DRUG METABOLISM AND TRANSPORT DURING REJECTION OF TRANSPLANTED LIVER

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Organ transplantation is an accepted therapy for diseases that result in chronic irreversible failure of various organs. During transplantation, the transplanted organ is subjected to two inflammatory processes, alloantigen-independent (ischemia/reperfusion injury) and alloantigen-dependent (rejection), both of which involve release of cytokines.

The objectives of this dissertation were to evaluate the effect of acute rejection of liver in rats on hepatic and extra-hepatic drug metabolizing capacity for phase I and II enzymes, to evaluate the effect of chronic rejection of liver in humans on hepatic metabolizing capacity of phase I and II enzymes, to evaluate the effect of acute and chronic rejection of liver on hepatic and extra-hepatic protein and mRNA expression of P-glycoprotein and to evaluate the effect of different cytokines on the constitutive and inducible hepatic CYP3A4 activity and protein expression in human hepatocytes.

Alloantigen-independent inflammation and altered blood flow caused a reduction in hepatic mRNA of different phase I and II enzymes. However, this reduction did not significantly alter protein levels of all CYP450 enzymes studied. Occurrence of rejection resulted in further

reduction in mRNA, protein levels and activity of all CYP450s studied. Syngeneic and allogeneic transplantation caused reduction in the metabolic capacity of extra-hepatic tissues and increased expression of P-gp in the liver. Chronic rejection of the liver in humans selectively altered the activity and protein expression of different phase I and II enzymes and increased P-gp protein expression. In human hepatocyte cultures, IL-1β, IL-6 and TNF-α decreased the activity and protein expression of both constitutive and induced forms of CYP3A4 enzyme. Pre-exposing or co-exposing the hepatocytes to cytokines reduced the ability of rifampicin to induce CYP3A4.

In conclusion, acute and chronic rejection of liver significantly altered the expression and activity of several drug metabolizing enzymes and transporters. The magnitude of these alterations was higher in acute rejection. Acute rejection also caused alterations in the metabolizing ability and transporters expression in renal and pulmonary tissues. Cytokines play a major role in modulating the activity of drug metabolizing enzymes and transporters and may contribute to the large inter-individual variation in the pharmacokinetics of drugs in transplant patients.

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Background:

Organ Transplantation:

Organ transplantation is an accepted therapy for diseases that result in chronic irreversible failure of organs such as liver, kidney, intestine, heart and lung. Organ transplant patients often receive multiple drug therapy that includes immunosuppressive drugs, antibiotics, antiviral agents, antifungal agents, antihypertensive agents and others. We have observed large variations in the pharmacokinetics of several drugs used in transplant patients. Following transplantation, patients undergo marked changes in their physiology, that may cause changes in absorption (due to changes in GI motility, altered splanchnic blood flow, change in bile composition and flow, changes in acid or enzymes secretion and presence of concomitant drugs), distribution (due to changes in organ blood flow, body fluid content, tissue partitioning and plasma protein binding) or elimination of drugs (due to changes in the intrinsic activity of the enzymes in the eliminating organs, the blood flow to the organ and the blood protein binding of the drug) (Venkataramanan *et al.* 1989).

Of the different organs that have been successfully transplanted, liver and kidney are directly involved in the elimination of most drugs. Liver is the primary site of metabolism of several drugs used by transplant patients. Liver transplantation was first carried out by Thomas E. Starzl in 1963 (Thalheimer and Capra 2002). The survival of patients following liver transplantation has improved over the past 10 years as a consequence of improved medical management and the introduction of newer immunosuppressive therapies. Presently, orthotopic liver transplantation is considered as a routine procedure. In the year 2000, 4934 liver transplantations were carried out in the United States alone (according to statistics by United Network for Organ Sharing, UNOS)

(http://www.unos.org). However, organ availability and rejection of liver are the two major factors that influence the number of patients who can be successfully saved by this procedure. The impact of organ shortage can be partially solved by some alternatives such as split-liver transplantation, living donor liver transplantation or hepatocyte transplantation. However, damage to the transplanted organ due to ischemia, reperfusion injury and rejection continues to be the major causes of graft dysfunction or loss following liver transplantation.

Ischemia and reperfusion injury:

In the early phase of reperfusion injury, cell death by necrosis is observed. In the intermediate phase, which can occur up to 6 hours after the reperfusion, Kupffer cells are activated and these activated cells produce different cytokines (including interleukin-1 (IL-1), IL-6, IL-10 and tumor necrosis factors-α (TNF-α)), reactive oxygen intermediates (ROIs) such as superoxide radical, hydrogen peroxide, hydroxyl radical, etc. and many other mediators that magnify the effect of the injury. In the late phase of injury there is infiltration of neutrophils into the liver due to the release of chemoattractants by the activated Kupffer cells. This leads to the release of other toxic mediators (Lichtman and Lemasters 1999). Despite the involvement of cytokines here, ROIs and subsequent lipid peroxidation are shown to be the important contributors to the ischemia and reperfusion injury.

Rejection of liver:

Recognition of the alloantigen by the host immune system leads to the rejection of the transplanted organ. Rejection of the liver graft can be classified as hyperacute, acute or chronic, based on the histopathology and the time of occurrence.

Hyperacute liver rejection can occur within minutes to days after transplantation due to the presence of preformed antibodies against the donors. These antibodies are directed against human lymphocyte antigen (HLA) molecules or blood group antigen. Hyperacute rejection can lead to necrosis of the transplanted organ. This type of rejection is rare in liver transplant patients. It can be avoided by cross matching the patients and by screening them for any antibodies that are specific to the donor (Bumgardner and Orosz 1999).

Acute liver rejection usually occurs within days to weeks after transplantation. Acute liver rejection is an immune cell-mediated pathological inflammatory response which is located within the allograft. It is characterized mainly histologically by the infiltration of the graft with lymphocytes, polymorphonuclear leukocytes and eosinophils. It is also characterized by the presence of damage to the bile duct. Endothelialitis of the blood veins and arteries may also be present. This is the most common type of rejection in liver transplant patients. Acute liver rejection is reversible upon achieving optimal immunosuppressive therapy in the transplant patient (Bumgardner and Orosz 1999).

Chronic liver rejection usually occurs slowly over months or years after transplantation. Histologically, it is characterized by the loss of the bile ducts (and hence the name, ductopenic rejection or vanished bile duct syndrome). It may be also characterized by interstitial fibrosis, hepatocellular ballooning, and dropout, and finally obliterative endarteritis. There is some evidence of the involvement of alloantibodies in chronic rejection, but the exact role of these alloantibodies in chronic rejection is not completely understood. Currently, there is neither a test

to predict the development of chronic rejection nor a drug to reverse this condition (Bumgardner and Orosz 1999).

Since rejection involves the release of different immunological and inflammatory mediators, it is important to understand the profile of the mediators that are released after transplantation and during the subsequent process of rejection.

Organ transplantation and immunological mediators:

In liver transplant patients, the liver graft is infiltrated with TNF-α positive monocytes and the plasma concentration of both TNF-α and IL-1β have been shown to increase 24-48 hrs before the rejection episode and continue to be elevated during the course of rejection (Imagawa *et al.* 1990; Tilg *et al.* 1990; Hoffmann *et al.* 1993). Hepatocytes express receptors for cytokines, growth factors and prostaglandins. The presence of these receptors makes the hepatocytes target for many of the circulating cytokines such as IL-1β, IL-6 and TNF-α that are released following inflammation. These cytokines are potent in triggering acute-phase protein synthesis and hence are known as acute-phase cytokines (Ramadori and Christ 1999).

IL-2 and IL-6 are good indicators of the occurrence of rejection in renal transplant patients (Kutukculer *et al.* 1995). The mRNA expression of IL-1β, IL-6 and TNF-α has been detected in samples from acutely rejecting renal tissues. However, the mRNA expression of IL-2 was not present in all cases (Krams *et al.* 1992). In heart transplant patients, mRNA expression of IL-1β, IL-2 and IL-6 was detected in the graft with the occurrence of even minimal evidence of rejection (Wu *et al.* 1992).

Although many groups have studied the effect of inflammation and infection, there is a tendency in the literature to regard them as one group, whereas inflammatory diseases have different plasma cytokines profiles than viral infections, bacterial sepsis or parasitic infections (Morgan 1997). It follows that different models of infection or inflammation will affect different subsets of enzymes in experimental animals (Morgan 1997). A thorough understanding of the effect of rejection on drug metabolism and transport is important in optimizing drug therapy in transplant patients. Before reviewing the effect of immune modulation and inflammation on hepatic drug metabolism, the pathways of drug metabolism will first be reviewed.

Drug Metabolism:

The blood/plasma concentration of a drug is determined by the process of absorption, distribution, metabolism and excretion. Drug metabolism is the process by which a drug is chemically converted to metabolites by various enzyme systems in the body. Drug metabolism is influenced by blood flow (Q), unbound fraction of the drug in blood (fu) and the intrinsic clearance of the drug in the metabolizing organ (Cl_{int}). In the eliminating organ, changes in blood flow will alter the clearance of drugs with a high extraction ratio, but will not alter the clearance of drugs with a low extraction ratio. Changes in fu or Cl_{int} will change the clearance of low clearance drugs, but will not affect the clearance of high clearance drugs. Changes in Cl_{int} are normally due to changes in the expression and activity of various drug metabolizing enzymes (Rodighiero 1999).

Drug metabolism pathways can be broadly classified into phase I and II pathways. Phase I reactions include oxidation, reduction, hydrolysis, etc. In most cases, the products of Phase I reactions are pharmacologically inactive. However, some of the metabolites produced may be active. For example, some drugs are given as prodrugs which must be converted to an active form once inside the body. In other situations, the metabolites are reactive with cytotoxic properties (e.g. the metabolites of dihydralazine act as hapten which activate the immune system and cause autoimmune hepatic injury) (Bourdi *et al.* 1994). Phase II reactions are the true detoxification pathways and result in a product that is more polar and readily excreted from the body. This category includes reactions such as glucuronidation, sulfation, acetylation, etc.

