ASSESSMENT OF THE IMMUNE RESPONSE IN KIDNEY TRANSPLANT PATIENTS

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Abbreviations

A
APC: Antigen Presenting Cell
ATP: Adenosine Triphosphate
AMP: Adenosine Monophosphate

B
B cell: Bursa of Fabricius/Bone marrow Cell

C
CMI: Cell Mediated immunity
CD: Complement Determinant
CO_2: Carbon dioxide
CMV: Cytomegalovirus
CNI: Calcineurin inhibitor

D
DNA: Deoxyribonucleic Acid
Dbn: Durban

E
ESRD: End Stage Renal Disease
EBV: Epstein - Barr virus

F
Fab fragment: Antigen binding Fragment of Antibody
FKBP: FK binding protein
FDA: Food and drug Administration

H
HLA: Human Leukocyte Antigen

I
Ig: Immunoglobulin
IL: Interleukin
IFN-γ: Interferon Gamma
ITAMS: Immunoreceptor Tyrosine-Based Activation Motif
ICAM: Intercellular Adhesion Molecule
IALCH: Inkosi Albert Luthuli central hospital
Inc: Increase

J
Jhb: Johannesburg

L
LFA: Lymphocyte function-associated antigen

M
MHC: Major Histocompatibility Complex
MMF: Mycophenolate Mofetil
mTOR: Mammalian "Target of Rapamycin"
MLR: Mixed lymphocyte response

N
NK: Natural Killer Cell
NF-AT: Nuclear Factor of Activated T cells

O
ODF: Organ donation fund

P
PBMC: Peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PHA: Phytohemagglutinin
pH: Power of Hydrogen Ion concentration
PPi: Inorganic pyrophosphate

R
RLU: Relative light per units
RPMI: Roswell Park Memorial Institute medium
RSA: Republic of South Africa

S
SANBS: South African National Blood Services
SEM: Standard error of the mean

T
T cell: Thymus cell
TCR: T cell Receptor
TH: T Helper cell
Tc: Cytotoxic T cell
TNF-α: Tumour Necrosis Factor
TB: Tuberculosis

U
UTI: Urinary tract infection
UKZN: University of Kwa-Zulu Natal

W
WBC: White Blood Cell

Z
ZAP 70: Zeta-associated protein

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Abstract

Background: Management of a transplant recipient involves the use of multiple immunosuppressant drugs. Currently there is no test that reflects the overall immune status of the patient. This results in under or over suppression of the immune system and consequently increases in morbidity and mortality rates. Evaluation of the proliferative response of PBMC's to a mitogen PHA by measurement of intracellular ATP was evaluated as a tool to assess the immune response in kidney transplant patients.

Method: PBMC's were separated from the blood samples of healthy controls and kidney transplant patients on cyclosporine, sirolimus, and tacrolimus based regimens by density gradient centrifugation, cells were counted and incubated overnight with and without PHA. The luciferin-luciferase enzyme reaction which induces bioluminescence and the Turner Biosystem luminometer were used to measure intracellular ATP levels in relative light units (RLU). An ATP standard curve was generated for each test.

Results: The ATP (ng/ml) levels measured in the transplant recipients were lower and statistically significantly different ($p < 0.0001$) than the healthy controls. No statistically significant difference was measured between the cyclosporine and sirolimus drug groups. Patients on tacrolimus gave a statistically significant ($p<0.0001$) stronger immune response than those receiving cyclosporine and sirolimus. Overall, the immune response results of kidney transplant patients were statistically significantly lower than the healthy control by 981 ng/ml. Linear regression analysis revealed no correlation between patient ATP (ng/ml) levels and therapeutic drug blood levels, immunosuppressant drug dosages, creatinine levels and white cell counts. The immune responses of patients who were

diagnosed with infection or were clinically stable were characterised as low or moderate, of interest, one patient who was diagnosed with rejection was found to have a strong immune response (>501 ng/ml ATP).

Conclusion: Future studies to determine the predictive value of the ATP assay in directing immunosuppressive therapy are required. The assay described in this study is simple, sensitive and rapid and has possible application in immunological monitoring in a variety of conditions that affects the immune system.

Keywords: kidney transplantation, immunosuppression, bioluminescence, lymphocyte, Adenosine Triphosphate (ATP), Phytohemmagglutinin (PHA)

Chapter 1

Introduction

A fully responsive immune system is essential for combating harmful agents in the body however in some instances this is undesirable for example an immunological response to a transplanted organ. The common approach for preventing or controlling immune reactions in transplant recipients is to administer immunosuppressive drugs. (Rowley et al., 1973)

Even though most of the technical problems of renal transplantation have been solved and despite the improvements in immunosuppressant protocols the variety of drug related complications i.e. opportunistic infections, malignancies, acute/chronic rejection and drug toxicities are still major causes of morbidity and mortality. (Truong et al., 2007., Sayegh and Carpenter, 2004) Studies have shown that early detection of these complications will result in improved graft survival and improve the recipients' quality of life. (Chávez et al., 2006., Penn and Starzl, 1972)

The complications mentioned above may be directly or indirectly related to the lack of an immunological test to assess the interaction between the immune suppressant drugs and the transplant recipients' immune system. (Truong et al., 2007) Thus to prevent these complications a tool is needed to monitor the changes in the immune system which will help manage immunosuppression therapy. A study by Shulick et al., (1993) highlighted

the importance of monitoring the immune system as a way to optimise immuno-suppressive therapy. (Schulick et al., 1993)

Lymphocytes, play a major role in the immune response against transplanted organs, hence most immunosuppressive therapy targets these cells. When these cells are undergoing suppression or death the levels of ATP which provides energy to the cells is significantly decreased like wise when cells are activated to proliferate there is a rapid increase in the levels of ATP hence ATP may be use as a biological marker to determine the viability of lymphocytes. (Crouch et al., 1993) Firefly bioluminescence which involves luciferin and luciferase enzyme reaction requires ATP as a co-factor to produce light. (Nakamura et al., 2006)

Hence an assay which involves ATP and bioluminescence was investigated as a potential non invasive diagnostic tool, to monitor the immune response of kidney transplant patients.

Literature Review

1.1 Historical overview

A half century has elapsed since the first organ transplantation. (Sayegh and Carpenter, 2004) In the early 1950's, there was a growing certainty that immunological mechanisms were involved in the destruction of transplanted kidney. Sir Peter Medawar identified the immunologic nature of skin allograft rejection in humans. These findings were based on extensive data from animal experimentation. (Medawar et al., 1953., Medawar, 1944)

Jean Dausset established that the major histocompatibility complex (MHC) genes are the most important markers of an individuals' biological identity and consequently play a vital role in transplantation immunology. Ralph Zinkernagel, Peter Gorer and Peter Doherty added to the growing pool of immunological knowledge. Peter Gorer established that the most important histocompatibility locus was antigene II, and named the locus histocompatibility II. Ralph Zinkernagel and Peter Doherty identified the role of MHC to signal foreign antigens to the immune system. (Sharma and Unruh, 2006)

These discoveries as well as findings in vascular surgical techniques (Murray, 1992) led to the first successful kidney transplantation. In 1954, Joseph Murray, John Merrill and Hartwell Harrison carried out the first transplantation between identical twins at the Peter Bent Brigham Hospital in Boston. The success was attributed to the lack of a rejection response. (Murray et al., 1955., Murray et al., 1956)

However, there still remained the problem of rejection of any kidney other than an identical twin's kidney, this marked the beginning of the evaluation of rejection and mechanisms to avoid it. Initial attempts at controlling rejection began with experiments involving total body irradiation. However these attempts resulted in poor outcomes. (Morris, 1970., Sayegh and Carpenter, 2004., Sharma and Unruh, 2006)

In the early 1960s, immunosuppression with 6 – mercaptopurine was first used to enable transplantation between non identical individuals. It was soon replaced by azathioprine which was less toxic. (Morris, 2004) Thomas E. Starzl in the early 1960's, established that a combination of corticosteroids and azathioprine led to much better outcome thus this combination was predominantly used for the next decade. (Sharma and Unruh, 2006)

The Swiss biochemist Jean-Francis Borel discovered cyclosporine in 1972. (Sharma and Unruh, 2006) The first clinical trials for cyclosporine were developed in the late 1980s. At the time success of renal transplantation was relatively poor thus the dramatic effect cyclosporine had on patient and graft survival was easily recognised. (Morris, 2004) However the setback of cyclosporine was its ability to cause acute and chronic nephrotoxicity. (Watson et al., 2005) In 1985 the first monoclonal antibody was introduced to treat acute rejection episodes however the toxicity restricted its use. (Morris, 2004)

Major developments that occurred in the 1990s included the introduction of an alternative to cyclosporine, tracrolimus, which was previously predominantly used in liver transplantation and mycophenolate mofetil (MMF), was found to be a more effective adjunctive agent than azathioprine. The monoclonal antibodies basiliximab and daclizumab which target the interleukin-2 receptor (CD25) were approved in 1998 because of their ability to reduce the risk of acute rejection. (Moore, 2000) In 1999 sirolimus was added to the list of immunosuppressive drugs. (Danovitch, 2001)

These findings and others have led to organ transplantation progressing from impossible to routine. However there are many challenges yet to overcome; of interest is to explore and improve on the powerful tools of biomedical technology currently available. (Murray, 1992)

1.2 Renal Transplantation

A diagnosis of end-stage renal disease (ESRD) is made when the kidney becomes permanently unable to cleanse the body of harmful substances. Consequently, death from the accumulation of waste in the body is imminent unless the person is placed on dialysis which is the replacement of the kidneys' excretionary role by artificial means or the patient receives a transplant. (Cianci et al., 1981., Ruth et al., 1987)

Kidney transplantation is the treatment of choice for patients with ESRD. (Soulillou, 2001) A successful kidney transplant improves the quality of life of such patients. (Andres, 2005., Bushell and Wood, 1999) Modern medicine has triumphed over many challenges to achieve successful organ transplantation. (Howard et al., 2002) and it has become a routine surgical practice. (Lechler et al., 2005) Renal allograft loss due to technical failure which historically was a major challenge is at present extremely rare. (Kaplan et al., 2003., Kaplan and Meier-Kriesche, 2004)

The diseases that most often associated with chronic renal failure are glomerulonephritis, diabetic nephropathy, chronic pyelonephritis, hypertension and polycystic kidney disease. (Cianci et al., 1981) Transplanted kidneys are implanted in the right or left iliac fossa. The renal artery is sutured to the internal or external iliac artery and the renal vein to the external iliac vein and the ureter is implanted in the bladder wall. (O'Callaghan and Brenner, 2000)

Approximately 55 000 organ transplants are performed worldwide each year and the estimated number of living organ recipients is in the region of 300 000. (Kowalski et al., 2003) Approximately 229 kidney transplants are performed each year in South Africa with just about five new transplants done per year at the Inkosi Albert Luthuli central hospital (IALCH) in KwaZulu-Natal. (Odf, June 2008)

There are many factors that contribute to the current success following renal transplantation which include better methods for preservation of the organs, the development of more improved immunosuppressive agents, better understanding of the rejection process (Suthanthiran and Strom, 1994) and improvement in surgical techniques. (Lechler et al., 2005)

Success is however limited by the restricted availability of donor organs, poor long term graft survival rates (> 5 years), developing methods to induce transplant tolerance and eliminating the need for continuous immunosuppression are the major challenges at present. (Lechler et al., 2005) The immunosuppressive drug regimens currently available have significantly improved short term graft survival, in most medical centers one year graft survival now has a success rate of 80 – 90% but the same cannot be said about long term graft survival. (Hariharan et al., 2000., Sayegh et al., 1999)

The most common cause of late graft loss is premature death of the recipient. Infections, stroke, cardiac disease and cancers are among the causes of death in transplant patients with transplanted organs that are still functional. (Ojo et al., 2000., Howard et al., 2002)

1.3 Transplantation Immunology

When an organ, such as a kidney, is transplanted from a donor into the recipient, the immune system of the recipient triggers a response against the new organ as it would to any foreign material, setting off a chain of events that can damage the transplanted organ. (Wood, 2006)

The human acquired/specific immunity consists of two distinct yet interactive types of immunity, humoral immunity and cell-mediated immunity (CMI). Humoral immunity is mediated by B lymphocytes and their production of antibodies, while CMI is mediated by T lymphocytes and their interactions with other immune cells. (Roitt, 1991) The actual recognition of transplantation antigens is predominantly by T lymphocytes and is referred to as allorecognition. (Aw, 2003)

Two distinct pathways of allorecognition have been described. The direct pathway involves receptors on the recipients T cells that directly recognise intact MHC on the cells of the transplanted organ. The T cell receptor (TCR) recognises unknown peptides bound in the groove of allogeneic MHC molecule. (Liu et al., 1993) This is thought to be due to molecular mimicry, the allogeneic MHC resembles self MHC. (Sayegh and Turka, 1998) The indirect pathway requires an antigen presenting cell that internalises the foreign MHC, processes and presents it to a particular subset of T cells via the recipients' MHC molecule. (Briscoe and Sayegh, 2002)

1.3.1 The Major histocompatibility complex

The Major histocompatibility complex (MHC) is the most significant genetic determinant of graft acceptance or rejection between genetically distinct individuals. In humans the MHC are referred to as Human Leukocyte antigen (HLA). The most intensively studied segment of the human genome, the Major histocompatibility complex occupies 4-6 megabases on the short arm of chromosome 6. HLA molecules are highly polymorphic. (Buckley, 2003., Germain, 1994)

The key role of the HLA molecules is to act as receptors which bind peptide fragments of foreign antigens and display it on the cell surface where they can be recognised by T cells. (Sayegh and Turka, 1998) The MHC genes, inherited in a classic mendelian fashion, have been divided into class I, class II and class III. (McCluskey and Peh, 1999., Buckley, 2003)

The class I HLA (HLA-A, HLA-B, and HLA-C) are found on virtually all nucleated cell surfaces. (Buckley, 2003) Peptides bound by class I HLA molecules are mostly derived from endogenous proteins and are recognised by cytotoxic CD8 T cells. (Aw, 2003)

In contrast, the class II HLA (HLA-DR, HLA-DP, and HLA-DQ) are usually only found on antigen presenting cells (APC) such as B lymphocytes, macrophages, dendritic cells, epithelial cells and monocytes. (Buckley, 2003) The HLA class II molecules are believed to play the predominant role in the initial immune response to foreign transplantation

antigens. Peptides bound to class II molecules are derived from proteolysis in acidic endosomal compartments and represent endocytosed proteins and are recognised by helper CD4 T cells. (Sayegh and Turka, 1998)

Class I HLA molecules consist of two polypeptide chains, a single heavy chain complexed to a smaller molecule known as β_2 microglobulin (β_2m) which are in a non-covalent association on the cell surfaces. The heavy chain is inserted into the plasma membrane and contains the antigenic portions. There are three domains of the class I heavy chain, formed in part by disulfide bonding to make loops (α_1, α_2, α_3). The amino acid sequence variable regions are on the first and second domains (α_1, α_2). (Buckley, 2003., McCluskey and Peh, 1999) (Refer to Figure 1.1)

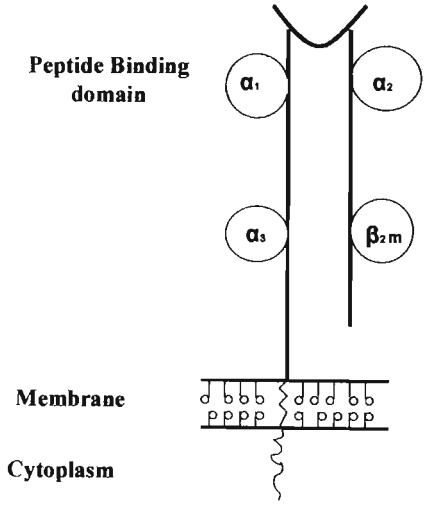

Fig 1.1: Schematic representation of HLA class I molecule

The overall protein structure of the class II molecules is similar to that of HLA class I. Class II HLA molecules consist of two membrane inserted, non-covalently associated glycosylated polypeptides called α and β chains. Each of these chains has two domains and again the polymorphic regions are mostly on the outer amino terminal, which is the peptide binding cleft (α_1,β_1). (McCluskey and Peh, 1999) (Refer to Figure 1.2)

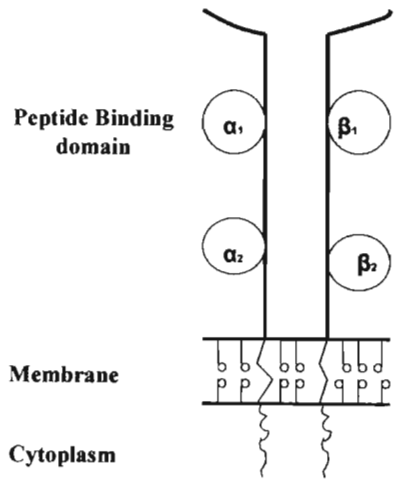

Fig 1.2: Schematic representation of HLA class II molecule

For reasons that are yet unknown foreign MHC molecules stimulate a much higher number of T cells (10–100-fold) than foreign antigen presented by self MHC hence the immune response against foreign MHC transplantation antigens are much stronger. (Wood, 2006) This makes matching of HLA between donor and recipients very important. It has been found that it is essential to match for the HLA A, B and DR antigens. (Rose and Hutchinson, 2006)

1.3.2 T lymphocyte alloantigen recognition

T lymphocytes recognition of alloantigens on antigen presenting cells (APC) is the key event that initiates allograft rejection. (Buckley, 2003)

T lymphocytes play a pivotal role in immune responses, they influence a majority of the cells in the immune system. The major functions include inducing B cells to produce antibody, recruit and activate mononuclear phagocytes and specialised cytotoxic T cells, secrete cytokines responsible for growth and differentiation of a range of cell types including macrophages and eosinophils. It also plays a role in the regulation of immune reactions. (Peakman and Vergani, 1997)

The T cell receptor (TCR) provides the specificity for an individual's T cell to recognise a particular non-self antigen. The TCR has to recognise the foreign antigen in combination with a polymorphic MHC molecule. This discriminating co-recognition event means that T cells are highly specific and genetically restricted to recognising HLA molecules of the individual from which they were derived. This concept is known as MHC restriction. The association between the antigen peptide and the T cell receptor is known as the T cell antigen recognition complex. (Roitt, 1991., Peakman and Vergani, 1997)

The TCR exists as a heterodimer of which there are two types the αβTCR and γδTCR. The αβTCR has been studied the most in humans and has been found to be present in 90% of peripheral T cells thus much more is known about the responsiveness of αβTCR. (Peakman and Vergani, 1997)

Each receptor consists of 2 different polypeptide chains termed the α and β chains bound by a disulfide bond. The T cell receptor is monovalent and is never secreted. Both chains of the TCR have a variable (V) region and a constant (C) region and a short hinge region with a cysteine residue that forms the inter-chain disulfide bond. Each chain spans the membrane by a hydrophobic transmembrane domain and ends in a short cytoplasmic domain. (Peakman and Vergani, 1997) (Refer to Figure 1.3)

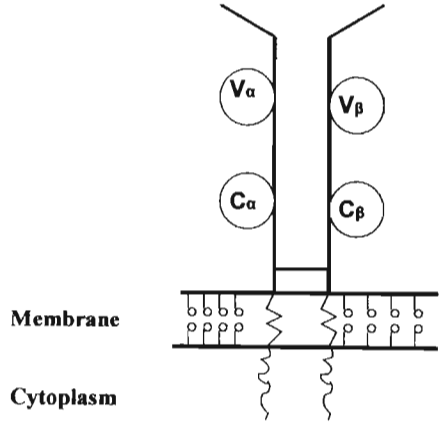

Fig 1.3: Schematic representation of the T cell receptor

On the T cell together with the expression of TCR a complex of molecules termed CD3 is also expressed, which are involved in transduction of an activation signal in the TCR. (Peakman M and Vergani D., 1997) T cells express either CD4 or CD8 glycoproteins on their cell surface. Helper CD4 T cells are considered to play a significant role in graft rejection. They are responsible for the production of the cytokines that stimulate an immune response against the graft. Cytotoxic CD8 T cells produce small amounts of cytokines thus their main contribution to graft rejection is through direct lysis of the donor cells. (Sayegh and Turka, 1998)

1.3.3 T lymphocyte activation

The binding of the TCR to the antigen MHC complex is insufficient to activate T cells thus the interaction between T lymphocytes and APC's involves binding of multiple T cell surface molecules and their counter receptors. These proteins on the T cell surface and on the surface of the APC act as costimulatory signals. (Lechler et al., 2005)

The initial signal of activation is provided by the TCR and the MHC peptide antigen complex which is stabilised by CD4 or CD8. The TCR signal is transduced via the CD3 complex. (Peakman and Vergani, 1997)

The costimulatory signal required for T-cell activation, is provided by the interaction between B7 on the APC and CD28/CTLA4 on the T cell surface which is the best characterised costimulatory molecules. (Suthanthiran and Strom, 1994) This interaction

bound intracellular stores and the subsequent activation of a calmodulin-dependent phosphatase called calcineurin while diacylglycerol in the presence of increased cytosolic free calcium binds to and activates protein kinase C a serine-threonine kinase protein that is sensitive to calcium and phospholipids. (Suthanthiran and Strom, 1994., Liu et al., 1998)

Calcineurin dephosphorylates the cytosolic inactive form of the nuclear factor of activated T cells termed "NF-AT". After dephosphorylation, NF-AT is translocated from the cytosol to the nucleus where it forms a complex with other DNA binding proteins, including fos and jun. The complex of DNA binding proteins, regulate gene transcription including the IL-2 gene which results in the growth and proliferation of the T-cell. This is an important target of immunosuppressant drugs which act on calcineurin, inhibiting the phosphatase activity. (Peakman and Vergani, 1997)

Production of the cytokine interleukin-2 (IL-2) through the signaling pathways, results in the IL2 receptors being inserted on their T cell surface which binds with IL2 in an initial step towards rapid proliferation of T cells with the best fit for the presented antigen. (Aw, 2003., Sayegh et al., 1999) (Refer to Figure 1.5)

