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The Efficacy of Leflunomide to Deplete Xenoreactive Antibody, and Prolong the Survival of Discordant Cardiac Xenotransplants *In Vivo.*

by

Jamie George Lucien

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Masters of Science**.

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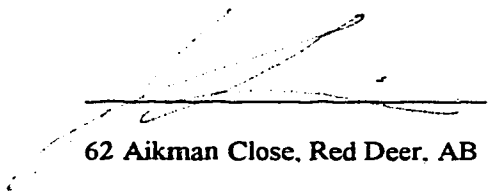

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The Efficacy of Leflunomide to Deplete Xenoreactive Antibody, and Prolong the Survival of Discordant Cardiac Xenotransplants *In Vivo*." submitted by Jamie George Lucien in partial fulfillment of the requirements for the degree of Masters of Science in Experimental Pathology

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Dear Mr. Simoneau:

In response to our telephone conversation on November 9, 1995, I have written you this letter. I have been granted permission by all authors of the following publications:

Lucien, J., Marath, A., Rayat, G., Thliveris, J., Koshal, A., and Yatscoff, R.W. The efficacy of leflunomide to reduce xenoantibody titers *in vivo*: An evaluation of the prolongation of discordant xenograft survival. Transplant Proceed. 1995. In Press.

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to use all material from these publications for the purpose of writing this thesis.

Sincerely,

Jamie Lucien

Table of Contents

The Efficacy of Leflunomide to Deplete Xenoreactive Antibody, and Prolong the Survival of Discordant Cardiac Xenotransplants *In Vivo*.

Xenotransplantation may provide much needed organs world wide to alleviate the suffering of many with end stage diseases. Unfortunately, hyperacute rejection remains a formidable barrier to the realization of clinical xenotransplantation. Leflunomide has proven to be an efficacious immunsuppressant used to inhibit T cell and B cell activation in several models. This study seeks to evaluate leflunomide in the context of the reduction of xenoreactive antibody and ultimately its ability to prolong the survival of discordant cardiac xenografts utilizing a pig to rabbit xenotransplant model. The first step involved the development of a high performance liquid chromatography technique to quantitate leflunomide active metabolite in blood or plasma. The within-day coefficients of variation of the assay at 1.0 and 10.0 mg/L were 5.7% and 7.0%, respectively. The between-day coeffients of variation were 12.2% and 14.7%, respectively. The sensitivity of the assay was 0.04 mg/L when 0.25 ml of sample were used. This method was used to investigate the blood distribution and pharmacokinetics of the drug. The blood distribution of leflunomide active metabolite was found to be mainly (>95%) in the lipoprotein free fraction of plasma at a range of concentrations from 0.4 mg/L to 100 mg/L. This is consistent with the results of a single dose pharmacokinetics study that demonstrated minimal distribution to the tissues as determined by volume of distribution (Vdss) ($Vdss_{I.V.}$ 0.09 ± 0.02 L/kg and $Vdss_{P.O.}$ 0.14 ± 0.03 L/kg). Additional findings demonstrated a longer mean residence time (MRT) for oral administration of leflunomide ($MRT_{P.O.}$ = 10.54 ± 2.6 h) than intravenous administration of leflunomide active metabolite ($MRT_{I.V.}$ = 6.76 ± 1.0 h). Similar areas under the curve (AUC) suggest 100% bioavailability ($AUC_{P.O.}$ = 421.16 ± 204.5 mg·h/L and $AUC_{P.O.}$ = 399.75 ± 126.9 mg·h/L). Xenotransplant studies resulted in an average survival time of control animals (n=8) of 18.79 ± 21.13 h. Animals given LEF I.V. or P.O. (n=6) at 5 mg/kg/d for 5 days prior to transplantation had a mean survival time of 27.66 ± 18.40 h, and 17.85 ± 18.44 h, respectively. Administration of LEF 5 mg/kg/d P.O. (n=3) for 21 days prior resulted in a mean survival time of 15.64 ± 14.18 h. None of these mean survival times were significantly different from that of controls ($p > 0.05$). However, 21 days administration of LEF was able to significantly reduce ($p < 0.05$) xenoreactive IgM

(XIgM) and IgG (XIgG) to 84.1 ± 12.7%, and 64.43 ± 26.14% of pretreatment levels respectively, at the time of surgery. This would suggest that LEF has the ability to inhibit the production of XIgM and XIgG, but the magnitude of this reduction is unable to prolong xenograft survival.

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List of Figures and Tables

Abbreviations and Symbols

ABTS	2,2'-axino-bis-3-ethylbenxothiazdine sulphonate
AUMC	Area under the moment curve
APC	Antigen presenting cell
AUC	Area under the concentration curve
BN	Brown-Norway
BQR	Brequinar sodium
BSA	Bovine serum albumin
°C	Degrees Celsius
C1q	Complement component 1q
C3	Complement component 3
C3b	Complement component 3 fragment b
C4	Complement component 4
C5a	Complement component 5 fragment a
Ca^{2+}	Calcium ion
CD	Cluster of differentiation
Cl	Clearance
CR1	Complement receptor 1
CsA	Cyclosporine A
CsG	Cyclosporine G
CV	Coefficient of Variation
CVF	Cobra venom factor
CyP	Cyclophillin
d	Density
DA	Dark Agouiti
Di-I-Ac-LDL	1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate

DNA	Deoxyribonucleic Acid
DSG	15-deoxyspergualin
$	Dollar
DMEM	Dulbecco's minimum essential medium
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbant assay
F	Bioavailability
Fc	Immunoglobulin gamma tails
FK506	Tacrolimus
FKBP	Tacrolimus binding protein
g	Gram
gu	Gauge
>	Greater than
x g	Relative centrifugal force
h	Hour
HDL	High density lipoproteins
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
IC_{50}	Concentration resulting in 50% of function
IL-1	Interleukin-1
IL-2	Interleukin-2
I.M.	Intramuscular
I.V.	Intravenous
kg	Kilogram
<	Less than
L	Liter
LDL	Low density lipoproteins

LEF	Leflunomide
LP	Lipoprotein
U	International unit
µg	Microgram
µL	Microliter
µmol	Micromole
µM	Micromolar
mg	Milligram
min	Minute
mL	Milliliter
mM	Millimolar
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
mmol	Millimole
MRT	Mean residence time
nm	Nanometer
O_2	Oxygen
PAEC	Porcine aortic endothelial cell
pH	Negative log of hydrogen ion concentration
PLC	Phospholipase C
%	Percent
P.O.	Oral administration
RAFT	Rapamycin Activation Factor
RAPA	Rapamycin/sirolimus
REJ	Time of rejection
SD	Standard deviation
SDS	Sodium docecyl sulphate

SLE	Systemic lupus erythematosus
$t_{½}$	Half-life
TCR	T cell receptor
TNF	Tumor necrosis factor
TOR	Target of Rapamycin
v	Volume
Vdss	Volume of distribution at steady state
VLDL	Very low density lipoproteins
w	Weight
Xab	Xenoreactive antibody/ xenoantibody
XIgG	Xenoreactive immunoglobulin gamma
XIgM	Xenoreactive immunoglobulin mu

I. Introduction: Overview of Xenotranplanation and Immunosuppression

A. Overview of Xenotransplantation

Advances in medical sciences and immunopharmacology have transformed the field of transplantation from a risky experimental procedure to an accepted form of medical treatment. The success and benefits that transplantation has to offer have attracted many centers in Canada to provide proper facilities and staff to serve an aging population. The investment in these organ transplant facilities is initially costly, but in the long run save thousands of dollars. The total cost for a kidney transplant was $42 405 plus the cost of immunosuppression, in 1992. In contrast, home dialysis costed $27 500 per year and hospital dialysis costed $40 250 per year. Thus, over three or more years the cost of dialysis far exceeds the cost of a transplant and immunosuppression. More importantly, transplantation allows for greater independence from the hospital and a greater quality of life for many transplant recipients. Unfortunately, the supply of organs for transplantation particularly for hearts and kidneys has risen more slowly than demand. This has resulted in longer waiting lists. In 1992, more than 2 000 patients were on the waiting list for kidney transplants. This figure does not include all potential recipients and those patients who died before a suitable donor organ could be found. Currently, organ transplantation is limited by the number of people who are willing to donate their organs. Three hundred and thirty cadaveric donors (1-73 years of age) supplied organs for 87% of all transplantations in 1992. This number is the lowest since 1985 (1).

The idea of using animals as a source of organs for human transplantation is an archaic one. In 1905, a French physician inserted slices of rabbit kidney into a nephrotomy in a child with renal insufficiency. At first, the child's volume of urine increased. Unfortunately, the child died of pulmonary congestion 16 days post transplant (2). This perhaps represents the first xenogeneic tissue transplant. In 1910, a German physician attempted a bilateral renal transplant using a nonhuman primate for a human recipient. The recipient died 32 h after transplantation. The autopsy revealed the presence of venous thromboses in the renal xenografts (3). This perhaps marks the first attempt at a concordant

xenotransplant. In 1923, an American physician attempted to transplant the kidney of a lamb into a patient with mercury bichloride poisoning. The patient died 9 days later. This represents one of the first discordant whole organ xenotransplants (4). In the years between 1920 and 1960 the interest in xenotransplantation decreased, as the medical community learned more about the immunological processes behind xenograft rejection (5). It was not until the 1960's and the advent of immunosuppressant agents that the interest in clinical xenotransplantation was renewed. In these attempts, human patients were given renal concordant xenotransplants from chimpanzee donors. Unlike earlier xenotransplants, patients were administered immunosuppressive agents, specifically azathioprine, actinomycin C, and steroids. In addition, donors were matched with recipients based on body size and ABO blood type. Xenograft and patient survival ranged between 2 and 9 months. However, this required local irradiation of the xenograft in order to reverse and control immunological rejection (6). The next xenotransplant was attempted in 1984, using a pediatric recipient and a baboon heart donor. This is better known as the "Baby Fae" heart transplant. Unfortunately, this transplant was lost to early antibody-mediated rejection. In the 1990's, there has been a renewed interest in xenotransplantation. This may be attributed to advancements in the fields of genetics, immunology, molecular biology, and immunosuppression. More importantly, the medical community has recognized the potential of clinical xenotransplantation to provide a final treatment to end stage organ diseases, and to fill the vacuum created by a world wide shortage of donated organs. As a result, more intense and novel investigations have been initiated to explore the possibility of making clinical xenotransplantation a reality.

Between 1950-1965, relatively few investigators were involved in xenogeneic transplantation, as determined by the number of papers in the field of xenotransplantation published per year. After Reemtsma's success in renal chimpanzee xenotransplants there was a resurgence in the interest in xenotransplantation. This was short lived and by 1980, the numbers of papers published had fallen back to pre-1965 levels. With the development of CsA in the mid-1980's the number of xenotransplant papers published increased, and from this point on the number of papers published per year has grown (5). Investigations into the immunology of xenotransplantation on a physiological and molecular level has

given the scientific community great insight into transplantation immunology and humoural immunology in general. In addition, recent developments and production of more efficacious immunosuppressant drugs that affect both cellular and humoural immunity has allowed the research community to take significant steps towards realizing clinical xenotransplantation.

B. Models of Xenotranplant Rejection: Concordant vs Discordant

The question of which animal species will ultimately be used in clinical xenotransplantation has directed researchers to explore several species combinations. All combinations may be grouped into two broad categories, namely concordant models and discordant models. In 1970, Calne first proposed the terms concordant and discordant based on the presence or absence of hyperacute rejection. In Calne's system of classification hyperacute rejection occured in discordant models only. These terms have been more refined based on current developments in immunology (7). Platt and Bach have recently proposed that the basis of dividing xenotransplant models into either concordant or discordant models center around three factors, and should a model meet these factors, it should be considered discordant. First, the presence or absence of natural xenoreactive antibodies directed against the donor xenograft in the serum of a naive recipient. Second, the inability of donor complement regulatory proteins to function in context of the complement system of the recipient. Thirdly, the inability of complement regulatory proteins of the recipient to function properly in the "environment" of the donor organ (8). Of these criteria, the presence of natural antibodies in the naive recipient is perhaps the most distinguishing characteristic between concordant and discordant models. Xenoreactive natural antibodies are a subset of antibodies that react with the antigens expressed on the donor endothelium. Binding of these antibodies leads to activation of complement via the classical pathway, and ultimately leads to a breakdown of the barrier and protective properties of the endothelial cells lining the vasculature of the xenograft. Since natural antibodies are preformed and are in the serum of the recipient before transplantation, these antibodies initiate hyperacute rejection.

C. Xenoreactive Antibody

Based on previous experience with hyperacute rejection in allograft models, and a better understanding of humoral immunity, a model of hyperacute rejection has been developed in which naturally occurring xenoreactive antibody plays a central role. Evidence to support this model is demonstrated in several observations. First, hyperacute xenograft rejection strongly resembles antidonor-antibody mediated hyperacute allograft rejection clinically and pathologically (9,10). Second, it has been demonstrated that xenoreactive immunoglobulin circulates in the blood of the recipient and rapidly binds to the vasculature of the xenograft in large deposits (11-15). Third, the administration of additional antidonor antibodies to the recipient accelerates xenograft rejection (16-18). Lastly, the removal of xenoreactive antibodies by plasmapheresis, organ perfusion, or specific immunoadsorption is able to deplete xenoreactive antibodies from the circulation of the recipient and prolong graft survival (19-25).

Xenoreactive antibody appears to be part of a family of naturally occurring antibodies found in the sera of unimmunized individuals. It has been proposed that natural antibodies along with complement form the first stage of development of our immune system. It has been estimated that 30% of B cell clones in normal individuals are producing natural antibodies (26). Natural antibodies have been proposed to serve a wide variety of functions. The B cells making natural antibodies have some unique characteristics, however the most interesting hypothesis is that the origin of natural antibodies is from a B-1 B cell (fetal tissue) as opposed to conventional B-2 B cells (adult bone marrow). Upon exposure to antigen, B-1 B precursors become long lived, self-replicating B-1 B cells that persist into adult life. In contrast, B-2 B precursors may tolerize in response to antigen by either undergoing anergy or apoptosis (27). It is this difference in the response to antigen that may explain why hematopoeitc chimerism between discordant species does not induce antibody tolerance whereas chimerism between concordant species produces tolerance (28). Natural antibodies from B-1 B cells are polyreactive, in contrast to elicit antibody from B-2 B cells. Polyreactive natural antibody maybe exclusively derived from CD5+ B-1 B cells. It has been demonstrated that xenoreactive antibody in human serum and antibody eluates from xenografts are polyreactive, as they bind to common ligands of polyreactive natural antibodies such as DNA and thyroglobulin (28,29). In addition, it has been demonstrated that these polyreactive

xenoantibodies are deposited in rejecting xenografts, and these xenoantibodies are broadly shared among primates. The specific isotype of the xenoreactive natural antibodies that are cytotoxic and more importantly are involved in hyperacute rejection is currently under debate. Although most natural antibody is IgM, B-1 B cells are influenced by cytokines and are able to undergo isotype switching to IgG and IgA (29). This would be consistent with studies that have demonstrated that IgG, IgM and IgA are all involved in xenograft rejection (30).

D. Complement: Alternate and Classical Pathways

The role that complement plays in hyperacute rejection remains as a crucial element in xenotransplantation, in the face of the scientific community focusing on isolating xenoantibodies and xenoantigens. The evidence that complement is involved in xenograft hyperacute rejection is compelling. First, recipients with transplanted discordant xenografts experience a decrease in complement titers (14,31). Second, complement factors are found on the vasculature of rejected xenografts (14,15). Lastly, depletion of essential complement factors with CVF (32-35) or by a congenital defect in the recipient's complement system (36,37) prolongs discordant xenograft survival. There are two possible mechanisms by which complement may be involved in hyperacute rejection. The first is antibody dependent activation of complement via the classical pathway (38). This involves deposition of xenoantibody and the binding of C1q to adjacent Fc tails, the formation of C2aC4b C3 convertase and eventually the formation of the membrane attack complex. Although there is some alternate pathway activation due to the highly reactive nature of C3 the primary mechanism of xenograft endothelial cell destruction is the deposition of C1q. The second mechanism is the antibody independent activation of complement via the alternate pathway. In this case, C3 is spontaneously activated via the carbohydrate complexes on the endothelial cell surface of the xenograft (39). In addition, C3 is an unstable thioester, and a small amount of C3 is spontaneously activated (C3 tickover). In both cases, C3b forms C3bBbP C3 convertase which activates more C3 in a positive feedback loop, leading to the eventual formation of the membrane attack complex. Normally damage to endothelial cells via alternate pathway activation is prevented by integral membrane proteins that accelerate the degradation of C3b (eg. membrane cofactor protein, decay accelerating factor) or by

prevention of the formation of the membrane attack complex (eg. homologous restriction factor, membrane inhibitor of reactive lysis). Thus it appears that, deposition of C3b on certain xenograft combinations are due to complement inhibitors not being able to function over species lines (40).

