example: cell count 2.25×10^7 cells /mL

volume 0.98 mL

Total cells $2.21x10^7$ cells

If the volume of the cell suspension was increased to 2 mL, the cell concentration would be 1.1×10^7 cells/ml. Since cell suspensions were frozen in 1 mL aliquots:

20% FCS	0.4 mL
10% DMSO	0.2 mL
Cell suspension	1.4 mL
Total	2.0 mL (i.e. 2 aliquots)

The cell suspension was mixed and placed on ice. Sterile vials were stored at -70^0 C. When the cell suspension was chilled to 4^0 C, DMSO was added drop-wise while mixing into the cell suspension. The cells were dispensed into the vials. The vials were placed into cryo-container and placed in -70^0 C freezer.

8.3. Antibodies and Dilution Assays

8.3.1. Antibodies

The primary monoclonal antibodies were obtained from VMRD Inc., Pullmann, Washington.

Four different types of antibodies were used:

1. anti-CD3 antibody (8E6;i.e. T-lymphocyte marker)
2. anti MHC II –DR-beta (MSA30)
3. anti-CD 45 (leukocytes)
4. antimonocytic antibody [Swine workshop cluster (SWC 1)].

The Gout anti-mouse (GAM) were obtained from Caltag, Inc.,and included:

1. FITC labelled IgG 1(used for CD3 staining)
2. PE labelled IgG 2a (used for MHC II staining)
3. FITC labelled IgG and IgM (used for leukocyte and monocyte staining).

8.4. FACS Analysis:

Two million cells were used for each antibody staining. Cells were incubated with primary antibodies at 4^0 C for 30 minutes, and then stained with secondary antibodies at 4 degree

Celsius for 30 minutes. After each incubation, cells were washed with 2% TC199 for 15 minutes and centrifuged twice at 1500 rpm. A maximum of 10,000 cells were counted by the flow cytometer. Leukocyte population was identified using swine anti-CD45 and anti-monocytic antibodies. Two secondary goat-anti mouse antibodies labeled with phycoerythrin (PE) and fluorescein isothyocyanate (FITC) were used for staining the lymphocytes bound to the primary antibodies.

8.5. Evaluation of Endomyocardial Biopsies

ISHLT modified evaluating criteria for determining the Grade of rejection in histopathological specimens.

8.6. Histopathology of the Transplanted Hearts

8.6.1. Specimen Preparation

1. Three to six EMB samples were obtained from the right ventricle samples of EMB were obtained from right ventricle (septum, free wall, apex)
2. The minimum dimension of the sample were 0.15 to 0.2 cm
3. Samples were fixed in 10% neutral buffered formalin (formaldehyde)
4. Tissue was processed for paraffin embedding and serially sectioned at 4 microns thickness
5. Slides were stained with H&E and two complementary slides are stained with Masson's trichrome (This was a minimum recommendation for adequate assessment of rejection).
6. Slides were evaluated at the pathology laboratories at Saint Paul's Hospital, Vancouver.
7. The specimens were evaluated by two independent pathologist, both blinded, according to modified ISHLT scale. (Table 1).

Table No. 1

ISHLT Grade	Histologic Appearance	Interpretation
0	No cellular infiltrate	No acute rejection
1 a	1 focus cellular infiltration without myocyte necrosis	Mild acute rejection
1b	> 1 focus of cellular infiltration without myocyte necrosis	Mild acute rejection
2	1 focus of cellular infiltration with myocyte necrosis	Focal moderate acute rejection
3a	2 foci of cellular infiltration with myocyte necrosis	Diffuse moderate acute rejection
3b/4	>2 foci of cellular infiltration with myocyte necrosis and myocardial edema	Severe acute rejection

VIII. Statistical Analysis

Statistical analysis was performed by non-parametric analog of unpaired t-test (Mann Whitney U-test) and multiple regression analysis for between a comparison of groups and correlation of different variables, respectively. The data are presented as Mean (plus – minus) standard deviation. $P<0.05$ is considered to be significant.

The use of parametric analysis requires the data to have normal distribution and equal variances ion groups. Mean and standard deviation are the key elements of parametric statistics. Since our data consisted of six observations per group only, one can not rely on parametric statistics because such a small sample of data does not necessarily demonstrate whether the distribution is normal or skewed. If the data deviates from normality, interpreting the mean and

standard deviation in terms of normal distribution produces a misleading picture. Therefore, the type of analysis such as non-parametric statistics, which use the ranks of observations rather than the actual measurements become suitable. Non-parametric statistics retain much of the information about the size of responses without making any assumptions about the distribution of the data. Non-parametric methods provide one with the opinion of assuming as little as possible when analyzing the data. Non-parametric statistical methods are 95% as powerful as parametric methods when the data are from normally distributed populations and more reliable when the data are not from normally distributed populations. Multiple regression analysis is always being used under the circumstances that not allow the scientist/biostatistician to predict the type of correlation and its development. I.e. there is no possibility to predict if the further relationship will be linear, logarithmic, or exponential.[142]

IX. Results

In the control group (A), results of our investigation of non-immusuppressed animals demonstrated no significant difference in the first two days (first and second day) after transplantation in comparison to the time before the transplant. However, the MHC II expression on peripheral T - lymphocytes increased on days 4 and 5 indicating a significant difference in comparison to pre-transplantation time and the first two days after transplantation. On the other hand, MHC II expression on peripheral T-lymphocytes in animals with immunosuppressive therapy showed a different feature. The MHC II expression on peripheral T-lymphocytes had a tendency to decrease on day 3 after transplantation and demonstrated a significantly different level on day 5 after transplantation in comparison to pre- transplantation period of time. Figure 1 depicts the comparison between the non-immunusuppressed and immuno – suppressed groups (A and B) on day 5. The MHC II expression on peripheral T –lymphocytes manifests a significant difference [($P< 0.03$) T-test] between groups A and B on day 5 after the transplantation. (Figure 1).