Phase I pathways:

Cytochrome P450: Cytochrome P450 is the most important phase I enzyme system that has been studied so far (Table 1) (Stegeman and Livingstone 1998). It metabolizes a variety of endogenous compounds (e.g. steroids, bile acids, prostaglandins), environmental chemicals, herbal components as well as drugs (Coon *et al.* 1992; Gonzalez and Lee 1996; Glue and Clement 1999). The term cytochrome P450 refers to a superfamily of heme-containing enzymes located on the membrane of the endoplasmic reticulum of the cell (Slaughter and Edwards 1995). In mammals, there are 17 distinct P450 gene families which encode for about 50-60 P450 genes in any species. The cytochrome P450 dependent metabolism of drugs is mainly mediated by enzymes of the CYP1, CYP2, CYP3, and CYP4 families (Waxman 1999). Among these enzymes, CYP3A is the most abundant isoform in human livers. It constitutes about 30% of the cytochrome P450 in human liver followed by CYP2C, CYP1A2, CYP2E1, CYP2A6 and

CYP2D6, which represent 20%, 13%, 7%, 4% and 2%, respectively (Shimada *et al.* 1994; Eagling *et al.* 1998; Glue and Clement 1999). CYP3A4 metabolizes about 53% of the commonly prescribed drugs, followed by CYP2D6, CYP2C, CYP1A2 and CYP2E1/2A6 accounting for 25%, 18%, 3% and 1%, respectively (Bertz and Granneman 1997). In rat livers, CYP2C11 constitutes about 54% of the cytochrome P450 in the liver followed by 17% and 2% of 3A2 and 1A2, respectively (Eagling *et al.* 1998)

CYP1A subfamily:

CYP1A1, an inducible CYP450 enzyme, is important for conversion of carcinogenic polycylic aromatic hydrocarbons (PAH) to epoxides, which can interact with DNA and cause carcinogenesis. It is located predominantly in extra-hepatic tissues such as lungs in humans. CYP1A2 is an inducible CYP which is closely related to CYP1A1. CYP1A2 is expressed in human and constitutes about 13% of total human liver cytochrome P450. CYP1A2 metabolizes many substrates including caffeine, phenacetin, etc. (Table 2) (Shimada *et al.* 1994; Venkatakrishnan et al. 2001). In rats, CYP1A2 constitutes 2% of the total CYP450 enzyme in the liver.

CYP2C subfamily:

In humans, this family consists of CYP2C8, CYP2C9, CYP2C18 and CYP2C19. It constitutes about 20% of total cytochrome P450 in human liver. CYP2C9 is the major enzyme in this subfamily and is involved in the metabolism of drugs such as flurbiprofen, S-warfarin, etc. (Table 2). The contribution of CYP2C19 is low as compared to CYP2C9. The contribution of CYP2C8 to the metabolism of drugs is very limited (Miners and Birkett 1998; Venkatakrishnan

et al. 2001). In rats, CYP2C11 is the major CYP2C isoform and constitutes about 54% of rat liver cytochromes P450s.

Table 1. Biological processes involving cytochrome P450.

Physiology	Steroids metabolism Fatty acids metabolism Bile acids metabolism Vitamin D metabolism
Pharmacology	Drug metabolism Drug interactions Polymorphisms
Toxicology	Activation of procarcinogens Endocrine disturbances Toxic metabolites of pesticides Toxic metabolites of pollutants

CYP2D subfamily:

In humans, CYP2D6 is the major isoform in this subfamily and constitutes about 2% of total human liver cytochrome P450s. It metabolizes different psychopharmacological and cardiovascular drugs. CYP2D6 substrates include desipramine, dextromethorphan, etc. (Table 2) (Shimada *et al.* 1994; Venkatakrishnan *et al.* 2001). In rats, CYP2D1 is the major isoform and it constitutes less than 2% of CYP450 in rat liver.

CYP2E subfamily:

CYP2E1 is inducible by ethanol and constitutes about 7% of total human liver cytochrome P450s. Its importance arises from its involvement in the metabolism of many toxicants and small molecules. It metabolizes chlorzoxazone, p-nitrophenol, etc. (Table 2) (Venkatakrishnan *et al.* 2001). In rats, CYP2E1 constitutes less than 2% of CYP450 in rat liver.

CYP3A subfamily:

This subfamily includes CYP3A4, CYP3A5 and CYP3A7. This is most abundant in both human liver and small bowel. It constitutes about 30% of human CYP450. It is also present in the kidneys. CYP3A4 is the major isoform in the liver as well as the small intestine of adult human. CYP3A5 is expressed in 20% of the population, while CYP3A7 is only expressed in fetal liver (Venkatakrishnan *et al.* 2001). CYP3A is involved in the metabolism of tacrolimus, cyclosporine, testosterone, etc. (Table 2). In rats, CYP3A constitutes 17% of the total CYP450 and includes CYP3A1, CYP3A2 and CYP3A23 (Waxman 1999).

Other CYP families:

Other CYP450 enzymes known to metabolize drugs include CYP1B, 2A, 2B and 4A. Cytochromes which belong to other P450 gene families (CYPs 5, 7, 8, 11, 17, 19, 21, 24, 26, 27, 39, 46, 51) are only important in metabolizing endogenous substrates such as biosynthesis of thromboxane and prostacyclins (CYPs 5 and 8), steroid hormone biosynthesis from cholesterol (CYPs 11, 17, 19, and 21) and biosynthesis of bile acids, vitamin D3 and cholesterol (CYPs 7, 24, 27 and 51) (Waxman 1999).

Table 2. Partial list of substrates for different cytochrome P450 enzymes*.

1A1	2C9	2D6	2E1	3A4
ethoxyresorufin, polycyclic aromatic hydrocarbons such as benzene (a)pyrene	diclofenac diazepam flurbiprofen ibuprofen irbesartan naproxen phenylbutazone phenytoin piroxicam proguanil S-warfarin, tolbutamide tenoxicam tienilic acid trimethoprim	amitriptyline bufuralol cinnarizine citalopram clomipramine clozapine codeine debrisoquine desipramine dextromethorphan encainide flecainide fluoxetine fluphenazine fluvoxamine imipramine metoprolol mexiletene mianserin nortriptyline ondanserton paroxetine perhexiline propafenone propranolol sparteine thioridazine timolol trifluperidol	4-nitroanisole 4-nitrophenol aniline benzene chlorzoxazone enflurane ethanol halothane isoflurane methylformamide nitrosamine organic solvents paracetamol pyridine styrene toluene	alfentanil amiodarone astemizole benzphetamine budesonide carbamazepine cortisol cyclophosphamide cyclosporine dapsone diazepam digitoxin diltiazem docetaxol erythromycin ethinylestradiol etoposide ifosphamide imipramine lansoprazole lidocaine loratadine losartan lovastatin midazolam nifedipine omeprazole paracetamol quinidine tacrolimus taxol teniposide terfenadine testosterone theophylline toremifene triazolam troleandomycin verapamil
1A2	2C19			
ethoxyresorufin acetanilide caffeine estradiol fluvoxamine imipramine mianserin paracetamol phenacetin propafenone theophylline	citalopram clozapine diazepam hexobarbital lansoprazole omeprazole pentamidine proguanil propranolol S-mephenytoin			

*Compiled from (Murray 1992; Shimada *et al.* 1994; Glue and Clement 1999; Guengerich 1999; Lewis 2001b; Venkatakrishnan *et al.* 2001)

Tissue distribution:

Cytochrome P450 enzymes are distributed in both hepatic and extra-hepatic tissues such as intestine, kidneys, lungs and brain (Krishna and Klotz 1994). Liver has the highest amount of cytochrome P450 enzymes including CYP1A, CYP1B, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP3A, CYP4A and other enzymes and it is the most important organ in the body that is involved in the metabolism of different endogenous and exogenous chemicals.

In kidneys, CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, CYP3A and CYP4A are expressed. The expression is much lower than what is found in the livers (Lohr *et al.* 1998). The intestine contains CYP3A4 as the major isoform. It also contains CYP2E1. Finally, CYP1A and CYP4A are expressed in lungs and CYP4A and CYP2E are expressed in the brain.

Other phase I enzymes:

Oxidations not involving P450 enzymes:

This includes alcohol dehydrogenase, aldehyde dehydrogenase, amine oxidase, xanthine oxidase, etc. These enzymes are mainly involved in metabolizing endogenous compounds and to certain extent also metabolize a few drugs (Gibson and Skett 1994a).

Other phase I enzymes:

This include reductive metabolism as the microsomal system can also catalyse the reduction of compounds such as azo-compounds, nitro-compounds, epoxides, etc. Hydrolysis is another phase I reaction. It includes ester hydrolysis, amide hydrolysis, etc. Finally, hydration in which water is

added to the compound without causing the dissociation of the compound as in case of hydrolysis (Gibson and Skett 1994a).

Phase II pathways:

Phase II reactions (i.e. glucuronidation, sulfation, acetylation, etc) are important steps in the hepatobiliary elimination of several xenobiotics. Phase II reactions generally lead to the formation of more polar metabolites that are subsequently removed from the body (Turner and Brouwer 1997).

Uridine diphospho glucuronosyltransferases: UDP-Glucuronosyltransferases (UGTs) are an important superfamily of phase II membrane bound enzymes located in the endoplasmic reticulum. These enzymes catalyze glucuronidation of endogenous and exogenous compounds including drugs, carcinogens, environmental pollutants, steroids, bile acids and bilirubin (Bock 1991; Meech and Mackenzie 1997; Burchell *et al.* 1998; Tephly *et al.* 1998). UGTs exist in two subfamilies designated 1A and 2B (Jedlitschky *et al.* 1999). Multiple members of these subfamilies exist in both humans and rats. Some examples of these subfamilies include UGT1A1 which metabolizes estradiol and bilirubin (Senafi *et al.* 1994), UGT1A6 which metabolizes acetaminophen (Bock *et al.* 1993), UGT1A9/10 which metabolizes mycophenolic acid (MPA) and UGT2B7 which metabolizes morphine (Table 3) (Coffman *et al.* 1997).

Table 3. Substrates for different UGTs*.