The destruction of discordant xenografts via the alternate pathway only occurs in a few select species combinations. In contrast, there is a large body of evidence that supports classical activation of complement in xenograft rejection. In pig to primate xenograft models, pig endothelial cells do not trigger the human alternate complement pathway as assayed by endothelial cell lysis assays, and endothelial cell activation assays. Immunohistochemistry of rejected cardiac and renal xenografts contain classical pathway components (eg. C4), but little alternate pathway components such as factor B or factor P (8). Thus in antibody dependent complement lysis models, the solution to preventing complement fixation in hyperacute rejection may be the removal of xenoreactive antibodies.

E. Endothelial Cells and Endothelial Cell Activation

In addition to physical damage to endothelial cell membranes by xenoreactive antibody and complement, there is also the possibility that hyperacute rejection may also be mediated by endothelial cell activation. Quiescent endothelial cells form a tight monolayer that provides an effective barrier to blood cells and plasma proteins. Under the influence of IL-1, TNF, and endotoxin, endothelial cells undergo a series of metabolic and structural changes. These changes promote platelet aggregation, fibrin generation, neutrophil adhesion, and increased permeability of the endothelial monolayer to plasma proteins and blood cells (40-44). Friedl et al. (45) described how C5a in the absence of xenoantibody causes the activation of endothelial cell-associated xanthine oxidase. It is currently unknown if C5a alone is sufficient to induce other characteristics of endothelial cell activation. The formation of the membrane attack complex in the endothelial cell membrane stimulates the synthesis of prostacyclin and causes endothelial cell membranes to express prothrombinase on its surface (45-47). In contrast to these studies that suggest that complement is the primary mediator of endothelial cell activation, additional studies have suggested that human serum containing xenoreactive natural antibodies and complement with porcine endothelial cells causes changes characteristic of endothelial cell activation, including the

synthesis of tissue factor and loss of thrombomodulin activity. Thus this suggests that it is a combination of xenoantibody and complement reacting with endothelial cells that may lead to endothelial cell activation (48).

A central feature of endothelial cell activation that is most relevant to hyperacute rejection is the loss of heparin sulfate on endothelial cells. The loss of heparin sulfate proteoglycan contributes to a number of the physiologic functions of endothelium including the barrier to efflux of blood components, the inhibition of thrombosis by binding and activating antithrombin III, and the inhibition of oxidant-mediated tissue injury (49-50). Thus, it has been proposed that the release of heparin sulfate may contribute to the manifestation of key characteristics of hyperacute rejection, namely the development of interstitial edema, hemorrhage, and thrombosis. Treatment of porcine endothelial cells with human sera leads to a rapid cleavage of endothelial cell-associated heparin sulfate and release from the cells as fragments of the proteoglycan. The release of heparin sulfate is a rapid process with 5% of total cell-associated heparin sulfate released in the first 4 min, and 50% released within 30 min. The release of heparin sulfate varied with the amount of IgM bound to endothelial cells, and the presence of complement. In this model, endothelial cell activation was not mediated by endotoxin, IL-1, nor the presence of donor or recipient isohemagglutins (51). Additional studies have demonstrated that delayed vascular rejection occurs in complement depleted animals, and lesions have been reported on organs perfused with decomplemented xenogenic serum, or xenoreactive antibody.

F. Accommodation to Xenografts

Hyperacute rejection is a powerful immune reaction that places a formidable barrier to the survival of xenografts. Similarly, hyperacute rejection is observed with allografts exposed to anti-ABO and anti-HLA class I antibodies. However, a number of clinical centers have overcome this problem of allograft hyperacute rejection by depleting antibody out of the recipient's circulation for a finite period (52-53). It was observed in these patients that the allograft continued to function despite the return of antibody and complement to the recipient's serum. In one center 14 of 18 human kidney allografts have survived across ABO incompatibility barriers. All of these transplants were parent to child. The anti-AB

antibodies were removed by plasmapheresis and neutralized by the infusion of blood group oligosaccharides. After 3 weeks the anti-AB antibodies returned, but no episodes of allograft rejection occurred (54). This phenomenon is called "accommodation" to signify the presumed establishment of a nonaggressive state between the antigraft antibodies and complement of the recipient and the donor graft (55).

The parallel between the set of natural antibodies that define the ABO system and natural occurring antibody has given hope to overcoming hyperacute rejection. The mechanism by which accommodation occurs is unknown. Some possible mechanisms have been proposed. First, the graft endothelial cells may develop resistance to complement injury over time. This may be accomplished by exposing endothelial cells to a small amount of antibody, instead of the massive amounts of antibody characteristically deposited during hyperacute rejection. A second possibility is that there is a change in the expression of the antigens on the surface of the endothelial cell membrane. A reduction in the amount or a change in the antigen structure of cell surface components may reduce the amount of xenoreactive antibody or perhaps reduce the amount of cytolytic xenoreactive antibody that binds to the surface of the xenograft's endothelial cells. The last proposed mechanism is that the repertoire of the xenoreactive antibody changes and less cytotoxic antibody is produced (8).

G. Approaches to Prolong Discordant Xenograft Survival

There are several approaches to the prolongation of discordant xenograft survival. Common approaches center around the inhibition of complement, and the depletion of natural antibodies from the recipient's serum. The inhibition of the reactivity of the complement system may be performed via exhaustion of the complement system, or the inhibition of key reactions in the complement cascade. CVF is a persistent C3b like compound that activates the complement system via the alternate pathway. Since it is stable and persists in the serum of the recipient, CVF exhausts the complement system for extended periods of time (56). The major liability of this process is that it removes the major killing effector and a major mediator of inflammation and opsonization from the immune defense system. Inhibitors of complement may either be free in the serum of the recipient or membrane bound to the donor xenograft.

C1q inhibitor has shown some promise to prevent hyperacute rejection via the classical pathway. Recently, soluble CR1 has been shown to delay hyperacute rejection (57). More sophisticated methods of transgenics have produced donors that have the membrane bound inhibitors of complement that are usually found on the surface of the recipient's tissues on donor cells (58-61). This method allows inhibition of complement at a level that leaves the complement system available to fight infection and other pathogens, but leaves the xenograft free of complement damage.

The depletion of antibody may be achieved in two approaches. First, one can physically remove xenoantibody from the recipient's serum by either plasma exchange (62-65), organ perfusion (65-67), antigenic column, and immunoadsorption (68). This provides an immediate depletion of xenoreactive antibody levels. However, these methods require repeated use, and the possibility of antibody rebound is quite high. The second method is to use immunosuppressive agents to suppress antibody synthesis (69-71). This requires a pre-transplant dosing period that may be several days or weeks; however, once antibody depletion is achieved and a therapeutic drug level is established the recipient need only to continue his course of immunosuppression. The combination of both T and B cell immunosuppression and specific depletion of xenoreactive antibody through the use of an antigen column or an immunoadsorbant column specific for xenoantibodies would appear to be the optimal approach (68). However, the development of a specific column and the discovery of an efficacious immunosuppressant that targets the synthesis of natural xenoantibody has yet to come.

H. Overview of the use of Immunosuppression

In the 1950's, the field of immunosuppression was largely represented by steroids. Steroids have a variety of nonspecific and anti-inflammatory and immunosuppressive effects. In the 1960's, azathioprine was implemented (72). However, the toxic effects were quite severe with these two substances, and the level of immunosuppression provided by both could not sustain transplants on their own. With the introduction of CsA in the 1980's, the field of immunosuppression has become more elegant and efficacious. In recent years, many new, small molecular weight substances have been identified and developed for suppression of the immune system. In fact, many of these new

immunosupprssivc drugs have been discovered between 1985-1995 (73). This surge in novel immunosuppression has facilitated a leap forward in the understanding of cellular immunology. As a result, the medical research community has a much clearer understanding of the fundamental processes underlining transplant immunology, and autoimmune disorders. The development of a better understanding of immune processes has allowed research into potential immunosuppressive agents to be more rational, and more efficient. The novel immunosuppressants can be roughly divided into four classes that reflect their mechanism of action.

First, there are the inhibitors of cytokine synthesis. These inhibitors suppress T-cell activation by impeding the signal to transcribe IL-2. The production of IL-2 and IL-2 receptor are essential in the autocrine stimulation of T-cell growth and clonal expansion. As a result of decreased IL-2 transcription, the T-cell is arrested in the G_0 phase of the cell cycle. CsA and FK506 are potent inhibitors of cytokine synthesis (74,75). Both CsA and FK506 bind to cytoplasmic proteins called immunophilins. CsA binds to the immunophilin CyP (76), and FK506 binds to immunophilin FKBP (77). Both of these binding proteins are rotomases that are involved in the folding of newly synthesized proteins. After binding to their respective binding proteins, CsA and FK506 binds to calcineurin (78). Calcineurin is a cytoplasmic phosphatase that plays a key role in Ca^{2+} dependent T cell activation signaling. Its function is to activate transcriptional activator proteins that translocate to the nucleus to promote gene expression and transcription of cytokine genes (79,80). By binding, calcineurin becomes inactive or the specificity of the enzyme is altered and cytoplasmic transcriptional factors are not activated.

Second, there are the inhibitors of growth factor signaling. Unlike CsA and FK 506, immunosuppressants such as RAPA do not block the transcription of cytokine genes, but they do inhibit signal transduction subsequent to the binding of cytokines and growth factors to their receptors (81,82). These immunosuppressants do not prevent the transcription of lymphokines or their receptors, but instead inhibit the cellular response to lymphokines. For example, RAPA acts later in the activation sequence of T cell activation than CsA and FK506. Most commonly this class of immunosuppressants block IL-2 responsiveness by interfering with intracellular signaling processes. Although the mechanism of action of RAPA is not well understood, the most recent studies have suggested that the target of the FKBP-RAPA

complex in lymphocytes may be a mammalian homologue to yeast TOR, RAFT. TORs and RAFTs are thought to be lipid kinases, as they have homology with the catalytic domain of the p110 subunit of PI-3 kinase. Lipid kinases are found associated with several src-like and receptor-like tyrosine kinases, and thus it is speculated that RAFT may be associated with the IL-2 receptor. Indirect evidence to support RAFT as the target of RAPA's immunosuppressive effect is the coincident activation of PI-3 kinases with the activation of growth factor receptors, suggesting that 3-phosphorylated phosphoinositide products are second messengers involved in controlling cellular growth and proliferation (83). Since the immunosuppressant targets an early signal transduction event, it is able to arrest Ca^{2+} dependent or Ca^{2+} independent lymphocyte activation.

Third, there are the inhibitors of DNA synthesis and DNA transcription. The mechanism of action of many of these immunosuppressants have been well defined. These immunosuppressants inhibit nucleotide biosynthesis, thus halting T and B cell proliferation by blocking the synthesis of DNA. The immunosuppressant MMF (84) and potentially LEF (85) selectively inhibit DNA synthesis by targeting key enzymes involved in purine and pyrimidine nucleotide biosynthesis via the de novo pathway. In lymphocytes, there is both a de novo and a salvage pathway for synthesis of purines and pyrimidines. However, in lymphocytes the salvage pathway is not as active as that in non-lymphoid cells, and thus inhibition of the de novo pathway halts nucleotide synthesis and ultimately production of DNA.

Fourth, there are the inhibitors of antigen processing, presentation, and reception. With the exception of steroids which have effects on both transcriptional events and antigen processing events, steroids, monoclonal antibodies such as OKT3 and DSG do not inhibit cytokine synthesis, cytokine action, nor DNA synthesis of T cells. Instead these immunosuppressants interfere with presentation of antigen, or costimulation processes required in T cell activation. Steroids bind to 97 kD intracytoplasmic and intranuclear receptor protein dimers, and displace heat-shock proteins that are involved in the transportation of antigen in the APC (86). Monoclonal antibodies directed against cell surface markers on T cells or APC's dampens antigen recognition and cytolytic function by binding costimulator molecules and thus preventing an important signal transduction effect (87). There are two possible explanations for the immunosuppressive action of DSG. First, DSG binds to heat shock proteins 70 and 90 that may be

involved in binding and intracellular transport of antigenic peptides within the antigen presenting cell (88,89). Thus, DSG acts like a peptide mimic. The second mechanism is that DSG may interfere with the interactions of heat shock proteins 70, 90, and 59 that are required to achieve proper activation of glucocorticoid receptors (89).

I. Mechanism of Action of LEF

LEF has been shown to inhibit both T and B cell responsiveness in several models. In most of these cases the parent compound LEF is given orally. This parent drug is then converted to its active metabolite. The initial conversion involves fracturing of the isoxazole ring. It is the active metabolite that is immunologically active, and constitutes greater than 95% of the circulating drug. Elimination of the active metabolite is via hepatic oxidizative metabolism and subsequent urine excretion. In one way MLR, leukocyte proliferation was inhibited by 50% at a concentration of 25-50 μmol/L LEF metabolite. LEF also inhibited anti-CD3/PMA and anti-CD28/PMA stimulated lymphocytes at concentrations of 65 and 75 μmol/L respectively. In these experiments significant levels of IL-2 were secreted (1000–4000 mg/L). This is in contrast to CsA and FK 506 which completely inhibit IL-2 production from lymphocytes. Addition of exogenous IL-2 failed to restore T cell proliferation in the presence of LEF metabolite. The loss of lymphocyte responsiveness was not due to an inhibition of IL-2 receptors being expressed on the cell surface of lymphocytes (90).

LEF is also able to suppress specific elicit antibody generated against a xenoantigenic source. In these experiments, Lewis rats were sensitized with a one time injection of human peripheral blood lymphocytes I.V. LEF metabolite was injected at a dose of either 3 or 10 mg/kg/d I.P. from the day of sensitization for 10 days. Rat serum was collected at regular intervals post-treatment and later titrated and added to human peripheral blood lymphocytes and quantitated for the binding of xenoreactive IgG and xenoreactive IgM onto the lymphocytes using flow cytometry. The results of this study showed that IgM had markedly stronger binding than IgG. In non-sensitized rats that receive 10 mg/kg/d LEF showed a 30% decrease in IgG and a 50% reduction in IgM binding on +11 days. Cessation of administration of LEF treatment resulted in a return of xenoreative antibody titer to normal. In addition, LEF was able to

show dose-dependent inhibition of xenogeneic sensitization in Lewis rat, both in IgG and IgM. By increasing the dose of LEF from 3 to 10 mg/kg/d, the titer of xenoreactive IgG dropped 32 times, and IgM titer dropped 16 times (91).

The mechanism of action of LEF is under debate at this point in time. Initial experiments found that LEF was able to inhibit epidermal growth factor (EGF) receptor tyrosine kinase activity. At concentrations of 20-30 μmol/L, LEF was able to inhibit autophosphorylation of EGF receptors due to a decrease in kinase activity. This concentration is similar to the same concentration required for inhibition of lymphocyte proliferation (25-50 μmol/L). The concentration range of 20-30 μmol/L inhibited EGF receptors both in intact cell and purified receptors. In contrast, many previously discovered inhibitors of EGF receptors inhibit purified preparations of such receptors at much lower concentrations (eg. Erbstatin IC_{50}= 6.0 μmol/L), but require much higher concentrations to inhibit the EGF receptor in intact cells (eg. Erbstatin IC_{50}= >130 μmol/L) (92).