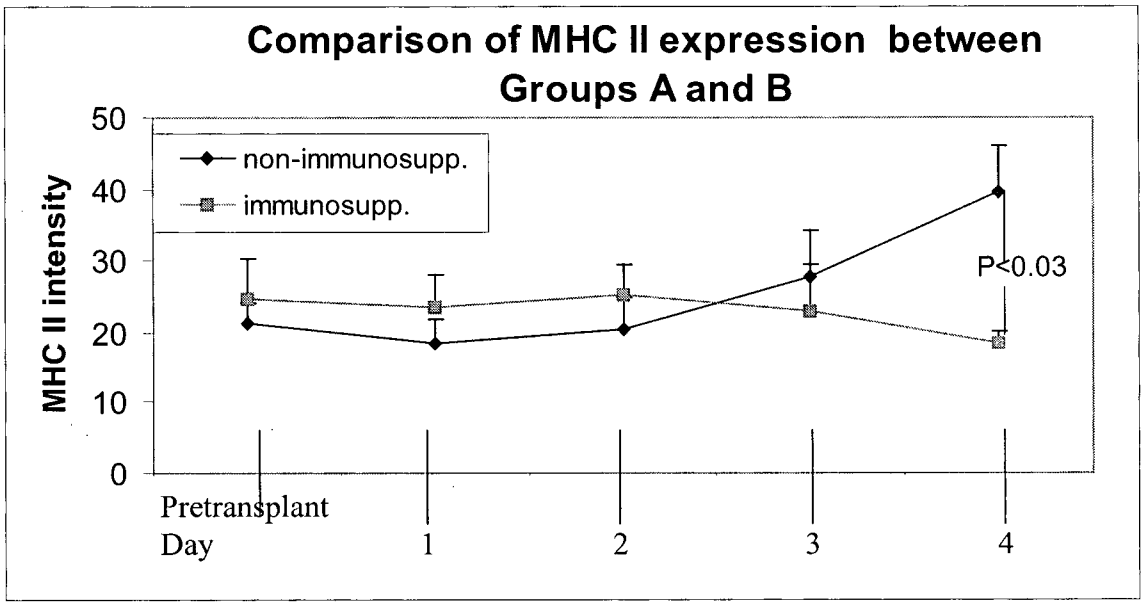

Comparison of MHC II expression between Groups A and B
MHC II intensity
50
40
30
20
10
0
non-immunosupp.
immunosupp.
P<0.03
Pretransplant
Day
1
2
3
4

Figure No.1

Results of the morphological assessment of the heart tissue obtained from ventricular wall, septum and apex are depicted in figure 2. These demonstrate a significant difference [septum: ($P<0.05$); apex: ($P<0.03$); free wall: ($P<0.01$)] in the grade of rejection between the groups A (red) and B (blue) [immunosuppressed and non- immunosuppressed animals] in all three regions of heart. Histological findings (rejection grades) for groups A and B are shown in the tables 2 and 3.

Figure No. 2

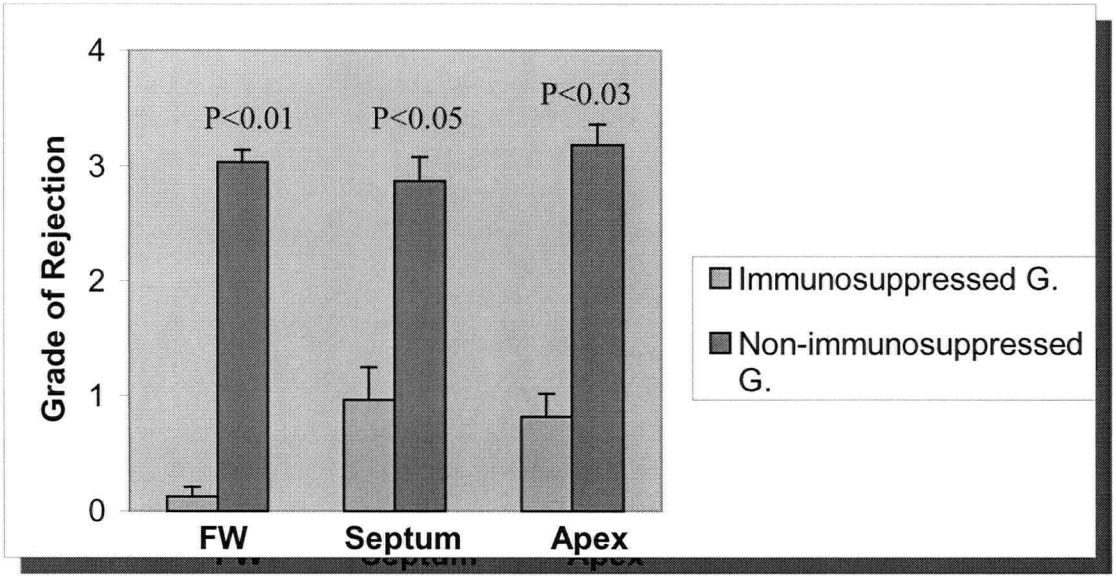

Grade of Rejection
4
3
2
1
0
P<0.01
P<0.05
P<0.03
FW
Septum
Apex
Immunosuppressed G.
Non-immunosuppressed G.

Table No. 2

(Grade of rejection in non-immunosuppressed animals- Group A on Day 5)

Group A	Apex	Free wall	Septum
Mean	4.033	4.006	3.752
St.deviation	0.582	0.321	0.594
St. error	0.238	0.131	0.242
Biopsy 1.	5.000	4.000	4.500
Biopsy 2.	4.000	3.400	3.375
Biopsy 3.	4.000	4.125	3.166
Biopsy 4.	3.500	4.350	4.500
Biopsy 5.	3.400	4.000	3.570
Biopsy 6.	4.300	4.180	3.400

Table No. 3

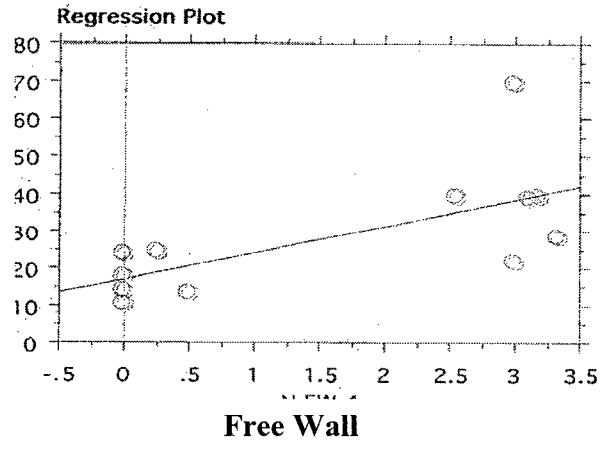
(Grade of rejection in immunosuppressed animals- Group B on Day 5)

Group B	Apex	Free wall	Septum
Mean	1.500	1.117	1.883
Std. Deviation	0.593	0.204	0.884
Std. Error	0.242	0.183	0.361
Biopsy No. 1	1.000	1.000	3.000
Biopsy No. 2	2.600	1.000	3.000
Biopsy No. 3	1.600	1.000	1.500
Biopsy No. 4	1.000	1.000	1.000
Biopsy No. 5	1.500	1.500	1.500
Biopsy No. 6	1.300	1.200	1.300

Since the aim of the study was to evaluate the relationship between the expression of MHC II on the peripheral T-lymphocytes and the grade of rejection in the transplanted organ, we performed the regression analysis in order to define this relationship.The regression analysis for both A and B groups had shown significant correlation between the histological findings from free wall and apex and MHC II expression on peripheral T-lymphocytes in our study. Regression analysis between the histological findings from septum and MHC II expression on peripheral T-lymphocytes did not reveal any significant correlation at all.

Values (Groups A and B together) of MHC II expression on the peripheral T - lymphocytes (on the Y axis) were plotted against the grade of rejection from the free wall heart tissue (X axis). Results of this regression analysis demonstrated a significant correlation between the expression of MHC II on the peripheral T-lymphocytes and degree of rejection ongoing in the free wall of the heart. ($R^2 = 0.683$; $P<0.01$); Figure 3; Table 4,5.

Figure No.3



Results of regression analysis performed on combined data (both groups A and B) where MHC II expression on the peripheral T-lymphocytes (Y axis) had been plotted against the grade of rejection from apex (X axis) demonstrated a significant correlation between the expression of MHC II on peripheral T-lymphocytes and degree of rejection. (R^2 = 0.734; R = 0.857; P<0.01).Figure 4; table 4,5.