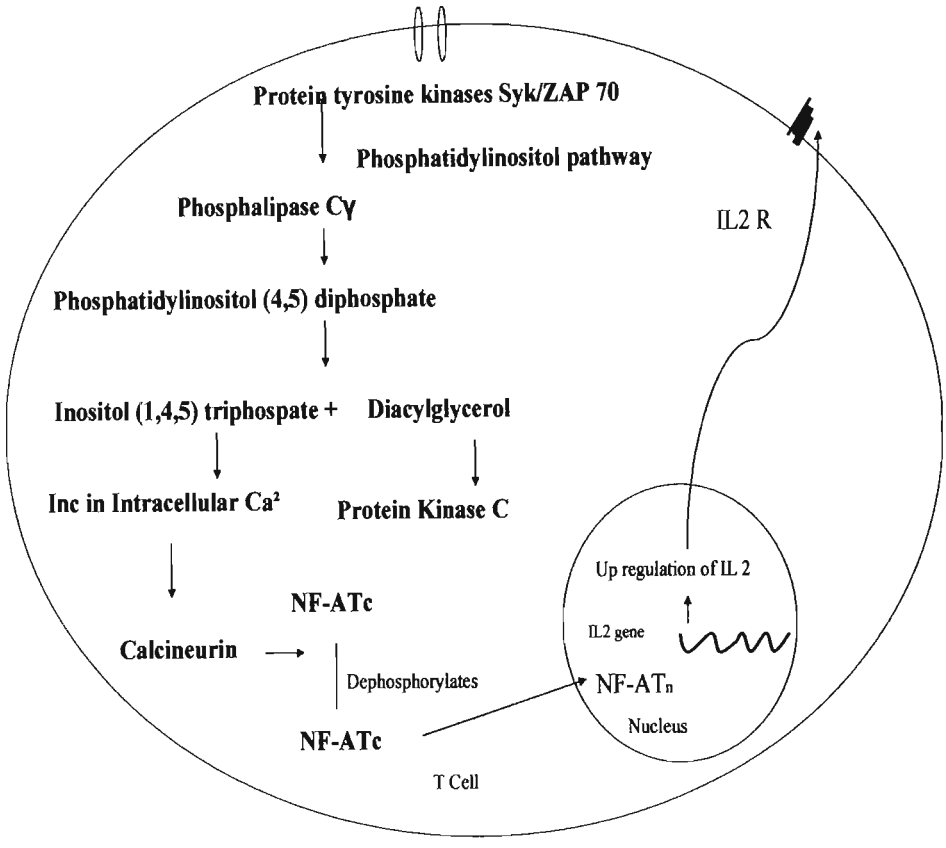

Fig 1.5: Schematic representation of T cell intracellular signaling

The cytokine IL-2 also plays a role in inducing naive or resting T helper cells to differentiate into one of two subclasses, T helper 1 (TH1) cells that assist the cellular or T cell response and T helper 2 (TH2) cells that assist the humoral or B cell response. This differentiation is driven by a process that is poorly understood however it is suggested that it depends on the different cytokines produced. (Peakman and Vergani, 1997., Roitt, 1991)

The TH1 subset secretes cytokines e.g. Interferon gamma (IFNγ) that facilitates a proinflammatory response, promotes the delayed type hypersensitivity reactions (DTHR), expansion of cytotoxic T lymphocytes and assists macrophages and natural killer cells to develop cytotoxicity against the graft. IFNγ also induces and intensifies class I and II MHC antigen expression of the graft. (Peakman and Vergani, 1997)

In contrast TH2 cells stimulate B lymphocytes to produce antibodies against the allografts functional cells. (Dallman, 1995) TH2 cells produce inhibitory products such as IL 4, IL 5, IL 6 and IL 10 which counter the effector activity of Th1 derived cytokines. (Peakman and Vergani, 1997)

The final outcome of cytokine production is the presence of antigen specific, graft infiltrating and destructive T cells. These interactions make the rejection of the transplanted tissue more efficient. (Suthanthiran and Strom, 1994)

In the absence of costimulatory signals T cells become unresponsive, fail to secrete cytokines and may undergo apoptosis. (Aw, 2003., Sayegh et al., 1999) Thus T cell accessory proteins and their ligands on the APC are targets for anti rejection therapy. (Buckley, 2003)

1.4 Immunosuppressive Drugs

The basic immunosuppressive regimen involves the use of multiple drugs each directed at a distinct site in the cascade of T cell activation, cytokine production and clonal expansion. Commonly transplant centers use regimens that have been developed according to the local response to the drugs. (Danovitch, 2001)

The factors that are taken into consideration when choosing the most favourable immunosuppressive regimen include whether it is a first or subsequent transplant, cadaver versus living related transplant, previous pregnancy, patients biological age, weight, ethnicity, the medical history of the patient and the degree of HLA matching. The risk of acute rejection is highest in the first weeks and months after transplantation therefore, the number and dosages of the immunosuppressive drugs is highest during this early period. (Cotran et al., 2000)

The components of a standard immunosuppressive protocol include sirolimus or calcineurin inhibitors (cyclosporine, tacrolimus) and corticosteroid. Azathioprine and mycophenolate mofetil (MMF) are used in combination with a calcineurin inhibitor to enhance the potency of the immunosuppressive protocol. Agents to prevent infection are also used which include trimethoprim sulfamethoxazole (TMP-SMX) and antivirals. (Danovitch, 2001)

Immunosuppressant drugs can be classified into 4 groups: corticosteroids, anti-metabolites, macrolides, and antibodies.

	Corticosteroids	**Anti-proliferative**	**Macrolides**		**Antibodies**
			mTOR Inhibitor	**Calcineurin Inhibitor**	
Names	Methylprednisolone Prednisone	Mycophenolate mofetil Azathioprine	Sirolimus	Cyclosporine Tacrolimus	Antithymocyte globulin Muromonab-CD3 Daclizumab Basiliximab
Method of action	Inhibition of production and release of IL-1	Inhibition of cell proliferation	Blocks production of or action of IL-2		Interfere with the function of T lymphocytes; Lysis of lymphocytes Impair function of cell surface markers necessary for recognition and processing of foreign antigens Causes an increase of suppressor T lymphocytes

Table 1.1: Description of Currently used immunosuppressive agents (Watson et al., 2005)

(* mTOR - mammalian "Targets Of Rapamycin")

1.4.1 Calcineurin Inhibitors

Cyclosporine is a small cyclic peptide of fungal origin. (Suthanthiran and Strom, 1994) Its chemical structure has eight amino acids, with a molecular weight of 1,203 daltons. It is isolated from a strain of the fungi *Hypocladium inflatum gams.* (Vaden, 1997)

Tacrolimus is a macrolide antibiotic extracted from *Streptomyces tsukubaensis.* It is a 23-membered ring compound with a molecular weight of 804 daltons. (Vaden, 1997)

These two immunosuppressant drugs have different structures but block T cell activation through similar mechanisms. Like cyclosporine, tacrolimus is highly lipophilic. Although there are some differences between these drugs they are however remarkably similar and both are highly effective. Tacrolimus is 10 to 100 times more potent than cyclosporine this may be due to the greater affinity for its binding protein. (Halloran, 2004)

After entering the cytoplasm, calcineurin inhibitors form complexes with their immunophilins. The immunosuppressive effects of cyclosporine and tacrolimus depend on the formation of a heterodimeric complex. (Suthanthiran and Strom, 1994)

Cyclosporine binds to cyclophilin and tacrolimus bind to the 12 kDa FK506- binding protein, FK binding protein -12. These complexes inhibit calcineurin activity and hence inhibiting the translocation of the NFAT family of transcription factors from the

cytoplasm to the nucleus of activated T-cells and thus hindering cytokine gene transcription. (Watson et al., 2005)

Inhibition of calcineurin blocks calcium-dependent T cell receptor signal transduction and subsequently inhibit de novo expression of nuclear regulatory proteins and T cell activation genes i.e. Cytokines like IL-2, IL-3, IL-4, IFN-γ and TNF-α , proto-oncogenes e.g. H-ras and c-myc and receptors for cytokines e.g. IL-2 receptor. (Suthanthiran and Strom, 1994) Calcineurin inhibitors have recently been found to block the JNK and p38 signaling pathways triggered by antigen recognition in T-cells. (Allison, 2000)

Cyclosporine and tacrolimus have many similar side effects but there are also important differences. Both drugs are associated with nephrotoxicity and this is one of the most important side effects particularly after renal transplantation. (Sayegh et al., 1999)

Cyclosporine and tacrolimus both cause hyperkalemia, hyperuricemia, and the hemolytic uremic syndrome. Hypertension, hirsutism, hyperlipidemia and gum enlargement are more commonly observed with cyclosporine and may be improved by switching to tacrolimus. The two major adverse events associated with tacrolimus have been neurotoxic reactions and diabetes mellitus. Tacrolimus is more toxic to pancreatic islets than is cyclosporine. Neurologic manifestations include confusion, seizures, headaches and sleep disturbances. These findings tend to be dose related. Thus, close monitoring of blood levels and knowledge of drug-drug interactions are important. (Cotran et al., 2000)

The clinical use of cyclosporine is complicated because blood concentrations achieved after a specific dose vary from patient to patient and within each patient over time. These variabilities are largely determined by differences in absorption, distribution, and metabolism. Cyclosporine is metabolized by the hepatic P450 enzyme system. (Karamperis et al., 2003) Several drugs interfere with cyclosporine metabolism and therefore may cause inadvertent overdosing or under dosing. Therefore to minimise the toxic effects of cyclosporine but maintain immunosuppression the dosage needs to be tailored to each patient. (Vaden, 1997)

Cyclosporine is usually administered orally in a single dose or twice daily. (Cotran et al., 2000) Absorption may be enhanced when cyclosporine is administered with a fatty meal. The microemulsion formation of cyclosporine (Neoral®) has a better and consistent bioavailiability. The advantage of this formulation is that drug absorption is independent of bile flow or the presence of a fatty meal, absorption is thus more rapid and complete. (Sayegh et al., 1999)

Tacrolimus undergoes extensive metabolism by the P450 enzyme system. (Karamperis et al., 2003) Tacrolimus has a narrow therapeutic index and it has poor aqueous solubility thus the absorption of oral tacrolimus is unpredictable and incomplete. The clearance of tacrolimus varies widely between patients. Increases in tacrolimus concentrations have been noted with concurrent administration of drugs such as erythromycin and methylprednisolone. Drugs shown to decrease tacrolimus concentrations include

dexamethasone and rifampin (Vaden, 1997) Thus it is important to monitor tacrolimus blood concentrations to optimise immunosuppression. (Zeevi et al., 2001)

1.4.2 mTOR Inhibitor (mammalian "Targets of Rapamycin")

Rapamycin/sirolimus is a macrocyclic triene antibiotic which was originally isolated from the actinomycete *Streptomyces hygroscopicus*. (Allison, 2000) It is highly lipophilic and passes through cell membranes easily. The major immunosuppressive action of sirolimus is disrupting the proliferation signal by growth promoting cytokines. (MacDonald et al., 2000)

It is structurally related to tacrolimus and binds to the same cytosolic immunophilin, the 12 kDa FK binding proteins. However, they work at distinctly different sites in the cell signaling pathway. The rapamycin and FK binding protein complex are highly specific inhibitors of mammalian target of rapamycin (mTOR). mTOR is a serine/threonine kinase involved in the phosphatidylinositol 3- kinase signaling pathway. Inhibition of mTOR has a profound effect on the cell signaling pathway required for cell-cycle progression and cellular proliferation. (Allison, 2000)

This antiproliferative effect of sirolimus is not specific to T cells. It also inhibits the proliferation of B cells, fibroblasts, endothelial cells, and vascular smooth muscle cells. The ability of sirolimus to inhibit growth factor action in both immune and non immune cells is an important and distinguishing characteristic of this drug. (Cotran et al., 2000)

The side effects of sirolimus are best categorised as metabolic, hematological, dermatological effects and effects related to growth factor inhibition. (Watson et al., 2005) Major side effects of sirolimus are thrombocytopenia, leukopenia and dyslipidemia. (Allison, 2000) Other effects include reduction in uric acid, and elevation in liver function tests. Skin rashes, particular acne, and mouth ulcers are more common in patients on mTOR inhibitors. (Watson et al., 2005)

1.4.3. Mode of Action

The complex between cyclosporine-cyclophylin and Tacrolimus (FK506)-FKBP binds to and inhibits the function of the enzyme calcineurin which has a serine/threonine phosphatase activity. As a result, calcineurin fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc), and thereby the transport of NF-ATc to the nucleus and the binding of NF-ATc to the nuclear component of the nuclear factor of activated T cells (NF-ATn). Consequently, T cells do not produce IL-2, which is necessary for full T-cell activation and proliferation. (Sayegh et al., 1999., Halloran, 2004)

Sirolimus inhibits late signals in T cell activation which are transduced by either the IL-2 receptor or CD28 costimulatory signal transduction pathways. It inhibits the progression of the T cell into the S phase of the cell cycle. (MacDonald et al., 2000) In contrast, cyclosporine and tacrolimus interfere with the early events in T cell activation, blocking T cell progression from the G0 and G1 phase. (Danovitch, 2001) (Refer to Figure 1.6)

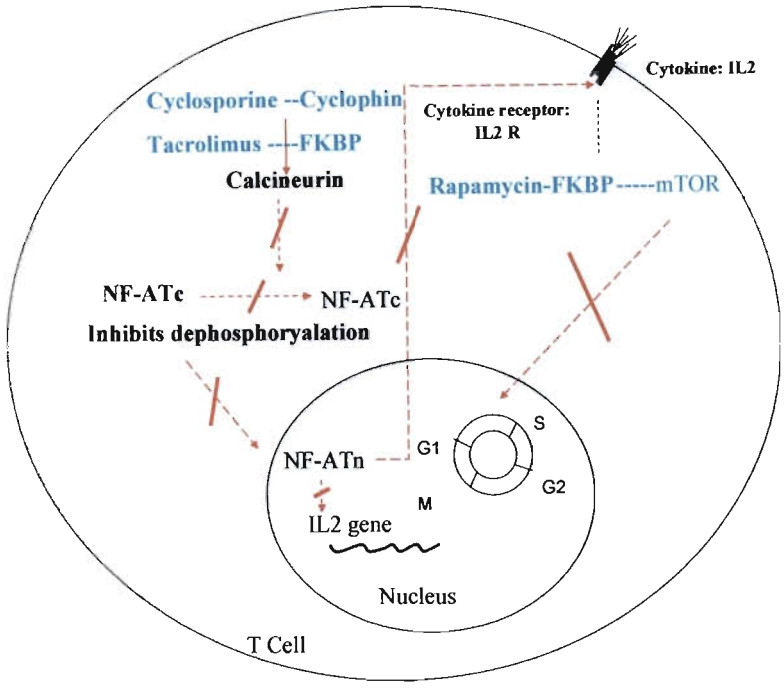

Fig 1.6: Schematic representation of mechanism of action of cyclosporine, tacrolimus and sirolimus

Therapeutic drug monitoring of immunosuppressant drugs are routinely performed however because of their narrow therapeutic ranges and the variation in blood levels of the drugs in different individuals and ethnicities there is no correlation between the amount of drug measured in the blood and the dose of drug administered. (Zheng et al., 2005., Kowalski et al., 2003)

The drug monitoring methods currently used do not take into account the effect that the administration of a combination of immunosuppressant drugs have on a patients' immune system. Certain drugs when administered together may be synergistic in that they work

together so the total effect is greater than the sum of the two (or more) or antagonistic in that they counteract or neutralise each others' effect. (Kowalski et al., 2006)

Appropriate utilisation of immunosuppressants is important for the success of transplantation. It is therefore important to monitor the effect of these drugs on the immune system and control drug concentrations that are too high or too low which creates the risk of complications. (Shaw et al., 1999)

1.5 Complications of Immunosuppression therapy

The immunosuppressant treatment necessary for long-term renal transplant function has a series of side effects. The major harmful consequences of long term immunosuppression are impairment of the hosts' immune defenses against infections, malignancies and increases in cardiovascular complications which can be life threatening for the patient. (Andres, 2005., Kriesche et al., 2003) Conversely, tapering of high levels of immune suppression results in antigen recognition and allo-immune activation thus reducing immunosuppression carries with it the risk of rejection. (Sayegh and Carpenter, 2004., Chávez et al., 2006)

Data accumulated over many years confirms the association between immunosuppressive drugs with infection and malignancy. (Fishman and Rubin, 1998., Soulillou and Giral, 2001) The leading cause of graft loss after the first year of transplantation today is premature death of the graft recipient due to infections which account for 11.7% of deaths and malignant conditions which account for 10.1% of deaths. Cardiovascular diseases are responsible for 30.1%. (Sayegh and Carpenter, 2004) The risk of cardiovascular disease and stroke can be linked directly or indirectly to the immunosuppressive therapy because of its association with hypertension, hyperglycemia anemia and lipid disorders. (Kriesche et al., 2003)

1.5.1 Rejection

Immunologic reactions against kidney transplants have become less common as more specific immunosuppressive agents have evolved. However, rejection is still a concern for allograft loss. Rejection can be classified into hyperacute, acute and chronic. (Sayegh and Carpenter, 2004)

1.5.1.1 Hyperacute rejection

This is a rare and early episode of rejection that causes swelling, rupture, and loss of the allograft within minutes or hours post transplantation. It is thought to be caused by preformed antibodies to antigens present in the donor kidney. Hyperacute rejection can be fatal and requires removal of the graft. Currently used pre-transplant cross-matching techniques which test the recipient for pre-existing antibodies against the graft, has dramatically reduced the occurrence of this type of rejection. (Wood, 2006., Kobrzycki, 1977)

1.5.1.2 Acute rejection

Acute rejection is an immunologic reaction against an allograft resulting in a rapid decline in renal function. Acute rejection usually occurs within one week or four months after transplantation. The majority of transplanted patients have at least one episode of acute rejection. (Kobrzycki, 1977)

Acute rejection typically manifests as a rapid rise in serum creatinine. Severe acute rejection episodes can cause fever, tenderness around the kidney and decrease in urine output. In some instances rapid weight gain, hypertension, an increase in proteinuria, a decrease in urinary sodium output and a decrease in creatinine clearance may occur. Histologically there may be extensive interstitial mononuclear cell infiltration, mild interstitial hemorrhage and oedema. (Cotran et al., 2000)

A study by Dharnidharka et al., (2004) shows that the improvement in post transplant care and the development of more potent immunosuppressive agents has led to an impressive decrease in acute rejection rates. (Dharnidharka et al., 2004)

The relation between acute and chronic rejection is not clearly understood however acute rejection is considered an important predictor of chronic rejection. (Hariharan et al., 2000)

1.5.1.3 Chronic rejection

This is the most common cause of failure of long term allografts. (Ojo et al., 2003) Chronic rejection is defined as an immunologic reaction against an allograft resulting in a gradual but persisting decline in renal function. Chronic rejection typically manifests as a gradual rise in serum creatinine, hypertension, decreasing creatinine clearance, and proteinuria. It is not a reversible process. (Kobrzycki, 1977)

Chronic rejection may be related to tissue injuries at the time of transplantation or during subsequent episodes of rejection and poor HLA matching. Using more living related donors and improving infection prophylaxis would lessen these two risk factors involved in chronic rejection. (Sayegh and Carpenter, 2004)

Chronic rejection is diagnosed by findings from percutaneous needle biopsy of the kidney transplant. Chronic rejection is typified by a gradual obliteration of the lumen of small arteries in the graft caused by endothelial thickening further histologic features include thickening of the intima of arterioles and arteries, sclerosis of glomeruli, and tubular atrophy. When a transplanted kidney is rejected the patient returns to dialysis while waiting for another transplant. (López et al., 2006) The means of intervention or preventing chronic rejection remains a challenge. (Sayegh and Carpenter, 2004)

Although newer immunosuppressive medications have greatly reduced the incidence of acute rejection the ideal therapy for both acute and chronic rejection would be to achieve a state of tolerance. Such a state will allow for indefinite allograft survival despite the recipients immune system being competent thus there would be no need for long term immunosuppression. (Sayegh et al., 2001)

1.5.2 Infection

Infection is one of the most important and common complications of renal transplantation. (Kobrzycki, 1977) Serious infectious complications develop in 15-44% of renal transplant recipient's within the first year of surgery. (Soulillou and Giral, 2001)

Transplant patients are at increased risk for infections in the first 6 months after transplantation when immunosuppression is relatively high to prevent acute rejection however infections can occur at any time and can vary considerably in severity. (Jamil et al., 1999) Infections add significantly to the morbidity and mortality of transplantation. Thus prevention of serious illness or death from infection depends on early detection. (Suthanthiran and Strom, 1994)

Signs of infection include a feeling of discomfort, cough and sore throat. Fever is the most common sign of infection however due to depression of the immune system it may be absent. (Soulillou and Giral, 2001)

When a transplant recipients' immune system is weakened they are susceptible to not only common bacteria that are encountered by surgical patients but also to opportunistic viral and fungal infections. (Kobrzycki, 1977) Post transplant infections can be caused by bacteria, viruses, fungi, mycobacteria, and other opportunistic microbes. Prophylactic treatment is administered to reduce the incidence of pulmonary infection (*pneumocystis carinii)* and urinary tract infections. Excessive immunosuppression also causes a

susceptibility to infectious diseases such as DNA viruses e.g. cytomegalovirus and Epstein-Barr virus (EBV). (Fishman and Rubin, 1998)

It has been found that some immunosuppressive molecules interact in a synergistic manner with anti infectious agents to increase their effect. (Soulillou and Giral, 2001) Evidence from the studies by Neyts et al., (1998) indicate that in addition to the level of exposure the type of drugs and the combinations used may be important variables affecting optimal prevention of viral infections in transplant recipients. (Neyts et al., 1998) Thus the solution may be to choose the best combination of therapies for each individual patient or complete drug withdrawal and induction of tolerance as suggested by Kasiske et al. (2000). (Kasiske et al., 2000)

1.5.3 Malignancy

Cancer is a major complication of renal transplantation that causes significant short and long-term mortality. (Andres, 2005) It accounts for 30% of deaths of renal transplant patients. (Mahony et al., 1995) Immunoregulatory mechanisms that normally protect against cancer development are suppressed by immunosuppression therapy therefore the risk of cancer in renal transplant recipients is twice that of age matched controls that have not had organ transplants as reported by Soullillou et al., (2001) (Soulillou and Giral, 2001)

A study by the Australian and New Zealand Transplant Registry (ANZTR) documents that kidney transplant patients have an increased risk of skin and non skin cancers and the risk of cancer increases with time and that many of the cancers that commonly occur in transplant recipients are virally mediated. Cincinnati Transplant Tumor Registry (CTTR) of transplantation found that the incidence of the rare non skin cancers which are rarely detected in the general population was greatly increased in transplant recipients. (Andres, 2005., Soulillou and Giral, 2001)

These types of cancer included lymphoma, lip cancer, Kaposi's sarcoma, carcinoma of the kidney, carcinoma of the vulva, cervix, esophagus and hepatic carcinoma. Cancers in transplant recipients were also found to be more aggressive and fatal than similar cancers in the general population. (López et al., 2006., Barret et al., 1993)

Skin cancers are the most frequent type of malignancy occurring in renal transplant recipients. Immunosuppression and exposure to ultraviolet sunlight act synergistically to increase risk to cancer as well as viruses e.g. human papillomavirus (HPV), Epstein Barr Virus (EBV), Human Herpes Virus 8 (HHV-8) cytomegalovirus, hepatitis B and C viruses. (Kobrzycki, 1977)

Non skin cancers that occur in renal transplant recipients are thought to occur as a result of latent viruses acquired from donor to recipient, during transplantation and activated by the collective immunosuppression. Post transplant lymphoproliferative disorder (PTLD), which is associated with EBV often progresses to non Hodgkins lymphoma which is the

second most common after skin cancers. Lymphomas are a major cause of cancer related mortality and morbidity. (Andres, 2005)

It is generally believed that immunosuppression as a whole rather than particular immunosuppressive agents increases the risk of cancer in renal transplant recipients. This view is changing as evidence mounts suggesting that the immunosuppressive agents themselves affect the cancer risk. For example Hojo et al., (1999) recently reported data suggesting that cyclosporine can increase cancer progression independent of its immunosuppressive effects in immune incompetent hosts. (Hojo et al., 1999) Other data suggests that cyclosporine and tacrolimus might promote PTLD by enhancing the viability of EBV infected B cells. (Beatty et al., 1998)

Since transplant patients typically require lifelong immune suppression the risks of rejection, cancer and infection associated with immunosuppressive agents continue to demand attention. Therefore to lower the risk of these complications the induction therapy and maintenance immune suppression regimen needs to be carefully controlled. Physicians strive endlessly to find the right balance between the level of immunosuppression required to prevent complications and the level that will minimise dose dependent side effects. (Soulillou and Giral, 2001)

This could be achieved with the development of diagnostic tools for directly assessing the impact of changing therapies on the immune system.