Tyrosine kinase activity is an essential component of T-cell activation signaling. Three major protein tyrosine kinases are involved in T cell activation; they are $p56^{lck}$, $p59^{fyn}$, and ZAP-70. All three of these protein tyrosine kinases are associated with the TCR-CD3 complex (93,94). However, $p56^{lck}$ also plays a major role in IL-2 receptor signal transduction. IL-2 receptors have no tyrosine phosphorylation activity and rely on the receptor associated $p56^{lck}$ to start essential signal processes cytosolically (95). Since, LEF renders lymphocytes insensitive to IL-2, it would appear that $p56^{lck}$ may be a target of LEF. When T cells are stimulated and exposed to LEF, T cells are able to produce IL-2 and upregulate IL-2 receptors. However, these cells are unable to respond to the presence of IL-2. Using ^{32}P-orthophosphate metabolic labeling and anti-phosphotyrosine immunoprecipitation, it has been demonstrated that incubation of T cells with LEF prior to IL-2 stimulation results in a dose dependent inhibition of tyrosine phosphorylation of a number of cellular substrates. Additionally, cell-free kinase assays demonstrate dose dependent inhibition of IL-2 stimulated $p56^{lck}$ activity by LEF. Although these studies do not show a direct relationship between $p56^{lck}$ and LEF, the evidence strongly suggests that $p56^{lck}$ activity is inhibited either directly or indirectly by LEF's pharmacological action (96,97). The mode by which LEF inhibits $p56^{lck}$ in IL-2 receptor signaling are two fold. First, primary T cells responding to antigen are inhibited,

and thus T cells requiring clonal expansion are inhibited. Secondly, those T cells that respond to antigen., but do not enter clonal expansion are not inhibited by LEF. This combination of effects suggests that LEF will inhibit those lymphocytes that will ultimately mount an immune reaction to the antigen (i.e. those cells undergoing clonal expansion), but will not interfere with T cells that are undergoing processes of anergy in the process of tolerization. This is unlike CsA which interferes with both processes. Thus, LEF may allow easier establishment of transplant tolerance.

More recent studies have suggested that LEF may inhibit DNA synthesis and DNA transcription much like BQR (98). Addition of the pyrimidines, uridine and cytosine, reverse the inhibitory effects of LEF, but addition of purines did not. At this time, this additional information has not been fully explored (85). A second study has demonstrated that LEF does block T-cell proliferation by inhibiting responsiveness to IL-2. However, there is some evidence that suggests that the mechanism of LEF is independent of receptor-mediated signal transduction. Firstly, LEF was found not to effect tyrosine phosphorylation at concentrations twice the IC_{50} for proliferation (IC_{50} for proliferation = 15 μM versus 30 μM). Additionally, in Jurkat T lymphoblasts in which IL-2 secretion is dependent on tyrosine phosphorylation of PLC-γ1, LEF was unable to prevent IL-2 secretion at a concentration of 50 μM which is more than twice the IC_{50} of proliferation for Jurkat T lymphoblasts (IC_{50} for proliferation = 16 μM). Direct cell cycle analysis of LEF treated lymphocytes demonstrate that LEF inhibits the progression of lymphocytes from G_1 to S, G_2, and M-phases. This was observed at concentrations of 30 μM which is slightly more that twice the IC_{50} for proliferation for these cells. It is speculated that LEF may not inhibit an enzyme directly involved in DNA replication (99).

J. The use of LEF in Autoimmune Disorders

LEF is an isoxazol derivative that has proven to be an effective agent in preventing and curing several autoimmune disorders. Early small animal research into the nature of LEF offered some initial clues that LEF may have anti-inflammatory and immunomodulating properties. Studies utilizing a Lewis rat adjuvant arthritis model followed by a "standardized arthritic assay" (100) demonstrated that LEF was

able to arrest the development of adjuvant arthritis, but could restore the diminished mitogen induced lymphocyte response of the diseased animals (101-102). This is a unique characteristic of LEF not often demonstrated by previously tested immunosuppressants. Further results demonstrated LEF's ability to significantly reduce edema, fibrinogen levels, and erythrocyte sedimentation as compared to CsA therapy (102-103).

LEF is also able to arrest murine SLE-like disease in mice. SLE is characterized by development of antibodies against self antigens, most commonly double stranded DNA. Treatment of LEF was able to dose dependently arrest the progression of SLE in afflicted mice and prevent the development of glomerulonephritis. This is due to the suppression of circulating immune complexes by inhibition of autoantibody synthesis. An additional benefit to LEF treatment was the inhibition of T-cell reactivity without the accumulation of lymphocytes in the lymph nodes and the spleen, a characteristic common to SLE afflicted animals (104).

K. The use of LEF in Transplantation

Currently, LEF has been tested for use as an immunosuppressant in animal transplantation only. In initial transplant studies LEF appeared not to be able to prevent transplant rejection. However past success of LEF to prevent chronic graft versus host disease in mice, and autoimmune disorders spurred on further transplant studies using a rat model. In these experiments, the kidneys of Brown Norway rats were transplanted into Lewis rat recipients. The untreated control animals rejected their kidney allografts within 8 days. Administration of CsA at 10 mg/kg P.O. prolonged allograft survival to the full 60 day duration of the study. Similarly, treatment of recipients with LEF for 30 days at a dose of 5 or 10 mg/kg/d P.O. resulted in allograft survival for the full 60 days of the study. This result suggested that LEF may be able to induce tolerance in the rat allograft model. CsA and LEF treated animals had normal kidney function throughout the study as determined by serum creatinine levels, and they showed no signs of chronic rejection at the end of the study. In contrast, azathioprine and prednisolone administered at 5 mg/kg/d I.V. were unable to protect the transplanted kidney any longer than the untreated control animals (8 days) (105).

Further studies using a skin allograft model between Dark Agouti/Lewis rats (MHC and non-MHC different) and Lewis/Fisher rats (non-MHC different) demonstrated a dosed dependent prolongation of graft survival. In addition to allograft kidney and allograft skin transplant models, LEF has also proven to be very efficacious in cardiac allograft survival. Utilizing a Brown-Norway/Lewis rat cardiac transplant model, LEF demonstrated an ability to indefinitely prolong allograft survival. In animals allowed to develop acute rejection over four days, LEF but not CsA was able to reverse the acute rejection episode. Acutely rejected cardiac allografts returned to histologically normal appearance 7 days after the initiation of the administration of LEF. Production of alloantibody responses as measured by microcytotoxicity assays and total IgG and IgM in rejecting animals also returned to base line levels approximately 9 days after initiation of LEF treatment. When both CsA and LEF were used in combination, graft survival improved even at low doses of each drug (106). The CsA/LEF combination at doses of 5 mg/kg/d CsA P.O. and 1.25, 2.5 and 5 mg/kg/d LEF P.O. was able to significantly increase ($p< 0.05$) graft survival compared with graft survival with either drug alone. Evaluation using the Cox proportional hazards model showed that this was a result of an additive effect at therapeutic doses (CsA/LEF 2.5 and 5 mg/kg/d P.O.) and a synergistic effect at lower doses (CsA 1.25 mg/kg/d and LEF 0.3 mg/kg/d P.O.). The additive and synergistic effect of CsA/LEF combination was not caused by reduced elimination as demonstrated by pharmacokinetic studies (106).

As stated above, early transplant results suggested that LEF may be able to induce tolerance. This property of LEF was investigated in a set of two experiments. The first involved, a series of skin allografts onto treated and naive Lewis rat recipients. Control animals were naive Lewis rats transplanted with DA skin grafts. Treated animals were Lewis rats treated with LEF and given a primary DA skin allograft; those rats that had not rejected their first skin allografts were transplanted with a second DA skin allograft and a third party BN skin allograft. All second DA skin allografts on treated animals survived for more than 55 days without further administration of LEF, and all primary DA skin allografts survived more than 120 days. In contrast, third party BN skin allografts were rejected rapidly in the same time frame as untreated controls (8 days). Similar results were found using a Lewis/F344 rat combination. The second set of experiments centered around the ability of spleen cells of control animals

that had rejected their skin allografts and LEF treated animals that had long-term skin graft survival to transfer tolerance to a naive recipient. Using a DA/Lewis rat combination 1X 10^8 spleen cells were isolated from control and treated animals and transferred to naive syngeneic Lewis rats. The next day these syngeneic rats were given DA skin allografts. Recipients given spleen cells from long-term LEF treated survivors had skin graft survival times of 37.1 ± 3.2 days while recipients given spleen cells from short-term survivors had skin graft survival time of 9.4 ± 1.4 days. Again similar results were found using a Lewis/F344 rat combination (107).

LEF was shown to be slightly less efficacious in the prolongation of renal dog allografts than initial studies that utilized small animal models. Monotherapy with LEF significantly prolonged dog renal allografts as compared to controls ($p< 0.05$) at doses of 8 and 16 mg/kg/d LEF P.O. Moderate doses of LEF of 8 mg/kg/d proved to be the most effective therapy to prolong renal dog allografts (27.6 ± 1 day) with some mild toxicity to the recipients. At the high dose of 16 mg/kg/d LEF P.O., LEF prevented acute allograft rejection (20.7 ± 2 days), but the dogs died of marked drug toxicity which was characterized by anorexia, and anemia. At a well tolerated non-toxic dose of 4 mg/kg/d LEF P.O., graft function was prolonged to 15.5 ± 5 days, however all dogs died of rejection. A combination of subtherapeutic doses of LEF at 4 mg/kg/d P.O. and CsA at 10 mg/kg/d proved to be a synergistic combination prolonging graft survival to 67.8 ± 16 days with no signs of toxicity. To determine LEF's contribution to the LEF/CsA combination, LEF was withdrawn at day 119 which resulted in rejection 14 days after withdrawal of drug treatment (108).

LEF has also been evaluated in a hamster to rat cardiac concordant xenograft model (109,110). In single and multiple drug therapy, LEF proved to be the most protective single agent and a LEF/CsA combination proved to be the most efficacious overall. Control animals survived 3.7 days, while RAPA (1.6 mg/kg/d I.P.), CsA (25 mg/kg/d P.O.), and FK506 treated (0.8 mg/kg/d I.P.) animals survived 7.2, 5.3 and 4.6 days respectively. Monotherapy with LEF (10 mg/kg/d P.O.) prolonged xenograft survival to 31.6 days. The combination of LEF (10 mg/kg/d P.O.) and CsA (12.5 mg/kg 3 times/week I.M.) was able to prolong xenograft survival to 54.3 days. Multiple dosing regimes of three or four immunosuppressants did not significantly prolong xenograft survival, but treated animals did suffer from anorexia and infection

due to overimmunosuppression. In tolerance studies, animals were given the combination of LEF and CsA and donor spleen and thymus cells I.V. This combination prolonged xenograft survival to 74.0 days, but did not induce tolerance (109). In a separate study utilizing the same hamster to rat model, monotherapy with LEF showed dose dependent prolongation of xenograft survival (110).

LEF is one of many new immunosuppressants that provide potent immunosuppression of T and B cell responsiveness. This new drug has proven to be promising in its application in allograft and concordant xenograft models. It is the purpose of the following set of experiments to test the efficacy of LEF to prolong the graft survival of a pig to rabbit discordant cardiac xenotransplant model.

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II. Objectives

Solid organ transplants provides an efficient and permanent solution to patients suffering from end stage diseases. Unfortunately, a shortage of organs leaves increasing numbers of patients on waiting lists. Xenotransplantation may provide a source for these much needed organs should one be able to control both humoral and cellular immunological reactions against the xenograft. The new immunosuppressant, leflunomide, has been proven to inhibit both T cell and B cell responsiveness in several animal models. In transplant studies, LEF has been shown to extend dog renal allografts indefinitely, and it has been shown to prolong the survival of concordant xenografts. More significantly, it has been shown to inhibit the synthesis of allogeneic and xenogeneic antibody. There are several methods of preventing the binding of xenoreactive antibody that mainly center around the physical removal of xenoreactive antibody from the blood. Although these methods provide an immediate drop in xenoreactive antibody levels they do not provide sustained depression of antibody levels. Hyperacute rejection remains a formidable barrier to the realization of clinical xenotransplantation. Hyperacute rejection is mainly antibody mediated. Binding of xenoreactive antibodies onto the endothelium of the graft results in rapid and irreversible destruction of the protective properties of the endothelial cell lining the vasculature of the graft. In the absence of antibody, slight though significant prolongation of discordant xenograft survival has been reported. Current studies into discordant xenotransplantation focus around dealing with xenoantibody and xenoantigens. One approach is to use selective immunosuppressants, such as leflunomide, to provide sustained B cell suppression and provide a means of overcoming hyperacute rejection.

It is the focus of this study to investigate the efficacy of the new immunosuppressant leflunomide to prolong discordant cardiac xenotransplants utilizing a pig to rabbit model. In order to achieve this end, the objectives of this study are the following:

1. To develop a method of analysis for the active metabolite of leflunomide in blood or plasma.
2. To investigate the blood distribution and pharmacokinetics of leflunomide in the New Zealand White rabbit.
3. To investigate the effect of leflunomide on xenoreactive antibody levels *in vivo*.
4. To evaluate the efficacy of leflunomide to prolong the survival of heterotopic cardiac xenotransplants in a pig to New Zealand White rabbit model utilizing both a short 5 day pretransplant and a longer 21 day pretransplant dosing regime.

III Chapter 1: The Measurement and Blood Distribution of Leflunomide Active Metabolite in Blood.

METHODS

A. Preparation and Handling of Blood Samples for Analysis of Leflunomide

A pool of whole blood was collected using EDTA as anticoagulant from a number of normal volunteers, and normal New Zealand white rabbits. Leflunomide prodrug (LEF prodrug) and its active metabolite (LEF metabolite) was obtained as a gift from Dr. R. Bartlett (Hoechst AG Werk Albert, Wiesbaden, FRG). Individual stock standards of LEF prodrug and LEF metabolite at a concentration of 10 g/L, were prepared in 95% (v/v) ethanol and normal saline respectively, and stored at -70°C. Stock solutions of 10 000 µg/L were freshly prepared. 17 ß-estradiol (Sigma Chemical Co. St. Louis, Mo, USA) was used as an internal standard and stored as a stock solution of 1 000 mg/L in 95% (v/v) ethanol and stored at -70°C. A working internal standard solution of 10 000 µg/L was made freshly.

B. Extraction of Samples for Analysis of Leflunomide Active Metabolite

Plasma or whole blood samples (250 µL) and 100 µL of the working internal stock solution (10 000 µg/L) were added to 10 mL glass extraction tubes, followed by, 4.0 mL of a 20 g/L potassium carbonate solution and 10 mL of ethyl acetate (A.C.S. specified) extraction solvent. These samples were shaken vigorously for 15 min. The top solvent layer (ethyl acetate) was transferred to a clean 13 X 100 mm borosilicate glass tube and evaporated to dryness under a gentle stream of air at room temperature. The dried extract was then reconstituted in 200 µL of acetonitrile:double distilled water (8:2 v/v) solution. The reconstituted samples were then vortexed for 60 sec and used for HPLC analysis.

Preliminary studies examined a number of different solvents for extraction recovery of a 4 000 µg/L combined concentration of LEF prodrug and LEF metabolite spiked human whole blood pool. Six different solvents were tested: 1. 100% ethyl acetate, 2. ethyl acetate:diethyl ether 6:1 (v/v), 3. ethyl acetate:diethyl ether 1:2 (v/v), 4. ethyl acetate:dichloromethane 1:2 (v/v), 5. ethyl

acetate:dichloromethane:diethyl ether 2:2:2 (v/v/v), and 6. dichloromethane. Based on the data of these preliminary studies ethyl acetate was subsequently chosen for the extraction of LEF metabolite.

C. High Performance Liquid Chromatography of Leflunomide Active Metabolite

Chromatographic separation by reverse phase HPLC was performed using a Hewlett Packard 1050 Series HPLC system. Chromatographic separation was performed isocratically using two heated Spherisorb-octyl C-8 columns joined in tandem (5 µm, 25 X 0.46 cm, Chromatography Sciences Company, Montreal, PQ, CN), preceded by a pellicular (30-40 µm) silica precolumn (Perisob A, Upchurch Scientific, Oak Harbour, WA, USA). The columns were heated and maintained at a temperature of 70°C using an external column heater (Jones Scientific, Wales, UK). Data from each chromatograph was integrated and analyzed using EZ-chrom software. For each sample, 50 µL of extract was injected onto the columns. The mobile phase acetonitrile:methanol:water 40:20:40 (v/v/v) was de-gassed under helium daily before use and delivered through the HPLC circuit at 1.0 mL/min. The eluted peaks were monitored at 280 nm and concentrations were calculated form the peak area ratios in relation to the internal standard.