1A1	1A6	1A9/10	2B7	2B17
bilirubin	acetaminophen	fenoprofen	ibuprofen	testosterone
estradiol		furosemide	ketoprofen	
morphine		ibuprofen	morphine	
		ketoprofen	naproxen	
		mycophenolic acid		

* Compiled from (King *et al.* 2000; Ritter 2000)

Regulation of drug metabolism:

Factors affecting drug metabolism:

Many endogenous and exogenous factors are known to increase (or) decrease the drug metabolizing capacity of the liver. These factors include age, gender, genetic makeup, drugs, environmental factors and disease states of the eliminating organ (Glue and Clement 1999). Increase in enzymatic activity is mainly observed due to induction of drug metabolizing enzymes, while inhibition of drug metabolizing enzymes leads to a decrease in the enzymatic activity.

Induction:

Induction is an adaptive mechanism by which an organism responds to foreign chemicals (including drugs) that might otherwise produce toxicity (Hasler *et al.* 1999). This is one of the important basis for many of the commonly encountered drug-drug interactions. Regulation of the levels of Cytochrome P450 enzymes in the liver and other extra-hepatic tissues are controlled by nuclear receptors (NRs) and other factors such as mRNA stabilization, and post translational modifications (Lewis 2001a). Nuclear receptors are a large superfamily of ligand-modulated transcription factors that mediate responses to steroid, retinoid and thyroid hormones (Moore 2000). This superfamily includes CAR (Constitutive Androstane Receptor), PXR (Pregnane X Receptor, also called PAR and SXR), PPAR (peroxisome proliferators-activated receptor), LXR (Liver X Receptor) and FXR (Farnesol X Receptor).

The regulation of CYP1 family of enzymes is mediated by AhR (Aryl hydrocarbone Receptor) and its nuclear translocator Arnt (Ah Receptor Nuclear Translocator). Once the inducer (or ligand) such as TCDD (tetrachlorodibenzo-p-dioxin) binds to the cytosolic AhR, this binding causes dissociation from the homodimeric HSP90. The ligand-bound AhR is then translocated into the nucleus where it heterodimerizes with the Arnt. This complex binds to the ligand (or xenobiotic) response elements (XRE) and the gene transcription for the CYP is turned on (Honkakoski and Negishi 2000). After translation, this protein is incorporated into the membrane of the ER. The regulation of CYP2B is mediated by CAR and its inducers include phenobarbital. CYP2E is regulated by post translational modifications and does not involve nuclear transcription factors and its inducers include ethanol. CYP3A is regulated by PXR and its inducers include rifampin and dexamethasone. CYP4 family is regulated by PPAR and its inducers include fibrate drugs. All these nuclear receptors share a common partner for heterodimerization which is the RXR (Retinoid X Receptor). This heterodimer binds to the regulatory region of the specific CYP and causes increased expression of enzyme proteins.

Inhibition:

Inhibition can occur by many mechanisms that involve destruction of the enzyme (heme ligation or heme adduct formation), inhibition of its synthesis, competitive or non competitive complexing between the enzyme and the inhibitor and finally mechanism based inhibition (Gibson and Skett 2001; Lewis 2001a). Inhibition of CYP enzymes during infection and inflammation is a complicated process that involves many factors and mediators which eventually lead to changes in the enzyme expression and function (Renton 2001). This can occur through molecular or non-molecular mechanisms.

Non-Molecular mechanisms:

Reactive oxygen intermediates (ROIs) are known to be produced after inflammation. These intermediates have been shown in many studies to be involved in pathways that lead to down-regulation of many CYP enzymes. Hydrogen peroxide and oxidative stress have been shown to play a role in down regulating mRNA expression of CYP1A1 and CYP1A2 in hepatocytes (Barker *et al.* 1994). Antioxidants have been shown to prevent the reduction in theophylline metabolism due to hydrogen peroxide and oxidative stress (El-Kadi *et al.* 2000).

Nitric oxide which is produced by the inducible nitric oxide synthase (iNOS) has also been shown to down regulate CYP enzymes. Inhibitors of NOS prevented animals from inflammation mediated down-regulation of CYP enzymes at both protein and mRNA levels (Khatsenko *et al.* 1993; Carlson and Billings 1996; Khatsenko *et al.* 1998). NO was shown to work mainly through two pathways. It can bind to the heme of the P450 and thus prevent the oxygen binding (direct and reversible effect). It can also cause irreversible effect (indirect) by the action of RNOS which are formed by autoxidation of NO (Wink *et al.* 1993; Wink and Mitchell 1998). However, many other investigators have shown that inhibition of NOS did not protect against down-regulation of CYP450 enzymes. For example, the inhibition of NOS in pig hepatocytes did not protect against cytokine (IL-1, IL-6 and TNF-α) mediated down regulation of CYP enzymes (Monshouwer *et al.* 1996). Similar results were also obtained in rat hepatocytes with CYP2C11 (Sewer and Morgan 1997).

In addition, xanthine oxidase down-regulates many CYP450 enzymes by producing super oxide radicals. But, more recent work has shown that depletion of xanthine oxidase did not protect the

enzymes from being down regulated (Ghezzi *et al.* 1984; Ghezzi *et al.* 1985; Mannering *et al.* 1988).

Molecular mechanisms:

It is well documented that control of enzyme inhibition occurs at pre-translational mechanisms. Cytokines play a major role in that mechanism by suppression of mRNA expression and eventually reducing the synthesis of the enzyme. This is documented by many studies showing a reduction in mRNA to precede the decrease in enzyme function. This reduction of mRNA expression can be due to gene transcription or mRNA destabilization.

Cytokines and drug metabolism:

Cytokines are soluble hormone-like proteins which are produced by different cells following stimulation with different inducers. In contrast to hormones, which are synthesized by endocrine tissues, cytokines are produced by a variety of cells. They can be classified broadly into interleukins (IL1-18), interferons (IFN-α,β and γ), growth factors (HGF, CSF, EGF, etc), chemokines (CXC, CCβ and Cγ) and tumor necrosis factors (TNF-α and β) (Simpson *et al.* 1997). The effect of cytokines on drug metabolism has been shown in many studies (Ferrari *et al.* 1993; Shedlofsky *et al.* 1994; Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carelli *et al.* 1996; Carlson and Billings 1996; Morel and Barouki 1998; Iber *et al.* 2000). IL-1, IL-2, IL-6, TNF and IFN are some important mediators that cause changes in the metabolic capacity of drug metabolizing enzymes.

IL-2, TNF-α and IFN-γ decreased the total P450 protein contents (Muntane-Relat *et al.* 1995; Tinel *et al.* 1995; Carlson and Billings 1996). The activity of CYP1A2 was reduced by IFN-α and β in humans. The effects of inflammation and infection on cytochrome P450 are largely due to stimulation of the cellular immune response (Morgan 1997). As early as in 1966, it was shown that various agents that stimulate the cellular immune response result in prolongation of barbiturate sleeping time (Wooles and Borzelleca 1966; Morgan 1997). Influenza virus infection (Chang *et al.* 1978) or injection of influenza vaccines (Renton *et al.* 1980) leads to elevation in plasma concentrations and half-lives of theophylline and other drugs (Morgan 1997). Administration of endotoxin significantly reduced the metabolism mediated by CYP1A2 in human volunteers. The reduction was maximized at the period when the concentration of TNF-α and IL-6 were at the peak (Shedlofsky *et al.* 1994). IL-1α, IL-6 and TNF-α caused reduction in the protein expression of CYP1A in humans (Muntane-Relat *et al.* 1995) while IL-1β, IL-2, TNF-α, IFN-β and IFN-γ decreased the protein expression of CYP1A in rodents (Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carelli *et al.* 1996; Carlson and Billings 1996). The mRNA expression of CYP1A1 was decreased in human by IL-1α, IL-6, TNF-α (Muntane-Relat *et al.* 1995) and in rats by IL-1β and TNF-α (Chen *et al.* 1995; Nadin *et al.* 1995; Carlson and Billings 1996; Morel and Barouki 1998).

The protein expression for CYP2B was decreased by IL-1β, IL-2, TNF-α, IFN-β and IFN-γ in both rodents and human (Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carelli *et al.* 1996; Carlson and Billings 1996). Also, its mRNA was reduced by IL-1β and TNF-α in cultured rat hepatocytes (Chen *et al.* 1995; Nadin *et al.* 1995; Carlson and Billings 1996).

The activity of CYP2C11 in rats was reduced by IL-1β and TNF-α (Ferrari *et al.* 1993; Nadin *et al.* 1995). In human volunteers, endotoxin administration significantly reduced the metabolism mediated by CYP2C19. The reduction was maximized at the period when the concentration of TNF-α and IL-6 were at the peak (Shedlofsky *et al.* 1994). The protein level of CYP2C11 was also down regulated in rats by different cytokines including IL-1β, IL-2, TNF-α and IFN-γ (Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carlson and Billings 1996). The mRNA expression of rat CYP2C11 was down regulated by IL-1β, IL-6 and TNF-α (Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Carlson and Billings 1996; Tapner *et al.* 1996). In addition, endotoxin down regulated the mRNA expression of CYP2C11 and CYP2C12 in rats (Shedlofsky *et al.* 1994).

Finally, the activity of CYP3A2 in rats was reduced by IL-1β and TNF-α (Ferrari *et al.* 1993; Nadin *et al.* 1995). Its protein expression was also down regulated by IL-1α, IL-1β, IL-2, TNF-α, IFN-α, IFN-β and IFN-γ in both human and rodents (Craig *et al.* 1990; Craig *et al.* 1993; Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carelli *et al.* 1996; Carlson and Billings 1996). The mRNA expression for this enzyme was also down regulated by IL-1α, IL-1β, IL-2, TNF-α and IFN-α in both human and rodents (Craig *et al.* 1990; Craig *et al.* 1993; Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carlson and Billings 1996).

The above discussion indicates that several cytokines released during inflammation and immune modulation in general appear to down regulate activity and expression of most of CYP450

enzymes. The mechanism by which cytokines regulate the transcription of different genes involves transcription factors such as NF-κB (nuclear factor) which is a signal transducer and activator of transcription. CYP2C11 has been shown to be controlled by a mechanism involving NF-κB (Iber *et al.* 2000). TNF-α down-regulated CYP1A1 by a mechanism involving NF-1 (Morel and Barouki 1998).