Figure No. 4

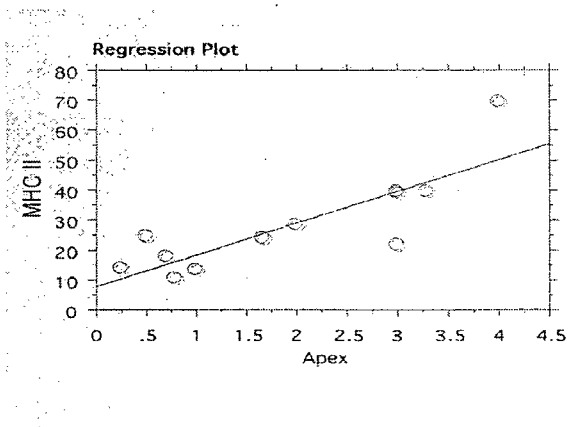


Table No. 4

MHCII expression in non-immunosuppressed animals – Group A

Group A	Day I	Day II	Day III	Day IV	Day V
Mean	21.383	18.567	20.450	27.932	39.550
Std. deviation	6.667	8.347	11.011	15.383	16.288
Std. Error	2.722	3.408	4.495	6.280	6.650
Animal No. 1	30.700	26.400	11.500	54.800	69.300
Animal No. 2	18.100	22.600	39.100	30.900	38.700
Animal No. 3	28.200	28.100	28.500	30.800	39.550
Animal No. 4	20.800	8.300	16.800	23.993	28.500
Animal No. 5	14.100	10.500	14.000	16.300	21.700
Animal No. 6	16.400	15.500	12.800	10.800	39.550

Table No. 5

MHC II expression in immunosuppressed animals – Group B

Group B	Day 1	Day 2	Day 3	Day 4	Day 5
Mean	24.833	23.529	25.278	22.960	18.542
Std. deviation	13.776	10.937	10.155	16.193	4.201
Std. Error	5.624	4.465	4.146	6.611	1.715
Animal No. 1	46.000	23.500	24.200	8.200	17.900
Animal No. 2	14.000	2.600	25.400	22.600	23.800
Animal No. 3	12.000	30.200	30.400	53.600	14.000
Animal No. 4	29.000	33.800	15.210	21.500	15.900
Animal No. 5	34.000	26.180	41.820	10.770	23.650
Animal No. 6	14.000	25.270	21.090	16.000	14.640

X. Discussion

Survival after cardiac transplantation has improved steadily over the past decade, with mortality at 1 year falling to as low as 10% in many centres.[103-4] This improvement in survival is due to better, more specific immunosuppressive agents such as cyclosporine and antilymphocyte antibodies. [95,104-5] Furthermore, early diagnosis of rejection by surveillance of endomyocardial biopsy, contributed to this improvement.[106-7] This early diagnosis is exceedingly important, and most clinicians experienced in transplantation are aware of the problem (very high mortality in patients with acute rejection associated with consequent allograft dysfunction).

Unfortunately, current procedures for this early diagnosis involves the purchase of up to twenty biopsy procedures for each patient, within the first year after cardiac transplantation. Furthermore, patient discomfort and potential risk result from the multiple invasive procedures. Accordingly, the search for non-invasive techniques for the diagnosis of cardiac allograft rejection continues in order to invent a reliable non-invasive technique for graft rejection diagnosis with full benefit for patients and health care professionals.

A perfect screening test for cardiac allograft rejection should be easy to administer repetitively, and should be non-invasive, low cost, and most importantly, it should have a high sensitivity. The need for specificity varies, depending on implications for further diagnosis. In the diagnosis of cardiac allograft rejection, the screening test must be nearly 100% sensitive, given the potentially fatal implications of missing acute rejection. Even if specificity is only 50%, this would result in a decrement of one half of the biopsies performed on a routine basis.[103]

Three general classes of possible screening tests for cardiac rejection are cardiac functional assessment, biochemical/immunological assays and myocardial imaging.

It has long been believed that systolic function as assessed by ejection phase indexes is neither sensitive nor specific for allograft rejection.[108-9] This has led to several studies investigating the use of diastolic indexes of left ventricular function as being potentially more sensitive in this

diagnosis.[110] It was first demonstrated that echocardiographic derived indexes of diastolic function were abnormal in patients with acute allograft rejection.

Later *Derumeaux et al* [110] proved that Doppler tissue imaging, using ejection fraction and diastolic myocardial wall velocity as diagnostic variables, could be a new tool for non-invasive acute rejection diagnosis. *Stork et al*[111] proved that sonographic evaluation of LV diastolic function PEV (peak velocity), PHT (pressure half-time), VTI- (velocity time integral) of early mitral flow, and IVRP (isovolumetric relaxation period) helps to detect cardiac rejection early and decreases the frequency of myocardial biopsy. In transplant recipients, significantly higher values than in 22 age-matched healthy controls were found for PEV (71 versus 56 cm/s; $P< 0.01$), PHT (51 versus 43 ms; $P<0.001$), VTI-E (72 versus 57 mm; $P<0.001$), and IVRP (90 versus 73 ms; $P<0.001$). During rejection, heart rate increased significantly from 78 to 91 beats per minute ($P<0.01$). Furthermore, a significant decrease was found for PEV from 73 to 63 cm/s ($P<0.01$), for PHT from 52 to 40 ms ($P< 0.001$), for VTI-E from 75 to 61 mm ($P<0.001$), and for IVRP from 90 to 74 ms ($P<0.001$) during cardiac rejection. Thus, sonographic evaluation of LV diastolic function helps to early detect cardiac rejection and to decrease the frequency of myocardial biopsy but can not be used for a detection for certain grade of rejection.

One exclusive study suggested that pacemaker evoked T-wave amplitude (ETWA) may be a sensitive non-invasive marker of cardiac allograft rejection. This clinical study conducted on 45 recipients with median duration of follow-up of 129 days showed the sensitivity value of 55% and specificty of 62%. [112] Another study showed that there are ECHO detectable differencesbetween rejection and non-rejection hearts. Since variations of ECHO variables are large, rejection may not be determined by ECHO alone. Thus a biopsy may still be required for final diagnosis of rejection especially in patients with confusing clinical findings.[113]

Subsequently, several scientists[114-16] demonstrated, that diastolic functions were prolonged in rejecting patients. These groups reported sensitivities from 78% to 88%.[114] Another

study, using radionuclide ventriculographic indexes of diastolic function reported similar results.[118] In addition, contaminating factors such as donor-recipient size matching may play an important role in diastolic properties of the allograft.[118-19]

Few animal studies had been carried out. Everett had conducted two different studies.[120] The first study demonstrated that intramyocardial unipolar peak-to-peak amplitudes obtained from plunge electrodes in the canine orthotopic heart transplant model are highly sensitive and specific (100%) for diagnosing rejection. The second study showed that R-wave amplitude analysis remains an accurate non-invasive means for the early detection of cardiac allograft rejection and should allow more selective use of EMB in the heterotopic heart transplant canine model.[121]

Imaging and characterization of the myocardium to diagnose rejection have been attempted including echocardiographic tissue characterization,[122] backscatter analysis,[123] indium labeled white cells, [124] and magnetic resonance imaging.[125] *FW Smart*[126] reported that serial magnetic resonance imaging with monitoring of change in signal intensity is a potential, non-invasive method of assessing clinically significant tissue rejection in patients with heterotopic hearts that are stable more than one month after transplantation. Although preliminary results in animal models have been promising, the results in patients have yielded sensitivities that are inadequate. Detection of myocardial rejection is difficult in patients with heterotopic heart transplantation because of the complex vascular anatomy present after transplant surgery. To determine whether magnetic resonance imaging might be useful for the assessment of heart rejection, eight patients with heterotopic heart transplantation were serially studied on 27 occasions In 30% instances a significant change in the magnetic resonance imaging signal occurred without clinical or biopsy evidence of rejection and vice versa. Thus, this study could be evaluated inadequate because of high degree of false positive or false negative results.