D. Temperature and Concentration Equilibration Experiments

Whole blood pools of 5 mL were spiked with LEF metabolite to final concentrations of 0.5, 5, 10 and 100 mg/L. Each concentration pool was then split into two aliquots which were left for 4 h to equilibrate at either 4°C or 22°C. After this initial equilibration, one set of samples was separated into its plasma and cell fractions by centrifugation for 10 min at 2000 X g (0 min time point), and a second set was left to incubate at 37°C for 120 min before separation by centrifugation (120 min time point). All separation by centrifugation was performed at the same temperature as the samples were incubated (4°C and 37°C respectively). Plasma and cell fractions (250 µL) from each separated sample were analyzed separately. All experiments were done in duplicate over three days.

E. Free Fraction Experiments

The free fraction of LEF metabolite in plasma was determined by ultracentrifugation using the method of Legg and Rowland (112). Briefly, rabbit plasma was spiked with LEF metabolite to final concentrations of 0.4, 1, 10, and 100 mg/L in 4 mL Beckman Quick-Seal polyallomer ultracentrifuge tubes (Beckman Inc. Palo Alto, CA). The tubes were subsequently ultracentrifuged at 150 000 X g for 15 h at 37°C using a Ti 50.1 fixed-angle rotor in a L8-70 Beckman ultracentrifuge (Beckman Instruments, Brea, CA). To minimize turbulence the centrifuge was allowed to come to a stop without breaking. Fourteen successive 280 µL fractions were obtained from top to bottom of each tube. Total protein concentration of each fraction was quantitated by a bicinchonic acid/Cu^{+} biuret reaction (BCA Protein Assay, Pierce Scientific, Richmond, VA). Fractions with a protein concentration less than 0.3 g/L were pooled and 1 mL of each pool was used to quantitate the free fraction of LEF metabolite. All experiments were done in triplicate over three days.

F. Lipoprotein Binding Experiments

Lipoproteins were separated using the method of Redgrave et al (113). Briefly, plasma samples were spiked to concentrations of 0.4, 1, 10, 100 mg/L, with LEF metabolite. The density of each plasma sample was adjusted to 1.21 g/L with solid potassium bromide. Four mL of plasma were then added to 13 mL ultracentrifuge tubes (Quick-Seal^{R}, Beckman Inc. Palo Alto, CA). This was successively overlaid with 3.0 mL of the following salt solutions, d=1.063 g/L, d=1.019 g/L, and d=1.006 g/L, respectively. The densities of all gradient solutions were confirmed by refractometry. The tubes were centrifuged for 24 h at 200 000 X g at 4°C. After centrifugation, fractions containing VLDL, LDL, HDL and the residual Lp-Free were collected. The identity of each collected fraction was then confirmed by lipoprotein electrophoresis as performed in the University of Alberta Hospital clinical laboratory. HPLC analysis of LEF metabolite was performed using 1.0 mL of each sample. All experiments were done in triplicate over three days.

RESULTS

A. Stability, Extraction, and Sample Chromatographs of Leflunomide Active Metabolite in Whole Blood and Plasma

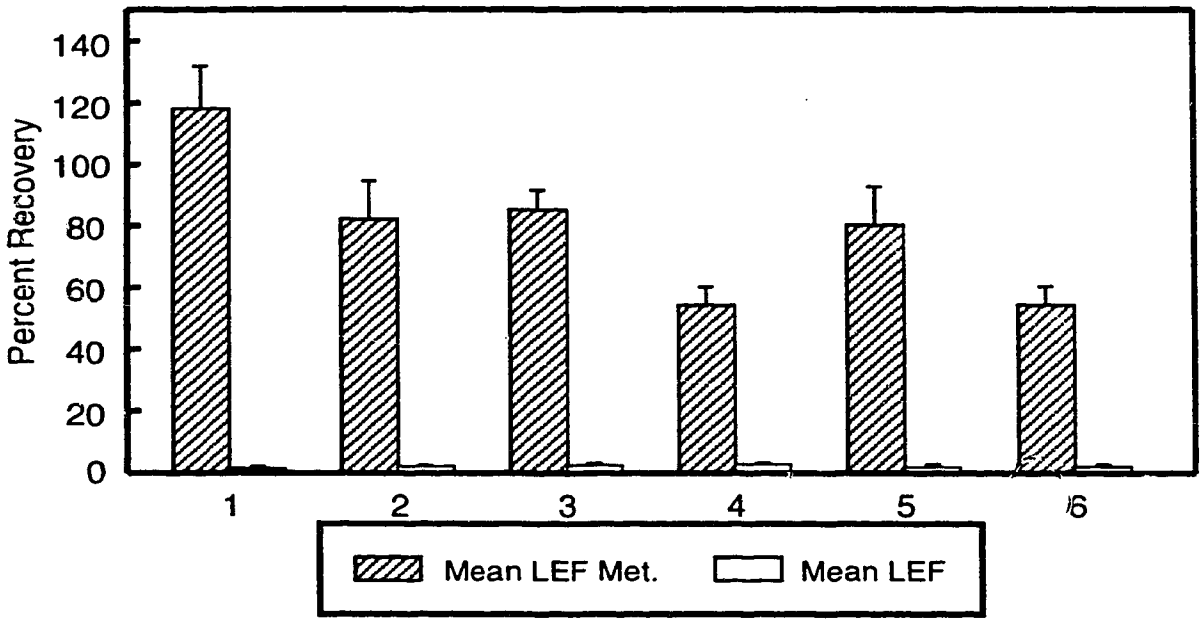
A 10 day preliminary study for LEF metabolite in human whole blood was determined by analyzing whole specimens spiked at a low (1 000 µg/L) and high concentration (10 000 µg/L) and stored at 4°C, -20°C, and -70°C. Samples stored at -20°C or -70°C demonstrated statistically insignificant ($p > 0.05$) changes ranging from a decrease of 4 ± 0.7% (mean ± SD) to an increase of 4 ± 2.5% (mean ± SD) from day 0. Only samples spiked to a concentration of 10 000 µg/L stored at 4°C demonstrated significant decreases in concentration of 5% at day 5 ± 1.9% (mean ± SD) and a decrease in concentration of 11 ± 1.9% (mean ± SD) at day 10, as compared to measured levels at day 0.

A number of extraction solvents were tested as shown in Fig. 1. The relative percent recovery for the six different solvents tested was found to vary from 115% ± 13.8% to 54 ± 6.1% (mean ± SD) for LEF metabolite, and less than 3% for LEF prodrug. The highest recovery was obtained for ethyl acetate. Subsequently ethyl acetate was chosen for solvent extraction of LEF metabolite.

Illustrations of representative chromatograms are shown in Fig. 2. Chromatogram A is an extracted whole blood specimen containing no LEF metabolite and no ß-estradiol. Chromatogram B is an extracted specimen containing 4 000 µg/L of ß-estradiol. Chromatogram C is an extracted specimen of 4 000 µg/L of both LEF metabolite and ß-estradiol. Chromatogram D is a representative chromatogram of human whole blood extracts, spiked with 4 000 µg/L of LEF metabolite and 4 000 µg/L of ß-estradiol. Similar results were found with rabbit whole blood and plasma specimens. No peaks of any significance were found to comigrate with LEF metabolite or the internal standard. In order to explore the possibility of comigration by other common immunosuppressants, 1 000 µg/L of RAPA, CsA, MMF and BQR was spiked into samples containing LEF metabolite and ß-estradiol. No instances of comigration were detected.

Figure 1.

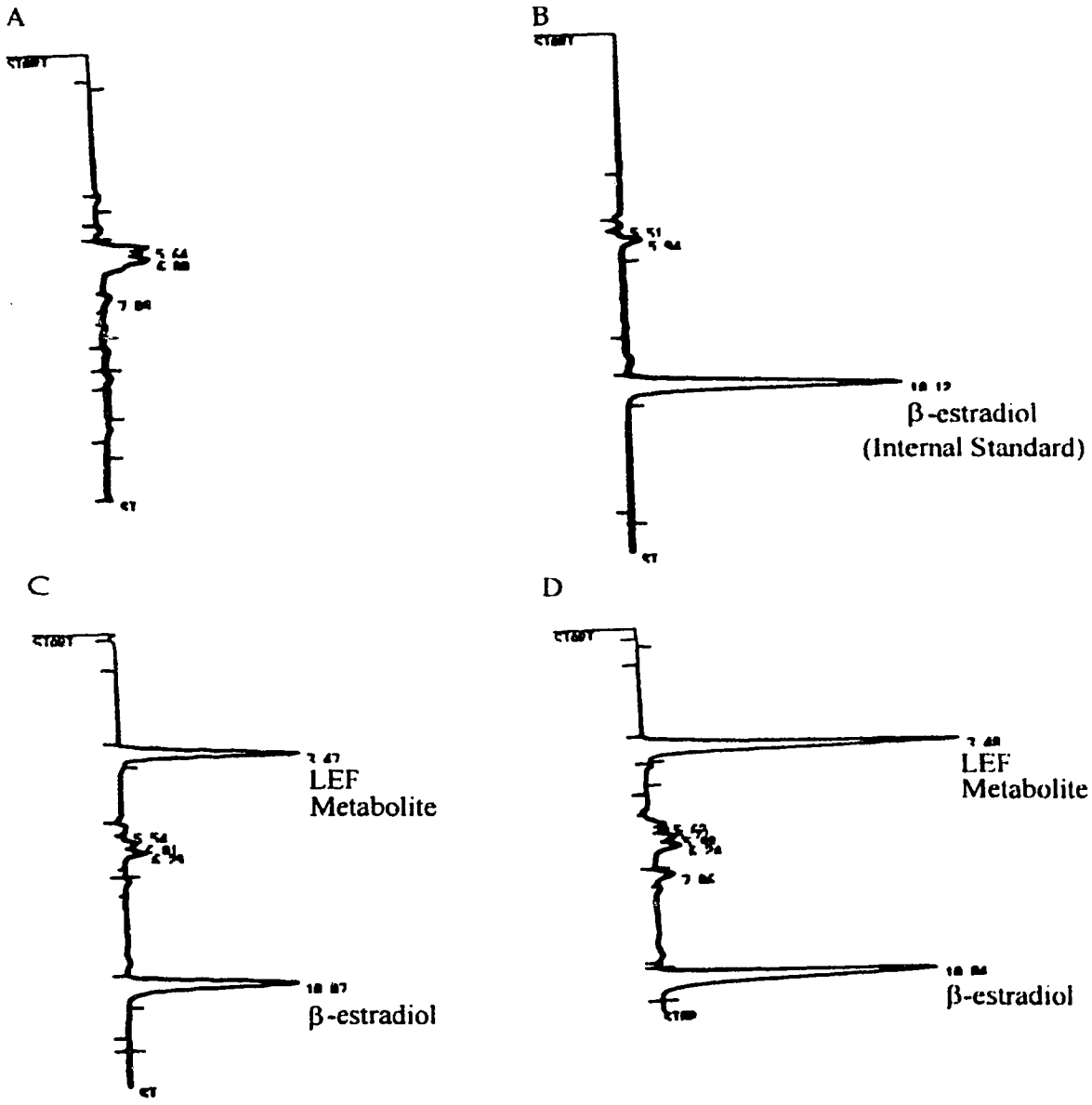
The Recovery of Leflunomide Prodrug and Leflunomide Active Metabolite from Human Whole Blood using Different Extraction Solvents.



A human whole blood specimen pool was spiked to a final concentration of 4 000 µg/L each of LEF and LEF metabolite. Spiked samples were subsequently extracted with the following solvents: **#1.** 100% ethyl acetate. **#2.** ethyl acetate:diethyl ether 6:1 v/v, **#3.** ethyl acetate:diethyl ether 1:2 v/v, **#4.** ethyl acetate:dichoromethane 1:2, **#5.** ethyl acetate:dichloromethane:diethyl ether 2:2:2, and **#6.** 100% dichloromethane. The extracts were quantitated by HPLC as described above.

Figure 2.

Representative Chromatograms of HPLC Method of Leflunomide Active Metabolite Analysis.



Chromatogram A is a representative chromatogram of an extracted whole blood specimen containing no LEF metabolite and no internal standard ß-estradiol. Chromatogram B is an extracted specimen containing 4 000 µg/L of ß-estradiol. Chromatogram C is an extracted specimen of 4 000 µg/L of both LEF metabolite and 4 000 µg/L of ß-estradiol. Chromatogram D is a representative chromatogram of a human whole blood extract spiked with 4 000 µg/L of LEF metabolite and 4 000 µg/L of ß-estradiol.

B. Linearity of HPLC Method of Detection of Leflunomide Active Metabolite

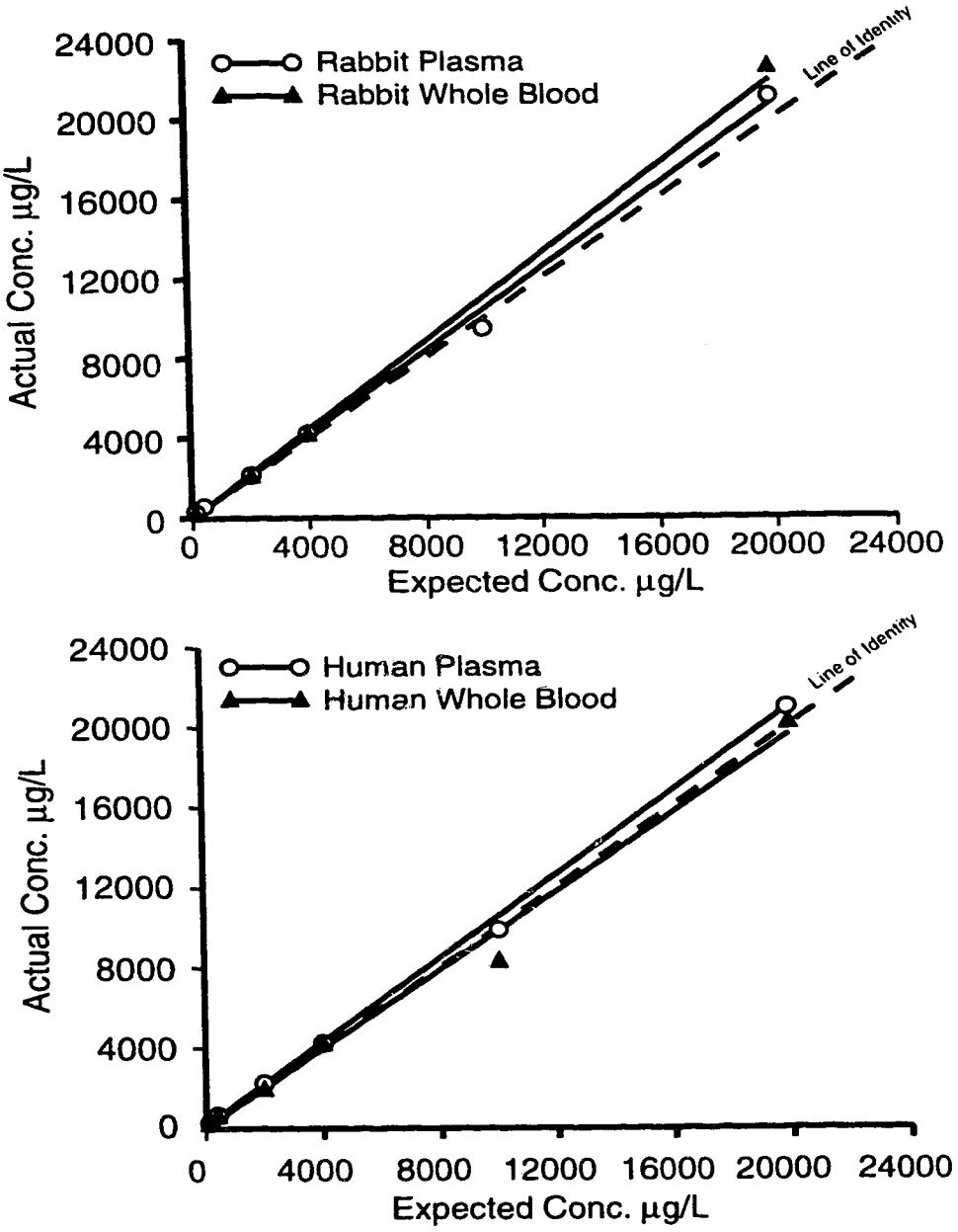
The linearity of this assay was determined using 250 µL of sample volume from human whole blood, human plasma, rabbit whole blood, and rabbit plasma pools spiked with LEF metabolite ranging from 400 µg/L to 20 000 µg/L. The assay was found to exhibit a linear relationship between the expected and actual LEF metabolite concentrations to the highest concentration tested 20 000 µg/L (Fig. 3.). The correlation coefficient and regression equation for rabbit plasma and whole blood were correlation coefficient (r^2) = 0.996, intercept (a)= 7.66, slope (b)= 1.041, and r^2= 0.992, a= 87.5, and b= 1.100. The correlation coefficient and regression equation for human whole blood and plasma were r^2= 0.998, a= -98.95, b= 1.041, and r^2= 0.990, a= -58.90, b= 0.980, respectively. A signal to noise ratio of < 1 was observed at 40 µg/L when 250 µL was used for analysis. Increasing the sample size to 1.0 mL produced an increase in the signal to noise ratio to > 3 at this concentration.