1.6 ATP Bioluminescence Assay

If transplantation is to be available with minimal risks and have optimal outcomes, challenges associated with immunosuppressive therapy need to be overcome. (Sayegh and Carpenter, 2004) In an editorial by Grimm P. (2006) he stated that many of the problems faced by transplant recipients will be improved by a test which determines overall cumulative immune suppression. (Grimm, 2006)

A patients' immunologic response varies as a result of the time since transplant, interaction with other drugs, drug metabolism (pharmokinetics), age, nutritional status, ethnicity, diabetes and stress. Currently there are no tests which look at the cumulative effect of these complex interactive factors. (Kowalski et al., 2003., Israeli et al., 2007)

The tools currently available for assessing the status of a transplanted organ include monitoring the concentration of immunosuppressant in the blood or plasma, organ function tests e.g. measuring markers in the blood such as creatinine, absolute lymphocyte counts and histological evaluation of allograft biopsies which is the "gold standard" for assessing the status of allografts. (Kowalski et al., 2006)

As a result of the pharmacokinetic differences between individuals the dose of a drug is not directly related to the levels measured in the blood and the test used targets a single drug which has limited value if the patient is on a multi drug regimen. (Kowalski et al., 2006) The main reason for drug monitoring of immunosuppressants is for preventing toxicity and measuring patient compliance not for preventing rejection or infection. (Caruso et al., 2001., Kowalski et al., 2006)

It is difficult to predict the outcome of a transplant based on the morphologic information obtained from a biopsy. (Mannon and Kirk, 2006) A biopsy does not give an accurate reflection of the patients overall immune status. The invasiveness and risk linked with the biopsy procedure is a major hindrance. (Israeli et al., 2007)

The methods currently employed to measure T lymphocytes function include counting the number of T cells e.g. flow cytometry which requires expensive equipment, removal of culture media and performing a number of cell washes. Methods for measuring the proliferation of lymphocytes include radioactive thymidine incorporation, this technique requires several days to produce results and dangerous radioactive material. (Sottong et al., 2000., Han and Bonventre, 2004., Kowalski et al., 2003)

The ATP bioluminescence assay is an in vitro assay. (Mollgard et al., 2000) It quantifies the metabolic activity of the cell as a measure of their cellular ATP content. (Crouch et al., 1993)

1.6.1 Adenosine Triphosphate (ATP)

ATP is an important intracellular energy source present in all metabolically active cells. (Redsven et al., 2007) All cells require ATP to remain alive and to carry out their specialised functions. (Crouch et al., 1993) The amount of ATP in a specific healthy cell is relatively constant (Lundin et al., 1986) thus cell injury (necrosis) and death (apoptosis) result in the rapid decrease in intracellular ATP. (Crouch et al., 1993) ATP has been shown to correlate with an increase or decrease in proliferation. (White et al., 1989) Therefore ATP can be used as a tool to assess the functional integrity of living cells. (Slater, 2001., Dexter et al., 2003)

The main source of ATP used by cells comes from respiration or glycolysis. Immune cells use ATP for both the housekeeping functions and their individual activities. Energy in the form of ATP is needed for cation transport, macromolecule synthesis and processes involved in targeting antigens. Without adequate energy supply, suitable immune function would fail. This explains why processes of energy metabolism are important targets of immunotherapy. (Buttgereit et al., 2000) ATP is an appropriate target for assessing the immune status of transplant patients on the immunosuppressants e.g. cyclosporine, tacrolimus and sirolimus as they are known to inhibit processes such as mitochondrial respiration that require cellular ATP. (Karlsson et al., 1997)

ATP dependent cellular functions of immune cells such as lymphocytes and macrophages include intracellular synthesis of lymphokines e.g. IL-2, transport, membrane insertion and release of receptors e.g. IL-2R and antigen presentation (Buttgereit et al., 2000).

1.6.2 Bioluminescence

Photon-emitting chemistries have increasingly become the preferred choice for many types of experimental procedures in the biological and medical fields of study because of the availability of highly sensitive devices that are able to measure the light emitted. (Nakamura et al., 2006) The photon emitting chemistries are grouped according to the way the energy needed for photon generation is obtained. Examples include the most commonly used fluorescence which relies on photons as the energy source, chemiluminescence which relies on chemical energy, radioluminescence which relies on radioactivity and electroluminescence which relies on electricity. (Wood, 2007)

Bioluminescence is a form of chemiluminescence (McElroy and Strehler, 1954) where the light yielding reaction is derived from a naturally catalysed process, found in glowing fireflies, jellyfish and bacteria. (Wood, 2007., Haneda and Johnson, 1958)

Dubois et al., in 1885 reported on the first experiment concerning the nature of the components needed for bioluminescence. His experiments were carried out on the luminous organ of beetles. He found that bioluminescence depends on the presence of an enzyme substrate system including extracts of heat labile luciferase and heat stable luciferin components which emit light when mixed together. (Haneda and Johnson, 1958) This early work of Dubois R was continued by E. Newton Harvey and his associates at Princeton. (McElroy and Strehler, 1954)

In 1947, McElroy demonstrated that the production of light by luciferase extracts from fireflies depends upon the presence of adenosine triphosphate (ATP). (McElroy and Strehler, 1954) The most well characterised firefly luciferase is that isolated from the North American firefly *Photinus pyralis*. (De Wet et al., 1986)

This enzyme reaction involves the mono oxidation of luciferin by firefly luciferase in the presence of ATP and Mg^{2+} and oxygen. This reaction results in the production of oxyluciferin, adenosine monophosphate (AMP), inorganic pyrophosphate (PPi), CO_2 and the emission of yellow-green light. (Squirrell et al., 2002) Factors such as heat and low pH is known to affect the reaction. (De Wet et al., 1986)

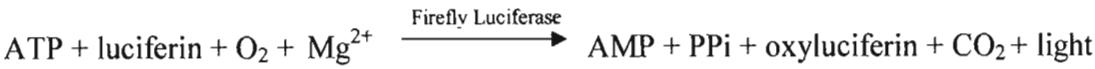

Fig 1.7: The luciferin/luciferase enzyme reaction (AMP: adenosine monophosphate, PPi: inorganic pyrophosphate, CO_2: Carbon dioxide)

Due to the sensitivity of the luciferin:luciferase system even small changes in ATP levels or changes in ATP levels in a small number of cells can be measured The light intensity from the reaction is proportional to the amount of ATP in the sample as little as 0.1 pg of ATP can be measured. (Redsven et al., 2007) It can therefore deliver 10 to 1000 fold higher assay sensitivity than fluorescence assays. (Wood, 2007)

Bioluminescent measurement of ATP has been used for assessing, cytotoxicity of cell lines and tumours and the quantification of biomass. (Bradbury et al., 2000., Dexter et al., 2003)

The success of organ transplantation relies on the ability to control the immune system. The ATP bioluminescence assay is proposed as a non invasive monitoring method of the immune system. (Chávez et al., 2006) Therefore reported in this document are our observations on the use of an ATP bioluminescence assay as a tool to assess the immune response in kidney transplant recipients.

Aim/Objectives

Aim

- To adapt and optimise an ATP bioluminescence assay

- To evaluate ATP bioluminescence as a potential tool to assess the effect of immune suppressant medication on the immune response in kidney transplant patients.

Objectives

- To describe the immune response distribution results obtained for the healthy individuals (control population) and patients on the immune suppressant drugs cyclosporine, sirolimus and tacrolimus.

- To describe the correlation between immune response distribution and measurement of drug levels in the blood, immunosuppressant dosage, white cell count (WCC) and creatinine levels

- To describe the association between immune response distribution, gender and time since transplantation.

- To describe the immune response distribution of patients and their clinical status

Chapter 2

Materials and Methods

2.1 Ethical consideration

This study has been approved by the Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal (UKZN), Biomedical Research Ethics Committee (BREC), Reference number: H172/05 and Postgraduate Education Committee. Reference number: PG049/07

2.2 Patient population

Patients were selected from the kidney transplant population attending the Inkosi Albert Luthuli Central Hospital (IALCH) renal transplant clinic. Patients who were on cyclosporine (n=22), tacrolimus (n=6) or sirolimus (n=10) as part of their immune suppressant regimen were selected. The other types of medication included a steroid prednisone and mycophenolate mofetil or azathioprine. Informed consent was obtained from all participating patients (Appendix 9/11). A questionnaire (Appendix 6) was used to obtain patient history via personal communication with the patient and from their medical records.

2.3 Control population

Blood obtained from South African National Blood Services (SANBS-10 Eden Rd, Paradise valley, Pinetown, RSA) and blood samples kindly donated by known healthy

individuals was utilised. The control population included healthy male or female adults who are eligible to donate blood according to the blood donation guidelines. (Appendix 8) Informed consent was obtained from all participating controls (Appendix 10/11).

2.4 Optimisation

The following parameters were investigated to ensure optimal results. The intracellular ATP content of 2500, 5000, 10 000, 50 000, 100 000 and 200 000 PBMC's were analysed after a 24 incubation period. Stimulation of cells by Phytohemmagglutinin (PHA) (Sigma®, Capital laboratory, New Germany, Dbn, RSA) prepared according to the manufacturers instructions (Appendix 5) to final concentrations of 1 µg/ml, 5 µg/ml and 10 µg/ml were investigated.

2.5 Sample collection

The control and patient whole blood specimens were collected in 4 ml purple top EDTA vacutainer tubes with the help of phlebotomist at the IALCH renal transplant clinic.
Each tube was dated and labeled with the patient name, hospital number and immunosuppressant taken by the patient. The tubes were gently inverted several times to ensure uniform distribution of blood cells and to ensure the blood mixed thoroughly with the anticoagulant. PBMC retrieval was completed within 30 hours of specimen collection and the blood collection tubes were stored at room temperature (18-25°C) before processing. This allowed good separation of the desired cells and subsequent cell proliferation. (Fitzgerald, 1972)

2.6 Peripheral blood mononuclear cell (PBMC) isolation

The technique of separating peripheral blood mononuclear cells (PBMC's) from whole blood was developed over 30 years ago (Boyum, 1968). Lymphocytes and monocytes can be selected from a variety of subjects and are used to study a variety of diseases. (Boyum, 1977) The technique for lymphocyte and monocytes separation used in this study was based on their density differences.

Approximately 4 ml of anticoagulated whole blood from each vacutainer tube was transferred to 15 ml centrifuge tubes and centrifuged at 2000 rpm for 10 min. The plasma fraction which formed following centrifugation was removed using a fine tip Pasteur pipette and dispensed into 2 ml nunc vials.

Thereafter phosphate buffered saline (PBS) (Whitehead Scientific (PTY) Ltd, Brakenfell, Cape Town, RSA) was added in a ratio of 1:1 or according to the amount of plasma removed. The 15 ml centrifugation tube was inverted a few times to ensure mixing and even distribution of the blood cells and PBS.

Histopaque 1077 (Sigma®, Capital laboratory, New Germany, Dbn, RSA) was gently dispensed into a sterile 15 ml polypropylene tube and allowed to warm up to room temperature. The blood and PBS mixture was gently layered onto the histopaque making sure not to break the interphase of the density gradient. The tubes were spun at 1400 rpm for 30 min at room temperature.

This resulted in the separation of the blood into its different cellular components according to their differences in density. The PBS, plasma fraction formed at the top of the tube followed by the mononuclear cells (buffy coat), the histopaque 1077, the granulocytes and the red blood cells (RBC) (Appendix 1). The PBS, RBC's and granulocytes fractions were discarded according to standard safety procedures.

The mononuclear cell layer was aspirated and place in a sterile 15 ml centrifuge tube and washed to remove residual histopaque. This was done by adding approximately 8 ml of PBS and centrifuged at 1500 rpm for 10 min at room temperature. Following the first wash step the mononuclear cell formed a pellet at the bottom of the tube this was resuspended in PBS and the washing step was repeated two more times Sterile distilled water was used to remove any contaminating RBC's and platelets in the pellet (Appendix 4).

Thereafter the pellet was immediately suspended in 1 ml Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum (FCS) (Highveld Biological (PTY) Ltd, Lyndhurst, Jhb, RSA) and HEPES, L-glutamine, penicillin, and streptomycin (Whitehead Scientific (PTY) Ltd, Brakenfell, Cape Town, RSA). Aseptic techniques, a laminar flow cabinet and 70% ethanol and bleach were used, to prevent contamination.

2.7 Cell counting

The PBMC's suspended in 1 ml RPMI 1640 medium supplemented with 10% foetal calf serum (FCS) were thoroughly vortexed and 10µl of cells were transferred to a Neubauer hemocytometer. The solution filled the area under the cover slip by capillary action. The counting grid was brought into focus on low magnification (10X). The cells in the four corner squares were systematically counted (refer to Figure 2.8). Precaution not to overload the chamber or move the cover slip was taken to prevent an inaccurate count. The cells were counted with the hemacytometer to ensure a uniform number of cells were used and to maintain consistency in the assay between patient samples.

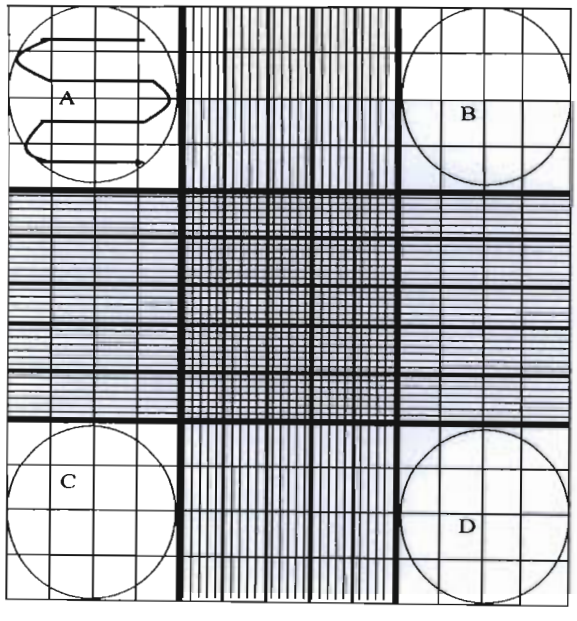

Fig 2.8: Hemacytometer counting chamber. Areas marked A, B, C, and D are used to count white blood cells.

The number of PBMC's per ml were calculated using the equation

PBMC counted in each block x 10^4-Volume conversion factor to 1ml

4

e.g. 185+200+240+1751/4 x 10000 = 2 000 000 cells/ml

If cells were for example suspended in 5ml (5000µl) of RPMI 1640 medium supplemented with 10% FCS, the total number of cells in 5 ml were calculated by multiplying the number of cells obtained per ml by the cell suspension volume i.e. 2 000 000 x 5000µl therefore you have 10 000 000 cells in 5000µl.

50 000 cells were required per well, cells enough for 20 wells were taken i.e. a total of 1 x 10^6 cells were removed from the cell suspension. The amount of the cell suspension that contained 1 x 10^6 was calculated ($1x10^6$.1000µl/$2x10^6$=500µl) which was suspended in 1100µl of RPMI and 10% FCS to make up 1,6 ml. Adding 80µl of this suspension to each well of a 96 well plate transferred 50 000 cells to each well.

2.8 Peripheral blood mononuclear cell (PBMC) stimulation

Lymphocytes separated from peripheral blood are stimulated by a commonly used mitogen, phytohemagglutinin (PHA) to proliferate. PHA is a plant lectin extracted from red kidney beans (*Phaseolus vulgaris*). PHA consists of two closely related proteins, called PHA-L and PHA-E. The letters L and E indicate that these proteins agglutinate leukocytes and erythrocytes respectively. As a mitogen PHA is able to trigger cell division (mitosis) in T-lymphocytes, however it produces a greater stimulatory effect on CD4 T lymphocytes than on CD8 T cells. (Ozerol et al., 1996)

PHA affects the permeability of the cell membrane to transport proteins. When T cells interact with PHA the metabolic activity of the cells increase and this increase is reflected in a significant increase in ATP levels. In this study PHA-M (mucoprotein) which is a mixture of glycoproteins found in the red kidney was used. (Nowell, 1960., Hamelryck et al., 1996., Forsdyke, 1967)

The white opaque 96 well polystyrene microtiter solid bottom assay plate (Cellstar, Lasec SA (PTY) Ltd, Marine parade, Dbn, RSA) was assembled so as to include one strip for the control sample, one strip for each patient sample and one strip for the ATP standard. 80 µl of culture media (RPMI with 10% FCS) containing approximately 50 000 cells were plated into the wells of each assay plate strip. White opaque 96 well solid bottom assay plates were used to prevent cross talk between wells and prevent the loss of luminescence signal which occurs with standard clear 96 well assay plates.

For the purpose of assessing ATP synthesis in response to the stimulant and the basal ATP activity, the first 3 wells of each of the patient strips and control strip were designated as non-stimulated wells (negative controls, wells 1-3), 20 µl of RPMI 1640 medium with 10 % FCS was added to these wells while 10 µl of RPMI 1640 and 10 µl of the mitogen, PHA solution (Sigma®, Capital Lab Supplies, New Germany, Dbn, RSA) at a final concentration of 1 µg/ml was dispensed into the next 3 wells, designated as stimulated wells (wells 4-6). (Refer to Figure 2.9)

The effects of incubating patient and control PBMC's with plasma isolated from the patients' blood samples and plasma from healthy individuals' blood samples was also investigated. In wells 7 to 12 of each patient and control strip containing 80 µl of PBMC's, 10 µl of PHA solution was added. Followed by 10 µl of plasma from a healthy control which was added to three of the six wells (wells 7-9) and 10µl of plasma from the patient was added to the remaining three wells (wells 10-12).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A Patient												
B												
C												
D												
E												
F Control												
G blank										1	1	1
H ATPStandard ng/ml (G10-H12)	10	10	10	100	100	100	1000	1000	1000	10000	10000	10000

Fig 2.9: Schematic representation of the plate map for the patient and control samples and ATP standard

Wells containing culture media only (blank) were used as a control to measure background luminescence i.e. ATP contamination. The background luminescence was subtracted from all readings.

Ultimately all the wells had an equal volume of 100 µl. The assay plate was then incubated overnight (15-18 hours) in a 37°C, 5% CO_2 incubator. Following incubation the 96 well microtiter assay plate was removed from the incubator, and equilibrated to room temperature for approximately 30 min.

2.9 ATP standard

The ATP (Sigma®- Capital Laboratory, New Germany, Dbn, RSA) standard was prepared by serial dilutions of 1 mg ATP according to manufactures instructions to concentrations of 10000 ng/ml, 1000 ng/ml, 100 ng/ml, 10 ng/ml and 1 ng/ml with distilled water. 100 µl of each level of the calibrator panel were dispensed into the designated wells (Refer to Figure 2.9). The amount of ATP produced is calculated from the ATP standard curve thus an ATP standard was run which each assay plate and a standard curve is generated for each test.

2.10 CellTiter Glo® Luminescent cell viability assay (Promega-Whitehead Scientific (PTY) Ltd, Brakenfell, Cape Town, RSA)

This test is a uniform single step method of determining the number of metabolically active cells in culture based on the amount of ATP detected. Intracellular ATP is measured using the firefly luciferin/luciferase reaction. This assay uses a stable form of luciferase based on the gene from the firefly *Photuris pennsylvanica*. A mutant form of the gene was created in which the characteristics that improve performance were selected, eliminating the problems caused by endogenous ATPases, pH, detergents and hence creating a reagent that has a sensitive and stable luminescence. The luminescence generated is proportional to the number of viable cells. The CellTiter Glo assay reagent was prepared according to manufactures instruction. (Promega, 2005)

An equal volume of 100 µl of the CellTiter Glo® assay reagent equal to that of the cell culture medium used, was dispensed into each well. The plate was then shaken on the Labnet Orbit P4 microplate shaker (Whitehead Scientific (PTY) Ltd, Brakenfell, Cape Town, RSA) for 2 min at 900 RPM to induce cell lysis and release the intracellular ATP to react. The cells were then incubated for 10 min at room temperature to stabilise the luminescent signal.

The cells were read with the GloRunner™ Microplate Luminometer (Whitehead Scientific (PTY) Ltd, Brakenfell, Cape Town, RSA, Turner Biosystem) at a wavelength of 550 nm for 1 sec per well. Luminescence was recorded in relative light units (RLU).

Turner Biosystems Glorunner™ microplate luminometer software was used to access and analyse the results.

2.11 Construction of an ATP standard curve

Microsoft Excel® program was used to draw up the standard curve. The average of the triplicate RLU readings for each known ATP dilution concentration was calculated. The Standard Curve was drawn with the known ATP concentration value on the X axis and the calculated RLU value on the Y axis. The straight line equation y=mx+c was used to determine the ATP values of the test samples.

2.12 Data analysis

A statistician (Ms Fikile Nkwanyana, College of Health Sciences, Tel: +27 31 260 4792) was consulted for help regarding the statistical analysis of the results. The data was analysed using a commercial statistics program (Graphpad prism 4). This is a descriptive study thus intra group statistics such as, mean, standard error of mean (SEM), median and minimum and maximum values were used to summarise the data. Chi-square test was employed to assess the statistical significance of differences between means. Values were considered statistically significant when p values <0.05.