The development of hybridoma technology by Kohler and Milstein[127] in 1975 not only led to their receiving of Noble prize but also to the development of monoclonal antibodies currently in clinical use.[103-4] One of the antibodies is the monoclonal antibody to cardiac myosin developed by Khaw and colleagues. The antibody itself or its Fab fragment has been found to myocytes in which the sarcolema is no longer intact and when labeled with Indium 111 has been demonstrated to be useful in the diagnosis of myocardial infarction[128] and myocarditis. *Yasuda T. Ballester, and Frist* [129-31] have extended their experience using indium labeled antimyosin antibodies in the diagnosis of acute cellular cardiac rejection. Average values for sensitivity in this studies were 90% and for specificity only 60%.

Frist et al.[131] conducted 20 studies of 7 days to 9 years after transplantation.

to evaluate antimyosin imaging as non-invasive means of detecting human cardiac rejection, the Fab fragment of murine monoclonal antimyosin antibodies was labeled with Indium-111 and given intravenously to 18 patients (age 45 +/- 12 years). The sensitivity, specificity, and overall accuracy of the technique were 80%. *Ballester and col.*[132] in their initial experience of 53 studies in 21 patients, an abnormal antimyosin uptake ratio (1.55) yielded a sensitivity of 95% for the diagnosis of rejection requiring treatment (moderate or severe rejection). The specificity was quite low (29%), with a majority of patients with no rejection also exhibiting antimyosin uptake ratios out of the normal range. In a follow up study of patients at least one year after cardiac transplantation, a negative antimyosin antibody scan assured the absence of rejection requiring treatment (sensitivity=100%), with four of the eleven patients with positive scans demonstrating clinically significant rejection (specificity=33%). Specificity, as anticipated, increased with an increasingly abnormal scan. Thus, both early and late biopsies after cardiac transplantation, appears to be nearly 100% sensitive but not specific. Based on these data, approximately one third of all biopsies on stable patients could therefore be avoided.[133]

The third major area of investigation has attempted to directly assay for increases in allo-immunologic activity. Study conducted by *RG Masters*[134] and colleagues suggested that brain natriuretic peptide (BNP) plasma levels might be a potential screening test for acute cardiac rejection. Generally, the plasma level associated with a rejection period is higher than 400 pg/mL at the stage of acute mild rejection (lymphocytic infiltrate without myocyte necrosis).[133-35]With the further understanding of immunologic activation, lymphocytes subsets, and activation markers, along with the development of flow cytometry techniques, several groups have investigated the possibility that the expression of activated antigens on circulating lymphocytes might predict rejection.[136-8]

Although Garner and his colleagues reported a sensitivity of 94% for the detection of rejection using cytoimmunologic monitoring, his results have not been confirmed by Roodman and May. McGhie[140] conducted the canine study using 111 Indium labeled antibody. This study proved that radiolabeled monoclonal antibodies to MHC II antigen can detect cardiac allograft rejection in this large animal model.

Carlquist and his colleagues[140] in their study on humans found that there is not highly significant correlation between class II antigen and interleukin –2-induced lymphocyte proliferation during acute cardiac rejection (35%-65%). In 1998 Winkler and col.[141] conducted a study analyzing MHC II expression on circulating T cells by flow cytometry in patients with acute rejection after organ transplantation. Peripheral lymphocytes obtained during serial blood drawings from 206 patients after kidney, heart or liver transplantation were analyzed for HLA – DR expression on CD8+T cells by two-color cytometry. Patients were investigated during stable course as well as during episodes of graft rejection or infection. The immunosuppressive the HLA-DR expression pattern observed. The significant increase in HLA-DR expression was observed during acute biopsy-proven graft rejection. In contrast to patients after liver transplantation, in kidney or heart transplant recipients with acute graft rejection the increase in

HLA-DR expression failed to reach statistical significance. In addition to this, an increase in HLA-DR+CD8+T cells was also detectable during bacterial or viral infections.

As mentioned above, in the past two decades many animal experiments investigating the relationship between the immune response to rejection and the grade of rejection have been conducted. The purpose of these studies was to find a method of early detection of acute, subacute and chronic stage rejection of transplanted organs. Current detection methods still depend on readings done by transplant pathologists. Three general classes of possible screening tests for cardiac rejection are cardiac functional assessment, biochemical/immunological assayes and myocardial imaging. Few studies had proven that increased level of MHC II antigen can detect cardiac allograftbeta antigens can up-regulate after the transplantation[97].

In our research project, the goal was to find a method to detect early stage of rejection, i.e. within the first five days, which was less invasive than current practices, caused less danger and discomfort to the patient and was more economical for medical institutions to perform.In our study we have proven that :

1. The MHC II expression on peripheral T-lymphocytes between the non-immunosuppressed and immunosuppressed groups (A and B) demonstrates a significant difference ($P<0.03$) on day 5.

2. Results of the morphological assessment of the heart tissue's ventricular wall, septum and apex demonstrate a significant difference in the grade of rejection between the non-immunosuppressed and immunosuppressed groups (A and B) on day 4 after transplantation [free wall: ($P<0.01$); septum: ($P<0.05$); apex: ($P<0.03$)].

3. There is a significant correlation between the grade of rejection and MHC II expression on T-lymphocytes in peripheral blood:

A. Regression analysis of combined data (Groups A and B) between MHC IIexpression and grade of rejection from the free wall [R^2 =0.683; P<0.01]demonstrated a significant correlation

B. Regression analysis between MHC II expression and grade of rejection in non immunosuppressed animals (Group A) demonstrated a significant correlation [P<0.01; R^2 =0.827]

C. Regression analysis of combined data (Groups A and B) between the MHC II expression and the grade of rejection in apex demonstrated a significant correlation as well [P<0.01; R^2 =0.734].