Drug metabolism in organ transplant patients:

During acute rejection of the liver, when several of the inflammatory mediators are released in the body, the clearance of antipyrine is significantly decreased in liver transplant patients (unpublished observations). In addition, in rats acute rejection of the liver also leads to impaired clearance of tacrolimus and reduced biliary excretion of sulfobromophthalein (BSP) (unpublished observations). A systematic evaluation of the various factors that contribute to the alteration in the metabolism of drugs in transplant patients has not been carried out. The primary objective of this dissertation is to evaluate the effect of acute and chronic rejection of liver on drug metabolism and transport.

Central vs. Peripheral Effects:

Central effect: It is generally known that disease of an organ results in impaired functional capacity of that organ. For example, urinary excretion of drugs is impaired in renal failure and drug metabolism is impaired in liver disease. In liver disease, the total content of CYPs is decreased but the magnitude of reduction is selective for individual enzymes. Moreover, the effect was related to the type of liver disease (with or without cholestasis) (George *et al.* 1995a; George *et al.* 1995b). The activity of CYP1A2, CYP2A6, CYP2E1, CYP3A4 and CYP2C19 (S-

mephenytoin) was decreased in patients with liver disease (Howden *et al.* 1989; Kraul *et al.* 1991; Murray 1992; Wilkinson 1996; Adedoyin *et al.* 1998; Marino *et al.* 1998). The reduction in CYP2A6 and CYP3A4 activity in liver biopsies has been shown to be proportional to the degree of liver damage (Kraul *et al.* 1991; Huang *et al.* 1993). On the other hand, liver diseases did not affect the activity of CYP2D6, CYP2C8, CYP2C9 and CYP2C19 (R-mephenytoin) (Howden *et al.* 1989; Adedoyin *et al.* 1998; Marino *et al.* 1998).

Peripheral effect: In addition to the local effect, from our knowledge about the release of inflammatory and immunological mediators in the body during conditions of inflammation, we also expect an effect on the functional capacity of peripheral organs as a consequence of any disease or impairment in an organ. This is documented by the effect of traumatic brain injury (TBI) and renal failure on hepatic drug metabolism. In patients suffering from TBI, the pharmacokinetics of several hepatically eliminated drugs is altered (Boucher and Hanes 1998). The clearance of antipyrine (Boucher *et al.* 1991), phenytoin (O'Mara *et al.* 1995) and phenobarbital (Heinemeyer *et al.* 1986; Wermeling *et al.* 1987) was increased following TBI. TBI in rats significantly reduced hepatic cytochrome P-450 activity after 24 hrs and reduced the activity of CYP2E1 after 48 hrs of injury. TBI increased the activity of CYP2E1 in kidneys at 24 hrs but did not change CYP2E1 activity in the brain (Poloyac *et al.* 2001). TBI in rats has been shown to decrease the mRNA expression of both CYP2C11 and CYP3A in the liver by 50% as compared to control. However, the same study did not show significant changes in either the activity or the protein expression of these enzymes (Toler *et al.* 1993).

The concentration of blood urea nitrogen has been shown to be correlated with the content of cytochrome P450 and its activity in the liver of rats with Chronic Renal Failure (CRF) (Uchida *et*

al. 1995). Recently, cytochrome P450 activity, protein content and mRNA expression of different CYP enzymes have been shown to be significantly reduced in liver of rats with CRF as compared to normal rats. CYP3A2 activity was also reduced by 50% as a result of CRF (Leblond *et al.* 2001). The above discussion points to the importance of evaluating the effect of organ rejection on not only hepatic metabolism but also metabolic capacity in other organs such as lungs, kidneys and small intestine.

Drug transporters:

Movement of drugs from one part of the body to another and from an organ to outside is facilitated by several transporters. Transporters are expressed in the liver, intestine, kidney, lung and brain and they regulate the absorption, distribution and elimination of exogenous and endogenous chemicals. The transporters of importance include multi-drug resistance protein (MDR, which encodes for P-Glycoprotein), multi-drug resistance associated protein (MRPs), organic anion transport protein (OATP), organic cation transporter (OCT) and bile salt export pump (BSEP). Of all these transporters, P-gp appears to be the most important transporter that is involved in modulating the bioavailability and disposition of many drugs.

P-Glycoprotein: The role of P-gp has been first reported for the anti-cancer drugs and its expression is associated with tumor cell resistance to the target drugs such as doxorubicin. P-gp pump is a transmembrane protein associated with a phenotype of multi-drug resistance (MDR1 and 2 in humans and mdr1a, 1b and 2 in rats) to certain anti-cancer agents (Ford and Hait 1990; Gottesman *et al.* 1995). This pump is distributed in different parts of the body other than the cancer cells, such as in liver, intestine, kidneys, lungs, brain, adrenal glands and lymphocytes

(Fojo *et al.* 1987; Sugawara *et al.* 1988; Lum and Gosland 1995). Recently, P-gp has been associated with low bioavailability of many other drugs such as cyclosporine, tacrolimus, hydrocortisone and diltiazim (Relling *et al.* 1994; Wacher *et al.* 1995; Zhang *et al.* 1998). It has been shown to efflux certain drugs out of the enterocytes which leads to re-exposure of the drug to the intestinal fluids and to the drug metabolizing enzymes which in turn leads to a reduction in the bioavailability.

Regulation of P-glycoprotein:

Expression and activity of P-gp (mdr gene) is controlled by many mechanisms including gene transcription and amplification, mRNA stabilization and processing, protein stabilization and plasma membrane incorporation. Of these mechanisms, effects on the mRNA level of mdr1 gene has been reported to be the most important (Sukhai and Piquette-Miller 2000).

Since transporters are involved in absorption and elimination of drugs used in transplant patients, it is important to evaluate the various factors that will alter the expression and activity of transporters as it relates to organ transplant patients.

Central hypothesis:

Rejection of a transplanted organ (liver) will lead to a decrease in metabolism and transport locally (liver) in that organ and also decrease the metabolism and transport in other organs (kidneys and lungs). In order to evaluate this hypothesis we first need to select an appropriate method to study drug metabolizing activity.

Methods for studying drug metabolizing activity:

Many methods have been used to study drug metabolism. It includes *in vivo* and *in vitro* methods. There are no ideal methods or a method of choice but each method has its own advantages and disadvantages and each can answer different questions.

In vivo methods:

a. *In vivo clearance:* In this case a probe is administered to the subject or animal and multiple blood or any relevant biological fluids are sampled during specific time intervals. Clearance of that probe is then calculated based on pharmacokinetics principles. This approach gives a good prediction of the status of metabolizing systems but many criteria must be fulfilled by the administered probe before it can be used as a marker for metabolizing enzymes. The probe should be absorbed to a high extent (ideally 100% bioavailable), distributed in the total body water, not bind to tissue or plasma proteins to any significant extent, only metabolized by the liver and not be eliminated by the renal route and follow a one compartment model (Gibson and Skett 1994b).
b. *Breath tests:* In these methods, the carbon dioxide (mostly labeled) excreted through lung after demethylation of certain drugs such as erythromycin is measured (Gibson and Skett 1994b).

In vitro methods:

These methods include (Table 4):

a. Whole liver perfusion: This system is the closest to the physiological situation since the liver (or any other organ) is intact. Thus, it gives a measure approximating the *in vivo* situation. However, it is experimentally demanding.

b. Liver Slices: This is the next better alternative to whole liver perfusion since it maintains the cell-cell interactions and it requires only slices of the liver as compared to a whole liver as in the case of whole liver perfusion. But, it is difficult to setup and maintain.

c. Isolated hepatocytes: Hepatocytes have both phase I and phase II enzymes. They are suitable to study enzyme induction and inhibition.

d. Sub-cellular fractions: It includes both S9 fraction and microsomes. They can be used to study enzyme inhibition but they are not suitable for studying induction.

Table 4. Methods for studying drug metabolism.

	Sub-cellular fractions	Hepatocytes cultures	Liver Slices	Whole liver perfusion
Closeness to *in vivo* situtaion	No cell-cell interaction and co-factors are lost during the preparation process	Cell-cell interaction maintained	Cell-cell interaction is still maintained. Also, the different types of liver cells are maintained	Closest to *in vivo*
Preparation and maintenance	Need to homogenize the tissue and to do differential centrifugations. Time consuming	Isolation of hepatocytes is time consuming and requires perfusing the liver with collagenase. Also, media need to be changed regularly	Easier than hepatocytes	Easy but required a suitable apparatus to maintain the flow through the organ
Stability	Can be stored for more than a year at -80C°	Can be maintained on cultures for more than a week	6 hours	2-6 hours

Hypothesis 1:

We hypothesize that rejection will cause local cellular injury that will lead to decrease in the activity of all drug metabolizing enzymes and transporters to a similar extent. It is not possible to study the effect of acute rejection in humans due to the fact that during acute rejection, patients receive many drugs (e.g. anti rejection drugs) to treat the rejection. In addition, it is not possible to directly evaluate the expression of various drug metabolizing enzymes as it is not possible to obtain liver samples from these patients during acute rejection, due to ethical considerations. For these reasons, an animal model (rat) will be used to evaluate the effect of acute rejection of the liver on hepatic metabolism as measured by activity, protein expression and mRNA expression of the following drug metabolizing enzymes; CYP3A2, CYP2C11, CYP2E1 and UGT1A9 (Chapter 3). The combination of Lewis and ACI rats that is commonly used in transplant research for evaluation of the immunology of acute rejection will be used in our studies. There are no suitable animal models for chronic rejection of liver. Human liver tissues will be used to evaluate the effect of chronic rejection of the liver in humans on hepatic metabolism as measured by activity and protein expression of CYP3A4, CYP2C9, CYP2E1 and UGT1A9 (Chapter 5).

Hypothesis 2:

We hypothesize that rejection leads to cytokine production. Exposure of these cytokines to other peripheral organs (kidneys and lungs) will lead to decrease in metabolism in these organs. The rat model for acute rejection will also be used here to evaluate the effect of acute rejection of liver in rats on renal metabolism as measured by activity, protein expression and

mRNA expression of CYP3A2, CYP2E1 and UGT1A10 and pulmonary metabolism as measured by mRNA expression of metabolizing enzymes (Chapter 4).