Chapter 3

Results

3.1 The format of the ATP bioluminescence Assay

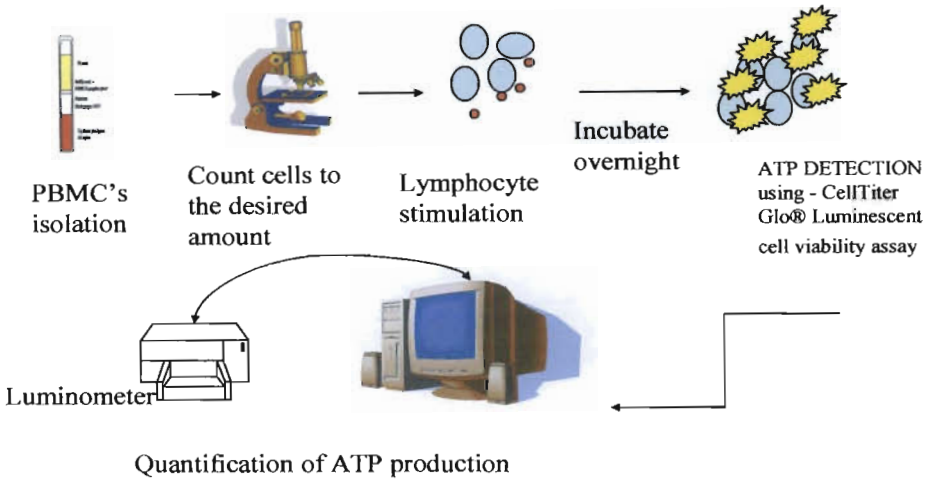

Fig 3.10: Format of the ATP bioluminescence Assay

3.2 Optimisation results

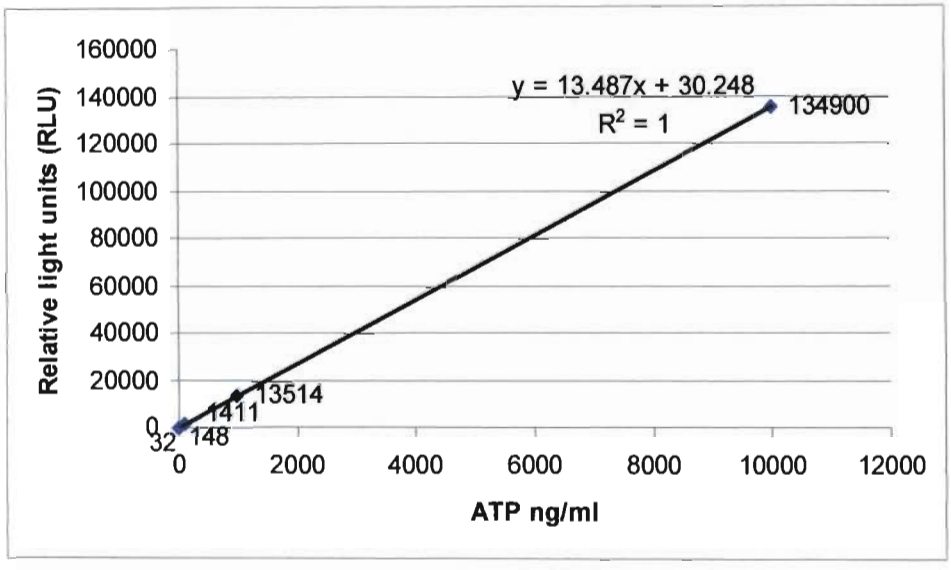

Fig 3.11: ATP standard curve (RLU vs. ATP ng/ml)

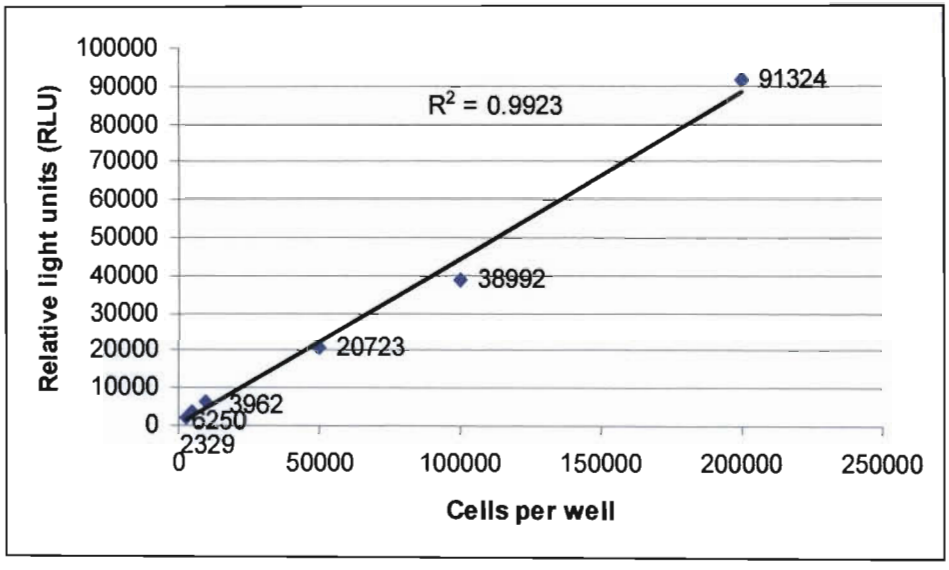

Fig 3.12: The relationship between Relative light units (RLU) and varying concentrations of PBMC's

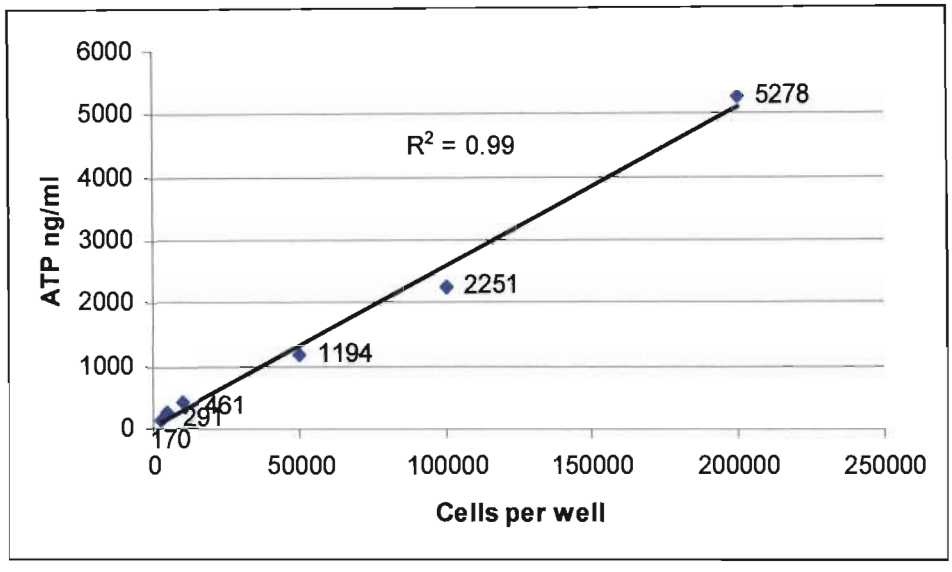

Fig 3.13: The relationship between ATP (ng/ml) and varying concentrations of PBMC's

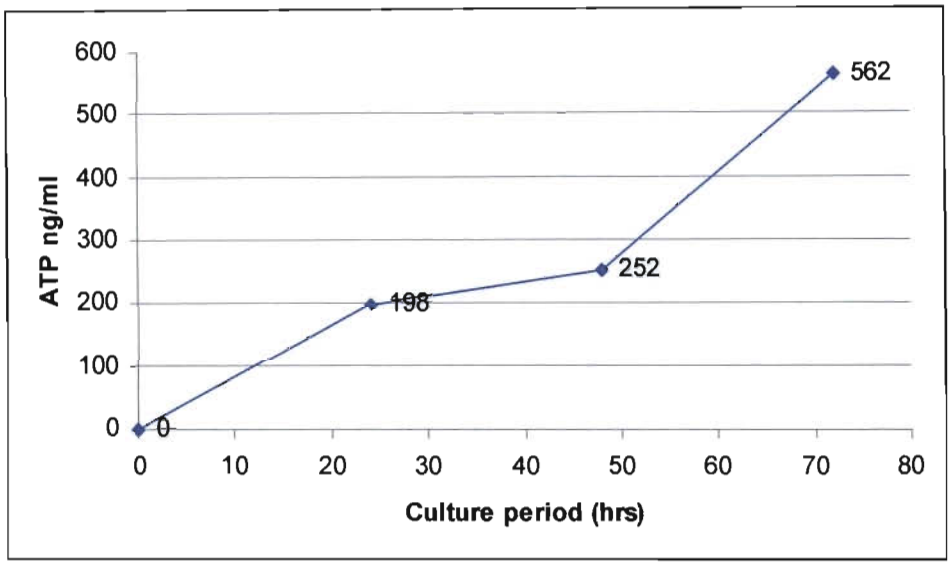

Fig 3.14: Changes in ATP level in PHA stimulated PBMC's cultured for 24, 48 and 72 hours

Table 3.2: The effect of various PHA concentrations on lymphocyte proliferation

PHA concentration	Unstimulated cells PBMC's only (ATP ng/ml – to measure basal ATP)	Stimulated cells PBMC's+PHA (ATP ng/ml)	Amount of ATP produced in response to stimulant PHA after subtracting the basal ATP value
0µg/ml	2852 ng/ml	-	-
1µg/ml	-	3193 ng/ml	341 ng/ml
5µg/ml	-	2959 ng/ml	107 ng/ml
10µg/ml	-	2817 ng/ml	-35 ng/ml

3.3 Clinical testing demographics

Table 3.3: Clinical testing demographics of kidney transplant patients

Kidney Transplant Patients						
	Cyclosporine		**Sirolimus**		**Tacrolimus**	
	Number of Samples	% of Total	Number of Samples	% Total	Number of Samples	% of Total
Gender						
Males	10	45	5	50	4	66
Females	12	55	5	50	2	33
Unknown	-	-	-	-	-	-
Total	**22**	100	**10**	100	**6**	100
Ethnicity						
Asian	14	64	6	60	4	66
African	2	9	4	40	1	17
Caucasian	4	18	-	-.	1	17
Coloured	2	9	-	-	-	-
Unknown	-	-	-	-	-	-
Total	22	100	10	100	6	100
Age range(yrs)	39-68		19-64		15-38	
Average Age	53.5		41.5		26.5	
Time since Transplant						
< 1 year	-	-	-	-	1	20
1-10 years	11	50	8	80	5	40
> 10 years	11	50	2	20	-	-
Unknown	-	-	-	-	-	40
Total	22	100	10	100	6	100

Table 3.4: Clinical testing demographics of control population

Healthy Controls		
	Number of Samples	% of Total
Gender		
Males	5	33
Females	5	33
Unknown	5	33
Total	15	100
Ethnicity		
Asian	10	67
African	-	
Caucasian	-	
Coloured	-	
Unknown	5	33
Total	15	100
Age range (years)	18-64	
Average Age	41	

3.4 The ATP bioluminescence assay results

Table 3.5: Immune response results obtained with the ATP bioluminescence assay in the control group, cyclosporine, sirolimus and tacrolimus groups (Chi square test was used to determine statistical significance of means)

	Unstimulated PBMC only (ATPng/ml) (measure of basal ATP activity)	**Stimulated** PBMC+PHA (ATPng/ml)	Amount of ATPng/ml produced following stimulation	% increase in ATP following stimulation	p-value (Control vs. immunosuppressant groups)
Control	2988±SEM216	4235±SEM 236	1247± SEM 132.2	47.69±SEM 7.24	
Cyclosporine	2406± SEM175	2649±SEM 178	243± SEM 26.56	11.40± SEM 1.67	p<0.001
Sirolimus	2250± SEM242	2505±SEM 255	255± SEM 44.39	11.99± SEM 1.88	p<0.001
Tacrolimus	2325±SEM 470	2691±SEM 501	366± SEM 109.0	21.62± SEM 10.78	p<0.001

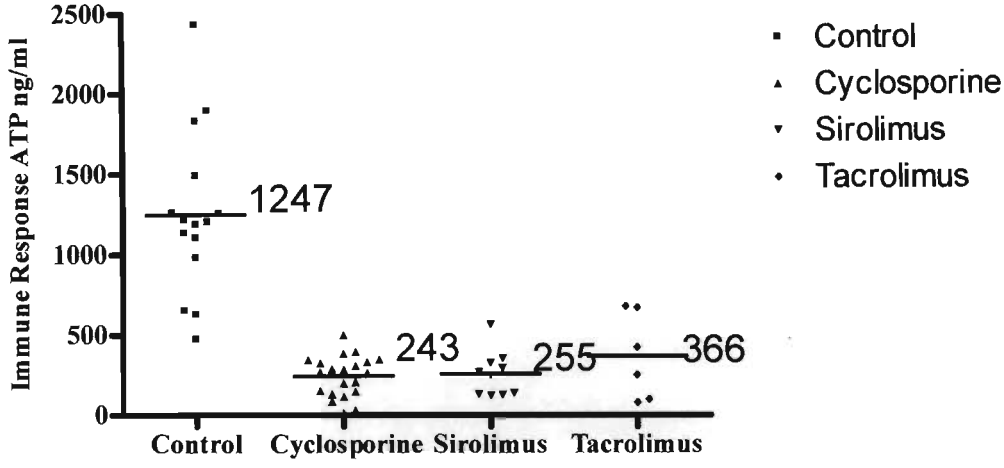

Fig 3.15: The average ATP produced following PHA stimulation of lymphocytes in the control group and the cyclosporine, sirolimus and tacrolimus treated patient groups (ng/ml)

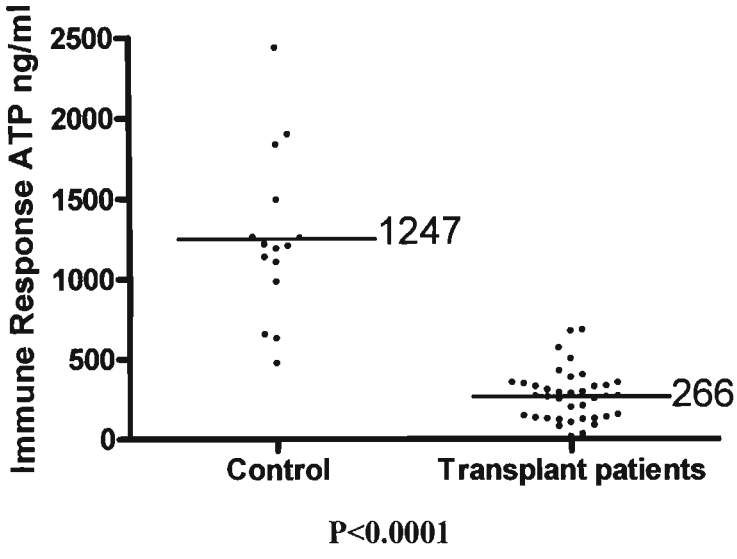

Fig 3.16: Evaluation of the immune response distributions between the control population (n=15) and all the kidney transplant patients included in the study (n=38)

Table 3.6: Statistical comparison of the immune response results according to the gender of transplant recipients and control population. (NS:not significant)

		Control Population		Transplant Recipient					
				Cyclosporine		Sirolimus		Tacrolimus	
		Mean	**p Value**	**Mean**	**p Value**	**Mean**	**p Value**	**Mean**	**p Value**
Gender	Male	911 (n=5)	P<0.05	225 (n=10)	NS	319 (n=5)	P<0.0001	424 (n=4)	P<0.0001
	Female	1051 (n=5)		259 (n=12)		191 (n=5)		250 (n=2)	
	unknown	1778 (n=5)		-		-		-	

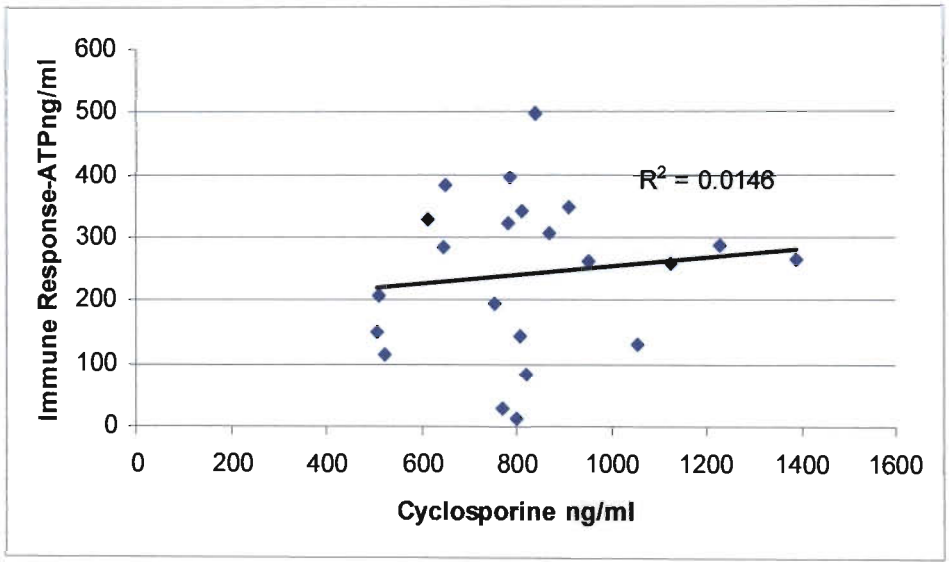

Fig 3.17: Assessment of ATP production of immune cells according to blood levels of cyclosporine

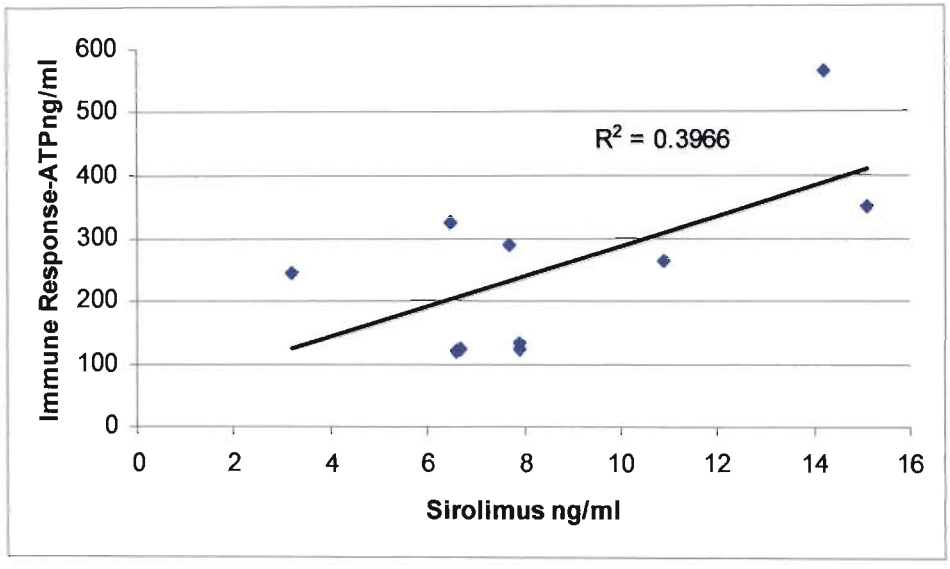

Fig 3.18: Assessment of ATP production of immune cells according to sirolimus blood levels

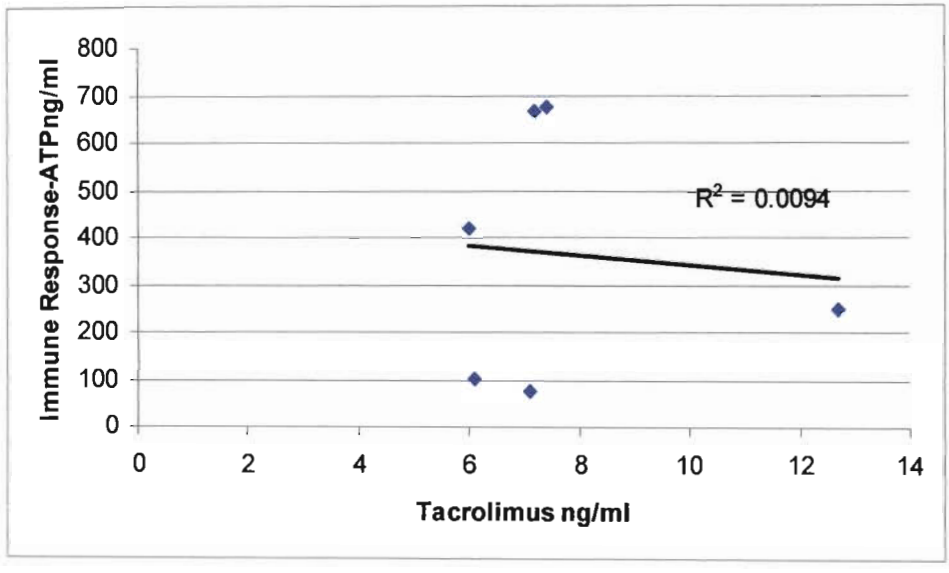

Fig 3.19: Assessment of ATP production according to tacrolimus blood levels

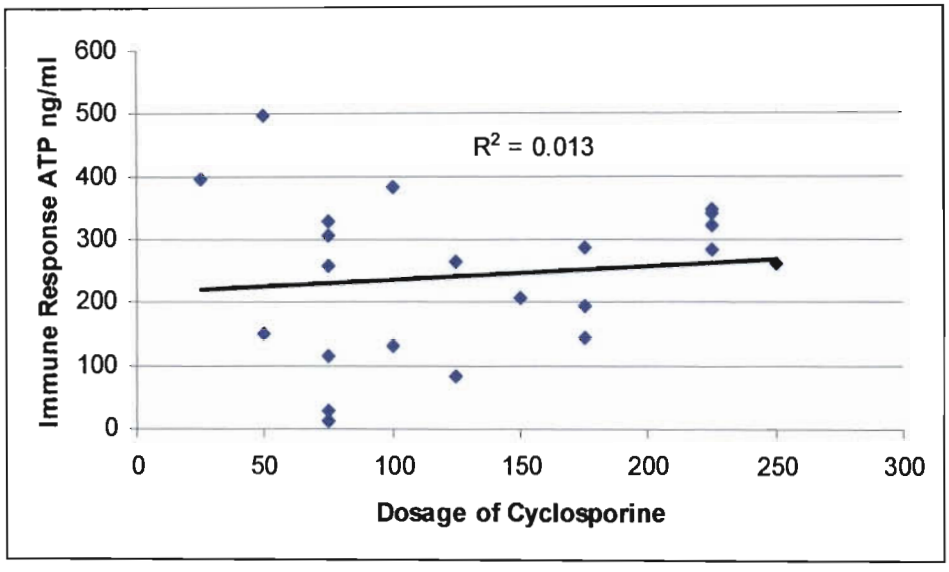

Fig 3.20: Assessment of immune response distribution according to dosage of cyclosporine (mg)