C. Relative Recovery and Coefficient of Variation of HPLC Analysis of Leflunomide Active Metabolite in Human and Rabbit Whole Blood and Plasma

The relative recovery of five different concentrations of LEF metabolite was determined with human and rabbit whole blood and plasma are shown in Table 1. The relative recoveries in relation recoveries in relation to the internal standard for the 400 µg/L concentration ranged from 78 ± 13.5% to 96 ± 29.1% (mean ± SD) and were found to be lower than those for the 4 000 µg/L and 10 000 µg/L concentrations which ranged from 99 ± 5.7% to 108 ± 4.8% (mean ± SD). Poor relative recoveries ranging from 21 ± 10.7% to 43 ± 20.4% (mean ± SD) were obtained for the lowest tested 40 µg/L concentration sample when 250 µL extraction volume was used. When 1.0 mL extraction volume was used for whole blood specimens, recovery at these concentrations was increased to 98 ± 9.2% (mean ± SD) and a CV range of approximately 8% to 12% (mean ± SD) for LEF metabolite. Similar results were obtained when plasma was used.

Figure 3.

The Linearity of the HPLC Method of Analysis of Leflunomide Active Metabolite in Blood.



Linearity of LEF metabolite HPLC assay. Results expressed as the mean of triplicate experiments. The line of identity is indicated. The correlation coefficient and regression equation for rabbit plasma and whole blood were correlation coefficient (r^2)= 0.996, intercept (a)= 7.66, slope (b)= 1.041, and r^2= 0.992, a= 87.5, b=1.100, respectively (Fig. 3A). For human plasma and whole blood r^2= 0.998, a= -98.95, b=1.041, and r^2= 0.990, a= -58.90, b= 0.980 respectively (Fig. 3B).

Table 1.

The Relative Recovery of Leflunomide Active Metabolite from Human and Rabbit Whole Blood and Plasma.

Concentration of LEF metabolite	40 µg/L	400 µg/L	4 000 µg/L	10 000 µg/L
Human Plasma	35 ± 25.2%	96 ± 29.1%	103 ± 8.5%	99 ± 9.3%
Human Whole Blood	21± 10.7%	83 ± 16.3%	107 + 6.8%	102 + 8.0%
Rabbit Plasma	43 ± 20.4%	93 ± 10.5%	105 +5.5%	99 + 5.7%
Rabbit Whole Blood	32 ± 26.3%	78 ± 13.5%	108 +4.8%	99 +6.0%

Data presented in Chart represents mean relative recovery ± SD. Four samples were analyzed for each group (n=4).

D. The Precision of HPLC Analysis of Leflunomide Active Metabolite in Human Whole Blood

The within-day and between-day CV of the assay at a low (1 000 µg/L) and high (10 000 µg/L) concentrations extracted from human whole blood controls is shown in Table 2. The within-day CV was 5.7% and 7.0% for the low (1 000 µg/L) and high (10 000 µg/L) range, respectively (n = 15). The between-day precision for these controls was 12.2% and 14.7%, respectively (n = 10). Similar results were found when low and high concentrations where extracted from rabbit whole blood.

E. The Blood Distribution of Leflunomide Active Metabolite

The proportions of LEF metabolite found in the plasma and cell fractions at two concentrations 0.5 mg/L and 10 mg/L are shown in Fig. 4. The distribution of LEF metabolite was found to be independent of temperature or concentration over the ranges tested. With increasing concentration of LEF metabolite at 0.5 mg/L and 10 mg/L , or temperature at either 4° C or 22° C, no significant differences were found in distribution between plasma and whole blood.

The majority of the drug was found in the lipoprotein free fraction of plasma (> 95%) as shown in Fig. 5. No detectable levels of LEF metabolite was found in free fraction of plasma containing drug up to 10 mg/L. At a concentration of 100 mg/L, a small amount of LEF metabolite was detected in the free fraction component, at < 1% of the total drug concentration (Fig 5.). Small quantities of drug was found bound to the lipoproteins independent of concentration. Among the plasma lipoproteins, the LDL fraction was found to contain the largest proportion of LEF metabolite (approximately 1.8% of total concentration). No detectable quantities of LEF metabolite (< 0.04 mg/L) were found in the other lipoproteins (VLDL and HDL).

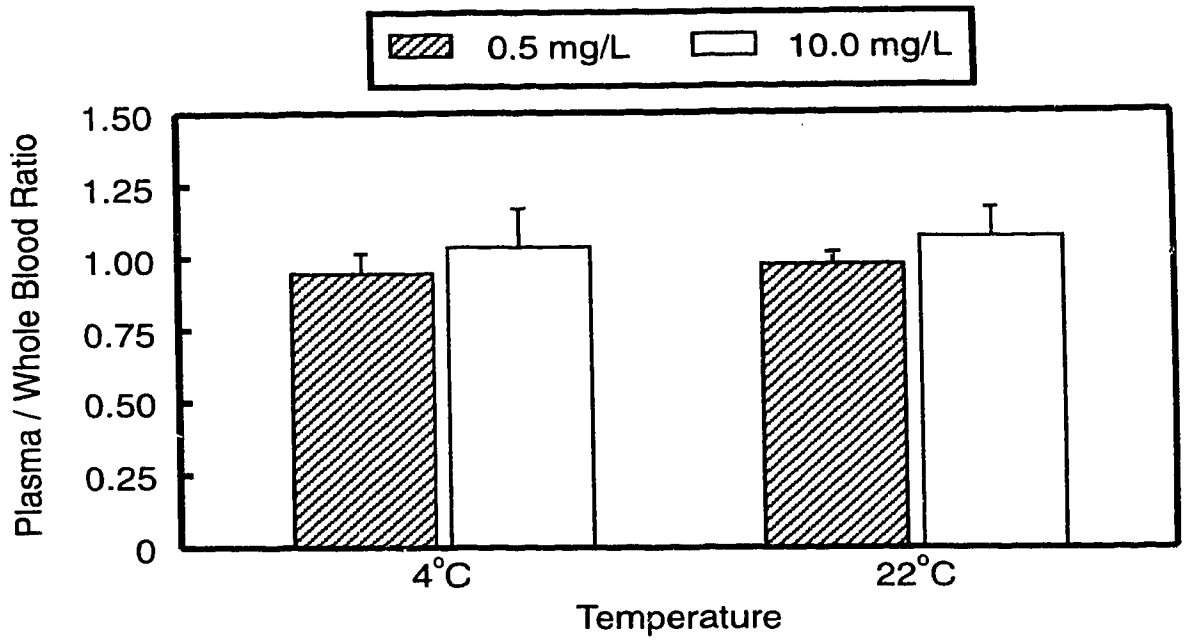
Table 2.

The Within-day and Between-day Precision for a Low and High Concentration of Leflunomide Active Metabolite Extracted from Human Whole Blood.

		Low Concentration	High Concentration
Within-day	Mean	986 µg/L	9447 µg/L
	SD	56.5 µg/L	695.6 µg/L
	CV	5.7%	7.0%
	n	15	15
Between-day	Mean	1006 µg/L	8146 µg/L
	SD	123 µg/L	1197.3 µg/L
	CV	12.2%	14.7%
	n	10	10

Figure 4.

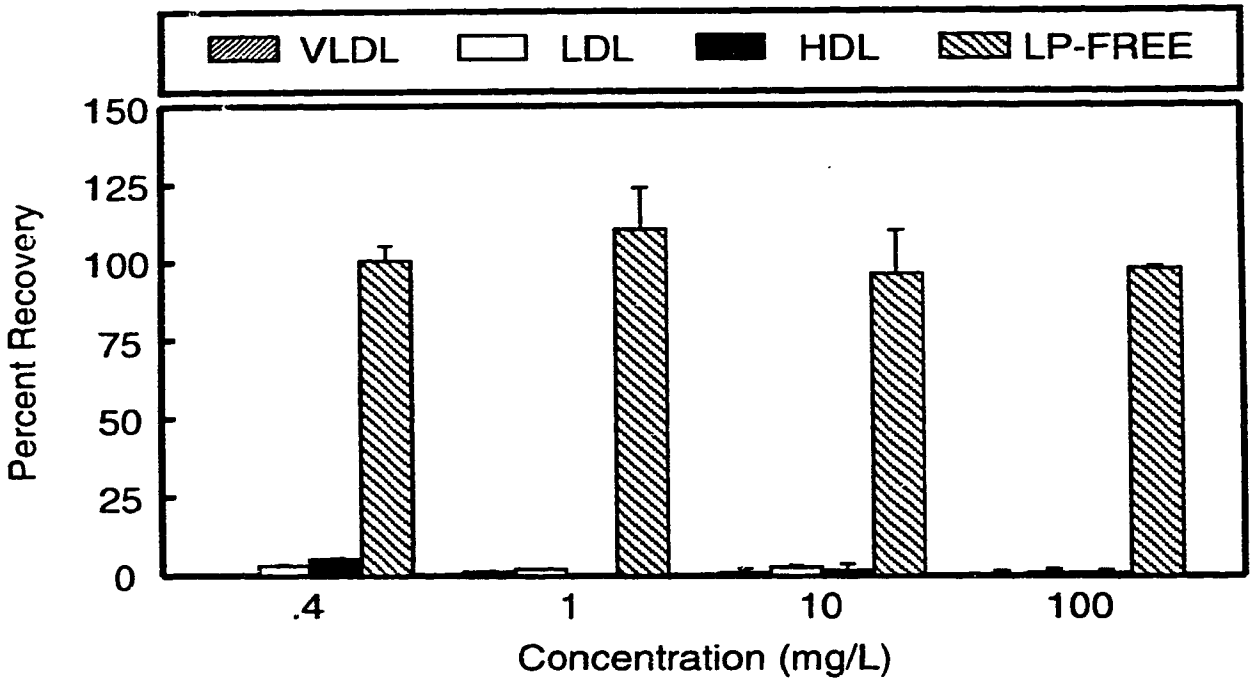
The Plasma to Whole Blood Ratio of Leflunomide Active Metabolite after Re-equilibration at 37°C for 120 Minutes.



Plasma to whole blood ratios of LEF metabolite after 120 min of time for re-equilibration at 37°C having an initial temperature of either 4°C or 22°C. Results are expressed as mean ± SD. Samples from each temperature and concentration group were run in duplicate over three days (n=6 for each group).

Figure 5.

The Overall Distribution of Leflunomide Active Metabolite amongst the Lipoproteins and Lipoprotein Free Components of Plasma.



The overall distribution of LEF metabolite amongst the very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL), and the residual lipoprotein-free proteins (Lp-Free) plasma components. Results are expressed as mean ± SD (n=6).

DISCUSSION

Currently, there are many HPLC techniques use to measure immunosuppressive drugs in a variety of tissue samples (114-117). In most cases the HPLC technique requires extraction of the drug utilizing either a liquid/liquid extraction method or preparatory column (118). In addition, many HPLC techniques used to detect immunosuppressive drugs have long run times (> 30 min) (114-116). In this HPLC technique total run times are < 15 min with LEF metabolite having an elution time of approximately 3.5 min and the internal standard having an elution time of approximately 10 min. The above HPLC method for measurement of LEF in biological specimens shows good precision, relative recovery and linearity. The within-day CV of this LEF HPLC method (low = 5.7%, high = 7.0%) is higher at high concentrations than the within day CV of a RAPA HPLC method described by Yatscoff et al. (116) (low = 8.1%, high = 1.9%). The between-day CV of this LEF HPLC method (low = 12.2%, high = 14.7%) is similar to that of the RAPA HPLC method (low = 14.4%, high = 9.8%). The relative recoveries of both the RAPA HPLC method and the LEF HPLC method are close to 100% for both low and high concentrations spiked into blood and plasma respectively. The limit of detection of the RAPA HPLC method (1.0 μg/L utilizing a 2.0 ml sample volume) is much lower than the limit of detection of the LEF HPLC method (40.0 μg/L utilizing a 1.0 ml sample volume). LEF was found to be most stable at either -20°C or -70°C with no significant changes ($p>0.05$) after 10 days. However, a significant ($p<0.05$) decrease (11 ± 1.9%) occured in samples spiked to 10 000 μg/L stored at 4°C. This is in contrast to RAPA which is stable at 4 °C and -40 °C for 30 days (116).

The distribution of LEF metabolite is unlike many immunosuppressive drugs. LEF metabolite appears to be primarily distributed in the plasma as indicated by plasma/whole blood ratios close to 1.0 (Fig. 4.). In plasma, LEF metabolite demonstrated minimal binding to lipoproteins (< 5%), and was found primarily (> 95%), associated with the lipoprotein free plasma fraction independent of concentration (Fig. 5.). In contrast, immunosuppressants such as CsA (119), CsG (120), and FK 506 (121) show temperature dependent distribution as demonstrated by an increase in plasma/whole blood

ratios as samples were taken from 4°C to 37°C. Free fraction studies demonstrated that the majority of LEF metabolite was protein bound, however this binding was saturatable at a concentration of 100 mg/L. In contrast to previously studied drugs, LEF appears to be highly hydrophilic, binding to the lipoprotein free fraction of plasma, independent of temperature or concentration. The high plasma/whole blood ratio of LEF (1.0-1.2) is in contrast to drugs with small plasma/whole blood ratios such as CsA (< 0.6) (119), CsG (0.7-0.8) (120), and RAPA (0.09) (122). A low plasma/whole blood ratio indicates a lipophilic immunosuppressant which preferentially distributes to the hydrophobic components of the blood. In blood, the majority of CsA and CsG are bound to erythrocytes (approximately 50%). Of plasma components CsA and CsG are primarily bound to lipoprotein; the majority of CsA and CsG are bound to HDL (approximately 50%) (119,120). A more lipophilic immunosuppressant, RAPA, is primarily bound to erythrocyte (94.5%) with very little found in plasma (122). In comparison to a second acidic immunosuppressant MMF, LEF metabolite, like MMF, is mainly distributed in the plasma fraction as reflected by a high plasma/whole blood ratio (1.5-2.0). MMF is primarily associated with non-albumin proteins in plasma, and exhibits concentration dependent binding to albumin (the greater the concentration of MPA, the smaller the proportion bound to albumin) (123). LEF metabolite, on the other hand, shows no such concentration dependence.

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VI. Chapter 2: The Single Dose Pharmacokinetics of Leflunomide.

METHODS

A. Drug Source and Administration

LEF and LEF metabolite were generously attained as described in Chapter 1. The oral formulation of LEF was prepared by suspending 5 mg/mL of LEF in a 2% carboxymethylcellulose low viscosity solution (Sigma). Administration of oral LEF was via a gastric tube directly to the lumen of the stomach. The I.V. formulation of LEF was prepared by dissolving LEF metabolite in I.V. saline to a concentration of 5 mg/mL. I.V. administration was via the right marginal ear vein.

B. Animal Procedure and Blood Sampling

Ten male New Zealand white rabbits weighing 3.5 - 4.75 kg were divided into two groups. Group 1 was given LEF metabolite 5 mg /kg I.V. and group 2 was given LEF prodrug 5 mg/kg P.O. The rabbits were housed in individual cages with food restricted 12 h prior to and 4 h after drug administration. Blood samples were taken from the left marginal ear vein at 0 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h after administration into EDTA tubes. Both whole blood and plasma samples were collected and frozen at -70° C.