Hypothesis 3:

Certain cytokines levels are elevated during rejection. **We hypothesize that cytokines will decrease the activity of drug metabolizing enzymes and will decrease the ability of enzyme inducing agents to increase drug metabolizing enzyme activity.** Human hepatocytes will be used to evaluate the contribution of cytokines to the observed changes in CYP3A4 metabolizing activity and protein expression (Chapter 7). Although the effect of cytokines on drug metabolism has been reported in the literature, studies are mainly directed toward measuring the effect of cytokines on protein expression and not on the activity of the metabolic enzymes. The use of human hepatocytes is advantageous as compared to other models such as liver microsomes as both induction and inhibition of metabolic enzymes can be studied in this system.

Hypothesis 4:

We hypothesize that cytokines released during the rejection of the liver will decrease the expression of transporters in liver and in other organs. We will evaluate the effect of acute (in rats) and chronic (in humans) rejection of liver on hepatic and extra-hepatic (kidneys and lungs in rats) expression of P-gp and other transporters (Chapter 6).

In all the proposed work, the choice of the enzymes to be studied was based on their abundance in the studied organ. CYP3A constitutes about 30% of total liver cytochromes P450s in humans followed by CYP2C with 20% and CYP2E with 7%. In rats, CYP2C constitute about 54% of

total liver cytochromes followed by CYP3A with about 17% and CYP2E with 2%. Also, the choice of these enzymes was based on their importance in metabolizing commonly prescribed drugs (CYP3A metabolizes 53% of the commonly prescribed drugs). The selected enzymes (CYP3A and UGT1A9/10) and P-gp are also important in the metabolism/transport of several immunosuppressive drugs.

Chapter 2

Materials and methods

Chemicals:

William's E medium, media supplements and insulin were purchased from BioWhittaker (Walkersville, MD). Gentamicin was obtained from Gibco Laboratories (Grand Island, NY). Testosterone and 6β-hydroxyltestosterone were purchased from Steraloids Inc. (Wilton, NH). Bovin serum albumin (BSA), chlorzoxazone, flurbiprofen, mycophenolic acid (MPA), NADPH, UDP-Glucuronic acid (UDPGA), MTT, dexamethasone, rifampicin, Folin reagent, NaOH, $MgCl_2$, Tris base, Tris HCl, SDS, 6-hydroxychlorzoxazone, 4'-hydroxyflurbiprofen, MPAG were purchased from Sigma (St. Louis, MO). Electrophoresis apparatus and reagents were obtained from Bio-Rad (Richmond, CA). Primary antibodies for CYP1A, CYP2C, CYP2D, CYP2E, CYP3A and UGT1A, secondary antibodies and control microsomes were obtained from Gentest (Woburn, MA). Trizol was obtained from Invitrogen (Carlsbad, CA). Microarray kits and RNA purifying kits was obtained from Clontech (Palo Alto, CA). Human IL-1β, IL-2, IL-6 and TNF-α were obtained from R&D systems (Minneapolis, MN).

All solvents and chemicals used were of HPLC grade or the highest available grade.

Falcon culture dishes (6-well plates, 60mm, 100mm) were purchased from Becton Labware (Franklin Lakes, NJ).

Methods:

Effect of acute rejection of liver in rats on hepatic metabolism:

The study protocol was approved by the institutional animal care and use committee (IACUC) at the University of Pittsburgh. Male ACI and Lewis rats (170-230 g) were used in the study. After

transplantation, this combination (ACI → Lewis) produces the most stringent acute rejection and this combination is considered to be an ideal model of acute rejection.

Groups were designed as follows:

1. Normal rats (n=4): livers from normal ACI rats.
2. Syngeneic Tx (n=4): ACI livers transplanted to ACI rats. This group served as a control for the potential effect of surgery, alteration in blood flow to the liver, ischemia and reperfusion injury on hepatic drug metabolism.
3. Allogeneic Tx (n=4): ACI livers transplanted to Lewis rats. This group evaluated the effect of the process of rejection in addition to surgery, altered blood flow to the liver, ischemia and reperfusion on hepatic drug metabolism.

Rats in group 2 and 3 were transplanted (by Dr. Noriko Murase's group in the Thomas E. Starzl Transplantation Institute) and monitored daily. On day 6, rats were sacrificed by decapitation. Blood samples were obtained from these rats for serum biochemistry. The occurrence of rejection was monitored by general health and well being of these animals and by liver biochemistry and histopathology. Livers were harvested on day 6 and stored at –80°C. Microsomes from those harvested organs were prepared as shown below and stored at –80°C.

Effect of chronic rejection of liver in humans on hepatic drug metabolism:

Human liver tissues were obtained from the University of Minnesota tissue bank. These samples were from normal individuals (organ donors) and from patients with chronic rejection of the liver. The occurrence of rejection was confirmed by liver biochemistry and histopathology.

Groups were designed as follows:

1. Normal livers (n=4): from normal individuals.
2. Rejected livers (n=4): livers from patients with chronic rejection of liver.

Microsomes from these liver samples were prepared as shown below and stored at –80°C.

Effect of acute rejection of liver in rats on metabolism in peripheral organs (kidneys and lungs):

For acute rejection studies in rats, groups were designed as follows:

1. Normal rats (n=4): kidneys and lungs from normal Lewis rats.
2. Syngeneic Tx (n=4): kidneys and lungs from Lewis rats transplanted with Lewis livers. This group served as a control for the potential effect of surgery, alteration in blood flow, cold ischemia and warm reperfusion injury on extra hepatic metabolism.
3. Allogeneic Tx (n=4): kidneys and lungs from Lewis rats transplanted with ACI livers. This group evaluated the effect of the process of rejection in addition to surgery, ischemia and reperfusion on extra hepatic metabolism.

Rats in group 2 and 3 were transplanted (by Dr. Noriko Murase's group in the Thomas E. Starzl Transplantation Institute) and monitored daily. On day 6, rats were sacrificed by decapitation. Blood samples were obtained from these rats for serum biochemistry. The occurrence of rejection was monitored by general health and well being of these animals and by liver biochemistry and histopathology. Kidneys and lungs were harvested and stored at –80°C. Microsomes from those harvested organs were prepared as shown below and stored at –80°C.

Orthotopic liver transplantation:

Orthotopic liver transplantation (OLTx) was carried out as described earlier (Kamada and Calne 1979). Briefly, after anesthetizing the rats with methoxyflurane (inhalation) and opening the abdomen by transverse incision, the donor's liver was perfused with cold lactate ringer solution through the aorta. The liver was then removed and placed in a cold ringer solution (50 ± 5 min). The cuff procedure was used for both infrahepatic inferior vena cava and the portal vein. The liver was then placed orthotopically into the recipient. The inferior vena cava was then anastomosed, the donor's portal cuff was inserted into the recipient portal vein, and the bile duct of the donor was inserted into the recipient's bile duct. The recipient animals were given 20 mg/day cefamandole for the first three days after the surgery.

Preparation of microsomes:

Microsomes from livers, kidneys and lungs were prepared by differential centrifugation. All instruments and buffers were kept on ice during the procedure. Tissue samples were placed in 4X their weight of Tris HCl buffer (0.05M Tris HCl, 1.5% KCl and 1mM EDTA, pH 7.4). First, tissues were cut with scissors and homogenized using an electrical homogenizer. This homogenate was then centrifugated at 9000g for 20 min at 4°C. Supernatants were then centrifuged at 100,000g for 1hr at 4°C. The pellets were reconstituted in the above buffer and recentrifuged again at 100,000g for 1hr at 4°C. the pellets were reconstituted using manual homogenizer in 2X their weight of a Tris Buffer (0.05M Tris HCl and 1mM EDTA, pH 7.4) containing 20% glycerol. Homogenates were then distributed into new labeled tubes (0.5 ml each) and stored at -80°C until used for incubation studies.

Total protein estimation:

The Microsomal/homogenate protein contents were determined according to the procedure of Lowry, using BSA as a standard. Standards and samples (400µl) were mixed with alkaline copper sulphate solution (2ml) and kept for 10 minutes. Then, Folin reagent (200µl) was added to each tube and kept for 30 min. The absorbance was measured at 750nm using a spectrophotometer (Lowry *et al.* 1951).

Determination of the CYP450 activity in the microsomes:

The content of CYP450 was measured using the method of Omura and Sato from the CO difference spectrum of dithionite-reduced samples using a molar extinction coefficient between 450 and 490 nm of 9100 m^2/mol and expressed as nmol/mg microsomal protein (Omura and Sato 1964).

Microsomal incubation:

Phase I:

CYP3A2 and CYP3A4:

The formation of 6β-hydroxytestosterone from testosterone was used as a measure of the activity of CYP3A enzyme system. The incubation was carried out in eppendorf tubes containing different concentrations of testosterone in acetonitrile (10-200 µM), 0.5 mg/ml microsomal protein with the fluid volume being adjusted to 0.5 ml by addition of 50 mM phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$. The tubes were pre-incubated for 5 minutes at 37°C in an oscillating water bath and then 1 mM of NADPH was added to initiate the reaction. The reaction was terminated 10 minutes later by placing the tubes in ice and adding equal volume (0.5 ml) of

ice-cold methanol. The tubes were centrifuged at 13,000 rpm for 5 minutes and the supernatants were transferred to new tubes and stored in -20°C until analysis of 6β-hydroxytestosterone.

CYP2C11 and CYP2C9:

The formation of 4'-hydroxyflurbiprofen from flurbiprofen was be used as a measure of the activity of CYP2C enzyme system. The incubation was carried out in eppendorf tubes containing different concentrations of flurbiprofen in acetonitrile (3.125-200 μM), 0.5 mg/ml microsomal protein with the fluid volume being adjusted to 0.5 ml by addition of 50 mM phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$. The tubes were pre-incubated for 5 minutes at 37°C in an oscillating water bath and then 1 mM of NADPH was added to initiate the reaction. The reaction was terminated 20 minutes later by placing the tubes in ice and adding equal volume (0.5 ml) of ice-cold acetonitrile. The tubes were centrifuged at 13,000 rpm for 5 minutes and the supernatants were transferred to new tubes and stored in -20°C until analysis of 4'-hydroxyflurbiprofen.