In summary, with this information provided by a simple blood test we were able to determine the grade of rejection and hence the quantity of immunosuppressive medication required to treat the patient. We were also able to easily monitor the effectiveness of that medication and the change in the grade of the rejection of the transplanted organ. The animal study has been conducted for a relatively short period of time (5 days per animal – 24 animals in total). Because of financial constraints we were not able to keep the animals for more than a five-day period. The results of this short study were so encouraging that a longer study of either small or large animals followed by clinical trials would be required to investigate the long term effectiveness and feasibility of this new diagnostic tool. This new technique is less invasive and very safe for the patient. Based on our results the technique is highly sensitive and specific. The current diagnostic techniques have, in average, an 80% sensitivity and specificity. Our results predict the possibility of almost 90% sensitivity and specificity for this new technique. It would be possible to extend this study for detection of rejection in transplanted organs such as liver, kidneys and pancreas. In addition the cost effectiveness of this technique could be of great

interest to medical institutions. For the above reasons we would highly recommend continuation of this study.

XI. Conclusion

During the early stages after transplantation (day one to day five) there is a significant correlation between the grade of rejection and MHC II expression on peripheral T-lymphocytes.

Based on the results of this study it can be speculated that:

1. This method can be used clinically as a less invasive indicator of rejection for patients after transplantation.
2. This method can be used to modulate immunosuppressive therapy more vigorously and cost- effectively.
3. Early deduction of rejection and modulation of immunosuppressive therapy with this less invasive and less costly method may be beneficial for the transplant patients and costless for the health care system.

XII. Bibliography

1. Balk AH et al. Effect of adopting a new histological grading system of acute rejection after heart transplantation. Heart. 1997; 78 : 603-60
2. Billingham ME et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group: the International Society For Heart Transplantation. J Heart Transplant.1990; 9:587-593
3. Winters GL, McManus B. Consistencies and controversies in the application of the International Society for Heart and Lung Transplantation working formulation for heart transplant biopsy specimens: Rapamycin Cardiac Rejection Treatment Trial Pathologists. J Heart Lung Transplant. 1996; 15:728-735
4. Morgan DC et al: New method for detection of heart Allograft rejection: validation of sensitivity and reliability in a rat heterotopic allograft model. Circulation, 1999; 100:1236 –1241
5. Winters GL. The challenge of endomyocardial biopsy interpretation in assessing cardiac allograft rejection. Curr Opin Cardiol. 1997; 12:146-152
6. Carrier M. Noninvasive assessment of cardiac transplant rejection:a critical look at the approach to acute rejection. Can J Surg. 1991; 34:569-572
7. Warnecke H et al: Noninvasive monitoring of cardiac allograft rejection by intramyocardial electrogram recordings.Circulation. 1986; 74(suppl.5, p. 2):PIII-72-III-76)
8. Everett JE et al. Noninvasive diagnosis of cardiac allograft rejection in an orthotopic canine model.Ann Thorac Surg.1996; 62:1337 – 1340;discussion 1340-1341

9. Popp RL et al. Ultrasonic studies for the early detection of acute cardiac rejection. Transplantation.1971; 11: 543-550

10. Mannaerts HF et al. Pulsed-wave transmitral Doppler do not diagnose moderate acute rejection after heart transplantation.J Heart Lung Transplant. 1993; 12:411-421

11. Gilberto GR et al. Acute rejection after heart transplantation: non-invasive echocardiographic evaluation. J Am Coll Cardiol. 1994; 23:1156-1161

12. Vion-Dury J et al. Early detection of heart transplant rejection using cardiac echography combined with with the assay of glycosylated residues in plasma by proton NMR spectroscopy. C R Acad Sci III. 1992;315: 479-484

13. Mullins PA et al. The use of exercise technetium –99m 2-methoxy-isobutyl-isonitrile (99mTc Sestamibi) perfusion scanning in the detection of acute rejection after cardiac transplantation. J Nucl Biol Med. 1992; 36:2-6

14. Frist W et al. Noninvasive detection of human cardiac transplant rejection with In-111 antimyosin (FAB) imaging. Circulation. 1987:76(pt. II): V-81-V.

15. Bocchi EA et al. Comparison between two strategies for rejection detection after heart transplantation: routine endomyocardial biopsy versus Gallium –67 cardiac imaging. Transplant Proc. 1997; 29:586-588

16. Doornbos J et al. MR imaging in assessment of cardiac transplant rejection in humans. J Comput Assist Tomogr. 1990: 1477-81

17. Carrel A. The transplantation of organs. N Y Med. 1914; 99:839-846

18. Guthrie CC. Blood vessel surgery and its application. New York, NY: Longmans Green; 1912:88

19. Hamilton DN, Reid WA. YuYu Voronoy and the first human kidney allograft. Surg Gynecol Obstet. 1984:159: 289-295

20. De Vito Dabs A et al. Transplantation in critical care: rejection following organ transplantation. American Journal of critical care. 2000:(V-9), 9: 1-2

21. Kuss R. Human renal transplantation memories: 1951-1981. In: Teraski P.,ed.History of Transplantation: Thirty-five recollections. Los Angeles, Calif: UCLA Tissue Typing Laboratory; 1991:42

22. Barnard CN. A human cardiac transplant. S Afr Med J. 1967; 41:1271-1274

23. Barnard CN. The first human to human heart transplantation. In: Teraski P,ed.History of Transplantation: Thirty-Five recollections. Los Angeles , Calif: UCLA Tissue Typing Laboratory; 1991:565

24. Dabbs A. de Vito, James H. Dauber, Hoffman LA:Transplantation in critical care, Rejectionfollowing organ transplantation: Ahistorical review. 2000 :(V-9), 6: 4

25. Hume DM, Merril JP, Miller BF, et al: Experience with renal hemotransplantation in the human: report in nine cases. J Clin Invest 34(2): 327-381,1955

26. Kolbeck PC, Johnson DR, Klassen LW, and Sharp JG: The pathophysiology of rejection,Transplant pathology, 1994, pp. 2-3.

27. Flye MW: History of transplantation; in Principles of organ transplantation, Philadelphia:WB Saunders Co;pp.6-7.

28. Gibson T, Medawar PB. Fate of skin hemografts in man. J Anat. 1942; 77:29-57

29. Dausset J, Rapaport FT. The life and times of the human histocompatibility complex. InFerrone S, Moller E, eds. The HLA System in Clinical transplantation. New York, NY: Sprin 1993:

30. Spiegelhalter DJ, Stovin PGA: An analysis of repeated biopsies following cardiac transplantation. Stat Med 2:33-40, 1983

31. Zerbe TR, Arena V: Diagnostic reliability of endomyocardial biopsy for assessment of cardiac allograft rejection. Hum Pathol 19:1307-1314, 1998

32. Kolbeck PC, McManuc MB, Markin RS: Transplant pathology, chapter 10: 197;ASCP 1994

33. Braun WE: Molecular, genetic, and clinical aspects of the HLA system. In: Cerillia GJ (ed): Organ transplantation and replacement. Philadelphia: JB Lippincott Co., pp. 162-170, 1988

34. Bollinger RR, Sanfilippo F: Immunogenetics of transplantation. In: Fly MW (ed): Principles of organ transpalantation. Philadelphia:W.B. Saunders Co., pp. 6-7, 1989

35. Kolbeck PC, McManuc MB, Markin RS: Transplant pathology, chapter 1, pp.3-8; ASCP 199

36. Kane KP et al: Molecular interaction required for triggering alloantigen – specific cytolytic T lymphocytes. J Immunol 142:4153-4160, 1989

37. Hosenpud JD: Noninvasive diagnosis of cardiac allograft rejection. Another of many searches of the grail. Circulation, 1992; 75: 368-370

38. Babbitt BP, Allen PM, Matsueda G, et al: Nature 317: 359-361,1985 Williams GM, Hume DM, et al: "Hyperacute renal-homograft rejection in man. N Engl J Med 279:611, 1968 (p.79)

39. Hadley GA, Linders B, Mohanakumar T: Immunogenicity of MHC class I alloantigens expressed on parenchymal cells in the human kidney. Transplantation 54:537-542, 1992 Knechtle SJ, Kolbeck PC, Tsuchimoto S: Hepatic transplantation into sensitized recipients. Transplantation. 43:8 – 12, 1987 (p.82)

40. Germain RN: The ins and outs of antigen processing and presentation. Nature 322: 687-689, 1986 Halloran PF, Wadgymar A, Ritchie S, et al: The significance of the anti-class I antibody response. Transplantation 27:175 – 179, 1979 (p.8).