C. Analysis of Leflunomide Metabolite Levels in Plasma

LEF metabolite levels were measured in plasma using the reverse-phase HPLC procedure described in Chapter 1. Briefly, to 250 µL of the plasma samples and 100 µL of a 10 000 µg/L internal standard was added to 4.0 mL of 20.0 g/L potassium carbonate solution. This was followed by 10 mL of ethyl acetate extraction solvent. After vigorous shaking for 15 min, the solvent layer was transferred to a clean 13X100 mm borosilicate glass tube, where it was evaporated to dryness under a gentle stream of air at room temperature. The dried extract was reconstituted in 200 µL of an (8:2 v/v) acetonitrile:water solution. Chromatographic separation by reverse-phase HPLC was performed using a Hewlett Packard

1050 Series HPLC system. Chromatographic separation was performed isocratically on two Spherisorb-octyl C-8 columns joined in tandem heated to a stable temperature of 70 °C. The mobile phase consisted of acetonitrile:methanol:dd H_2O (37:20:43 v/v/v), and was degassed with helium daily before use.

D. Pharmacokinetic and Statistical Analysis

Plasma drug levels-time curves for both I.V. and P.O. routes of administration were fitted using PCNONLIN V 4.2 Model 200 (Statistical Consultants Inc., Lexinton; KY, USA.) to a noncompartamentalized open model. Terminal elimination half-life ($t_{\frac{1}{2}}$), AUC, AUMC and MRT were all calculated using PCNONLIN V 4.0. Additional calculations for volume of distribution at steady state (Vdss), and total body clearance (CL) were done outside PCNONLIN 4.2, using the equations:

$$CL = \frac{(\text{Dose } \mu g/kg)}{(\text{AUC } \mu g\ h/L)} \times \frac{1000\ mL/L}{60 min/h} = mL/min/kg \qquad (124)$$

$$Vdss = \frac{\text{Dose } \mu g/kg \times \text{AUMC } (\mu g/L\ h)\ h}{(\text{AUC } \mu g\ h/L)^2} = L/kg \qquad (125)$$

AUC was calculated using the trapezoidal rule and the extrapolated AUC from the last data point to time infinity was calculated by dividing the last experimental blood concentration value by the terminal slope. AUMC was calculated from the product of AUC and time. The differences between the pharmacokinetic parameters derived by the I.V. and P.O. routes of administration were analyzed using a paired t-test ($p < 0.05$ was considered significant).

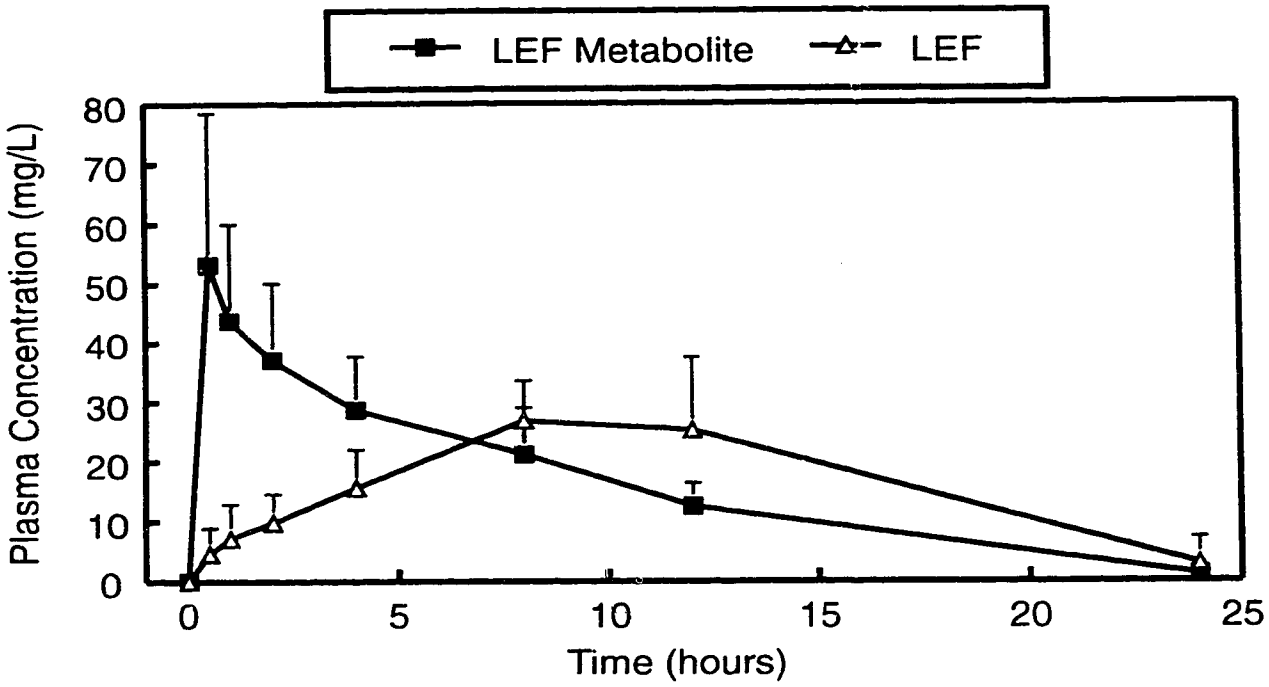
RESULTS

Single Dose Pharmacokinetics of Leflunomide Active Metabolite

The mean concentration versus time profiles for the oral dose of 5 mg/kg and intravenous dose of 5 mg/kg are shown in Fig. 6. Peak LEF metabolite levels are reached approximately 8 to 10 h post oral

Figure 6.

The Mean Plasma Concentration versus Time Profile of Leflunomide Metabolite After Administration of Leflunomide P.O. and Leflunomide Metabolite I.V.



dose. The AUC, $t_{\frac{1}{2}}$, CL, MRT, and Vdss as determined by PCNONLIN are summarized in Table 3. There was no significant difference in the AUC's between P.O. and I.V. routes of administration ($p > 0.05$), suggesting that there is 100% bioavailability of the LEF prodrug ($F = 1.0$). Consequently, clearance values for the oral route of administration can be represented as CL instead of CL/F. There was also no significant difference ($p>0.05$) for the $t_{\frac{1}{2}}$, and CL between the two routes of administration. The MRT and Vdss were significantly increased after P.O. administration as compared to I.V. administration.

DISCUSSION

To date, there is little data in the literature regarding the pharmacokinetics of LEF. This animal model was chosen since the size of the rabbit facilitates pharmacological and immunological studies without compromising the health of the rabbit. In addition, our laboratory has developed considerable experience in using this animal model in the pharmacokinetic evaluation of other immunosuppressive drugs (126,127). The dose of 5 mg/kg was chosen based on previous studies that demonstrated prolonged renal allograft survival in dogs with minimal toxicity (128). The absorption of LEF as monitored by LEF metabolite was relatively slow with peak levels being obtained in approximately 7 hr. It is presently unknown if the rate limiting step to the absorption of LEF is the diffusion of LEF metabolite or LEF prodrug across the gut wall. The MRT P.O. was significantly longer ($p < 0.05$) than MRT I.V. suggesting that P.O. administration of LEF may result in more prolonged immunosuppressive concentrations of the drug being achieved throughout the dosing interval. Since the immunosuppressive affect of leflunomide is dependent on its presence in the blood, increased MRT would equate to longer periods of immunosuppressive action. The low Vdss suggests low compartmentalization of LEF metabolite in cells and a high distribution in extracellular compartment. This is consistant with drug distribution studies that demonstrate a high plasma/whole blood ratio. Similar AUC between oral and intravenous routes of administration suggest that LEF is 100% bioavailable. The 100% bioavailability of LEF P.O., and an increased MRT of LEF P.O.as opposed to LEF metabolite I.V suggest that oral administration of LEF be the perferred method of administration of this immunosuppressant.

Table 3.

The Steady State Leflunomide Metabolite Pharmacokinetic Parameters (Mean ± SD), after Single Doses of Leflunomide Prodrug P.O. and Leflunomide Metabolite I.V.

LEF Prodrug (P.O.)

Rabbit	AUC (mg · h/l)	t½ (h)	CL (ml/min/kg)	MRT (h)	Vdss (l/kg)
1	368.49	3.37	0.226	10.29	0.14
2	307.92	1.55	0.271	7.74	0.13
3	403.09	4.09	0.207	11.20	0.14
4	772.38	7.71	0.108	14.48	0.11
5	253.90	2.69	0.328	8.98	0.18
Mean ± SD	421.16 ± 204.5	3.88 ± 2.3	0.228 ± 0.1	10.54* ± 2.4	0.14* ± 0.1
CV	48.5%	60.3%	36.5%	24.3%	21.4%

LEF Metabolite (I.V.)

Rabbit	AUC (mg · h/l)	t½ (h)	CL (ml/min/kg)	MRT (h)	Vdss (l/kg)
1	602.75	5.92	0.138	8.50	0.07
2	286.65	2.23	0.291	5.74	0.1
6	437.71	2.20	0.190	6.38	0.07
7	315.27	3.18	0.264	6.45	0.10
8	356.40	2.37	0.234	6.72	0.09
Mean ± SD	399.75 ± 126.9	3.18 ± 1.6	0.223 ± 0.1	6.76* ± 1.0	0.09* ± 0.1
CV	31.8%	49.7%	29.9%	15.4%	22.2%

* Significant differences between pharmacokinetic parameters from the two treatment regimes were assessed by a two tailed t-test ($p<0.05$).

Previous studies have examinined the pharmacokinetics of other immunosuppressive drugs in the rabbit model. The clearance of LEF metabolite was lower than that of CsA (129), RAPA (127), and MMF (126) in this model. LEF was found to have a relatively smaller Vdss (0.14 ± 0.1 L/kg) compared with other immunosuppressants such as CsA (3.16 ± 0.3 L/kg) (129), RAPA (2.84 ± 0.23 L/kg) (127), and MPA (4.75 ± 2.65 L/kg) (126). A relatively small Vdss is probably the major determinant of the short $t_{½}$ and inferred rapid disposition of LEF metabolite in this animal model compared to other immunosuppressants, such as CsA (129), and RAPA (127). On the other hand, LEF metabolite has a longer $t_{½}$ than MPA (126), which has a relatively larger CL value. This would suggest that the dosing interval for LEF would have to be more frequent than for CsA or RAPA, but less frequent than for MPA. The similar AUC from the P.O. and I.V. routes of administration suggest that LEF prodrug was 100% bioavailable, and hence the former route of administration would be preferred. If a similar situation is shown to exist in humans, this would have a significant advantage in clinical transplantation since oral as compared to I.V. administration would be more optimal. A therapeutic range for this drug has not yet been established; hence it has not known been determined if there is a correlation with immunosuppressive efficacy and toxicity. It is presently unknown if routine monitoring of LEF metabolite is required for dose adjustment in order to optimize immunosuppression and minimize drug induced side effects. However, the blood distribution and pharmacokinetics will facilitate refinement of the dosing regime once additional information regarding the mechanism(s) of action, and concentration /effect of the drug is investigated in animal and clinical studies.

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VI. Chapter 3: The Efficacy of Leflunomide to Reduce Xenoantibody *In Vivo* and an Evaluation Leflunomide's Ability to Prolong Discordant Xenograft Survival.

METHODS

A. Animal Procedures and Drug Administration

Cardiac xenotransplants were performed utilizing newborn domestic pigs (1.0 -1.5 kg) as the donors and male New Zealand white rabbits (3.2 - 4.0 kg) as recipients. Animals were housed separately under standard laboratory conditions.

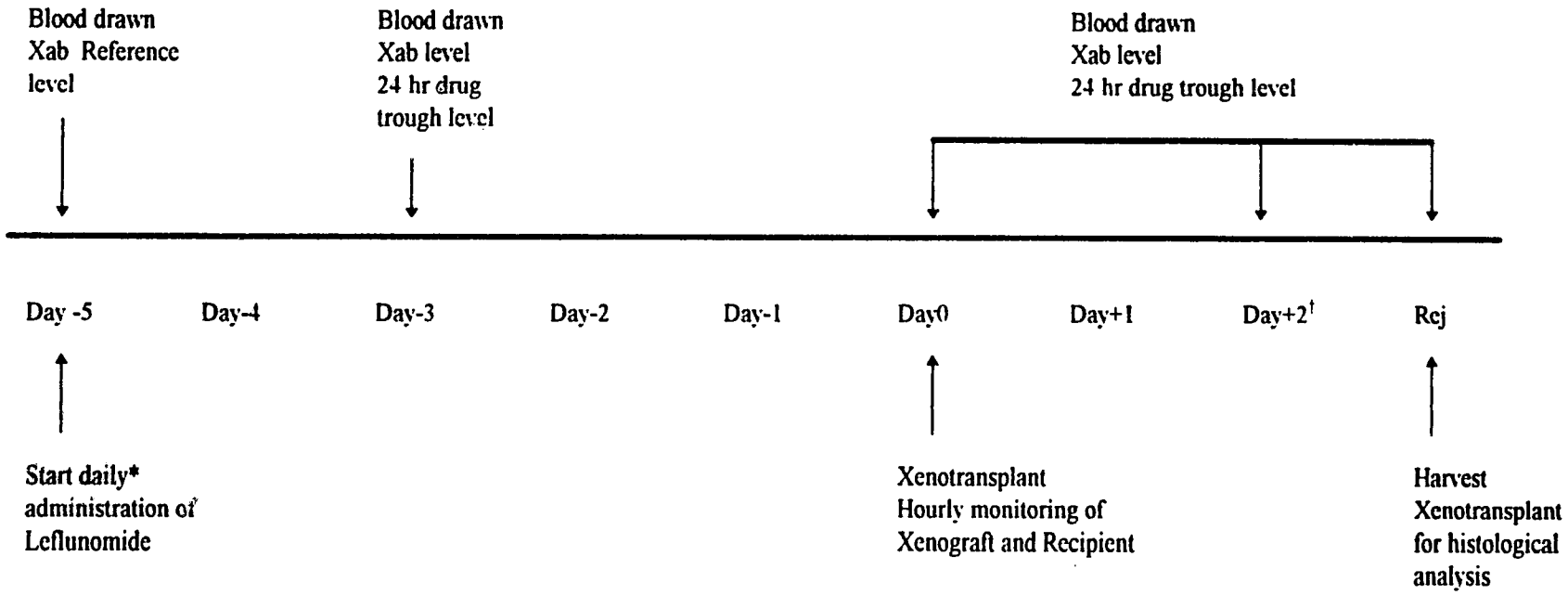
LEF prodrug and LEF metabolite were obtained as described in Chapter 1. The prodrug was suspended in a 2% (w/v) low viscosity carboxymethyl cellulose (Sigma, St. Louis, MO) solution, at a concentration of 5 mg/mL. The drug was given orally once a day at the same time point via a gastric feeding tube. The LEF metabolite was dissolved in 0.9% (w/v) intravenous saline (Baxter) to the concentrations of 5 mg/mL and 10 mg/mL, and aliquoted in a sterile fashion. The metabolite was administered I.V. via the marginal ear vein of the rabbit xenograft recipient once a day at the same time point.

B. Blood Sample Collection and Storage

Blood samples were collected from the rabbit recipients at prescribed times pre and post transplant (Fig. 7.). Whole blood samples collected into tubes containing EDTA as an anticoagulant on Day-5, Day -3, Day 0, every second day of xenograft survival, and at REJ for short term dosing regime. For chronic dosing, blood samples were collected into tubes containing EDTA on Day -21, Day -14, Day -7, Day 0, every second day of xenograft survival and at the time of rejection. Blood specimens were centrifuged at 2000 X g and the plasma was drawn off and stored at -70°C.

Figure 7.

Summary of the Xenotransplant Procedure.



*In one group, dosing commenced 21 days prior to surgery.
†Blood was drawn every second day post-transplant until rejection occured.

C. Analysis of Leflunomide Metabolite Levels in Plasma

Twenty four hour trough plasma LEF concentrations were measured on the days described above. Drug concentrations of the active metabolite of LEF were measured by reverse-phase HPLC using the method previously described in Chapter 1.