CYP2E1:

The formation of 6-hydroxychlorzoxazone from chlorzoxazone was used as a measure of the activity of CYP2E enzyme system. The incubation was carried out in eppendorf tubes containing different concentrations of chlorzoxazone in acetonitrile (10-500 μM), 0.5 mg/ml microsomal protein with the fluid volume being adjusted to 0.5 ml by addition of 50 mM phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$. The tubes were pre-incubated for 5 minutes at 37°C in an oscillating water bath and then 1 mM of NADPH was added to initiate the reaction. The reaction was terminated 15 minutes later by placing the tubes in ice and adding equal volume (0.5 ml) of

ice-cold methanol. The tubes were centrifuged at 13,000 rpm for 5 minutes and the supernatants were transferred to new tubes and stored in -20°C until analysis of 6-hydroxychlorzoxazone.

Phase II:

UGT1A9/10:

The formation of MPAG from MPA was used as a measure of the activity of UGT1A9/10 enzyme system. The incubation was carried out in eppendorf tubes containing different concentrations of MPA in DMSO (0.05-2.5 mM), 1 mg/ml microsomal protein, 4mM Saccharolactone, Brij58 (0.1mg/mg of microsomal protein) with the fluid volume being adjusted to 0.2 ml by the addition of 50 mM Tris buffer (pH 7.4) containing 10 mM $MgCl_2$. The tubes were pre-incubated for 5 minutes at 37°C in an oscillating water bath and then 4mM of UDPGA was added to initiate the reaction. The reaction was terminated 30 minutes later by placing the tubes in ice and adding equal volume (0.2 ml) of ice-cold methanol. The tubes were centrifuged at 13,000 rpm for 5 minutes and the supernatants were transferred to new tubes and stored in -20°C until analysis of MPAG.

Preparation of tissue homogenates:

Tissue homogenates were prepared for evaluation of P-gp expression. Approximately 150 mg of liver tissues were thawed, cut and homogenized with 5 volumes of 1X Laemmli buffer. Homogenates were centrifuged at 3000 rpm for 20 min at 4°C. The supernatants were sonicated, centrifuged at 14000 rpm for 15 min at 4°C and the new supernatant were distributed into multiple tubes and kept at -80°C.

Western blotting:

Proteins (15-30µg) from microsomal samples, tissue homogenate or from pooled sonicated hepatocyte samples were separated by electrophoresis on 10% SDS-PAGE and transferred electrophoretically to PVDF (polyvinylidene difluoride) membranes. These membranes were blocked overnight at 4°C with TBS-T buffer containing 5% (w/v) blocking grade non-fat powder milk. The membranes were then incubated for 1.5 hrs or overnight (depending on the enzyme studied) with the primary antibodies. After washing, the membranes were incubated with secondary antibodies conjugated with horse radish peroxidase for one hour at the room temperature. The peroxdiase activity was detected by ECL detection with luminal. The bands were analyzed by Quantity One image processing software version 4.03 (Bio-Rad Laboratories, Melville, NY).

RNA isolation:

Total RNA was isolated using Trizol reagent (Carlsbad, CA) according to the manufacturer's instruction. Extracted RNA was treated with both DNase I which was provided with the microarray kit (Clontech Laboratories Inc., Palo Alto, CA) and with phenol-chloroform in order to reduce DNA contamination. The concentration and purity of the RNA was determined using spectrophotometer. The integrity of the RNA was determined by evaluating the bands for ribosomal RNA (18S and 28S bands) on 1.5% agarose gel electrophoresis.

Microarray analysis:

This work was done according to the manufacturer's instruction (Clontech Laboratories Inc., Palo Alto, CA) shipped with Atlas Rat Toxicology 1.2 array (contains 1,176 cDNA fragments

corresponding to different metabolizing enzymes, transporters, stress markers, cytokines and others). Four micrograms of the pooled purified RNA from each group was converted to the ^{32}P-labeled cDNA using Moloney Murine Leukemia Virus reverse transcriptase (MMLV), master mix and primer mix specific to the array (Atlas Rat Toxicology 1.2 array). The cDNA was purified by passing through G-50 sephadex columns (Eppendorf 5-Prime, Boulder, CO). The radioactivity was measured using scintillation counter (Packard 1500 Tri-carb, Perkin Elmer live Sciences, Downes Grove, IL). The membranes were first prehybridized with ExpressHyb solution (provided with the kit) containing heat-denatured salmon testes DNA (100μg/ml) for more than one hour. The membranes were then hybridized over night with cDNA probes at 68°C. The membranes were washed with different washing solutions. The membranes were wrapped individually with plastic wrap and exposed to phosphoimaging screen (Bio-Rad Laboratories, Melville, NY). The hybridization intensity was then analyzed by Bio-Rad image processing software version 4.03. The intensity was normalized to one of the positive control included on the microarray (GAPDH). This technique was used to evaluate the mRNA expression of metabolizing enzymes (CYP1A1, CYP1B1, CYP2A1, CYP2A3, CYP2B1, CYP2C, CYP2D18, CYP2E1, CYP3A, CYP4A10, UGT1A1, UGT1A7 and the UGT family), transporters (MDR1, MDR3, MRP1, MRP2 (cMOAT), BSEP(SPGP) and OCT) and cytokines (IL-1α, IL-β, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-15, IL-18, TNF-α, INF-α, INF-γ, TGF-α and TGF-β).

Effect of Cytokines on hepatic metabolism in human hepatocytes:

Human hepatocytes were used for this study. The effect of four cytokines (IL-1β, IL-2, IL-6 and TNF-α) on the activity and expression of CYP3A4 was evaluated. These cytokines were selected

based on the importance of these cytokines during the process of rejection of transplanted organs.

Isolation of human hepatocytes:

Hepatocytes were isolated from donor livers by a three-step collagenase perfusion technique which was described earlier by Strom et al (Strom *et al.* 1996). Briefly, liver perfusion was achieved by placing catheters into the large hepatic veins (one catheter for the left lobe and 2-3 catheters for the right lobe). The liver was the placed into a sterile plastic bag and maintained at 37°C in a water bath. First, buffer 1 (HEPES, NaCl, KCl, EGTA and glucose) was perfused for 20 min, followed by buffer 2 (same as buffer 1 but without EGTA) for 10 min. Buffer 3 (HEPES, NaCl, KCl, $CaCl_2$, glucose, BSA, Collagenase P, Dnase I) was then perfused through the liver for 25-30 min. Tissue was then placed in a sterile container containing ice-cold buffer 2 and chopped with sterile scissors. Hepatocytes are now released into the buffer. The suspension was filtered to remove larger pieces of tissues. Three centrifugation steps (50 x g for 5 min each) were then carried out in order to enrich the hepatocytes relative to nonparenchymal cells. The final pellet was then resuspended in culture media and viability was assessed by trypan blue exclusion test. This suspension was then diluted to obtain 1 million cells/ml.

Assessment of the viability of human hepatocytes:

The viability of the hepatocytes was assessed by Trypan Blue exclusion test in which cell suspension and trypan blue (4%) were mixed in equal volumes. This suspension was then placed on a hemocytometer and the cells were observed under a light microscopy. The number of live and dead cells were counted in two fields (live cells exclude the die and were not stained, while

dead cells stained blue in color). The viability was determined by dividing the number of live cells by the total number of cells and the result was then multiplied by 100. ([Live cells# / Total cells#] X100 = % viable). When the viability of the cells was low, a density gradient separation using percoll was used to remove dead cells. The viability of the cells used in our studies ranged from 66 to 85, as determined by trypan Blue exclusion test.

Hepatocytes culture:

Hepatocytes was suspended in Williams E medium (HMM) in the presence of 0.1μM insulin, 0.1 μM dexamethasone, 0.05% gentamicin and 10% bovine calf serum (BCS). This hepatocyte suspension was plated on six well plates which were covered with collagen. The hepatocytes were maintained for 4-6 hours to allow them to attach to the collagen. The medium was then replaced with fresh medium with out the BCS. Fresh medium was added every 24 hours and hepatocytes were maintained at 37°C at all times in the presence of 5% CO_2 and 95% O_2.

Hepatocytes incubation:

Twenty four or 48 hrs after plating of the hepatocytes, the hepatocytes were maintained in fresh medium containing the treatment agent. This process was repeated daily until the last day of the treatment.

The effect of each cytokine on the activity of CYP3A4 as evaluated by the formation of 6β-hydroxytestosterone from testosterone was determined at multiple concentrations (10-300 pg/ml) of cytokines. The selection of the cytokines concentrations was based on the serum levels of these cytokines in patient undergoing acute organ rejection.

After establishing a dose-response curve, additional studies were carried out to simulate potential clinical scenarios in terms of time course of cytokine release. The hepatocytes were pretreated with cytokines and/or inducers at different time points and using different orders of pre-treatment (Table 5). This allowed us to measure both the effect of cytokines on the basal activity of the enzyme and on the induction potential of classic inducers in presence of the cytokines.

At the end of the last day of treatment, the hepatocytes were washed with fresh medium (blank) for one hour. The hepatocytes were incubated with fresh medium containing testosterone (300 μM). After 30 minutes, the medium was collected and frozen at -20°C until analysis. The hepatocytes were collected from the plates using scrapers in the presence of 150μl phosphate buffer (0.1 M, pH 7.4) for protein measurement. Samples were frozen until further protein determination and western blotting. Rifampicin was used as a positive control for induction as shown in Table 5.

Table 5. Different treatment orders used in the human hepatocyte culture studies.

days	1	2	3	4	5	6
A	Plate	HMM	HMM	HMM	HMM	test with testosterone
B	Plate	HMM	HMM	Rif	Rif	test with testosterone
C	Plate	Cyto	Cyto	Cyto	Cyto	test with testosterone
D	Plate	Cyto	Cyto	Rif	Rif	test with testosterone
E	Plate	Cyto	Cyto	Cyto+Rif	Cyto+Rif	test with testosterone

HMM=hepatocytes maintenance media, Cyto=cytokine, Rif=Rifampicin

Twenty four hours after the hepatocytes plating (day 1), treatment were started (day 2 – day 5) according to the above table. After that, testosterone was incubated with the hepatocytes for evaluating the CYP3A4 metabolic capacity.