41. Townsand A, Bodmer H: Antigen recognition by class I restricted T lymphocytes. Ann review Immunol 7:579-599, 1989Scornic JC, LeFor WM, CicciarelliJC, et al: Hyperacute and acute kidney graft rejection due to antibodies against B cells. Transplantation 54:61-64, 1992 (p.84)

42. Kolbeck PC, McManuc MB, Markin RS: Transplant pathology, chapter 1, pp.6-14; ASCP 1994 Fly MW (ed): Principles of organ Transplantation. Philadelphia: W.B. Saunders CO., pp. 6-7, 1989 (p.85)

43. Bier BE et al: The biologic roles of CD2, CD4, and CD8 in T cell activation. Ann rev Immunol 7:579-599, 1989Braun WE: Molecular, genetic, and clinical aspects of the HLA system. In: Cerillia GJ (ed): Organ Transplantation and Replacement. Philadelphia:J.B. Lippincott Co., pp.162-170, 1988 (p.86)

44. Opelz G: Correlation of HLA matching with kidney survival in patients with or without cyclosporine treatment. Transplantation 40:240,1985

45. Cicciarelli J, Teraski PI, Mickey MR: The effect of zero HLA class I and Iimismatching in cyclosporine-treated kidney transplant patients. Transplantation 43:636, 1987

46. Gilks WR, Gore SM, Bradley BA: Renal transplant rejection: transient immunodominance of HLA mismatches. Transplantation 50:141-146, 1990

47. Kupiec –Weglinski JW, de Sousa M, Tilney NL: The importance lymphocyte migration patterns in experimental organ transplantation. Transplantation 40:1-6, 1985

48. Kirby JA, Ikuta S, Clark K, et al: Renal allograft rejection: investigation of alloantigen presentation by cultured human renal epithelial cells. Immunology 72:411-417, 1991

49. Hadley GA, Linders B, Mohanakumar T: Immunogenicity of MHC class I alloantigens expressed on parenchymal cells in the human kidney. Transplantation 54:537-542, 1992

50. Reiherz EL, Meuer SC, Fitzgerald KA, et al: Comparison of T3- associated 49-and 43-kilodalton cell surface molecules on individual human T cell clones: evidence for peptide variability in T cell receptor structures. Proc Natl Acad Sci USA 80:4104-4108, 1983

51. Kolbeck PC, McManuc MB, Markin RS: Transplant pathology, chapter 1, pp.6-14; ASCP 1994

52. Giles RC, deMars R, Chang CC, et al: Allelic polymorphism and transassociation of molecules encoded by the HLA-DQ subregion. Proc Natl Acad Sci USA 82:1776-1780, 1985

53. Fly MW: Immunosuppressive therapy. In Fly MW (ed): Principles of organ transplantation. Philadelphia, WB Saunders, 1989, pp. 155-175

54. Fly MW: History of transplantation. In Fly MW (ed): Principles of organ transplantation. Philadelphia, WB Saunders, 1989, pp. 1-17

55. Kolbeck PC, Johnson DR, Klassen LW, Sharp JG: The pathophysiology of rejection. In Kolbeck KC, Markin RS, McManus BM (eds): Transplant pathology. Chicago, ASCP, Press, 1994, p. 38

56. Reinke P, Fietze E, Docke WD, Kern F, Ewert R, Volk HD.: Late acute rejection in long-term renal allograft recipients. Diagnostic and predictive value of circulating activated Tcells. Transplantation. 1994 Jul1 5;58(1):35-41.

57. Triebel F, de Roquefeuil S, Blanc C, Charron DJ, Debre P.: Expression of MHC class II and Tac antigens on IL2-activated human T cell clones that can stimulate in MLR, AMLR, PLT and can present antigen. Hum Immunol. 1986 Mar;15(3):302-15.

58. Kolbeck PC, Johnson DR, Klassen LW, Sharp JG: The pathophysiology of rejection. In Kolbeck KC, Markin RS, McManus BM (eds): Transplant pathology. Chicago, ASCP, Press, 1994, pp. 1-18

59. Fly MW: Immunosuppressive therapy. In Fly MW (ed): Principles of organ transplantation. Philadelphia, WB Saunders, 1989, pp. 155-175

60. Barry JM: Immunosuppressive drugs in renal transplantation. Drugs 44:554 –556, 1992

61. Philip TA at al., Toxicology and adverse effects of drugs used for immunosuppression in organ transplantation. In Philip TA, Gerson B. (eds): Clinics in laboratory medicine, WB Saunders Comp., pp.755-764,1998

62. Constanzo MR: Immunosuppression in organ transplantation. In Kolbeck KC, Markin RS, McManuc MB, (eds): Transplant Pathology. Chicago, ASCP Press, 1994, pp. 45-61

63. Kolbeck PC, Johnson DR, Klassen LW, Sharp JG: The pathophysiology of rejection. In Kolbeck KC, Markin RS, McManus BM (eds): Transplant pathology. Chicago, ASCP, Press, 1994, pp. 45-60

64. Russel SW, Salomon DR: Macrophage effector and regulatory functions. In: Reif AE, Mitchel MS, eds. Immunity to cancer, New York, NY: Academic Press,pp. 205-216,1985Fly

65. Fisher G, Wittmann – Leibold B, Klang K, et al: Cyclophilin and peptidyl- propyl cis –trans isomerase are probably identical proteins. Nature, 337:476-478, 198

66. Handschumacher RE, Harding M, Rice J, et al: Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science, 226:554-546,1984

67. Emmel EA, Verweij CL, Durand DB, et al: Cyclosporine specifically inhibits function of nuclear proteins involved in T cell activities. Science, 246:1617-1620,1989

68. Reem GH, Cook LA, Vilcek J: Gamma interferon synthesis by human thymocytes and T lymphocytes inhibited by cyclosporine A. Science, 22:63-65, 1983

69. Haydon GH, Hayes PC: New immunosuppressive treatment in transplantation medicine. Balliers Clinical Gastroenterol 8:455-464, 1994

70. Morris RE: Mechanism of action of new immunosuppressive drugs. Ther Drug Monit 17:564-173,1994

71. Allison AC, Kowalski WY, Muller CJ, et al: Mycophenolic acid and brequinar, inhibitors of purine and pyrimidine synthesis block the glycosylation of adhesion molecules. Transplant Proc 25 (suppl 2): 67-70, 1993