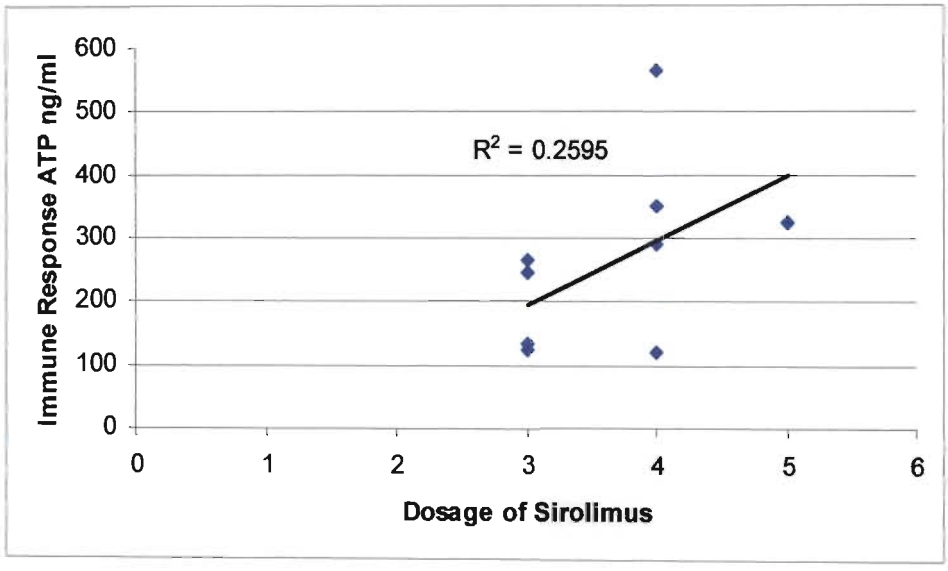

Fig 3.21: Assessment of the immune response distribution according to dosage of sirolimus (mg/24hr)

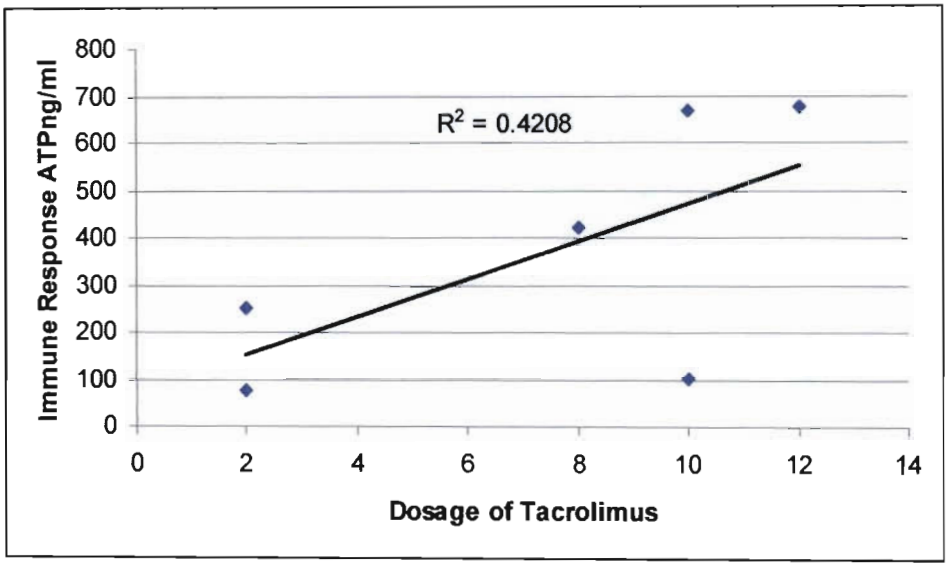

Fig 3.22: Assessment of the immune response distribution according to dosage of tacrolimus (mg)

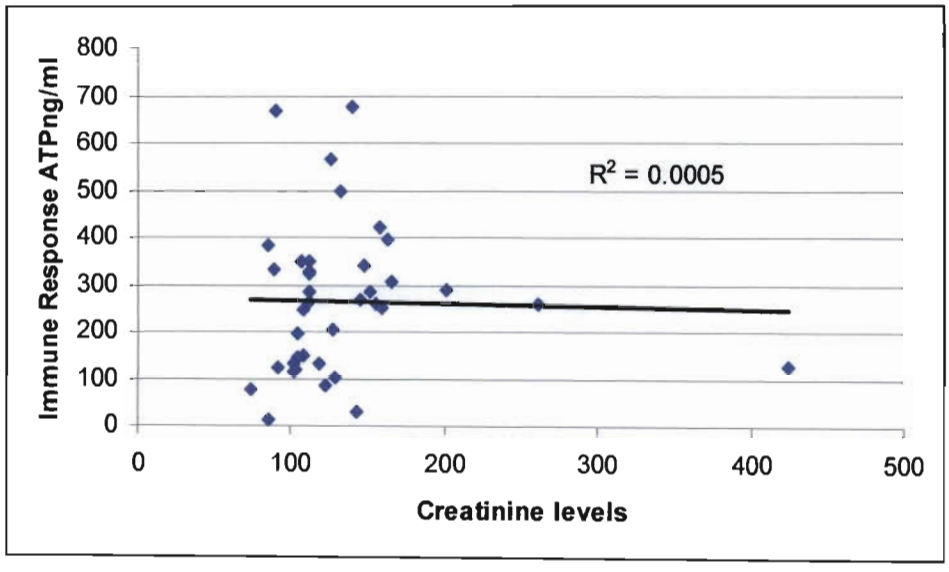

Fig 3.23: Assessment of the immune response distribution according to creatinine levels (µmol/l).

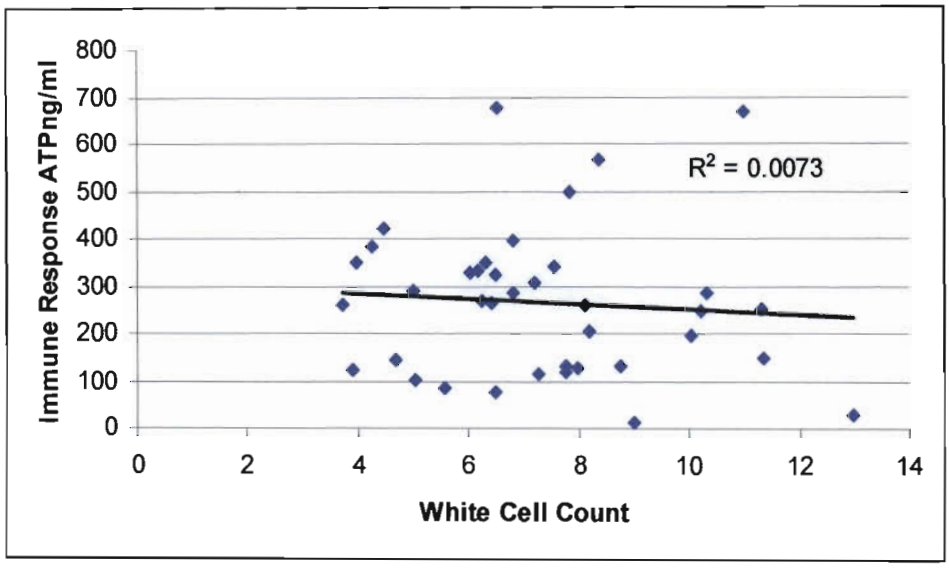

Fig 3.24: Assessment of the immune response distribution according to white cell count (WCC) of the transplant recipients (x 10^9/l).

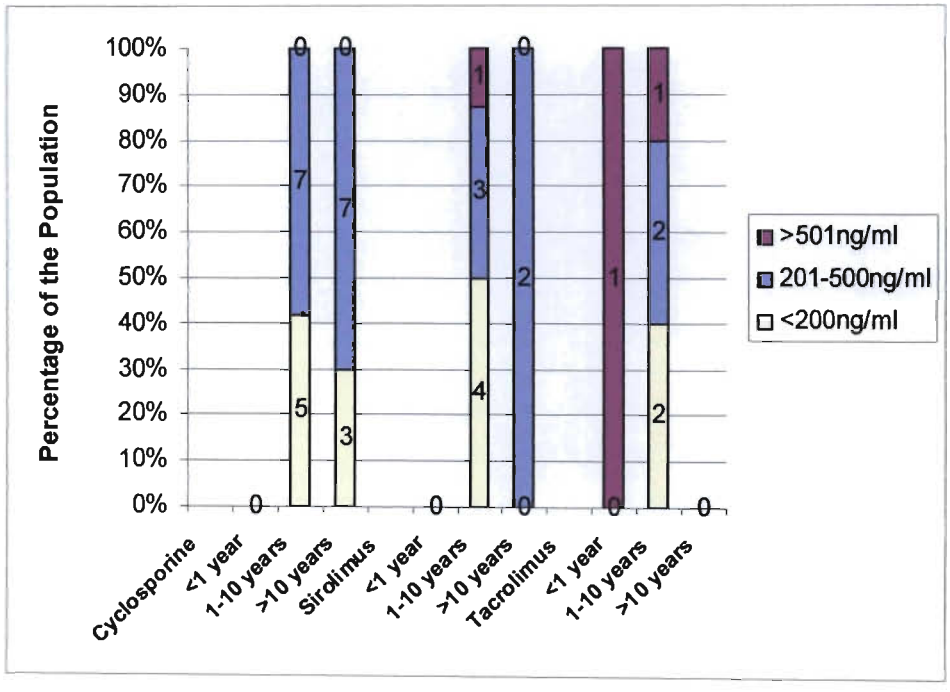

Fig 3.25: Immune cell response range vs. time since transplantation

Table 3.7: Evaluation of the immune response ranges and the clinical outcomes of transplant recipients

ATP levels (ng/ml)	**Stable**	**Infection**	**rejection**
>525 (n=3)	2	-	1
524-225 (n=20)	15	5	-
<224 (n=15)	12	3	-

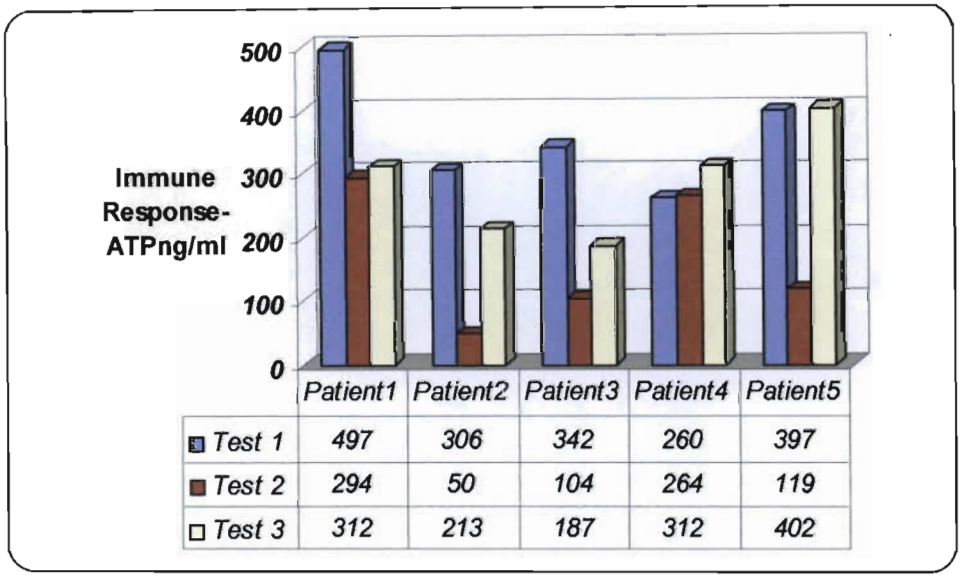

Fig 3.26: Immune response distribution of patients (n=5) over 3 months. (Low: <200, Moderate: 201-500, Strong: >501)

3.5 Evaluation of ATP bioluminescence Assay including plasma

Table 3.8: Immune response results obtained with the ATP bioluminescence assay in the control group, cyclosporine, sirolimus and tacrolimus groups in the presence of plasma isolated from the patients' blood (Chi square test was used to determine statistical significance of means)

	Unstimulated PBMC only (ATPng/ml) (measure basal ATP activity)	**Stimulated** PBMC+PHA+10µl patient plasma (ATPng/ml)	Amount of ATP (ng/ml) produced following stimulation	% increase/ decrease in ATP following stimulation
Control	2988 ±SEM216	3762 ±SEM 196	774 ± SEM 59.16	30.5 ±SEM5.0
Cyclosporine	2406 ± SEM175	2510 ±SEM 207.5	48 ± SEM 114.2	3.4 ± SEM 6.3
Sirolimus	2250 ± SEM242	2336 ±SEM 229.5	86 ± SEM 202.7	4.1 ± SEM 13.4
Tacrolimus	2325 ±SEM 470	2149 ±SEM 100.5	-176 ± SEM 426.5	6.2 ± SEM 28.1

Table 3.9: immune response results obtained with the ATP bioluminescence assay in the control group, cyclosporine, sirolimus and tacrolimus groups in the presence of plasma isolated from the blood of healthy individuals (Chi square test was used to determine statistical significance of means)

	Unstimulated PBMC only (ATPng/ml) (measure basal ATP activity)	**Stimulated** PBMC+PHA+10µl control plasma (ATPng/ml)	Amount of ATP (ng/ml) produced following stimulation	% increase/ decrease in ATP following stimulation
Control	2988 ±SEM 216	3313 ±SEM 200.1	325 ± SEM 78.35	13.7 ±SEM 3.5
Cyclosporine	2406 ± SEM175	1915 ±SEM 149.9	-547 ± SEM 139.3	-31.4 ± SEM 9.3
Sirolimus	2250 ± SEM242	1834 ±SEM 151.4	-416 ± SEM 130.8	-21.2 ± SEM 6.7
Tacrolimus	2325 ±SEM 470	1252 ±SEM 64.07	-1073 ± SEM 452.7	-83 ± SEM 35.5

Chapter 4

Discussion

Transplant recipients require lifelong immune suppression medication to prevent rejection. During personal communications with the patients included in this study, side effects of the medication/immune suppressants administered was cited as their number one concern.

A comment made by one of the patients was that "taking the medication is like a double ended sword, on the one hand it helps with making the new kidney work while on the other it makes us very sick, the side effects will be the end of me rather than my donors' kidney!" Some of the debilitating ailments mentioned by the patients included skin cancer, increase in hair growth, darkening of the skin, increase in acne, bone dystrophy requiring hip replacements, diarrhea, decrease in appetite, dental and gum complaints, loss of weight and palpitation.

This comment re-emphasised that there is a dire need for better diagnostic tools to monitor immune suppression and prevent the development of such complications and ultimately improve the transplant recipients' quality of life.

Thus the aim of the study was to evaluate the ATP bioluminescence assay as a potential diagnostic tool to assess the effect of immune suppressant medication on the immune response in a cross section of the transplant patient population attending IALCH renal

transplant clinic. Ultimately the study explores ATP as a determinant of viability and thus the level of immune suppression of lymphocytes.

As shown in figure 3.10 the steps involved in the ATP bioluminescence assay included isolation of peripheral blood mononuclear cells which were counted to a specific number and plated and incubated overnight with and without the stimulant PHA, following which the intracellular ATP content of the lymphocytes was measured with the use of the CellTiter Glo® luminescent cell viability assay which lysed the cells to release the intracellular ATP and produce light via the luciferin/luciferase enzyme reaction.

The luminescent signal was read by a luminometer. The concentration of ATP (ng/ml) was calculated from a standard curve that was run with each assay plate. Hence, the assay looks at the ability of lymphocytes to function according to their response to a mitogen and subsequent ATP production.

Although the method to culture peripheral blood mononuclear cells is well established (Boyum, 1968) the measurement of lymphocyte intracellular ATP to determine immune function via bioluminescence with the use of a luminometer to my knowledge, is not well established in the South African context.

The following components of the assay were optimised before subjecting patient samples to the assay these included generating a standard curve with a suitable range of ATP (ng/ml) values, testing different incubation periods, investigating a range of PHA concentrations to obtain one that elicited sufficient proliferation of T cells and evaluating the production of ATP (ng/ml) by varying concentrations of PBMC's.

An ATP standard was included in each microtiter plate in order to generate a standard curve from which the intracellular ATP levels of control and patient test samples could be directly calculated after each assay run. The relative light units (RLU) measured for control and patient samples were converted to ATP (ng/ml) based on equivalent RLU values in the ATP standard curve. As depicted in Figure 3.11 the known ATP ng/ml is plotted on the X axis and the amount of light produced measured in relative light units (RLU) on the Y axis. The ATP standard ranged from 1 ng/ml to 10 000 ng/ml. To assess linearity of the curve the correlation coefficient (r^2) was calculated.

Our findings correlated with previous studies that showed that the measure of intracellular ATP is directly proportional to the number of living cells in the sample. (Ishizaka et al., 1984., Bulanova et al., 1995., Kangas et al., 1984)

The relationship between RLU which is a measure of the intracellular ATP detected by the luminescent signal and the number of cells in culture showed a linear relationship (r^2= 0.99). Conversion of the RLU values to the corresponding ATP (ng/ml) values with the ATP standard curve calibrations confirmed that this assay exhibited a linear

correlation between ATP produced and the increasing concentrations of cells i.e. 2 500 to 200 000 (refer to Figure 3.12 and 3.13). This finding highlights the sensitivity of the ATP bioluminescence assay.

The increase in ATP in response to the stimulant PHA was measured after incubation periods of one (24 hr), two (48 hr) and three (72 hr) days. An increase in ATP production was demonstrated over the three incubation periods. There was a 198 ng/ml ATP level increase after a 24 hour culture, after 48 hours there was a slight increase to 252 ng/ml of ATP and after 72 hours of culture a marked increase in the ATP level (562 ng/ml of ATP) was found (refer to figure 3.14). A 24 hour incubation period was selected for the study because a change in ATP levels is able to be measured within approximately 24 hours as depicted above which is relatively short time period in comparison to other proliferation tests which require 3-10 days e.g. radioactive thymidine incorporation (Assounga and Jones, 1996).

A study by White et al., (1989) illustrated that there are early changes in the levels of intracellular ATP in mitogen stimulated lymphocytes. Under the influence of the mitogen, T lymphocytes undergo activation which includes the clustering of surface receptors, increased uptake of metabolites and ions and increased metabolism of phospholipids. These initial events are followed by DNA synthesis, synthesis of cytokines and finally mitosis. (White et al., 1989) These processes require ATP hence increases in intracellular ATP levels induced by mitogens in lymphocytes is related to cell activation. (Buttgereit et al., 2000., Sottong et al., 2000)

The mitogen phytohemagglutinin (PHA) was used as it is a potent stimulus and allows the study of the functional activity of lymphocytes in vitro as it is able to elicit a response in even highly suppressed lymphocytes. (Kowalski et al., 2003) Table 3.2 shows the results of testing various concentrations of PHA; it was found that high concentrations (5 µg/ml, 10 µg/ml) of PHA-M exhibited a toxic effect in the stimulated test wells in comparison to non stimulated cells. That is 107 ng/ml and -35 ng/ml of ATP which is less than the basal level of ATP was measured in response to 5 µg/ml and 10 µg/ml concentrations of PHA respectively. A significant increase in ATP (ng/ml) was measured in response to a lower dose of PHA (1 µg/ml) following 24 hour incubation (ie. 341 ng/ml). Hence 10 µl of PHA at a final concentration of 1 µg/ml was used. (Bulanova et al., 1995)

Table 3.3 and table 3.4 summarises the demographics of the populations included in the study. 38 kidney transplant patients and 15 apparently healthy adults were recruited. The inclusion criteria for controls were apparently healthy male and females of different ethnicities i.e. Indian, African, Caucasian and Coloured between the ages of 18 and 64 who were able to donate blood according to the blood donation guidelines.

The inclusion criteria for the transplant recipients were men and women of different ethnicities i.e. Indian, African, Caucasian and Coloured between the ages of 15-70 years who were recipients of a cadaver, living related or living unrelated kidney transplant. The inclusion criteria were not restricted by the time period since the transplant or the type of

immunosuppressant regimen. The dosage of the different immunosuppressant drugs varied within and between patients.

The nature of patients recruited is a reflection of the dynamics of the patients attending the IALCH renal transplant clinic. Majority of the transplant population are of the Indian race and majority of the patients are on cyclosporine that is approximately 200 in comparison to approximately 30 on sirolimus and approximately 10 patients on tacrolimus.

Table 3.5 represents the immune response results obtained with the ATP bioluminescence assay in the control group, cyclosporine, sirolimus and tacrolimus groups. For each of the groups both stimulated and non-stimulated PBMC's test wells were included for each test sample.

The average ATP (ng/ml) measured for the unstimulated PBMC's in the apparently healthy controls group was 2988 ng/ml (n=15, $\pm$SEM216) and the mean ATP (ng/ml) measured for the unstimulated PBMC's of the kidney transplant patients on the immune suppressants cyclosporine was 2406 ng/ml (n=22, $\pm$SEM175), sirolimus 2250 ng/ml (n=10, $\pm$SEM242) and tacrolimus 2325 ng/ml (n=6, $\pm$SEM470).

Statistically significant difference was measured between the mean unstimulated ATP (ng/ml) result of the control group and the unstimulated ATP (ng/ml) results of each of the immunosuppressant drug groups ($p<0.001$), no statistical significant result was

measured between the drug groups. The unstimulated PBMC's tested in the control group produced on average 661 ng/ml more ATP than the unstimulated PBMC's tested in each of the drug groups.

The mean ATP production compared between stimulated PBMC's in apparently healthy controls (n=15, mean:4235 ng/mlATP, ±SEM236) and kidney transplant patients on the immune suppressants cyclosporine (n=22, mean:2649 ng/mlATP, ±SEM178), sirolimus (n=10, mean: 2505 ng/mlATP, ±SEM255) and tacrolimus (n=6, mean:2691 ng/ml ATP, ±SEM501) differed by on average 1620 ng/ml ATP.

Statistically significant difference was measured between the mean ATP (ng/ml) results of the stimulated cells in the control group and the mean ATP (ng/ml) results of the stimulated cells in each of the immunosuppressant drug groups ($p<0.001$), no statistical significant result was measured between the drug groups.

In view of the fact that all somatic cells contain ATP and will therefore exhibit a baseline level of ATP, unstimulated test wells were set up for each test sample to measure basal ATP activity. We therefore proposed that calculating the difference between the unstimulated and stimulated results was a measure of the ATP produced by lymphocytes that were activated to proliferate in response to PHA, this value was used to characterise the immune response of the individuals tested. The percentage increase between unstimulated cells and stimulated cells was also calculated.