Total protein estimation:

The hepatocyte protein content was determined according to the procedure of Lowry as described above.

Analysis of the metabolite formation:

The formation of different metabolites were measured as an index of enzyme activities:

- CYP3A 6β-hydroxytestosterone
- CYP2C 4'-hydroxyflurbiprofen
- CYP2E1 6-hydroxychlorzoxazone
- UGT1A9/10 MPAG

HPLC was used to measure the metabolite formation in the incubation media as follows:

HPLC analysis of 6β-hydroxy testosterone:

One hundred µl of the incubation medium was injected onto a LiChrospher 100 RP-18 column (4.6 X 250 mm X 5µm) at room temperature. A mobile phase of methanol:water (60:40) was used at a flow rate of 1.2 ml/min. The UV detector was set at 242 nm. The retention time for 6β-hydroxytestosterone was 5.8 minutes. The standard curve was linear over a concentration range of 0.25-5 µg/ml. The CV was less than 7.8%.

HPLC analysis of 4'-hydroxy flurbiprofen:

One hundred µl of the incubation medium was injected onto a Brownlee Spheri-5® C18 column (4.6 X 100mm) at room temperature. A mobile phase of acetonitrile:20mM K_2HPO_4 (45:55), pH

3 was used at a flow rate of 1.0 ml/min. The fluorescence detector was set at 260 nm for excitation and 320 nm for emission. The retention time for 4'-hydroxyflurbiprofen was 2.55 min. The standard curve was linear over a concentration range of 5-200 ng/ml. The CV was less than 15%.

HPLC analysis of 6-hydroxy Chlorzoxazone:

Seventy five µl of the incubation medium was injected onto Supelco C18 column (4.6 X 150mm, 5mm) at room temperature. A mobile phase of acetonitrile:0.25% acetate buffer (18:82), pH 4.8 was used at a flow rate of 1.0 ml/min. The UV detector was set at 287 nm. The retention time for 6-hydroxychlorzoxazone was 6.4 minutes. The standard curve was linear over a concentration range of 0.078-5 µg/ml. The CV was less than 12.9%.

HPLC analysis of MPAG:

Thirty µl of the incubation medium was injected onto a Alltech Hypersil BDS C18 (4.6 X 250mm, 5mm) at room temperature. A mobile phase of acidified water (with 0.05% o-phosphoric acid):acetonitrile (75:25) was used at a flow rate of 1.0 ml/min. The UV detector was set at 254 nm. The retention time for MPAG was 11 min. The standard curve was linear over a concentration range of 1-100 µg/ml. The CV was less than 3.7%.

Data analysis:

Parameters calculated:

Vmax, Km and Cl_{int}:

The metabolite formation rate vs. substrate concentration was fitted to Michaelis-Menten equation. Vmax and Km were calculated for the activity of various enzymes in the microsomal systems using Prism software version 3.02 (GraphPad Software Inc., San Diego, CA). The Cl_{int} was calculated as (Vmax/Km). In case of kidneys and lungs, only one concentration (highest concentration) of substrate was used as there were not enough microsomes from these organs in order to incubate with multiple substrate concentrations for all the probes tested.

Statistical Analysis:

Log transformation of original data was done followed by one way ANOVA/Tukey test or t-test as appropriate to evaluate the presence of significant differences between the groups at a p-value ≤ 0.05. Correlation between different parameters was carried out using Pearson and Spearman correlation analysis. This was carried out using Prism software version 3.02 (GraphPad Software Inc., San Diego, CA). Sample size was chosen based on power calculation using Pass software version 6.0 (NCSS, Kaysville, UT). At $\alpha = 0.05$, the proposed sample size will allow the achievement of a power $\geq 80\%$ for all the studied enzymes.

Chapter 3

Effect of acute rejection of liver in rats on the hepatic drug metabolizing enzymes activity and expression

Introduction:

Acute rejection of liver is an immune cell-mediated pathological inflammatory response that takes place within the allograft. It usually occurs within days to weeks after transplantation. It is characterized histologically by infiltration of the graft with lymphocytes. Acute rejection is associated with elevated levels of several cytokines in serum and tissues (Bumgardner and Orosz 1999). Cytokines which are responsible for the occurrence of acute rejection of the liver are known to decrease drug metabolism (Morgan 1997). Transplant patients often receive multiple drug therapy and a number of these drugs are metabolized in the liver. For instance, the immunosuppressive drugs commonly used in transplant patients such as tacrolimus, sirolimus and cyclosporine are metabolized by CYP3A4, a phase I enzyme, while mycophenolic acid (MPA) is metabolized by UGT1A9/10, a phase II enzyme.

We hypothesized that cytokine mediated changes in the activity of drug metabolizing enzymes will contribute to the large variability in the pharmacokinetics of immunosuppressive drugs observed clinically. As a first step, we evaluated the effect of acute rejection on the expression and activity of various hepatic drug metabolizing enzymes. The specific aims of this study were to evaluate the effect of acute rejection of the liver in rats on the hepatic drug metabolizing capacity, protein expression and mRNA expression for phase I (CYP3A2, CYP2C11 and CYP2E1) and phase II (UGT1A9) enzymes.

Experimental methods:

The study protocol was approved by the IACUC at the University of Pittsburgh. Male ACI and Lewis rats (170-230 g) were used in the study. ACI-Lewis combination is a strong responder to each other and is the standard animal model used to evaluate the activity of immunosuppressive drugs.

Groups design:

Groups were designed as described in Chapter 2. Rats in the syngeneic and allogeneic groups were transplanted (by Dr. Noriko Murase's group in the Thomas E. Starzl Transplantation Institute) and monitored daily. On day 6, rats were sacrificed by decapitation. Blood samples were obtained from these rats for serum biochemistry. The occurrence of rejection was monitored by general health and well being of these animals and by liver biochemistry and histopathology. Livers were harvested on day 6 and stored at –80°C. Microsomes from those harvested organs were prepared as shown in chapter 2 and stored at –80°C.

Determination of enzyme activity:

Microsomal proteins were obtained by differential centrifugation. Microsomal proteins were incubated with different substrates (testosterone for CYP3A2, flurbiprofen for CYP2C11, Chlorzoxazone for CYP2E1 and MPA for UGT1A9) as described in chapter 2.

Determination of enzyme expression:

Enzyme protein expression for each enzyme was evaluated by Western blotting as described in chapter 2.

Determination of mRNA expression:

Total RNA was isolated using Trizol reagent and mRNA expression was characterized by microarray analysis as described in chapter 2.

Statistical Analysis:

Sample size was chosen based on power calculation using Pass software version 6.0 (NCSS, Kaysville, UT). At $\alpha = 0.05$, the proposed sample size of $n = 4$ rats will allow for the achievement of a power $\geq 80\%$ for all the enzymes studied. After log transformation of the data, one way ANOVA/Tukey test was used to evaluate the presence of significant differences between the groups at a p-value ≤ 0.05. Correlation between the enzyme activity and protein expression was determined using Pearson and Spearman correlation analysis using Prism software version 3.02 (GraphPad Software Inc., San Diego, CA).

Results:

Characterization of rejection (histopathology and biochemistry):

The presence of acute rejection was assessed by histopathology and biochemistry. In the control group, the morphological state of the hepatic architecture appeared normal. Morphological zonation of the hepatic units and morphology of hepatocytes and sinusoidal cells were normal. Portal triad and bile ducts were also normal. There were no mononuclear Cells (MNC) infiltration in the liver. In the syngeneic transplant group, the liver architecture was modified by increased number of bile ducts per portal triad. Portal areas were infiltrated by MNC and less by granulocytes, however, there was only minimal damage associated with the infiltration. There were some infiltrating cells (MNC and granulocytes) in morphological contact with sinusoidal cells. Occasionally, there were small necrotic foci, randomly scattered within parenchyma, which was often accompanied by neutrophilic infiltration. However, the volume of this necrotic damage does not suggest any functional impact. Sinusoidal cells are morphologically activated, with some characteristics of pathology (degeneration, necrosis/apoptosis, and detachment). The rate of hepatocytes proliferation was higher than normal. In summary, the liver pathology corresponds to syngeneic orthotopic liver transplantation (OLTx). In the allogeneic transplant group, the liver architecture was significantly modified by enlargement of the pericentral and periportal areas predominantly due to accumulation of MNC and less of granulocytes. There was significant disarrangement of hepatic plates. Glisson' capsule in some areas is thickened and infiltrated. Significant necrotic damage was present in all specimens. This damage involved both parenchymal and non-parenchymal cells. Almost all sinusoidal cells were necrotic, degenerating or had already disappeared. This damage was accompanied by significant MNC and less by granulocyte infiltration. Most of the vessels showed endothelial damage and detachment.

Perivascular accumulation and intravascular adhesion/accumulation of MNC, predominantly macrophages, also were evident in all vessels. All bile ducts showed signs of damage. In conclusion, this pathology corresponds to the acute rejection of the allogeneic OLTx.

Figures 1 and 2 show the results of serum biochemistry in different groups. Serum AST and bilirubin were elevated in the allogeneic transplant group in comparison to syngeneic transplant group and control group.

mRNA expression of different cytokines in rat livers:

Table 6 shows the mRNA expression of different cytokines in liver tissues from rats in syngeneic transplant group and allogeneic transplant group in comparison to the control group as measured by DNA microarrays. Cytokine levels were increased marginally in liver tissues from rats in the syngeneic transplant group and markedly in the liver tissues from allogeneic transplant group.

mRNA expression of inducible nitric oxide synthase (iNOS) and anti oxidant enzymes:

The mRNA of iNOS was not changes in both syngeneic and allogeneic transplant groups as compared to control (103 and 104% as compared to control, respectively). The mRNA expression for glutathion-s-transferase (GST) was 97 and 35% for syngeneic and allogeneic transplant groups, respectively. The mRNA expression for Catalase (CAT) was 102 and 14% for syngeneic and allogeneic transplant groups, respectively. The mRNA expression for superoxide dismutase (SOD) was 52 and 52% for syngeneic and allogeneic transplant groups, respectively. Finally, the mRNA expression for heme oxygenase (HO) was 474% and 895 for syngeneic and allogeneic transplant groups, respectively.