72. Morris RE: Mechanism of action of new immunosuppressive drugs. Ther Drug Monit, 17: 564-569, 1995

73. Makowka L, Chapman F, Cramer DV: Historical development of brequinar sodium as a new immunosuppressive drug for transplantation. Transplant Proc 25 (suppl2):2-7,1993

74. Morris RE: New small molecule immunosuppressants for transplantation: Review of essential concepts. J Heart and Lung Transplant 12: S275 – 286, 1993

75. Reem GH, Cook LA, Vilcek J: Gamma interferon synthesis by human thymocytes and T lymphocytes inhibited by cyclosporine A. Science, 22:63-65, 1983

76. Kolbeck PC, Johnson DR, Klassen LW, Sharp JG: Pathology of heart allograft rejection. In Kolbeck KC, Markin RS, McManus BM (eds): Transplant pathology. Chicago, ASCP, Press, 1994, pp. 197-205

77. Caves PK, Stinson EB, Graham AF, et al: Percutaneous transvenous endomyocardial biopsy. Jama 225:289 –291, 1973

78. Caves PK, Stinson EB, Billingham ME, et al: Percutaneous transvenous endomyocardial biopsy in human heart recipients (experience with a new technique). Ann Thorac Surg 16:325 –336, 1973

79. Williams GM, Hume DM, et al:"Hyperacute renal –homograft rejection in man. N Eng J Med 279:611, 1968

80. Miller LW:Listing criteria for cardiac transplantation: results of an American Society of Transplant Physicians-National Institutes of Health conference. Transplantation. 1998 Oct 15; 66(7):947-51.

81. Mills RM, Naftel DC, Kirklin JK, Van Bakel AB, Jaski BE, Massin EK, Eisen HJ, Fishbein DP, Bourge RC:Heart transplant rejection with hemodynamic compromise: a multiinstitutional study of the role of endomyocardial cellular infiltrate. Cardiac Transplant Research Database. J Heart Lung Transplant. 1997 Aug; 16(8):813-21

82. Knechtle SJ, Kolbeck PC, Tsuchimoto S: Hepatic transplantation into sensitized recipients. Transplantation. 43:12, 1987

83. Haloran PF, Wadgymar A, Ritchie S, et al: The significance of the anti class I anibody response. Transplantation 27:175-179, 1979

84. Scornic JC, Le For WM, Cicciarelli JC, et al: Hyperacute and acute kidney graft rejection due to antibodies against B cells. Transplantation 54:61-4, 1992

85. Fly MW (ed): Principles of organ Transplantation. Philadelphia: W.B. Saunders CO.,pp. 6-7, 1989

86. Braun WE: Molecular, genetic, and clinical aspects of the HLA system. IN: Cerillia GJ (ed): Organ Transplantation and Replacement. Philadelphia: JB. Lippincott Co. pp.162-170, 1998

87. Marelli D, Laks H, Kobashigawa JA, Bresson J, Ardehali A, Esmailian F, Plunkett MD, Kubak B.: Seventeen-year experience with 1,083 heart transplants at a single institution. Ann Thorac Surg. 2002 Nov; 74(5):1558-66

88. Billingham ME: Diagnosis of cardiac rejection by endomyocardial biopsy. Heart transplant 1:25-30, 1982

89. Muller J, Warnecke H, Spiegelsberger S, Hummel M, Cohnert T, Hetzer R. Reliable noninvasive rejection diagnosis after heart transplantation in childhood. J Heart Lung Transplant. 1993 Mar-Apr;12(2):189-98

90. Dengler TJ at al: Diagnostic efficiency of rejection monitoring after heart transplantation with cardiac troponin T is improved in specific patient subgroups. Clin Transplant. 2003 Jun; 17(3):284-91.

91. Fishbein MC, Kobashigawa J.: Biopsy-negative cardiac transplant rejection: etiology, diagnosis, and therapy, Curr Opin Cardiol. 2004 Mar; 19(2):166-8

92. Hsu DT, Spotnitz HM: Echocardiographic diagnosis of cardiac allograft rejection.Prog Cardiovasc Dis. 1990 Nov-Dec;33(3):149-60

93. Frist W, Yasuda T, Segall G, Khaw BA, Strauss HW, Gold H, Stinson E, Oyer P, Baldwin J, Billingham M, et al. Noninvasive detection of human cardiac transplant rejection with indium-111 antimyosin (Fab) imaging. Circulation. 1987 Nov;76(5 Pt 2):V81-5. Hosenpud JD: Noninvasive diagnosis of cardiac allograft rejection. Another of many searches of the grail. Circulation, 1992;75: 368-30

94. Dengler TJ, Zimmermann R, Braun K, Muller-Bardorff M, Zehelein J, Sack FU, Schnabel PA, Kubler W, Katus HA.:Elevated serum concentrations of cardiac troponin T in acute allograft rejection after human heart transplantation. J Am Coll Cardiol. 1998 Aug; 32(2):405-12.

95. Billingham ME, Joshi A, Masek MA, Brown BW Jr, Weiss LM: "Quilty" revisited: a 10-year perspective.Hum Pathol. 1995 May;26(5):547-57! Bristow MR, Gilbert EM et al: Use of OKT3 monoclonal antibody in heart transplantation: Review of the initial experience. J Heart Transplant 1988; 7: 1-11

96. Qayumi AK, Nikbakth-Sanghari M, Godin DV, et al. The relationship of ischemia reperfusion injury of transplanted lung and the up-regulation of major histocompatibility complex II on host peripheral lymphocytes. J Cardiovascular and Thoracic surgery. 1998: 5 : 164-169

97. Nikbakht-Sangari M, Qayumi AK. Ischemia reperfussion injury induces the expression of an immune response gene, MHCII – Dr-beta, in the host peripheral T lymphocytes via an alloantigen –independent pathway. UBC, 1998

98. Iain McGhie, MD; Branislav Radovancevic, MD; Pavel Capek, MD; Warren H. Moore, MD; Leela Kasi, PhD; Lamk Lamki, MD; Fred J. Clubb, Jr, DVM, PhD; O. Howard Frazier, MD; ; James T. Willerson, MD . Major Histocompatibility Complex Class II Antigen Expression in Rejecting Cardiac Allografts (Detection Using In Vivo Imaging With Radiolabeled Monoclonal Antibody). Circulation. 1997; 96:1605-1611.)

99. Clegg DO, Pincus SH, Zone JJ. Circulating HLA-DR T cells: Correlation with genetic rather than clinical variables. 1986; 5: 870-4.

100. Rodney GE, Luehrman LK, and Thomas DW. In vitro primary immunization of human peripheral blood lymphocytes to KLH. Evidence for HLA-D region restriction. J. Immunol. 1979; 123:2250-2254

101. Triebel F, De Roquefeuil S, Blanc C, Charon DJ, and Debre P. Expression of MHC II and Tac antigens on IL-2 –activated T cell clones that can stimulate in MLR, AMLR, PLT and can present antigen. Human immunology 1986; 15:302-315.