Figure 3.15 depicts the average increase in ATP (ng/ml) by lymphocytes in response to PHA stimulation. Each dot represents the results of the individuals tested. The mean increase in ATP (ng/ml) by lymphocytes in response to PHA stimulation in the group of healthy donors was 1247 ng/ml (±SEM132.2, median: 1200, min: 471, max: 2431) with a percentage increase of 47.69% (±SEM 7.24)

The mean increase in ATP (ng/ml) by lymphocytes in response to PHA stimulation in the cyclosporine group was 243 ng/ml (±SEM26.56, median:263, min:13, max:497) compared to 255 ng/ml (±SEM44.39, median:256, min:121, max:564) and 366 ng/ml (±SEM109.0, median:336, min:78, max:677) increase in ATP for the sirolimus and tacrolimus groups respectively. Cyclosporine had a percentage increase of 11.4% while sirolimus and tacrolimus had percentage increases of 11.99% and 21.62% respectively

Statistically significant difference was measured between the mean increase in ATP (ng/ml) calculated for the control group and each of the immunosuppressant drug groups ($p<0.001$). No statistically significant difference was measured between the mean increase in ATP measured for the cyclosporine and sirolimus groups. These two immunosuppressant drugs appear to have a similar immunosuppressive effect (potency) on patients' immune system despite the fact that the mode of action on lymphocytes differ.

A statistically significant difference was measured between the tacrolimus group and the cyclosporine as well as the sirolimus group. Tacrolimus treated patients demonstrated on average 123 ng/ml and 111 ng/ml (ATP) stronger response to PHA stimulation than the cyclosporine and sirolimus treated patients respectively. However it must be noted that fewer patients who received tacrolimus (n=6) based regimen was included in the study.

Bearing in mind tacrolimus is considered to be a more potent immune suppressant drug (Kowalski et al., 2003) in comparison to the other two test drugs because of its' greater affinity for calcineurin, one would have expected the results to reflect the opposite i.e. lower lymphocyte response to PHA stimulation. A possible explanation for these results is the age of the patients who were recruited in this group. They were fairly young, an average age of 26 years old with the youngest being 15 and oldest 34 in comparison to patients in the other drug groups, cyclosporine with an average age of 53 years old and sirolimus 41 years old. It has been documented that as one ages the immune system weakens, and another contributing factor could be the fact that the patients in the tacrolimus group were made up of more recent transplants, less than 10 years old, hence having a stronger immune response. (Tsukada et al., 1999., Martins et al., 2005)

Overall the immune response results of the kidney transplant patients (n=38) which averaged 266 ng/ml ($\pm$SEM 25.75, median 263, min 13, max 677) was significantly statistically lower ($P<0.0001$) than healthy controls by 981 ng/ml of ATP (refer to Figure 3.16).

Table 3.6 illustrates the comparison between ATP (ng/ml) measured and gender of transplant recipients and apparently healthy controls. The immune response of males and females transplant recipients were not statistically different in the cyclosporine population ($P=0.2742$) however in the tacrolimus and sirolimus patient groups statistical significant difference was measured ($P<0.0001$).

Male patients showed a 34 ng/ml lower ATP level than females in cyclosporine group this same trend is not seen in the sirolimus and tacrolimus groups where males had a 128 ng/ml and 174 ng/ml respectively higher ATP level than their female counterparts.

The dosages of immunosuppressant drugs administered to the male and female patients in the sirolimus and tacrolimus groups were analysed to determine if they differed to explain the statistical significant difference in the mean ATP (ng/ml) measured for patients in these drug groups. No major difference was observed, male patients were not given higher or lower doses of immunosuppressant drugs in comparison to their female counter parts.

However, the small size of the sample of patients in the sirolimus and tacrolimus groups, dictates that no clinical significance should be drawn from these results unless they are confirmed in a larger patient sample.

Healthy male controls gave statistically significant lower ($P<0.05$) immune responses than females by 140 ng/ml ATP. Females are known to have a more active immune system than males. They have significantly higher numbers of CD4 T lymphocytes than males, which most probably contributes to their increased immune response. Unfortunately, in some individuals their immune responses are exaggerated to such an extent that it leads to the development of autoimmune diseases e.g. systemic lupus erythematosus (SLE) which has a female to male ratio of approximately 9:1. (Whitacre, 2001., Trune and Kempton, 2002)

In this study we did not investigate the differences between ATP production and patients of different ethnicities, because the majority of the patients attending the IALCH renal transplant clinic and hence recruited into this study were of the Asian/Indian designation. However, it is important to investigate this issue in future studies, more insight into ethnicity dependent variations of ATP production could support individualised immune suppression strategies.

Measuring the level of immunosuppressive drugs in the blood is a common diagnostic method to determine mainly drug toxicity and patient compliance. (Kowalski et al., 2003) An assessment was made of the levels of cyclosporine, sirolimus and tacrolimus measured in whole blood obtained from patients' medical records according to the ATP bioluminescence assay results of the patients in the respective immunosuppressant drug groups. (Refer to figure 3.17, 3.18, 3.19)

No correlation was observed between patients' ATP (ng/ml) results and therapeutic drug levels measured in the cyclosporine (r^2=0.0146), sirolimus and tacrolimus groups r^2=0.2825 and r^2=0.0017 respectively. This highlights the importance of measuring the cumulative effect of the drugs on immune system.

This lack of correlation may be attributed to differences in each individuals' pharmacokinetic responses. Each patient immune system responds differently to immune suppressant regimens based on their age, ethnicity, presence of medical conditions such as diabetes, osteoporosis, cardiovascular disease and the strength of the patients immune system. (Danovitch, 1999., Ojo et al., 2000)

The method used for determining the therapeutic drug level targets one drug and does not take into account the effect of other drugs administered e.g. steroids, antiproliferatives or antimetabolites. (Jusko and Thomas, 1995., Yatscoff et al., 1990) A study by Israeli M. et al., 2007 found ATP based assay to have a higher level of correlation with patient clinical status than calcineurin inhibitor blood levels (CNI). (Israeli et al., 2007)

In accordance with previous studies, regression analysis indicated no correlation was observed between ATP production of lymphocytes from transplant recipients and immunosuppressive drug dosages (Figure 3.20, 3.21, 3.22), creatinine levels (Figure 3.23) and white cell counts (WCC) (Figure 3.24) (Chávez et al., 2006).

The lack of an association between the drug dosages, creatinine levels and WCC may be as a result of the difference in each patients' pharmacokinetic response to the immunosuppressive drugs, (Kowalski et al., 2003) also factors that effect immune response such as stress and inflammation in the graft may not be reflected in creatinine levels or WCC. Creatinine is a late indicator of rejection it is elevated after cellular infiltration has taken place therefore it is a poor indicator of complications within the graft. WCC fails to measure the viability of the cells; a high count does not correlate with increased ATP production as the test does not take into account whether the cells are functionally compromised. (Johnston and DW, 1999., Kowalski et al., 2006)

In order, to interpret the ATP results a standard range of values is required to compare the results to and characterise the individuals' immune response as low, moderate or strong. For this study the immune cell response of healthy controls and patients on cyclosporine, sirolimus and tacrolimus were characterised according to these three different ranges that were arbitrarily chosen based on the mixture of ATP results observed <200 ng/ml was considered a low immune response, 201-500 ng/ml a moderate immune response and >501 ng/ml a strong immune response. Future studies are required to establish a more precise range to our setting/laboratory by testing the assay on a larger population of known healthy and immunocommpromised individuals.

In figure 3.25 the immune cell response of patients on cyclosporine, sirolimus and tacrolimus were characterised according to the three different ranges and were plotted against the time since transplantation. The ATP bioluminescence assay results do not directly quantify the level of immune suppression therefore the results are described in conjunction with clinical presentation (Kowalski et al., 2003) to give a complete picture of the patients' immune status.

Within the past five years approximately 30 new transplantations were carried out at the IALCH thus the number of patients in the, one year group were far and few between. Majority of the patients who have had a transplant for more than one year, appeared to have a moderate or low immune response, possibly indicating that the effectiveness of the immunosuppressive therapy on the immune system improves over time despite the patients being on lower doses of the drugs/maintenance therapy. (Kowalski et al., 2003)

Over time the continuous consumption of immunosuppressant drugs results in reduced immune response to the newly transplanted organ but the immune system becomes suppressed to such an extent that it leads to an increase in incidences of infection, malignancies, drug toxicities etc. (Zeevi et al., 2005., Dantal et al., 1998)

In the cyclosporine group no patient gave a strong immune response over the three time periods. The vast majority (n=14), were in the moderate immune response range. Three of the patients who had an immune response characterised as moderate presented with urinary tract infection (UTI) and one with suspected bronchopneumonia at the time of the test, the rest were considered to be clinically stable.

Five (40%) of the twelve patients who received their kidney transplant within 1-10 years and three (30%) of the ten patients who received a transplant more than 10 years ago had a low immune response (<225 ng/ml). Seven of these patients with a low immune response were considered clinically stable at the time of the test only one patient was diagnosed with UTI.

Within the sirolimus group four (50%) of the eight patient's in the 1-10 years transplantation group had a low immune response (<225 ng/ml). Three of these patient's were clinically stable and one presented with CMV infection and recurrent UTI.

The patient who gave a strong immune response (>525 ng/ml) in the 1-10 years transplantation group was considered to be clinically stable. The remaining three were in the moderate immune response range; one of these patients displayed symptoms of UTI and suspected TB and the other two patients were considered clinically stable.

One patient in the Tacrolimus group was newly transplanted and displayed a strong immune response with an ATP (ng/ml) level of 667 ng/ml, his clinical status showed raised WCC, urea and creatinine levels. A biopsy that was carried out did not point to acute rejection however acute rejection was suspected based on his clinical picture. He had no symptoms of infection.

Of the remaining five patients on tacrolimus in the 1-10 year time period, one was tested to have a strong immune response and two each with a moderate and low immune response. The patient with the strong immune response showed no signs of rejection however he was treated for dermatological related conditions. Of the two patients with the low immune response, one was clinically stable and the other presented with recurrent Klebsiella UTI and the patients in the moderate range were clinically stable.

Table 3.6 represents the results of the transplant patients in all three drug groups that were pooled and classified according to their clinical status (rejection, infection and stability) and the immune response standard range. Patients diagnosed with an infection were more frequent in the moderate (n=5) and low (n=3) immune response range. Of the patients diagnosed as clinically stable twelve patients (n=12) were included in the low immune response zone and fifteen patients (n=15) in the moderate immune response range.

Infection was confirmed via microbiological culture. The mean ATP levels for patients with infection was 248 ng/ml ATP (±SEM40.52, n=8) and those that were stable 257 ng/ml ATP (±SEM28.82, n=29). No statistically significant difference was measured between the means.

It has been reported by Gautam et al., (2006) that a single determination of the ATP level has limited diagnostic value, serial determination using the patients as his or her own control provides more information of patients at risk of infection or rejection. (Gautam et al., 2006)

Figure 3.26 depicts the immune response distribution of five patients who were tested monthly for three months. All five patients maintained an immune response status within the low and moderate immune response range over the time period. (refer to appendix 15)

Patient one and patient four maintained a moderate immune response over the three months. Patient one had no signs of infection or rejection over the three months his medical history indicated, he had gingival enlargement (cyclosporine toxicity) and recurrent haematuria. Patient four clinical indicators also shows no sign of rejection however had a recent history of septic wound post removal of a Tenckhoff dialysis catheter removal from the abdomen however healing was good.

Patient two and three displayed moderate to low immune response, patient two was diagnosed with a UTI and patient three presented with a cough, night sweats and sinusitis and a rapid loss of weight bronchopneumonia was suspected during the test period.

Patients 5 results over the three months ranged between moderate and low, the clinical history showed no indicators for rejection however diagnosis of influenza, UTI and infection of the upper gum was made.

The results of some patients interchanged between low and moderate immune response ATP range between each month, with no distinct changes in the clinical status, possibly since the patients were rejection free the ATP levels did not change dramatically therefore further studies is required in a larger cohort of patients preferably in pre and post transplantation when the risk of rejection is higher (Kowalski et al., 2006) to accurately determine the predictive value of the assay.

The response of PBMC's to the stimulant PHA in the presence of plasma isolated from patient's blood and plasma from healthy individual's was also investigated. (Refer to table 3.8 and table 3.9) We found that the level of ATP (ng/ml) measured in the presence of patient's plasma in both the control and immunosuppressant drug groups were lower than the levels of ATP (ng/ml) measured without plasma. It was also found that plasma isolated from healthy individuals incubated with PBMC's from the control group as well as from patients in the immunosuppressant drug groups resulted in the lowest level of ATP (ng/ml) measured.

In the control group, PBMC's incubated with plasma from patients on either cyclosporine, tacrolimus or sirolimus a mean of 774 ng/ml level of ATP (30.5% increase) was measured as compared to 1247 ng/ml ATP (47.69% increase) in the absence of plasma from patients. Surprisingly in the presence of plasma from the healthy controls a lower level of 325 ng/ml ATP (13.67% increase) was measured.

In the cyclosporine group when patients' PBMC's were incubated with their plasma, a mean of 48 ng/ml ATP (3.4% increase) was measured as compared to 227 ng/ml ATP (11.4% increase) in the absence of plasma. Surprisingly in the presence of plasma from the healthy control there was no increase in ATP (-547 ng/ml ATP; -31.4% decrease), proliferation was inhibited.

In the sirolimus group when patient PBMC's were incubated in the presence of their plasma, a mean of 86 ng/ml level of ATP (4.1% increase) was measured as compared to 255 ng/ml ATP (11.99% increase) in the absence of plasma. Surprisingly in the presence of plasma from the healthy controls there was no increase in ATP (-416 ng/ml ATP; -21.2% decrease), proliferation was inhibited.

In the tacrolimus group when patient PBMC's were incubated with their plasma, no increase in ATP was measured (-176 ng/ml ATP; -6.2% decrease) as compared to 366 ng/ml ATP measured (21.62% increase) in the absence of plasma from patients. In the presence of plasma from the healthy control there was also no increase in ATP (-1072 ng/ml ATP; -83.1% decrease), proliferation was inhibited.

It appears that plasma isolated from patients on immunosuppressant drugs and more so plasma from healthy controls contains factors which suppress the response of lymphocytes to PHA stimulation. The concept that plasma proteins are capable of inhibiting immune response was first demonstrated by Kamrin et.al., (1959) Plasma lipoproteins carry out lipid transport and bioregulatory roles. (Hui et al., 1980)

Suppression of lymphocyte proliferation by human plasma lipoproteins has been observed in a variety of studies. (Curtiss and Edgington, 1980) Alpha-globulin-enriched fractions from human plasma have been shown to possess immunosuppressive properties. (Mowbry, 1963)

A study by Cooperband et al., (1972) reported that α globulin fraction of plasma may play a role in the regulation of the immune response. It is able to suppress immune responses in vivo and prevents lymphocyte proliferation in vitro. The study demonstrated that α globulin is elevated when patients are undergoing renal rejection and α globulins isolated from these patients suppress PHA induced lymphocyte stimulation in vitro. It is assumed that the mechanism by which α globulin functions to prevent stimulation of lymphocytes may be by noncompetitive inhibition of activation. This implies that there is a receptor for the α globulin and another for the mitogenic stimulus. The mitogen receptor will not carry out its metabolic events when the α globulin receptor is activated. (Cooperband et al., 1972)

Inhibition of lymphocyte stimulation by PHA has been observed with plasma lipoproteins that regulate several aspects of lymphocyte function and metabolism. All the classes of lipoprotein fractions; Very Low Density Lipoproteins, Intermediate Density Lipoproteins, Low Density Lipoproteins, and High Density Lipoproteins were shown to inhibit the mitogen-induced stimulation of lymphocyte proliferation in a study by Morse et al., (1977) (Morse et al., 1977., Cuthbert and Lipsky, 1983., Hui et al., 1980)

Plasma lipoproteins, particularly those containing apolipoproteins B and E, have been shown to suppress mitogen activation and subsequent proliferation of peripheral blood T lymphocytes.(McCarthy et al., 1987)

Hence a possible explanation for the suppressed immune response and decreased ATP levels measured in the presence of plasma from healthy controls may be as a result of lipoproteins in the plasma. Future studies are required to confirm this as well as optimise the ATP bioluminescence assay to include plasma in the assay.

The suppressed response to plasma from patients may be attributed to the fact that plasma plays a role in the distribution of the drug in the body. The drugs may occur in plasma as free and unbound or bound to plasma proteins. Hence when incubated with the PBMC's the presence of the immunosuppressant drugs in the plasma results in a further suppression. (Wasan, K.M, 998)

Hyperlipidemic plasma has also been shown to have an inhibitory effect on lymphocyte proliferative responses to mitogenic stimulation in culture in several clinical conditions including nephrotic syndrome. (Lenarsky et al., 1982., Martini et al., 1981) Hyperlipedemia is a known side effect of cyclosporine, tacrolimus as well as sirolimus.(Sayegh et al., 1999)

The assay under investigation is similar to an FDA approved assay known as ImmunKnow™ immune cell function assay manufactured by Cylex Inc®, (Columbia, MD, USA) which briefly involves overnight incubation of whole blood with PHA, after which CD4 cells are selected using paramagnetic particles coated with a monoclonal antibody to the CD4 epitope followed by lysis of the cell and ATP measurement. (Kowalski et al., 2003)

The protocol of the assay evaluated in this research study differs from the ImmunKnow™ immune cell function assay in that it does not involve the use of whole blood or separation of CD4 T cell.

Studies by Barten et al., (2002) and Copeland et al., (1988) stated that using whole blood when stimulating the cells with PHA allows the lymphocytes to be maintained in the presence of the immunosuppressive drugs which are partitioned between the red cells membrane (Barten et al., 2002., Copeland and Yatscoff, 1988) in conjunction Zeevi et al., (1996) reported that a prolonged incubation time of 5-7 days with out red blood cells results in lymphocytes recovering from suppression and hence an increase in the recall responses and alloreactivity of T cells. (Zeevi and Duquesnoy, 1996., Kowalski et al., 2003)

However, in view of the fact that the assay under investigation requires only an overnight incubation of PBMC's with PHA in the absence of red blood cells, the concern of the lymphocytes reverting to their original strength is negligible.

The protocol of the assay under evaluation did not involve the isolation of CD4 T lymphocytes but rather makes use of peripheral blood mononuclear cells (PBMC's) i.e. a mixture of lymphocytes and monocytes. It has been stated by the Cylex Inc research group that CD4 T cells were isolated as they play a major role in rejection. (Kowalski et al., 2003)

Purified peripheral blood mononuclear cells were utilised based on the view that using the mixture of cells has more clinical relevance, allowing the lymphocytes to be maintained in an environment comparable to which the cells interact in vivo.

CD4 T cells do not work in isolation; they play an immunoregulatory role via cytokines (IL2) therefore suppression of CD4 T cells results in overall suppression of the cells it interacts with. By removing a specific subset of T cells from a mixed population of cells results in the accessory cells which are required for the response of T cells to antigens not to be present. PHA is not sufficient to fully activate CD4 T cells to proliferate it requires co-stimulation by other immune cells such as monocytes. (Grimm, 2006., Halvorsen et al., 1988., McCarthy et al., 1987)

Magnetic separation of specific immune cells as in the ImmunKnow™ immune cell function assay is expensive and requires special equipment. Separation of lymphocytes by magnetic affinity techniques may result in changes in the lymphocytes which may affect further tests on the isolated cells. The separation of a subset of lymphocytes from whole blood requires a further wash step to remove cells that are bound non-specifically.

It is also difficult to preserve the sterility of the sample once the cells are removed from the original environment. (Wier, 2007)

Transplant recipients are on a variety of immunosuppressant drugs which inhibit a range of immune cells. While cyclosporine and tacrolimus specifically target T lymphocytes, sirolimus on the other hand has a non specific antiproliferative effect it targets the cell cycle which includes all dividing cells and azathioprine and mycophenolate mofetil which prevents the expansion of both T cells and B cells. (Sayegh et al., 1999) Hence it was decided to not limit the study by isolating CD4 T cells.

Potential drawbacks of the study: repeated cultures using the lymphocytes of one individual showed variation in the ATP levels calculated after stimulation. It must be noted that many factors other than pharmacologic immune suppression may influence PBMC's and specifically T cell function, results may vary as T cells are affected by the time of day the blood sample is taken (circadian/diurnal variation), fatigue and stress. (Promega, 2005., Grimm, 2006) Good reproducible results are dependent on appropriate handling of reagents, cell counts and plating techniques. Bioluminescence reagents are sensitive to extremes in temperature and bright light.

All material of cellular origin will give a response with an ATP bioluminescence method, i.e. somatic/non microbial and microbial cells all contain ATP. ATP is fairly heat stable, autoclaving may not be sufficient therefore in order to prevent contamination necessary precautions need to be taken. (Redsven et al., 2007)

To ensure a uniform number of cells, cells were counted using a hemacytometer, this technique has substantial accuracy error due to its' subjective nature and it may result in an over estimation or under estimation of the number of viable cells, it is also laborious allowing only a few tests to be performed simultaneously. However the advantages of counting cells using a hemacytometer is that it is a cost effective method to distinguish viable cells using light microscopy and only a small fraction of cells are required. (Trauth and Keesey, 1996)

The ATP bioluminescence assay requires fresh blood. The blood sample needs to be used within 30 hours to ensure good proliferation of cells following mitogen stimulation. The use of fresh blood, however, is a potential drawback as it does not allow the processing of a large number of samples in a short period of time and collection of fresh blood samples over a long period of time may be affected by seasonal and experimental variations. The use of cryopreserved cells allows better planning of a study, replication of experiments to confirm results, and the option of repeating a test if there are inaccuracies. It will also allow retrospective investigations on the predictivity of ATP of the status of the immune system by using the blood sample obtained before the first clinical signs of a complication occurred. (Zijno et al., 2007)

A paper by Bohler T et al., 2007 which looked at biomarkers for pharmocodynamic monitoring of immunosuppressive drugs questions the role of ATP as a efficacy biomarker, since ATP reflects the overall energy metabolism of cells it is not specific in determining the effects of immunosuppressive drugs. The pharmocodynamic monitoring biomarkers considered to be more specific and investigated in the study were intracellular cytokine expression (IL2 and TNFα) which respond to immune suppressants in a more specific way. (Bohler et al., 2007) Future research may involve the evaluation of the immunomodulatory effects of currently used immunosuppressive regimens on peripheral blood T cell cytokine production.

Chapter 5

Conclusion

The assay described in this study is part of the growing interest and research in the development of quick, convenient and reliable assays to monitor the immune system. The immunological markers currently under investigation include serum CD30, specific anti donor antibodies, interferon gamma enzyme linked immunosorbent assay (ELISA), perforin granzyme B in urine and proteomic techniques. (Israeli et al., 2007., Rodrigo et al., 2008) The ultimate aim is to improve on currently available diagnostic tests which will allow for early and non invasive detection and prevention of complications as well as improve on strategies to obtain the optimal immunosuppressive dose.