D. Xenoreactive Antibody Measurement

Plasma samples on each day of collection were analyzed for Xab using a PAEC ELISA (130). Both XIgG and XIgM were examined on different plates and compared to reference pretreatment Ig levels. Porcine aortic endothelial cells from Cell Systems Co (Kirkland,WA) were cultured in 96 well plates coated with 1% (w/v) BSA or Cell Systems attachment factor. Conformation of PAEC cell type was performed by Cell Systems. The process of confirmation of the PAEC involved two processes. The first is the determination of the presence of the acetyl LDL receptor by the ability of isolated cells to incorporate Di-I-Ac-LDL. The acetyl LDL receptor is found primarily on macrophages and endothelial cells . The second process is the determination of the presence of Factor VIII antigen on the surface of the cultured cells utilizing an immunofluorescent staining technique specific for Factor VIII antigen (131). PAEC were cultured to confluence and then fixed using a 0.1% (v/v) gluteraldehyde solution. Experiments were performed using an ELISA Starter Kit (Pierce Chemical, Richmond, VA). This involves washing the fixed PAEC three times with wash buffer consisting of 0.05% (v/v) Tween 20 in 0.14 M sodium chloride solution (pH=7.4). The PAEC were then incubated in blocking buffer consisting of 1% (w/v) BSA in 0.14 M sodium chloride solution (pH=7.4) at room temperature for 1 hour to reduce nonspecific binding by the primary and secondary antibodies. Test serum (rabbit serum) was serially diluted and incubated with the PAEC for 1 hour at room temperature. This was followed by three applications of the washing buffer, and incubation with either horseradish peroxidase coupled goat anti-rabbit IgG or goat anti-rabbit IgM (Southern Biotechnology, Birmingham, AL) for 1 hour at room temperature. The PAEC was then washed three times with wash buffer, followed by addition of horseradish peroxidase substrate One Step™ ABTS (2,2'-axino-bis-3-ethylbenxothiazdine sulphonate) (Pierce Chemical, Richmond, VA). After 30 minutes incubation at room temperature 1% (w/v) SDS was added to stop the reaction. The absorbancy was read

at 405 nm using a plate reader (Molecular Devices, Menlo Park, CA). Results are expressed as the percentage of absorbance of a 1:32 titer of rabbit serum with the pre-treatment xenoreactive antibody levels being assigned a value of 100%. This titer was chosen since it gave an optimal absorbance reading of one absorbance unit.

E. Surgical Procedure

The heterotopic cardiac xenotransplants were performed by a surgery fellow and assisted by a technician or graduate student following a previously described method (132). Briefly, to minimize the size discrepancy between anastomosing vessels, heterotopic cardiac xenotransplants were perfomed using large male New Zealand White rabbits as recipients (3.2 - 4.0 kg) and small newborn domestic pigs as donors (1.0 - 1.5 kg). The rabbit recipient was placed under general anesthesia using isofluorane gas (4% at 1.5 L O_2/min). The recipient was placed in a supine position and its neck was prepared for surgery and sterily draped. A 4 - 6 cm incision was made over the sternocleidomastoid muscle, and the external jugular vein and the common carotid artery were exposed and dissected free. 100 U/kg of heparin was given I.V. via the external jugular vein. The external jugular vein and the common carotid artery were clamped off using bull dog vascular clamps at the proximal ends of the vessels. The external jugular and the common carotid were transected at an angle distally. The common carotid was threaded through the lumen of a 1 cm segment of 12 gu Angiocath, and the external jugular was threaded through the lumen of a 1 cm segment of 14 gu Angiocath. Each vessel was then stretched back over its Angiocath segment, and secured with one tie of 3-0 silk at the distal end of the cuff and a 4-0 silk tie at the proximal end of the cuff.

The pig donor was then placed in a supine position under halothane gas and the heart was exposed via a midsternotomy. Heparin at 100 U/kg was administered intravenously. The vena cava and the ascending aorta were cross clamped and cold cardioplegia solution (5-10 mL) was injected into the root of the ascending aorta proximal to the cross clamp. Once the heart ceased to beat, the inferior vena cava, superior vena cava, ascending aorta, pulmonary arteries, and pulmonary veins were transected and the heart removed and placed in a saline slush. The pulmonary veins, inferior vena cava and the superior

vena cava were "gang tied" following perfusion of the heart with 20 mL of cold cardioplegia solution. The xenograft was attached to the rabbit's circulation by anastomosing the rabbit's common carotid to the xenograft's ascending aorta, and the rabbit's external jugular vein to the xenograft's pulmonary artery. Patency of the anastomoses was achieved by using a 4-0 silk tie positioned in between the two ties used to secure the cuff onto the rabbit's vessels. Once the xenograft was in place, the graft was de-aired using a 25 gu needle and a 10 mL syringe. Upon reperfusion of the xenograft's coronary system, the heart contractility was established and the skin was closed.

F. Post - surgical Monitoring

Animal care procedures were performed in accordance with protocols established by the Canadian Council for Animal Care and approved by the University of Alberta Animal Ethics Committee. Post-surgical care included monitoring both the rabbit recipients for post-surgical complications, and monitoring their cardiac xenografts for their strength of contraction and heart rate. The animals were examined every 30 minutes for the first 1.5 hours, and on the hour thereafter. Hourly monitoring continued until graft rejection or the recipient was sacrificed due to complications. A xenograft was considered rejected if it ceased to beat. At this time, a blood sample was drawn for measurement of xenoantibody titer, and the recipient sacrificed to allow for xenograft removal for histological examination. Total xenograft survival time was calculated from time of perfusion and steady heart rate until the estimated time point of xenograft rejection.

G. Histopathology

The rejected cardiac xenograft was removed from the recipient and fixed in formalin. The formalin fixed samples were embedded in paraffin, sectioned and stained with hematoxylin-eosin for examination by conventional light microscopy. Untransplanted newborn pig hearts were collected and used as controls to distinguish any morphological changes due to the surgical procedure.

H. Statistical Analysis

Student t-test was used for statistical evaluation of graft survival data and xenoantibody concentration changes. P values less than 0.05 ($p < 0.05$) were considered significant. A Pearson's correlation coefficient was used to determine the association between plasma LEF level, survival time, and XIgG and XIgM concentrations.

RESULTS

A. Xenograft Survival and Leflunomide Drug Levels

Monotherapy of LEF could not significantly ($p > 0.05$) prolong xenograft survival as compared to control animals. However, a greater percentage of animals survived longer in LEF treated animals with the exception of 5 day pretransplant administration of LEF 5 mg/kg/d P.O. (Table 4.). The mean plasma LEF metabolite trough concentrations for animals administered LEF or LEF metabolite at a dose of 5 mg/kg/d was 10.3 ± 6.5 mg/L. Animals receiving LEF showed no toxic side effects from drug administration, and no animals included in this study died before xenograft rejection had occurred. In addition, no significant relationship ($p > 0.05$) was found between LEF metabolite plasma trough levels and survival times, as determined by a Pearson's correlation coefficient.

B. Xenoantibody Concentration

Chronic dosing of LEF at 5 mg/kg/d P.O. for 21 days was able to produce a significant decrease ($p < 0.05$) in XIgG (a reduction of $40.5 \pm 25.8\%$) and XIgM concentrations (a reduction of $29.1 \pm 7.3\%$) from pretreatment levels (Fig. 8.). This level of reduction of Xab was unable to produce a prolongation of xenograft survival time (Table 4.). Comparison of inter-recipient Xab concentrations at time of rejection showed no significant difference (Fig. 9.). Mean survival times, mean XIgM concentrations and mean XIgG concentrations are represented in Table 4. No significant correlation was found between treatment regime and XIgG and XIgM concentrations, nor Xab level and survival time, as determined by a Pearson's correlation coefficient. No adverse affects were observed over the 5 or 21 day pretransplant

Table 4.

Comparison of Survival Time and Xenoantibody Concentration for Various Leflunomide Dosing Groups.

Group	n	Mean Survival Time ± SD	% Survival >12 h	Mean of REF† XIgM ± SD	Mean of REF† XIgG ± SD
Controls	8	18.79 ± 21.13 h	37.5	81.8 ± 10.0%	79.2 ± 12.5%
LEF I.V. 5 mg/kg/d	6	27.66 ± 18.40 h	66.7	86.5 ± 17.1%	96.0 ± 24.9%
LEF I.V. 10 mg/kg/d	4	21.43 ± 20.57 h	50.0	86.4 ± 19.3%	73.1 ± 9.6%
LEF P.O. 5 mg/kg/d	6	17.85 ± 18.44 h	33.3	71.2 ± 14.4%	78.6 ± 22.5
LEF P.O.* 5 mg/kg/d	3	15.64 ± 14.20 h	66.7	70.9 ± 7.3%	59.5 ± 25.8%

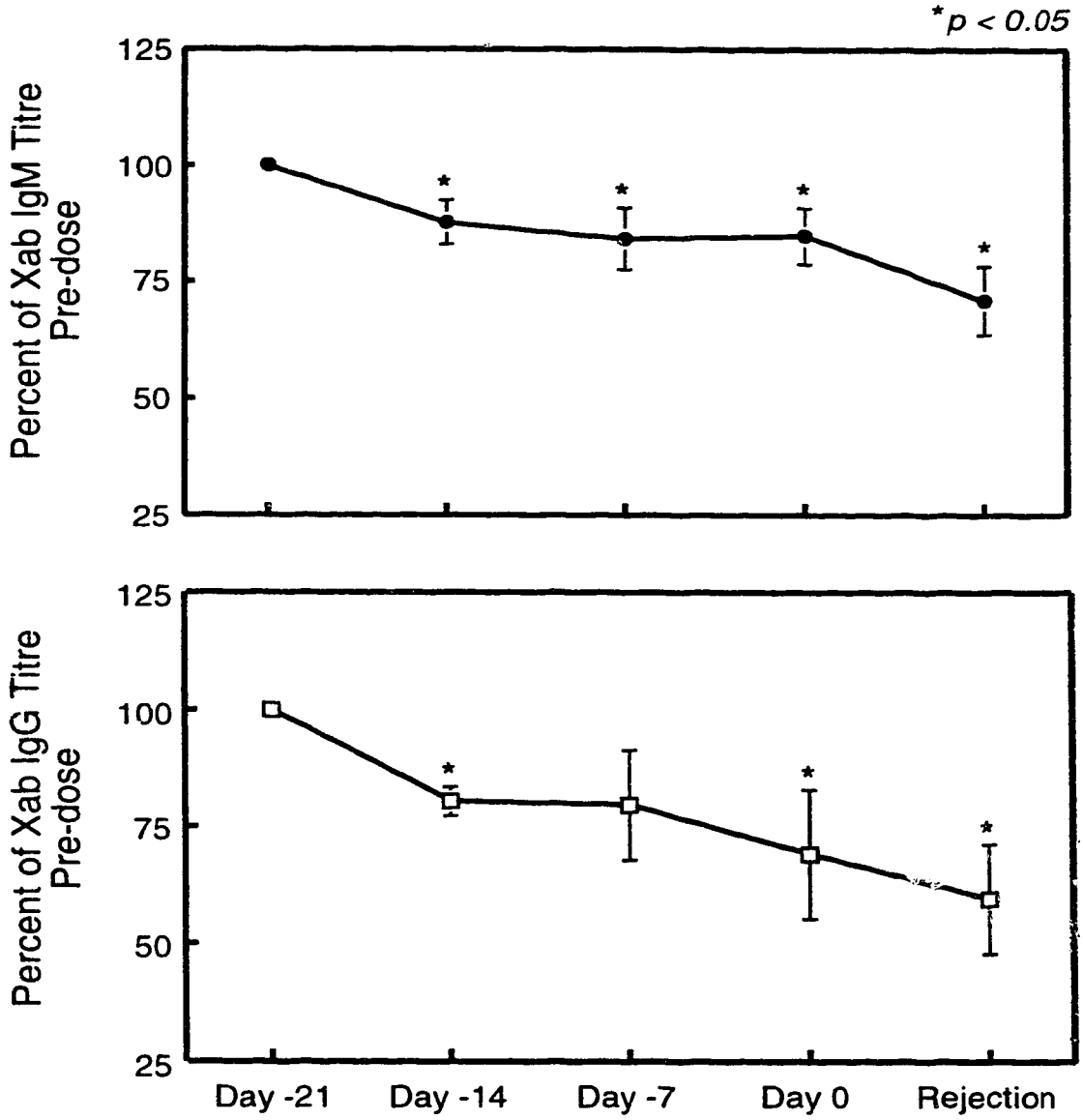
* Dosing commenced 21 days prior to surgery.

† From time of initiation of dose to time of rejection.

Comparing survival times and xenoantibody concentrations demonstrated that chronic dosing of LEF at 5 mg/kg/d for 21 days could not produce a statistically significant drop in Xab over controls. In addition, these reductions in Xab concentrations did not correlate with increased survival times, as determined by a Pearson's correlation coefficient.

Figure 8.

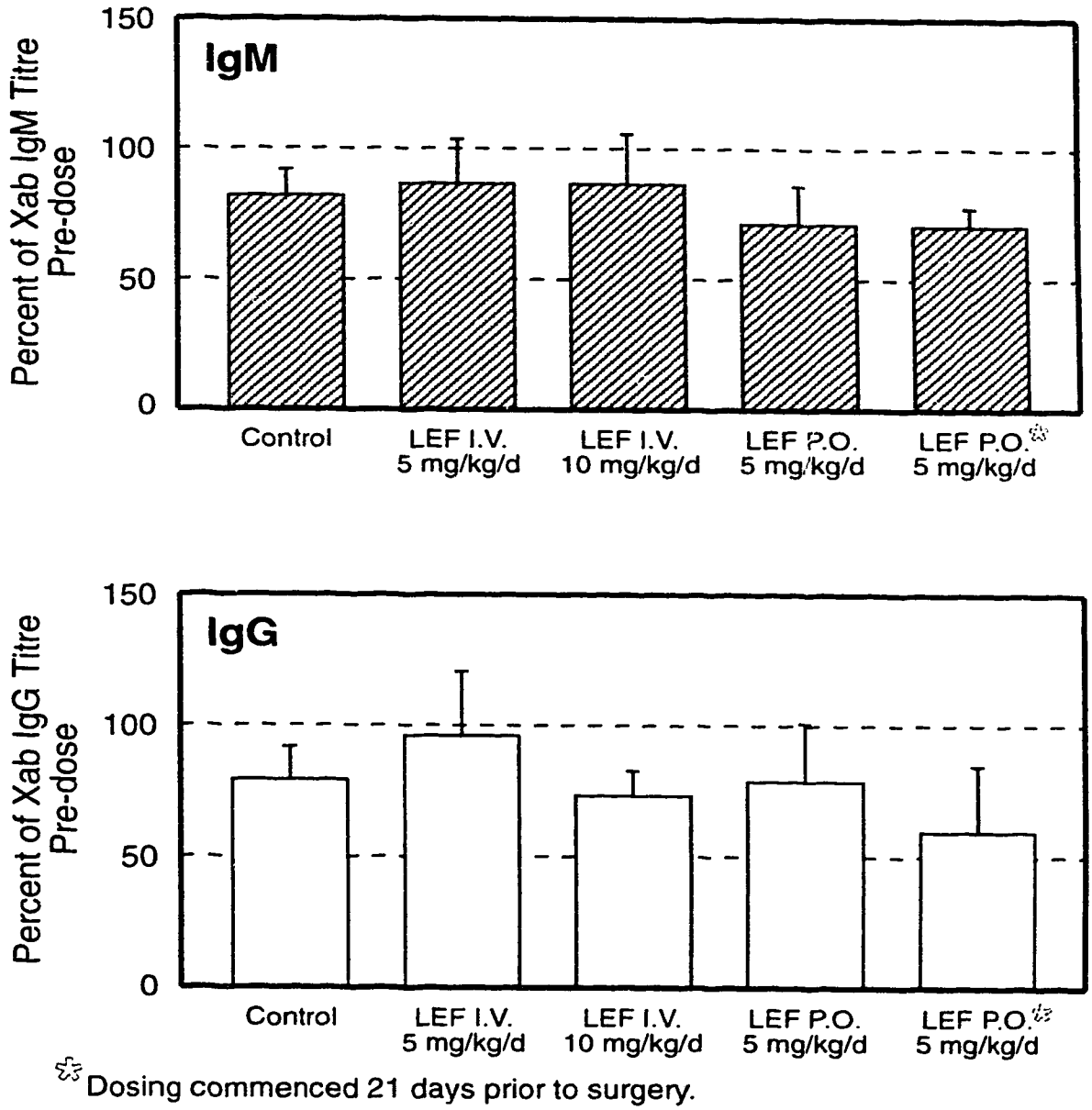
Xenoreactive IgG and Xenoreactive IgM Concentrations over 21 Days of Dosing of Leflunomide.