102. Saalmueller A and Maurer S. Major histocompatibility antigen class II expression on T cells is independent of the regulation of Tac and the intduction of proliferation. Clin. Exp. Immunology. 1991; 86: 544-549

103. Hosenpud JD: Noninvasive diagnosis of cardiac allograft rejection. Another of many searches of the grail. Circulation, 1992; 75:368-9

104. Bristow MR, Gilbert EM et al: Use of OKT3 monoclonal antibody in heart transplantation: Review of the initial experience. J Heart Transplant 1988;7: 1

105. Modry DL, Jamieson SW, Baldwin JC et al: Cyclosporine in heart and lung transplantation. Can J Surg 1985; 28:274-280, 282

106. Caves PK, Schulz WP, Dong E et al: New instrument for transvenous cardiac biopsy. Am J Cardiol 1974:33:264-267

107. Rose AG: Endomyocardial biopsy diagnosis of cardiac rejection. Heart failure 1986; 2:64-72

108. Stinson EB et al: Changes in left ventricular mechanical and hemodynamic function during acute rejection of orthotopically transplanted hearts in dogs. J Thora Cardiovasc Surg 1974; 68:783-

109. Paulsen W et al: Left ventricular function of heart allografts during acute rejection: An echocardiographic assessment. J HeartnTransplant, 1985; 4:525-529

110. Derumeaux G et al: Accuracy of Doppler tissue imaging for the diagnosis of a rejection after heart transplantation, 2000, Abstract

111. Stork T et al: Noninvasive diagnosis of cardiac allograft rejection by means of pulsed Doppler and M-Mode ultrasound. Journal of Ultrasound in Medicine. 10(10):569- 75,1991

112. Brainbridge AD et al: The utility of pacemaker evoked T wave amplitude for the non-invasive diagnosis of cardiac allograft rejection. Pacing & Clinical Electrophysiology. 22(6Pt 1):942-6, 1999

113. Ruiz CE et al: Comparison of M-Mode Echo with endomyocardial biopsy in pediatric heart transplantat patients with and without rejection. Abstract. Loma Linda University Children's hospital & Medical centre, Loma Linda, CA

114. Valantine H et al: Changes in Doppler echocardiographic indexes of left ventricular function as potential markers of acute cardiac rejection. Circulation 1987; 76(suppl V): V-86-V-92

115. Haverich A et al: Noninvasive parameters for detection of cardiac allograft rejection. Clin Transplantation 1987; 1:151-158

116. Desruennes M et al: Doppler echocardiography for the diagnosis of acute cardiac allograft rejection. J Am Coll Cardiol 1988; 12:63-70

117. Tatum JL et al: Radionuclide detection of abnormal ventricular filling patterns in rejecting human allografts. Clin Nucl Med 1989; 14: 175-178

118. Hausdorf G: Diastolic function after cardiac and heart lung transplantation. Br Heart J 1989; 62:123-132

119. Hosenpud JD et al: Relation between recipient: donor body size match and hemodynamics three months after heart transplantation. J Heart Transplant 1989; 8:241-243

120. Everett JE et al: Noninvasive diagnosis of cardiac allograft rejection in an orthotopic canine model. Annals of Thoracic surgery. 62(5):1337-41,1996

121. Everett JE et al: Noninvasive diagnosis of cardiac allograft rejection: the effect of procainamide. Journal of Investigative Surgery. 8(3):195-201,1995 (May-June)

122. Wear KA et al: Ultrasonic characterization of acute cardiac rejection from temporal evolution of echocardiograms. J Heart Transplant 1986; 5:425 – 429

123. Masuyama T et al: Serial measurements of integrated ultrasonic bockscatter in human cardiac allografts for the recognition of acute rejection. Circulation 1990; 81:829-839

124. Eisen HJ et al: Noninvasive detection of rejection of transplanted hearts with Indium 111-labeled lymphocytes. Circulation 1987; 75:868-876

125. Revel D et al: Magnetic resonance imaging of human orthotopic heart transplantation: Correlation with endomyocardial biopsy. J Heart Transplant 1989; 8:1139-14

126. Smart FW, Young JB, Weilbaecher D, Kleiman NS, Wendt RE 3rd, Johnston DL.:Magnetic resonance imaging for assessment of tissue rejection after heterotopic heart transplantation. J Heart Lung Transplant. 1993 May-Jun; 12(3):403-10.

127. Kohler G, Milstein C: Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256:495-497

128. Khaw BA et al: Scintigraphic quantification of myocardial necrosis in patients after intravenous injection of myosin – specific antibody. Circulation 1986; 74: 501-508

129. Yasuda T: Indium 111-monoclonal antimyosin antibody imaging in the diagnosis of acute myocarditis. Circulation 1987; 76:306-311

130. Ballester M: Early postoperative reduction of monoclonal antimyosin antibody uptake is associated with absent rejection –related complications after heart transplantation. Circulation 1992; 85:61-6.

131. Frist W et al: Noninvasive detection of human cardiac transplant rejection with Indium-111 antimyosin (Fab) imaging. Abstract. Circulation. 76 (Pt 2): V81-5, 1987

132. Ballester-Rodes M et al: Patterns of evolution of myocyte damage after human heart transplantation detected by Indium-111 monoclonal antimyosin. Am J Cardiol 1988; 62:623-627

133. Womble JR et al: Urinary polyamide levels are markers of altered T Lymphocyte proliferation/loss and rejection in heart transplant patients. Transplant Proc 1984; 16:1573-1571

134. Frist W, Yasuda T, Segall G, Khaw BA, Strauss HW, Gold H, Stinson E, Oyer P, Baldwin J, Billingham M, et al. Noninvasive detection of human cardiac transplant rejection with indium-111 antimyosin (Fab) imaging. Circulation. 1987 Nov; 76(5 Pt 2):V81-5.

135. Carrier M et al: Urinary polyamines as markers of cardiac allograft rejection: A clinical evaluation. J Thorac Cardiovasc Surg 1988; 96:806-810

136. Garner RJ et al: Immune monitoring of blood in heart transplant recipients: Application of flow cytometry. Semin Diagn Pathol 1989; 6:83-90

137. Roodman ST et al: Role of interleukin 2 receptors in immunologic monitoring following cardiac transplantation. Transplantation 1988; 45:1050-105

138. May RM et al: Cytoimmunologic monitoring after heart –lung transplantation. J Heart Transplant 1990; 9:133-135

139. McGhie AI et al: Major histocomopatibility complex class II antigen expression in rejecting cardiac allografts: detection using in vivo imaging with radiolabeled monoclonal antibody. Circulation. 96(5): 1605-11, 1997

140. Carlquist JF et al: Correlation between class II antigen (DR) expression and interleukin

2-induced lymphocyte proliferation during acute cardiac allograft rejection. Transplantation. 1990 (4); 582-588

141. Winkler M et al: Analysis of MHC class II expression on circulating T-lymphocytes by flow cytometry in patients with acute rejection following solid organ transplantation. Infusionstherapie und Transfusionmedizin. 1998; 25 (6): 360-363

142. Glantz SA: Primer of bio-statistics. Fourth edition. McGraw Hill, New York, 1997