The proposed bioluminescence method for the evaluation of the proliferative response of T lymphocytes by measuring ATP production was shown to be sensitive and simple in keeping with previous work by research groups namely Cree and Andreotti, 1997., White et al., 1989., Crouch et al., 1993., Ishizaka et al., 1984. The assay is non radioactive, it is a simple straight forward protocol and employs standard equipment and standard cell culture formats. The results obtained with the ATP bioluminescence assay evaluated in this study further validated ATP as a marker for monitoring lymphocyte function.

The kidney transplant patients on immunosuppressive drugs gave a significantly lower immune response than controls. Clinical findings of high frequency of various infections in the patient population support the overall findings. However determining the correlation between the immune response invitro and invivo requires a larger population. The predictive value of the ATP bioluminescence assay investigated in this study was insufficient for directing immunosuppressive therapy.

The future challenges include developing the assay further by performing follow up studies to improve the method and to validate the role of the assay in managing immune suppression in a larger patient population. The ATP bioluminescence assay has potential to be used in the assessment of the immune response in other scenarios e.g. the effect of debilitating infectious diseases like HIV/Aids on the immune system and monitoring vaccine, cancer therapy etc.

Chapter 6

References

SANBS,2008. Blood donation guidelines In:www.sanbs.org.za Date accessed: June 2008.

Allison, AC. 2000. Immunosuppressive drugs: the first 50 years and a glance forward. Immunopharmacology, 47, 63-83.

Andres, A. 2005. Cancer incidence after immunosuppressive treatment following kidney transplantation. Critical Reviews in Oncology/Hematology, 56, 71-85.

Assounga, AG., Jones, GS. 1996. In-vitro Immunosuppression with Phosphorylated 2 Aminothiazoles. Saudi Journal of kidney diseases and Transplantation, 7, Suppl 1: S152-S153.

Aw, MM. 2003. Transplant Immunology. Journal of Paediatric Surgery, 38, 1275 - 1280.

Barret, WL., First, MR., Aron, S., Penn, I. 1993. Clinical course of malignancies in renal transplant recipients. Cancer, 72, pg2186.

Barten, MJ., van Gelder, T., Gummert, JF., Shorthouse, R., Morris, RE. 2002. Novel assays of multiple lymphocyte functions in whole blood measure. New mechanisms of action of mycophenolate mofetil in vivo. Transplant Immunology, 10, 1-14.

Beatty, PR., Krams, SM., Esquivel, CO., Martinez, OM. 1998. Effect of cyclosporine and tacrolimus on the growth of Epstein Barr virus transformed B cells lines. Transplantation, 65, pg1248.

Bohler, T., Nolting, J., Kamar, N., Gurragchaa, P., Reisener, K., Glander, P., Neumayer, H., Budde, K., Klupp, J. 2007. Validation of immunological biomarkers for the pharmacodynamic monitoring of immunosuppressive drugs in humans. Therapeutic Drug Monitoring, 29, 77-86.

Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. Journal clinical Laboratory investigations, 21, 77-89.

Boyum, A. 1977. Separation of Lymphocytes, Lymphocyte Subgroups and Monocytes: A review. Lymphology, 10, 71-76.

Bradbury, DA., Simmons, TD., Slater, KJ., Crouch, SPM. 2000. Measurement of the ADP:ATP ratio in human leukaemia cell lines can be used as an indicator of cell viability, necrosis and apoptosis. Journal of Immunological Methods, 240, 79-92.

Briscoe, DM., Sayegh, MH. 2002. A rendezvous before rejection: Where do T cells meet transplant antigens? Nature Medicine, 8, 220 - 222.

Buckley, R. 2003. Transplantation immunology: Organ and bone marrow. Journal Allergy Clinical Immunology, 111, S733.

Bulanova, EG., Budagyan, VM., Romanova, NA., Brovko, LY., Ugarova, NN. 1995. Bioluminescent assay for human lymphocyte blast transformation. Immunology Letters, 46, 153-155.

Bushell, A., Wood, KJ. 1999. Permanent survival of organ transplants without immunosuppression: Experimental approaches and possibilities for tolerance induction in clinical transplantation. . Expert Review in Molecular Medicine In: http://www.expertreviews.org/99001179h.htm Date accessed: Jan 2007

Buttgereit, F., Burmester, GR., Brand, MD. 2000. Bioenergetics of immune functions:fundamental and therapeutics aspects. Immunology Today, 21, 192-199.

Caruso, R., Perico, N., Cattaneo, D., Piccinini, G., Bonazzola, S., Remuzzi, G., Gaspari, F. 2001. Whole Blood calcineurin activity is not predicted by cyclosporine blood concentration in renal transplant recipients. Clinical Chemistry, 47, 1679–1687.

Chávez, RC., De Echegaray, S., Santiago-Delpı´n, EA., Rodrı´guez, AT., Camacho, B., Alfaro, T., Saavedra, M., Carrasquillo, L., Caraballo, G., Morales, LA. 2006. Assessing the Risk of Infection and Rejection in Hispanic Renal Transplant Recipients by Means of an Adenosine Triphosphate Release Assay. Transplantation Proceedings, 38, 918-920.

Cianci, J., Lamb, J., Ryan, RK. 1981. Renal Transplantation. American Journal of Nursing, 81, 354-355.

Cooperband, SR., Badger, AM., Davis, RC., Schmid, K., Mannick, JA. 1972. The effect of immunoregulatory alpha globulin upon lymphocytes in vitro. Journal of Immunology, 109, 154-163.

Copeland, KR., Yatscoff, RW. 1988. Use of a monoclonal antibody for the therapeutic monitoring of cyclosporine in plasma and whole blood. Therapeutic Drug Monitoring, 10, pg453.

Cotran, RS., Kumar, V., Colins, T. 2000. The Kidney. Robbins Pathologic basis of disease, 6th edition. W.B. Saunders company The Curtis Center, Independence Square West, Philadelphia, Pennsylvania

Cree, IA., Andreotti, PE. 1997. Measurement of cytotoxicity by ATP based Luminescence Assay in primary cell cultures and cell lines. Toxicology in Vitro, 11, 553-556.

Crouch, SPM., Kozlowski, R., Slater, KJ., Fletcher, J. 1993. The use of ATP bioluminescence as a measure of cell proliferation and cytocoxicity. Journal of Immunological Methods, 160, 81-88.

Curtiss, LK., Edgington, TS. 1980. Differences in the characteristics of inhibition of lymphocyte stimulation by 25-Hydroxycholesterol and by the immunoregulatory serum lipoprotein LDL-In. Journal of immunology, 125, 1470-1474.

Cuthbert, JA., Lipsky, PE. 1983. Immunoregulation by low density lipoproteins in man: low density lipoprotein inhibits mitogen stimulated human lymphocyte proliferation after initial activation. Journal of Lipid Research, 24, 1512-1524.

Dallman, MJ. 1995. Cytokines and Transplantation: TH1/Th2 regulation of the immune response to solid organ transplants in the adult. Current opinion in Immunology, 7, 632-638.

Danovitch, GM. 1999. Choice of immunosuppressive drugs and individualisation of immunosuppressive therapy for kidney transplant patients. Transplantation Proceedings, 31 (suppl 8A), 2S-6S.

Danovitch, GM. 2001. Immunosuppressive medications for renal transplantation: A multiple choice question. Kidney International, 59, 388 - 402.

Dantal, J., Hourmant, M., Cantarovich, D., Giral, M., Blancho, G., Dreno, B., Soulillou, JP. 1998. Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporine regimens. Lancet, 351, 623–628

De Wet, JR., Wood, KV., Helinski, DR., DeLuca, M. 1986. Cloning Firefly Luciferase. Methods in Enzymology, 133, pg3.

Dexter, SJ., Camara, M., Davies, M., Shakesheff, KM. 2003. Development of a bioluminescent ATP assay to quantify mammalian and bacterial cell number from a mixed population. Biomaterials, 24, 27-34.

Dharnidharka, VR., Stablein, DM., Harmon, WE. 2004. Post-Transplant Infections Now Exceed Acute Rejection as Cause for Hospitalisation: A report of the NAPRTCS. American Journal of Transplantation, 4, 384-389.

Drovan, D., Krentz, A. 2002. Oxford handbook of clinical and laboratory investigation, University Press, New York, USA

Fishman, JA., Rubin, RH. 1998. Infection in organ transplant recipients. New England Journal of Medicine, 338, pg1741.

Fitzgerald, MG. 1972. A satisfactory quantitative test of lymphocyte response to phytohemagglutinin for the definition of normal control values and recognition of immunological defects. Journal of clinical pathology, 25, 163-168.

Forsdyke, DR. 1967. Quantitative Nucleic Acid Changes during Phytohaemagglutinin-Induced Lymphocyte Transformation in vitro Dependence of the response on phytohaemagglutinin/serum ratio. Biochemical Journal, 105, pg679.

Fülöp, T., Larbi, A., Gilles Dupuis, G., Pawelec, G. 2003. Ageing, autoimmunity and arthritis: Perturbations of TCR signal transduction pathways with ageing – a biochemical paradigm for the ageing immune system. Arthritis Research Therapy, 5, 290–302.

Gautam, A., Morrissey, PE., Brem, AS., Fischer, SA., Gohh, RY., Yango, AF., Monaco, AP. 2006. Use of an immune function assay to monitor immunosuppression for treatment of post transplant lymphoproliferative disorder. Pediatric Transplantation, 10, 613-616.

Germain, RN. 1994. MHC dependent Antigen processing and peptide presentation. Providing ligands for T lymphocyte Activation. Cell, 76, 287-299.

Grimm, P. 2006. Use of an immune function assay to monitor immunosuppression. Pediatric Transplantation, 10, 533-535.

Halilovic, J., Kenna, GD. 2006. Immunosuppressants used in solid organ transplantation. U.S Pharmacist, 31, pg8.

Halloran, PF. 2004. Immunosuppressive drugs for kidney Transplantation. New England Journal of Medicine, 351, 2715-2729.

Halvorsen, R., Leivestad, T., Gaudernack, G., Thorsby, E. 1988. Role of Accessory cells in the activation of pure T cells via the Tcell receptor -CD3 complex or with PHA. Scand. journal of Immunology, 27, 555-563.

Hamelryck, T., Dao-Thi, M., Poortmans, F., Chrispeels, M., Wyns, L., Loris, R. 1996. The crystallographic structure of phytohemagglutinin-L. Journal Biological Chemistry, 271, 20479-20485.

Han, WK., Bonventre, JV. 2004. Biological markers for the early detection of acute kidney injury. Current Opinion in Critical Care, 10, 476–482

Haneda, Y., Johnson, FH. 1958. The luciferin-luciferase reaction in a fish, Parapriacanthus Beryciformis, of newly discovered Luminescence. Biochemistry: Haneda and Johnson, 44, 127-129.

Hariharan, S., Christopher, P., Johnson, CP., Bresnahan, BA., Taranto, SE., McIntosh, MJ., Stablein, D. 2000. Improved graft survival after renal transplantation in the United States, 1998 to 1996. New England Journal of Medicine, 342, 605-612.

Hojo, M., Morimoto, T., Maluccio, M. 1999. Cyclosporine induces cancer progression by a cell -autonomous mechanism. Nature, 397, pg530.

Howard, RJ., Patton, PR., Reed, AI., Hemming, AW., Van der werf, WJ., Pfaff, WW., Srinivas, TR., Scornik, JC. 2002. The changing causes of graft loss and death after kidney transplantation. Transplantation, 73, 1923-1928.

Hui, DY., Harmony, JAK., Innerarity, TL., Mahley, RW. 1980. Immunoregulatory Plasma Lipoproteins: Role of Apoprotein E and Apoprotein B. Journal of biological chemistry, 255, 11775-11781.

Ishizaka, A., Tono-oka, T., Matsumoto, S. 1984. Evaluation of the Proliferative Response of Lymphocytes by Measurement of Intracellular ATP. Journal of Immunological Methods, 72, 127-132.

Israeli, M., Yussim, A., Mor, E., Sredni, B., Klein, T. 2007. Preceding the rejection: In search for a comprehensive post transplant immune monitoring platform. Transplant Immunology, 18, 7-12.

Jamil, B., Nicholls, K., Becker, GJ., Walker, RG. 1999. Impact of acute rejection therapy on infections and malignancies in renal transplant recipients. Transplantation, 68, pg1597.

Johnston, A., DW, H. 1999. Therapeutic drug monitoring of immunosuppressant drugs. Journal of Clinical Pharmacology, 47, 339-350.

Junqueira, LC., Carneiro, J., Kelley, RO. 1995. Basic Histology, 8th edition, Appleton and Lange Norwalk, CT.

Jusko, WJ., Thomas, AW. 1995. Consensus document: therapeutic monitoring tacrolimus (FK-506). Therapeutic Drug Monitoring, 17, pg606.

Kangas, L., Gronroos, M., Nieminen, AL. 1984. Bioluminescence of Cellular ATP: A new method for evaluating cytotoxic agents in vitro. Medical Biology, 62, 338-343.

Kaplan, B., Meier-Kriesche, HU. 2004. Renal Transplantation: A Half Century of Success and the Long Road Ahead. Journal American Society Nephrology, 15, 3270–3271.

Kaplan, B., Schold, J., Meier-Kriesche, HU. 2003. Poor predictive value of serum creatinine for renal allograft loss. American Journal of Transplant, 3, 1560–1565.

Karamperis, N., Povlsen, JV., Hojskov, C., Poulsen, JH., Pedersen, AR., Jorgensen, KA. 2003. Comparison of the Pharmacokinetics of Tacrolimus and Cyclosporine at Equivalent Molecular Doses. Transplantation Proceedings, 35, 1314-1318.

Karlsson, H., Depierre, JW., Nassberger, L. 1997. Energy levels in resting and mitogen-stimulated human lymphocytes during treatment with FK506 or cyclosporine A in vitro. Biochimica et Biophysica Acta, 1319, 301–310

Kasiske, BL., Chakkera, H., Louis, T., Ma, JZ. 2000. Immunosuppression Withdrawal in Renal Transplantation. Transplantation Proceedings, 32, 1506-1507.

Kobrzycki, P. 1977. Renal Transplant Complications. American Journal of Nursing, 77, 641-643.

Kowalski, R., Post DR., Mannon RB., Sebastian A., Wright HI., Sigle G., Burdick J., Elmagd KA., Zeevi A., Cepero ML., Daller JA., Gritsch., Reed EF., Jonsson J., Hawkins D., Britz JA., 2006. Assessing relative risk of infection and rejection: A meta-analysis using an immune function assay. Transplantation, 82, 663-668.

Kowalski, RJ., Post, D., Schneider, MC., Britz, JA., Thomas, J. 2003. Immune cell function testing: an adjunct to therapeutic drug monitoring in transplant patient management. Clinical Transplant, 17, 77-88.

Kriesche, HUM., Baliga, R., Kaplan, B. 2003. Decreased renal function is a strong risk factor for cardiovascular death after renal transplantation. Transplantation, 75, 1291-1295.

Lechler, RI., Sykes, M., Thomson, AW., Turka, LA. 2005. Organ Transplantation- How much of the promise has been realized. Nature Medicine, 11, 605-613.

Lenarsky, C., Jordan, SC., Ladisch, S. 1982. Plasma Inhibition of Lymphocyte Proliferation in Nephrotic Syndrome: Correlation with Hyperlipidemia. Journal of Clinical Immunology, 2, 276-281.

Liu, J., Framer, JD., Lane, WS., Friedman, J., Weissman, I., Schreiber, SL. 1991. Calcineurin is a common target of Cyclophilin - Cyclosporin A and FKBP-FK506 Complexes. Cell, 66, 807-815.

Liu, KQ., Bunnell, SC., Gurniak, CB., Berg, LJ. 1998. T Cell Receptor–initiated Calcium Release Is Uncoupled from Capacitative Calcium Entry in Itk-deficient T Cells. Journal of experimental medicine, 187, 1721-1727.

Liu, Z., Sun, YK., Xi, YP., Maffei, A., Reed, E., Harris, P., Suciu-Foca, N. 1993. Contribution of Direct and Indirect Recognition Pathways to T Cell Alloreactivity. Journal of experimental medicine, 177, 1643-1650.

López, MM., Valenzuela, JE., Álvarez, FC., López-Álvarez, MR., Cecilia, GS., Paricio, PP. 2006. Long-term problems related to immunosuppression. Transplant Immunology, 17, 31-35.

Lundin, A., Hasenson, M., Persson, J., Pousette, A. 1986. Estimation of Biomass in growing cell lines by Adenosine Triphosphate Assay. Methods in Enzymology, 133, 27-42.

MacDonald, A., Scarola, J., Burke, JT., Zimmerman, JJ. 2000. Clinical Pharmacokinetics and Therapeutic Drug Monitoring of Sirolimus. Clinical Therapeutics, 22, B101-B121.

Mahony, JF., Caterson, RJ., Coulshed, S., Stewart, JH., Sheil, AG. 1995. Twenty and 25 years survival after cadaveric renal transplantation. Transplant Proceedings, 27, 2154-2155.

Mannon, RB., Kirk, AD. 2006. Beyond histology: Novel tools to diagnose Allograft dysfunction. Clinical Journal American Society of Nephrology, 11, 358-364.

Martini, A., Vitiello, MA., Siena, S., Capelli, V., Ugazio, AG. 1981. Multiple serum inhibitors of lectin-induced lymphocyte proliferation in nephrotic syndrome. Clinical experimental Immunology, 45, 178-184.

Martins, P, N,A ., Pratschke, J., Pascher, A., Fritsche, L., Frei, U., Neuhaus, P., Tullius, SG. 2005. Age and Immune Response in Organ Transplantation. Transplantation, 79, 127-132.

McCarthy, BM., Okano, IY., Nakayasu, ZT., Macy, M., Watson, SR., Harmony, JAK. 1987. Plasma lipoproteins and transferrin regulate the proliferation of a continuous T lymphocyte cell line. Journal of Lipid Research, 28, pg1067.

McCluskey, J., Peh, CA. 1999. The human leucocyte antigens and clinical medicine: an overview. Reviews in Immunogenetics, 1, 3-20.

McElroy, WD., Strehler, BL. 1954. Bioluminescence. MMBR, 18, 177-194.

Medawar, PB. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits. Journal of Anatomy, 78, pg176.

Medawar, PB., Billingham, RE., Brent, L. 1953. Actively acquired tolerance of foreign cells. Nature, 172, 603-606.

Mollgard, L., Tidefelt, U., Engberg, BS., Lofgren, C., Paul, C. 2000. In vitro chemosensitivity testing in acute non lymphocytic leukaemia using the bioluminescence ATP assay. Leukemia Research, 24, 445-452.

Moore, R. 2000. Redefining Immunosuppressive Strategies. Transplantation Proceedings, 32, 1460-1462.

Morris, PJ. 1970. Kidney Transplantation Principles and Practice, 3rd edition. W. B. Saunders Company Harcourt Brace Jovanovich, Inc. The Curtis Center Independence Square West Philadelphia, PA.

Morris, PJ. 2004. Transplantation - A medical miracle of the 20th century. New England Journal of Medicine, 351, 2679-2680.

Morse, JH., Witte, LD., Goodman, ADS. 1977. Inhibition of lymphocyte proliferation stimulated by lectins and allogeneic cells by normal plasma lipoproteins. Journal of experimental medicine, 146, 1791-1803.

Mowbry, HF. 1963. Ability of large doses of an alpha2 plasma protein fraction to inhibit antibody production. Immunology, 6, 217-225.

Murray, JE. 1992. Human Organ transplantation: Background and Consequences. Science, New Series, 256, 1411-1416.

Murray, JE., Merrill, JP., Harrison, JH. 1955. Renal homotransplantation in identical twins. Surgical Forum, 6, pg432.

Murray, JE., Merrill, JP., Harrison, JH., Guild, WR. 1956. Successful homotransplantation of the human kidney between identical twins. Journal of American Medical Association, 160, 277-282.

Nakamura, M., Niwa, K., Maki, S., Hirano, T., Ohmiya, Y., Niwa, H. 2006. Construction of a new firefly bioluminescence system using Luciferin as substrate. Tetrahedron Letters, 47, 1197-1200.

Neyts, J., Andrei, G., De Clercq, E. 1998. The novel immunosuppressive agent mycophenolate mofetil markedly potentiates the anti herpes virus activities of acyclovir, gangiclovir and penciclovir in vitro and in vivo. Antimicrobial agents Chemotherapy, 42, pg216.

Nowell, PC. 1960. Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. Cancer Research, 20, 462-466.

O'Callaghan, CA., Brenner, BM. 2000. Kidney at a glance. Blackwell Science, Oxford, UK.

Odf. June 2008. Transplant statistics. In: http://www.odf.org.za Date accessed: 6/9/2008.

Ojo, AO., Hansen, JA., Wolfe, RA. 2000. Long term survival in renal transplant recipients with graft function. Kidney International, 57, 307-313.

Ojo, AO., Held, PJ., Port, FK., Wolfe, RA., Leichtman, AB., Young, EW., Arndorfer, J., Christensen, L., Merion, RM. 2003. Chronic Renal Failure after Transplantation of a Non renal Organ. New England Journal of Medicine, 349, 931-940.

Ozerol, H., Senol, M., Ageitos, A., Talmadge, JE. 1996. The Investigation of the responses to phytohemagglutinin in the patients of non-Hodgkin's Lymphoma who were candidates for high dose chemotherapy, Autologous Bone Marrow, or Peripheral Stem Cell Transplantation. Journal of Turgut Özal Medical Center, 3, 306-310.

Peakman, M., Vergani, D. 1997. Basic and Clinical Immunology, Churchill Livingston, New York.

Penn, I., Starzl, TE. 1972. Malignant Tumors arising de novo in immunosuppressed organ transplant recipients. Transplantation, 14, pg407.

Promega. 2005. CellTiter Glo Luminescent Cell viability Assay. In:www.promega.com/tbs/ Date accessed:02/2005.

Redsven, I., Kymalainen, HR., Pesonen-Leinonen, E., Kuisma, R., Ojala-Paloposki, T., Hautala, M., Sjoberg, AM. 2007. Evaluation of a bioluminescence method, contact angle measurements and topography for testing the clean ability of plastic surfaces under laboratory conditions. Applied Surface Science, 253, 5536-5543.

Rodrigo, E., Velasco, SP., Velaro, R., Ruiz, JC., Fresnedo, FG., Hoyos, L., Pinera, C., Palomar, R., Cobian, LF., Arias, M. 2008. Intracellular ATP Concentrations of CD4 cells in kidney transplant patients with and without infection. Clinical Transplantation, 22, 55-60.

Roitt, I. 1991. Essential Immunology, 7th edition. Blackwell Scientific Publications Pty Ltd, 54 University Street, Carlton, Victoria

Rose, ML., Hutchinson, JV. 2006. Transplant immunology II: overcoming acute and chronic rejection. Surgery, 24, pg79.

Rowley, DA., Fitch, FW., Stuart, FP., Köhler, H., Cosenza, H. 1973. Specific Suppression of Immune Responses. Science, 181, 1133-1141.