Comparison of xenoreactive IgG (XIgG) (1a.) and IgM (XIgM) (1b.) concentrations over 21 days dosing of LEF at 5 mg/kg/d: Chronic daily administration of LEF was able to produce a significant drop in Xab concentrations ($p<0.05$) over 21 days. Fig. 1a. demonstrates a drop to 64.4 ± 26.1% of preadministration XIgG concentration at the time of transplant and a drop to 59.5 ± 25.8% at the time of rejection. Fig 1b. demonstrates a drop to 84.3 ± 12.7% of preadministration XIgM concentration at the time of transplant and a drop to 70.9 ± 7.3% at the time of rejection. Xab concentrations were measured using a PAEC ELISA. The data is represented as mean ± SD, n=3.

Figure 9.

Comparison of Xenoreactive IgG and Xenoreactive IgM at the Time of Rejection.



Comparison of xenoreactive IgG (XIgG) (2a.) and IgM (XIgM) (2b.) concentrations at the time of rejection: Administration of LEF in any treatment regime did not produce a decrease in XIgG (2a.) nor in XIgM (2b.) that reached significance ($p>0.05$), as compared to control animals at the time of rejection. Xab concentrations were measured using a PAEC ELISA. The data is represented as mean ± SD. dosing regime of 5 mg/kg/d LEF or the 5 day pretransplant dosing regime of 10 mg/kg/d LEF, regardless of the route of administration.

D. Histopathological Analysis

Examination of formalin fixed sections of rejected xenograft biopsies demonstrated microvascular thrombosis, edema and hemorrhage in all rejected tissues. In 13/27 cases, focal infiltrates without the presence of necrosis were found. Of those specimens with infiltrates, eosinophils were a consistent finding which suggests the presence of antibody-antigen complexes present in the xenograft. All of these demonstrated endothelial swelling, but no vasculitis was found. No such observations were seen with histological control tissues (untransplanted newborn pig hearts) (Fig. 10.).

DISCUSSION

The efficacy of LEF to reduce Xab concentrations and result in prolongation of discordant cardiac xenografts utilizing newborn pig donors and New Zealand rabbit recipients were evaluated in this study. The results show that LEF alone cannot significantly prolong discordant xenograft survival as compared to untreated control animals. Histological examination of the cardiac xenografts revealed classical signs of humoral rejection, namely microvascular thrombosis, edema, and hemorrhage (133,134). Very few foci of infiltrating lymphocytes were found in all examined tissues. LEF was unable to significantly increase xenograft survival time with increasing dosage (5 mg/kg/d to 10 mg/kg/d), or increasing the number of days of pretreatment prior to xenotransplantation (5 to 21 days). However, increasing the number of days of administration of LEF prior to surgery from 5 days to 10 days leads to a significant reduction of XIgG and XIgM levels to 59.5 ± 25.8%, and 70.9 ± 7.3% of pretreatment levels, without inducing common side effects associated with LEF ($p < 0.05$). This reduction of Xab was unable to prolong xenograft survival, and it did not provide a significant nor strong correlation with increasing survival time of the xenograft. Thus far only two other major studies that utilize pharmacological means alone to deplete Xab have been conducted (132,135). These two studies have also found that pharmacological reduction of Xab has not resulted in sustained discordant xenograft survival. Previous

Figure 10.

The Morphology of rejected xenograft biopsies.

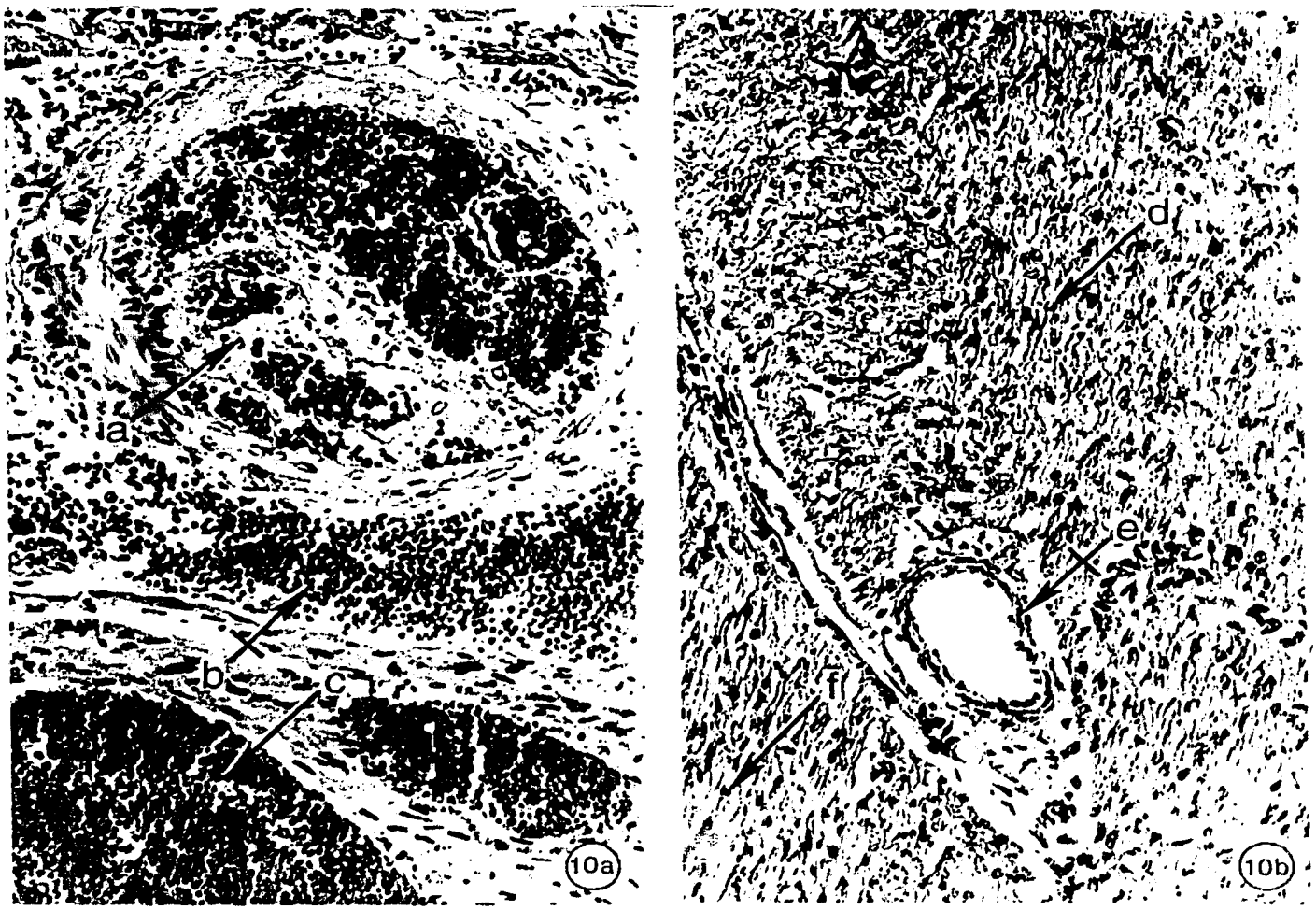


Fig. 10a. is a representative section of the rejected xenograft biopsies. These slides demonstrated fibrin deposition (a), hemorrhage (b), microvascular thrombosis (c), and edema. Few cellular infiltrates were discovered. Fig. 10b. is a representative section of histological control tissue. These slides showed no remarkable change in morphology (d-f). Thus any change in morphology may be attributed to the rejection of the xenograft and not to the surgical procedure.

studies in this laboratory have shown that a RAPA/MMF dosing combination (n=5, 24.3 ± 11.9 h) was able to significantly prolong ($p < 0.05$) discordant cardiac xenografts over a control group (n= 11, 7.98 ±13.85 h). No significant difference ($p > 0.05$) was found in xenoantibody levels, although a trend to decreased concentrations was noted (132). The leflunomide study has demonstrated that LEF could produce similar mean survival times as the RAPA/MMF combination. However, the control animals in this study demonstrated longer survival times. In a second study, the efficacy of cyclophosphamide and DSG were tested in a discordant guinea pig to rat model. Both cyclophosphamide and DSG were able to decrease xenoreactive antibody levels when in combination with splenectomy. Control animals had xenoreactive antibody titres of 1:256 to 1:1024. In contrast cyclophosphomide and DSG treated recipients had lower xenoreactive antibody titres of 1:32 to 1:64. The survival times of splenectomized rats administered either cyclophosphamide or DSG did significantly prolong graft survival ($P= 0.001$ and $P = 0.015$ respectively). Control animals had a mean xenograft survival time of 20.1±8.2 min (n=8). Splenectomized rats administered cyclophosphamide at 50 mg/kg I.P. had a mean xenograft survival time of 37.8 ± 6.5 min (n=5). Splenectomized rats administered DSG at 5 mg/kg I.M. had a mean xenograft survival time of 36 ± 11.58 min (n=5). Histological examination of the rejected xenografts revealed focal IgM and more wide spread C3 deposition on capillary endothelium. Interstitial deposits of IgG were also revealed (135).

A key element to the survival of discordant xenografts is the depletion of Xab prior to transplantation and the maintenance of low Xab titer thereafter. It is becoming increasingly clear that pharmacological means of depleting Xab requires administration of drugs that reduce antibody concentrations, such as LEF, well in advance of transplantation (> 21 days). Current experience with immunoabsorption in conjunction with conventional immunosuppression has demonstrated longer prolongation of xenografts (10-22 days) (136-138). Unfortunately, the use of conventional immunosuppressants was still unable to maintain low levels of Xab, and reuse of the immunoabsorptive column was required periodically. Currently, the threshold to which Xab concentrations must be reduced at the time of transplantation is still unknown. In future, combining of an immunoabsorptive column and

novel immunosuppressants which inhibit T and B cell activation, as well as a longer time pretreatment with immunosuppressive agents, will need to be evaluated so that there is a balance between humoral and cellular immunosuppression, toxicity, and immunodeficiency.

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VI. Conclusions

Current investigations into immunosuppression and xenotransplantation have yielded some limited success in realizing clinical xenotransplantation. However, hyperacute rejection still remains a formidable barrier. Hyperacute rejection is a prevelant feature in discordant xenotransplant models. This potent rejection process is characterized by thrombosis, interstitial edema, and hemorrhage. Hyperacute rejection is initiated by xenoreactive antibodies binding to the endothelial cells of the xenograft. This in turn leads to antibody dependent complement fixation, and destruction of the barrier properties of the endothelium of the xenograft. A second mechanism by which xenoreactive antibody may result in damage to the graft is through the activation of endothelial cells. Endothelial cell activation is a process by which endothelial cells undergo a series of metabolic structural changes that promote platelet aggregation, fibrin generation, neutrophil adhesion, and permeability of the endothelial monolayer to plasma proteins and blood cells. Xenoreactive antibody plays a prominant role in the hyperacute rejection of xenografts. Many approaches are taken to deplete xenoantibody from the serum of the recipient. The use of columns and other similar techniques to deplete xenoreactive antibody provides only temporary depletion of xenoantibody, thus necessitating repeated use of the column. An alternative to depelete Xab would be to use immunosuppressive agents that suppress antibody synthesis. LEF is both a T cell and B cell suppressant that has been reported to inhibit both allogeneic and concordant xenogeneic antibody synthesis. This study investigates the use and efficacy of LEF in a discordant cardiac xenograft model.

The first step in this study was to develop a method of analysis for LEF active metabolite in tissue specimens. This would prove to be valuable since little was known about LEF's blood distribution, pharmacokinetics and its therapeutic index in the rabbit model. As a result, a reverse-phase HPLC technique was developed to detect LEF active metabolite in blood and other tissues. The assay was relatively rapid taking less than 15 min per sample, as compared to other HPLC techniqes for CsA, and RAPA which have running times of greater than 30 min. The sensitivity of the assay was 400 µg/L when

a 250 µl specimen was used, and 40 µg/L when a 1.0 mL specimen volume was used. The assay showed good analytical recovery, linearity, and precision for an HPLC method.

Studies into the blood distribution of LEF demonstrated that the LEF active metobolite was mainly distributed in the plasma, as indicated by plasma/whole blood ratios of 0.93 to 1.12. LEF active metabolite showed no temperature nor concentration dependence in its distribution. No detectable amounts of LEF were found in the free fraction indicating that the majority of LEF active metabolite was protein bound. However this binding was saturable, at concentrations of 100 mg/L or greater. The active metabolite was found to be mainly associated (>95%) with the non-lipoprotein fraction of plasma with less than 5% bound to all lipoprotein classes. The fact that little of the active metabolite is found in the free fraction with the majority bound to non-lipoprotein components of plasma suggests that LEF may be taken up by the cell via active transport mechanisms. In other words, it probably does not diffuse across the membrane. This may also explain its reported low toxicity as these transport mechanisms may be only located on a few cell types.

The pharmacokinetics of LEF demonstrated that the prodrug given orally had 100% bioavailability (F=1.0), as demonstrated by similar AUC between oral administration of prodrug and intravenous administration of LEF active metabolite. Orally dosing the prodrug also demonstrated that LEF had a significantly ($p < 0.05$) longer MRT than intravenous injection of LEF active metabolite. Thus, oral administration may be optimal since an increase in the presence of immunosuppressive concentrations would equate to longer periods of immunosuppressive action. In comparison to other immunosuppressants, LEF has a low Vdss both with oral administration of LEF and intravenous administration of LEF active metabolite. This suggests a low distribution of LEF active metabolite to the blood which is in agreement with the high plasma/whole blood ratio found in the blood distribution studies.

Administration of LEF was shown to significantly ($p < 0.05$) reduce both IgG and IgM Xab levels over the 21 day oral dosing period. A reduction of XIgG and XIgM of 40.5 ± 25.8% and 29.1 ± 7.3% respectively from pretreatment levels was demonstrated with 21 day pre-surgery administration of LEF. No other dosing regime was able to significantly reduce Xab levels. However, this reduction in Xab

was not able to significantly ($p > 0.05$) prolong xenograft survival as compared to untreated control animals, nor was there a strong correlation between drug levels and xenograft survival time. In addition, no correlation was found between Xab level and survival time suggesting that the reduction in XIgG and XIgM produced by LEF in this study was not sufficient, and that lower Xab levels may be needed to prolong xenograft survival. Hyperacute rejection was confirmed in all xenograft specimens and was characterized by microvascular thrombosis, edema, and hemorrhage, all classical signs of humoral rejection. Previous studies that use immunosuppression to prolong xenograft survival have also met with limited success. The combination of RAPA/MMF was found to be able to prolong xenograft survival as compared to controls with a modest reduction in Xab of $20 \pm 20.8\%$ that was not found to be significant ($p>0.05$). It should be noted that LEF was able to provide greater reductions in Xab levels with similar survival times. However, the survival time of the untreated control animals in each study was quite different (RAPA/MMF study 7.98 ± 13.85 h versus LEF study 18.79 ± 21.13 h). DSG has also been able to reduce Xab as demonstrated by lower Xab titers in treated animals (1:32 to 1:64) as opposed to control animals (1:256 to 1:1024). However, it could not prevent hyperacute rejection. Histopathology revealed focal IgM deposits and wide spread C3 deposition.

Several studies have demonstrated that Xab plays a key role in discordant xenograft hyperacute rejection. Thus, the first step on the road to clinical xenotransplanation must deal with the reduction of Xab levels. This may be achieved with the use of immunoabsorbant columns and/or the use of immunosuppressant drugs. The attraction of the use of immunosuppressive agents to deplete antibody is that these agents may be administered outside of a hospital setting with less trauma and the inconvenience of repeated use of an immunoabsorbant column. Unfortunately, many of todays immunosuppressant agents are unable to reduce antibody levels sufficently to prevent hyperacute rejection on their own. Perhaps the best combination would be the use of an immunoabsorbant column with an immunosuppressant that selectively targets antibody production of B cells. LEF has proven to be a B cell immunosuppressant that has demonstrated a large therapeutic index with quite specific action on lymphocytes. Thus, it seems that LEF would be an ideal immunosuppressant to combine with use of an immunoabsorbant column.