Ruth, RJ., Wyszewianski, L., Campbell, DA. 1987. The Future of Kidney Transplantation: The Effect of Improvements in Survival Rate on the Shortage of Donated Kidneys. Medical Care, 25, 238-249.

Sayegh, MH., Carpenter, CH. 2004. Transplantation 50 years later - Progress, Challenges and Promises. New England Journal of Medicine, 351, 2761-2766.

Sayegh, MH., Denton, MD., Magee, CC. 1999. Immunosuppressive strategies in transplantation. Lancet, 353, 1083-1091.

Sayegh, MH., Turka, LA. 1998. The Role of T cell Costimulatory Activation Pathways in Transplant Rejection. New England Journal of Medicine, 338, 1813-1821.

Sayegh, MH., Womer, KL., Lee, RS., Madsen, JC. 2001. Tolerance and Chronic Rejection. Philosophical Transactions: Biological Sciences, 356, 727-738.

Schulick, RD., Weir, MB., Miller, MW., Cohen, DJ., Bernas, BL., Shearer, GM. 1993. Longitudinal study of in vitro CD4 helper cell function in recently transplanted renal allograft patients undergoing tapering of their immunosuppressive drugs. Transplantation, 56, pg590.

Sharma, S., Unruh, H. 2006. History of Transplantation In: emedicine http://www.emedicine.com/med/topic3497.htm Date access: Jan 2006.

Shaw, LM., Holt, DW., Keown, P., Venkataramanan, R., Yatscoff, R. 1999. Current opinions on Therapeutic Drug Monitoring of Immunosuppressive drugs. Clinical Therapeutics, 21, pg1632.

Slater, KJ. 2001. Cytotoxicity tests for high throughput drug discovery. Current opinion in Biotechnology, 12, 70-74.

Sottong, PR., Rosebrock, JA., Britz, JA., Kramer, TR. 2000. Measurement of T Lymphocyte response in whole blood cultures using newly synthesized DNA and ATP. Clinical and Diagnostic Laboratory Immunology, 7, 307-311.

Soulillou, JP. 2001. Immune Monitoring for rejection of kidney transplants. New England Journal of Medicine, 344, 1006-1007.

Soulillou, JP., Giral, M. 2001. Controlling the Incidence of Infection and Malignancy by Modifying Immunosuppression. Transplantation, 72, SS89-SS93.

Squirrell, DJ., Price, RL., Murphy, MJ. 2002. Rapid and specific detection of bacteria using bioluminescence. Analytica Chimica Acta, 457, 109-114.

Suthanthiran, M. 2000. T-Cell Antigen Recognition and Costimulatory Pathways: Implications for the Induction of Transplantation Tolerance. Transplantation Proceedings, 32, 1451-1452.

Suthanthiran, M., Strom, TB. 1994. Renal Transplantation. New England Journal of Medicine, 331, 365-376.

Trauth, BC., Keesey, J. 1996. Guide to cell proliferation and apoptosis methods. Boehringer mannheim Indianapolis, USA.

Trune, DR., Kempton, JB. 2002. Female MRL.MpJ-Faslpr autoimmune mice have greater hearing loss than males. Hearing Research, 167, 170-174.

Truong, DH., Darwish, AA., Gras, J., Wiers, G., Cornet, A., Robert, A., Mourad, M., Malaise, J., De Goyet, J., Reding, R., Latinne, D. 2007. Immunological monitoring after organ transplantation: Potential role of soluble CD30 Blood level measurement. Transplant Immunology, 17, 283-287.

Tsukada, KO., Tsukada, T., Isobe, K. 1999. Accelerated development and aging of the immune system in p53-Deficient Mice. Journal of Immunology, 163, 1966-1972.

Vaden, SL. 1997. Cyclosporine and Tacrolimus. Seminars in Veterinary Medicine and Surgery, 112, 161-166.

Wange, RL., Samelson, LE. 1996. Complex Complexes: Signaling at the TCR. Immunity, 5, 197-205.

Watson, CJE., Taylor, AL., Bradley, JA. 2005. Immunosuppressive agents in solid organ transplantation: Mechanisms of action and therapeutic efficacy. Critical Reviews in Oncology/Hematology, 56, 23–46

Whitacre, CC. 2001. Sex differences in autoimmune disease,. In: Nature Publishing Group http://immunol.nature.com Date accessed: 29/08/2008.

White, AG., Raju, KT., Keddie, S., Abouna, GM. 1989. Lymphocyte activation: changes in intracellular adenosine triphosphate and deoxyribonucleic acid synthesis. Immunology Letters, 22, 47-50.

Wier, MI. 2007. Methods for measurement of lymphocyte function-Patent number US 7,169,571 B2. United States, Cylex Inc., Columbia, MD (US).

Wood, KV. 2007. The Bioluminescence Advantage. Promega Notes, 96, 3-5.

Wood, PJ. 2006. Immunology of transplantation. Anaesthesia and intensive care medicine, 7, pg184.

Yatscoff, RW., Copeland, KR., Faraci, CJ. 1990. Abbott TDx monoclonal antibody assay evaluated for measuring cyclosporine in whole blood. Clinical Chemistry, 36, pg1969.

Young, B., Heathe, JW. 1995. Wheater's functional histology, Church hill Livingstone.

Zeevi, A., Ahmed, M., Venkataramanan, R., Logar, AJ., Rao, AS., Bartley, GP., Robert, K., Dodson, FS., Shapiro, R., Fung, JJ. 2001. Quantitation of Immunosuppression by Tacrolimus Using flow cytometric analysis of Interleukin 2 and Interferon inhibition in CD8 and CD8 Peripheral blood T cells. Therapeutic Drug Monitoring, 23, 354-362.

Zeevi, A., Britz, JA., Bentlejewski, CA., Guaspari, D., Tong, W., Bond, G., Murase, N., Harris, C., Marsha, A., Martin, D., Post, DR., Kowalski, RJ., Elmagd, KA. 2005. Monitoring immune function during tacrolimus tapering in small bowel transplant recipients. Transplant Immunology, 15, 17-24.

Zeevi, A., Duquesnoy, R. 1996. Cellular and molecular method a immunologic monitoring of solid organ transplant. Transplant pathology internet services statement of purpose.

Zheng, YZ., Peng, Y., Wang, S. 2005. Immunosuppressants: Pharmacokinetics, methods of monitoring and role of high performance liquid chromatography/mass spectrometry. Clinical and Applied Immunology Reviews, 5, 405-430.

Zijno, A., Saini, F., Crebelli, R. 2007. Suitability of cryopreserved isolated lymphocytes for the analysis of micronuclei with the cytokinesis-block method. Mutagenesis, 22, 311-315.

Chapter 7

Appendices

Appendix 1

Components of blood following centrifugation

Blood is composed of erythrocytes or red blood cells, platelets and leukocytes. When blood is removed from the circulatory system and centrifuged it separates into different layers because of the different densities of the cells. Plasma forms the yellow translucent supernatant, erythrocytes make up the lower layer in the centrifugation tube and the white or grayish layer immediately above the separation medium is made up of the peripheral blood mononuclear cells (PBMC's) which consists of lymphocytes and monocytes. Above this layer is a thin layer of platelets. (Young and Heathe, 1995)

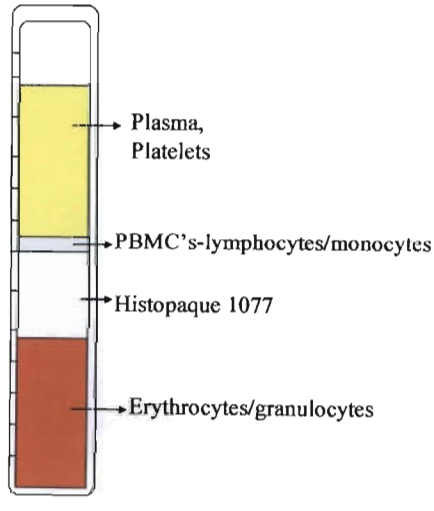

Fig 7.27: Schematic representation of blood following centrifugation

Appendix 2

Leukocytes/ White blood cells (WBC)

Leukocytes play an important role in the bodys' defense against foreign agents. Each type of leukocyte has specialised functions and their roles varies between specific and non specific immune defense. There are five types of leukocytes present in circulation which are divided into two groups. Granulocytes, which include neutrophils, basophils and eosinophils. Agranulocytes or mononuclear cells which include lymphocytes and monocytes. The concentration of leukocytes in the blood changes according to gender, age and physiologic conditions. In normal adults there are approximately 10 000 leukocytes per microliter of blood. (Junqueira et al., 1995)

Lymphocytes

Lymphocytes are characterised by a round densely stained nucleus and small amount of pale basophilic non granular cytoplasm. In blood 80% of the lymphocytes are T lymphocytes, a far smaller percentage of circulating lymphocytes are represented by B lymphocytes. The life span of T lymphocytes last for several months or years while B lymphocytes survive for about 10-20 days. T lymphocytes like B lymphocytes do not produce antibodies but rather bind to foreign antigens. (Junqueira et al., 1995)

Appendix 3

Immunosuppressant drugs classification and dosage

Table 7.10: Immunosuppressant drugs classification and dosage (*- dosage depends on the medical condition being treated, severity of the condition and presence of other medical conditions, age, weight) (Halilovic and Kenna, 2006)

Generic name	Brand name	Dosage*
Cyclosporine, CyA	Neoral®	± 5 mg/kg/day
Rapamycin, Sirolimus	Sirolimus®	± 8 mg/day
Tacrolimus, Fk506	Prograf®	± 0.2 mg/kg/day
Prednisone	-	± 5-60 mg/day
Mycophenolate mofetil	CellCept®	± 1000 mg/twice day
Azathioprine	Imuran®	± 1-3 mg/kg

Appendix 4

Red blood cell lysis

Sterile distilled water was used to remove any contaminating red blood cells (RBC's) and platelets. A few drops of distilled water was added and resuspended with the cell pellet after about 10 seconds, approximately 10ml of PBS was added. The RBC's swell when placed in distilled water, because of osmosis and eventually burst.

Appendix 5

Preparation of PHA

The PHA lyophilised powder was reconstituted with PBS according to manufactures instruction and further diluted as follows.

100µl of the original 1mg/ml solution was added to 9900µl PBS to make a 1:100 dilution, thus, giving a final concentration of 1µg/ml. The solution was aliquoted into labeled 1ml eppendorff tubes and stored at -20°C.

Appendix 6

Table 7.11: **Questionnaire**

Hospital Number	KZ-
Date Information is taken	
Translator	
Participant Personal Information	
Age:	
Gender:	
Race:	
Participant Medical History	
Date of Transplant:	
Type/Dosage of immunosuppressive therapy: (Cyclosporine, Sirolimus, Tacrolimus)	
Type/Dosage of additional therapy	
Blood levels of immunosuppressive drugs at time of sample collection:	
Monitoring function of transplanted kidney:Creatinine	
White cell count (WCC)	
Clinical Examination: Rejection/ infection/Stable	

Appendix 7

Normal range for white cell count and creatinine levels

Table 7.12: Normal range for white cell count and creatinine levels (Drovan and Krentz, 2002)

Investigation	**Normal Range**
White cell count	$4.0\text{-}11.0 \times 10^{9}$ /l
Creatinine	60-125 μmol/l

Appendix 8

Blood donation guidelines

- Weight >50kg
- Age: 17 - 65
- Good Health
- Have a sexually safe lifestyle (SANBS,2008)

Appendix 9

Information document for participants

Greetings, my name is Saleha Omarjee, I am a medical science masters student and I plan on doing research on kidney transplant patients. Research is just the process to learn the answer to a question. In this study I want to learn more about the immune response in kidney transplant patients.

The title of my study is Assessment of the Immune Response in kidney Transplant Patients

I am inviting you to take part in my research study.

Your role in this study would be to give a blood sample during your routine visit to the IALCH renal transplant clinic

Risks of being involved in the study are minimal as a blood sample which is routinely taken, will be drawn from you.

Your participation in this study is voluntary. If you refuse to participate, you will not be penalised. Please note that you may stop participation at any time without penalty.

Great care will be taken to ensure that all your personal information remains confidential although absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Contact details for further information:

Miss Saleha Omarjee email:201292883@ukzn.ac.za, tel:031-260 4225

Supervisor: Prof A. Assounga email: assoungaa@ukzn.ac.za, tel: 031-260 4217

Contact details of BREC Administrator or Chair – for reporting of complaints/ problems:
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Administrator: Ms P Ngwenya email: ngwenyap@ukzn.ac.za
Chair: Email: Prof D R Wassenaar email: c/o ngwenyap@ukzn.ac.za

Appendix 10

Information document for control

Greetings, I, Saleha Omarjee a medical science masters student, intends on doing research on kidney transplant patients. Research is just the process to learn the answer to a question. In this study we want to assess the immune system of kidney transplant patients.

Study title: Assessment of the Immune Response in kidney Transplant Patients

I am inviting you to kindly participate in this research study as a control. The definition of a control is "A standard against which other conditions can be compared in a scientific experiment".

The inclusion criteria, to be a control is an apparently healthy male or female adult who is eligible to donate blood according to the established blood donation guidelines.

If you have consented to be a control a blood sample will be taken and utilised in my study as a comparison to the blood samples of kidney transplant patients who are on various immunosuppressant therapies attending the Inkosi Albert Luthuli Renal unit.

Risks: of being involved in this study is minimal.
Benefits: By donating a blood sample you will be assisting in increasing scientific knowledge and improving the quality of life of kidney transplant recipients.

Great care will be taken to ensure that all your personal information remains Confidential although absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Your participation in this study is voluntary. If you refuse to participate, you will not be penalised. Please note that you may stop participation at any time without penalty.

Contact details of Researcher: Miss Saleha Omarjee, email:201292883@ukzn.ac.za, tel:031-260 4225

Supervisor: Prof A. Assounga, email:assoungaa@ukzn.ac.za

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Administrator: Ms P Ngwenya email: ngwenyap@ukzn.ac.za
Chair: Email: Prof D R Wassenaar email: c/o ngwenyap@ukzn.ac.za

Appendix 11

Consent to Participate in Research

You have been asked to participate in a research study

You have been informed about the study by Prof AGH. Assounga and Saleha Omarjee

You may contact Saleha Omarjee at 031-260-4225 during office hours if you have questions about the research or if you are injured as a result of the research.

You may contact the Medical Research Office at the Nelson R Mandela School of Medicine at 031-260 4604 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalised or lose benefits if you refuse to participate or decide to stop.

If you agree to participate, you will be given a signed copy of this document and the participant information sheet which is a written summary of the research.

The research study including the above information, has been described to me orally. I understand what my involvement in the study means and I voluntarily agree to participate.

_____ _____

Signature of Participant Date

_____ _____

Signature of Witness (Where applicable) Date

_____ _____

Signature of Translator (Where applicable) Date

Appendix 12

Imvume yokubamba Iqhaza ocwaningweni

Uceliwe ukuthi ubambe iqhaza ocwaningweni

Uchazeliwe ngalolu cwaningo ngu Prof. AGH. Assounga and Saleha Omarjee

Lapho kufanele: uchazeliwe ngezinxephezelo ezingase ezikhona noma ukwelashwa uma kwenzeka uthola ukulimala ngenxa yalolucwaningo.

Ungaxhumana no Saleha Omarjee kule nombolo : 031 260 4225 ngazozonke izikhathi uma uma unemibuzo eqondene nalolu cwaningo.

Ungathintana nehhovisi lezocwaningo (I medical research office) elise Nelson R. Mandela Medical School kulenamba: 031 260 4604 uma unemibuzo eqondene namalungelo akho njengo muntu obambe iqhaza kulolu cwaningo.

Awuphoqelekile ukuthi ubambe iqhaza kulolu cwaningo kungokuthanda kwakho, futhi ungeke uhlawuliswe noma ulahlekelwe uma ungabami iqhaza noma unquma ukuhoxa kulolu cwaningo.

Uma uvuma ukusebenzisana nathi, uzonikwa ikhophi esayiniwe yalolu cwaningo(yaledocuument) kanye nepheshana elinolwazi oluthize lwababame iqhaza. Lokhu kuzobe kune ncazelo efinqiwe noma emfushane ngalolucwaningo.

Ngichazeliwe ngomlomo ngenhloso yalolucwaningo kanye nalolwazi olongenhla. Ngiyakuqonda okufanele ngikwenze kulolucwaningo futhi ngiyavuma ngokuthanda kwami ukuba yinxenye yalolu cwaningo.

_____ _____

Isignisha yakho(signature) Usuku (date)

_____ _____

Isignisha kafakazi Usuku
(Uma kufanele)

_____ _____

Isignisha katolika Usuku
(Uma kudingekil

Appendix 13

Table 7.13: Results obtained for each individual included in the cyclosporine group

	Group	gender	Age	race	date of transplant	ATP ng/ml	percentage increase of ATP	clinical status (before-during-after the test	Drugs blood levels ng/ml	WCC x 10^9/l	creatinine µmol/l
1	Cyclosporine	Male	48	Indian	2001	497	21.41%	haematuria, gum hypertrophy	842	7.85	133
2	Cyclosporine	Male	44	Indian	1998	115	3.69%	stable	523	7.25	102
3	Cyclosporine	Male	48	African	2001	144	3.71%	stable, warty lesion on abdomen	809	4.68	105
4	Cyclosporine	Male	54	Indian	2002	260	9.41%	stable, septic wound	1125	3.72	261
5	Cyclosporine	Male	66	Indian	1992	286	15.86%	stable	1229	10.3	152
6	Cyclosporine	Male	45	White	1999	131	13.07%	recurrent UTI, papules on the face	927	7.76	118
7	Cyclosporine	Male	58	Indian	1993	349	36.09%	stable	911	6.32	112
8	Cyclosporine	Male	39	White	2000	283	14.13%	stable	648	6.8	112
9	Cyclosporine	Male	55	Indian	1998	150	5.94%	stable	506	11.34	108
10	Cyclosporine	Male	54	Indian	1991	30	2.68%	stable	771	12.97	143
11	Cyclosporine	Female	56	Indian	1999	306	7.67%	stable, UTI	871	7.19	166
12	Cyclosporine	Female	45	Indian	1993	265	8.65%	Gastritis	1390	6.4	112
13	Cyclosporine	Female	27	White	2001	342	12.64%	bronchopneumonia	814	7.55	148
14	Cyclosporine	Female	53	Indian	1997	397	14.99%	UTI, gum infection	786	6.82	163
15	Cyclosporine	Female	40	Coloured	1998	84	2.95%	stable	819	5.57	122
16	Cyclosporine	Female	45	White	1991	206	9.23%	stable	512	8.18	127
17	Cyclosporine	Female	41	Coloured	1989	261	11.18%	UTI	953	8.1	155
18	Cyclosporine	Female	68	Indian	1996	13	0.51%	stable	798	8.99	85
19	Cyclosporine	Female	57	Indian	1996	330	17.53%	stable	625	6.15	89
20	Cyclosporine	Female	47	Indian	2005	323	18.58%	neurotoxicity, angina	784	6.5	112
21	Cyclosporine	Female	43	African	1999	383	11.46%	stable	651	4.24	85
22	Cyclosporine	Female	67	Indian	1993	197	9.31%	stable	756	8.78	100

Appendix 14

Table 7.14: Results obtained for each individual included in the sirolimus group

	Group	gender	age	Race	Date of transplant	ATP (ng/ml)	Percentage Increase of ATP	clinical status (before-during-after the test)	drug blood level (ng/ml)	WCC x 10^9/l	creatinine µmol/l
1	Sirolimus	Male	24	Indian	2004	564	21.08%	stable	14.2	8.37	126
2	Sirolimus	Male	23	Indian	1999	126	3.91%	stable	6.7	7.96	425
3	Sirolimus	Male	36	Indian	1995	266	8.34%	stable	10.9	6.24	146
4	Sirolimus	Male	19	African	2003	350	19.81%	stable	15.1	3.98	107
5	Sirolimus	Male	61	Indian	1988	291	16.84%	stable	7.7	5.01	201
6	Sirolimus	Female	50	African	2001	327	11.27%	UTI, recurrent cough, inc weight loss	6.5	7.74	185
7	Sirolimus	Female	64	African	1998	246	14.83%	stable, gum hypertrophy	3.2	10.19	109
8	Sirolimus	Female	37	Indian	2002	121	8.43%	CMV infection, UTI	6.6	7.77	103
9	Sirolimus	Female	23	Indian	2005	134	4.91%	stable	7.9	8.76	102
10	Sirolimus	Female	35	African	2006	125	10.48%	stable	7.9	3.89	92

Appendix 15

Table 7.15: Results obtained for each individual included in the tacrolimus group

	Group	gender	age	race	Date of transplant	ATP (ng/ml)	Percentage Increase of ATP	clinical status (before-during-after the test)	drug blood level (ng/ml)	WCC x 10^9/l	creatinine µmol/l
1	Tacrolimus	male	15	Indian	1998	102	4.85%	stable, recurrent UTI	6.1	5.04	129
2	Tacrolimus	male	38	Indian	2007	677	19.01%	stable, papules on the face	7.4	6.53	140
3	Tacrolimus	male	16	Indian	2008	667	74.03%	stable	7.2	11	90
4	Tacrolimus	male	21	White	2005	250	17.08%	stable	12.7	11.3	160
5	Tacrolimus	Female	35	black	1999	422	11.04%	stable, hypopigmentation face and thorax	9.4	4.45	158
6	Tacrolimus	Female	35	Indian	1998	78	3.72%	stable	7.1	6.47	74

Appendix 16

Table 7.16: Results obtained for each of the five patients from the cyclosporine group tested over a three month period (Pred: predisinone,, Aza: azatheropine Mmf: mycophenolate mofetil)

	Patient 1			Patient 2			Patient 3			Patient 4			Patient 5		
Cyclosporine dosage (mg)	50mg			75mg			225mg			75mg			25mg		
Other drug dosage	Pred 5mg Aza 75mg			Mmf 1000mg			Mmf 750mg Pred 5mg			Pred 7.5mg Aza 50mg			Pred 2.5mg Mmf 1000mg		
Drug Blood levels ng/ml	842	1055	427	871	-	337	814	221	662	1125	797	606	786	639	383
Creatinine µmol/l	133	124	97	-	166	219	148	105	146	261	216	211	163	128	173
WCC x 10^9/l	7.85	8.12	8.66	7.19	6.32	6.61	7.55	9.08	6.91	3.72	4.51	4.95	6.82	5.51	6.66
Clinical status	haematuria, gum hypertrophy			UTI			Suspected Bronchopneumonia			Septic wound			UTI-gum infection		
ATP level	497	294	225	306	50	213	342	104	187	260	264	312	397	119	402
Percentage increase of ATP	18%	15%	10%	7%	4%	11%	11%	3%	7%	9%	19%	12%	13%	3%	11%