CYP450 contents in rat livers:

Table 7 shows the CYP450 contents in the livers from three different groups of rats. All groups were significantly different from each other. The CYP450 contents were reduced in the syngeneic transplant group to 43% of control. Acute rejection of the liver further reduced the CYP450 contents to 24% of control.

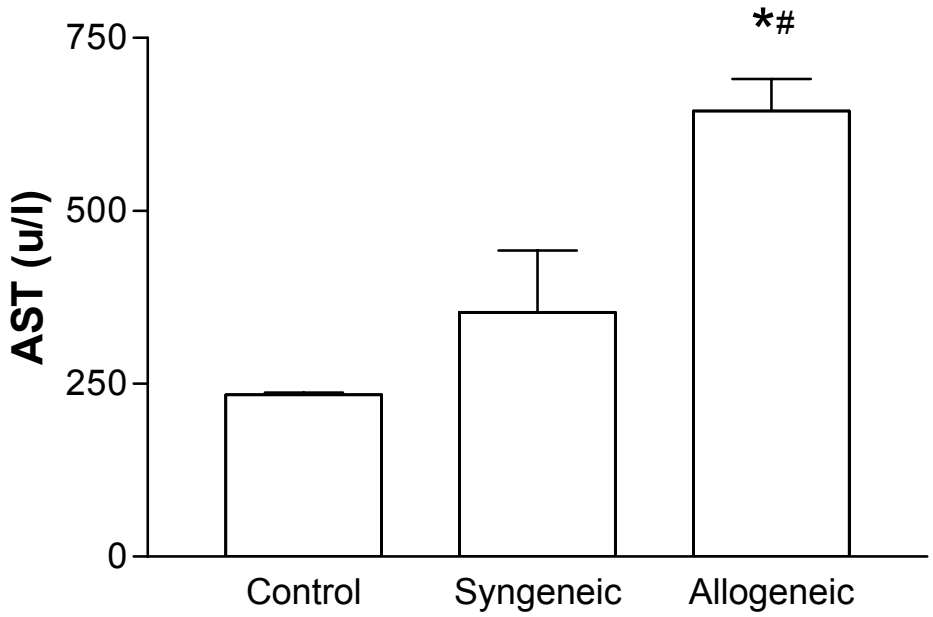

Figure 1. Serum levels (mean ± SE) of Aspartate Aminotransferase (AST) in rats in control group, syngeneic transplant group and allogeneic transplant group (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

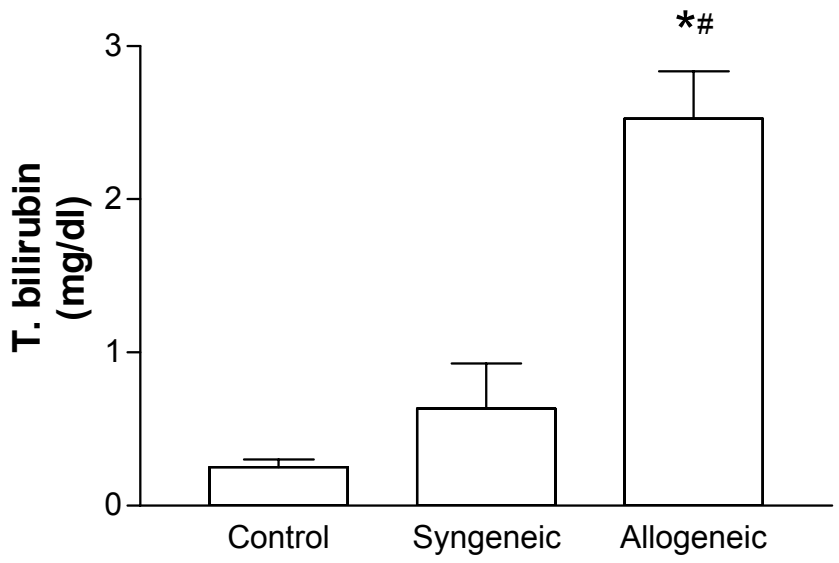

Figure 2. Serum levels (mean ± SE) of total bilirubin in rats in control group, syngeneic transplant group and allogeneic transplant group (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

Table 6. mRNA expression of different cytokines in liver tissues from rats in syngeneic transplant group and allogeneic transplant group in comparison to control group.

Cytokine/group	Syngeneic	Allogeneic
• Pro-inflammatory: IL-1α, IL-1β, 1L-12, IL-15, IL-18, TNF-α • Anti-inflammatory: IL-13	↔	↑↑
• Pro-inflammatory: IFN-γ • Anti-inflammatory: TGF-β	↑	↑↑
• Pro-inflammatory: IL-2	↑	↑
• Pro-inflammatory: IL-5, IFN-α • Anti-inflammatory: IL-4, TGF-α	↑	↔

↔ indicates no difference from control.

↑ indicates at least 30% difference from control

↑↑ indicates at least 30% from syngeneic and 60% difference from control

Table 7. CYP450 content in livers from rats in control, syngeneic transplant and allogeneic transplant groups (n=4 for each group).

	Control	Syngeneic	Allogeneic
P450 content (nmol/mg protein)	0.70 ± 0.07	0.30 ± 0.06*	0.17 ± 0.01* #

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

* Significantly different from control group.

Significantly different from syngeneic transplant group.

CYP3A2 enzyme activity, protein expression and mRNA expression in rat livers:

Figure 3 shows the CYP3A2 enzyme kinetics in the microsomes from the livers of rats in three different groups. The Vmax in the syngeneic group was significantly reduced (30% of control). Acute rejection further reduced the Vmax for CYP3A2 to 10% of control (Figure 4). The Vmax was significantly different in all the groups. The Km values were 57±14 μM for control, 118±22 μM for syngeneic and 52±9 μM for allogeneic group. There were no significant differences in Km values between the control and the allogeneic groups or the control and the syngeneic groups. The Km value was significantly higher in the syngeneic group compared to the allogeneic group. The Cl_{int} values were 131±19 for control, 21±7 for syngeneic transplant group and 12±3 μl/min/mg protein for allogeneic transplant group. The Cl_{int} for syngeneic and allogeneic groups were significantly different from control.

The CYP3A2 protein expression was also reduced, but not to the same magnitude as the enzyme activity (Figure 5). Protein expression was not different between control and syngeneic groups (75% of control) but the allogeneic group was significantly different from control (25% of control). There was a significant (r^2 = 0.77) correlation between enzyme activity and protein expression when data from all the groups was evaluated (Figure 6). The mRNA expression of CYP3A2 was reduced to 75% and 6% of the control in the syngeneic transplant group and the allogeneic transplant group, respectively.

CYP2C11 enzyme activity, protein expression and mRNA expression in rat livers:

Figure 7 shows CYP2C11 enzyme kinetics in the microsomes from the livers of rats in three different groups. The activity of the syngeneic group was reduced to 37% of the control. Acute

rejection further reduced the CYP2C11 activity to 11% as shown in Figure 8. The Km values were 36±24 μM for control, 44±16 μM for syngeneic transplant and 60±16 μM for allogeneic transplant group. The Km values were only significantly different between control and allogeneic groups. The Cl_{int} values were 14±4 for control, 5±1 for syngeneic transplant and 1±0.1 μl/min/mg protein for allogeneic transplant group. The Cl_{int} values were all significantly different from each other.

The CYP2C11 protein expression was not significantly different between syngeneic and control groups. The CYP2C11 protein expression was significantly reduced in the allogeneic group (18% of control) as shown in Figure 9. The correlation between the CYP2C11 activity and enzyme protein expression was significant (r^2 = 0.74) as shown in Figure 10. The mRNA expression was reduced to 11% and 0% of control values for syngeneic and allogeneic groups, respectively.

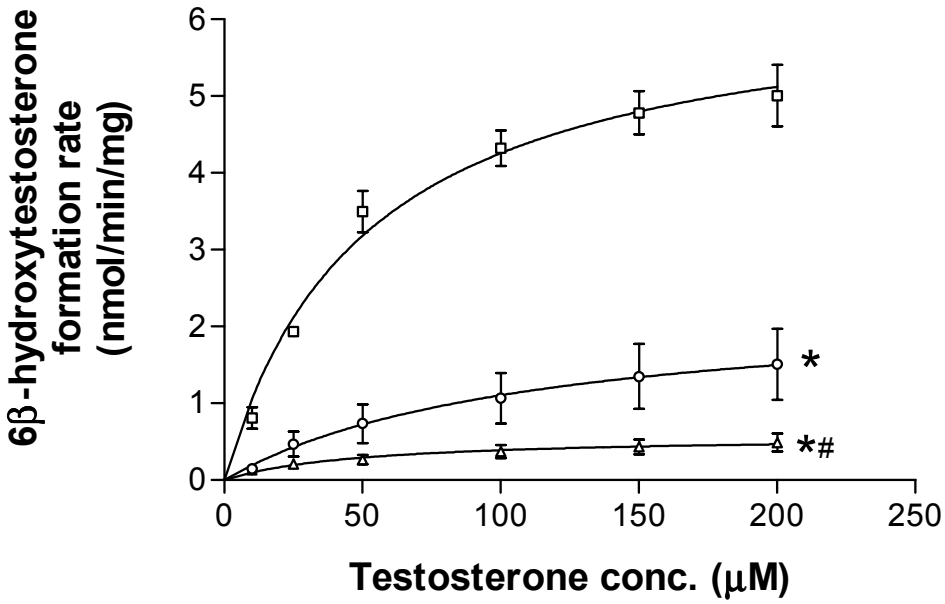

Figure 3. CYP3A2 enzyme activity as measured by the formation of 6β-hydroxytestosterone (nmol/min/mg protein) from testosterone in liver microsomes from rats in control group (□), syngeneic transplant group (○) and allogeneic transplant group (Δ) (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group at the highest concentration used.**

 # Significantly different from syngeneic transplant group at the highest concentration used.

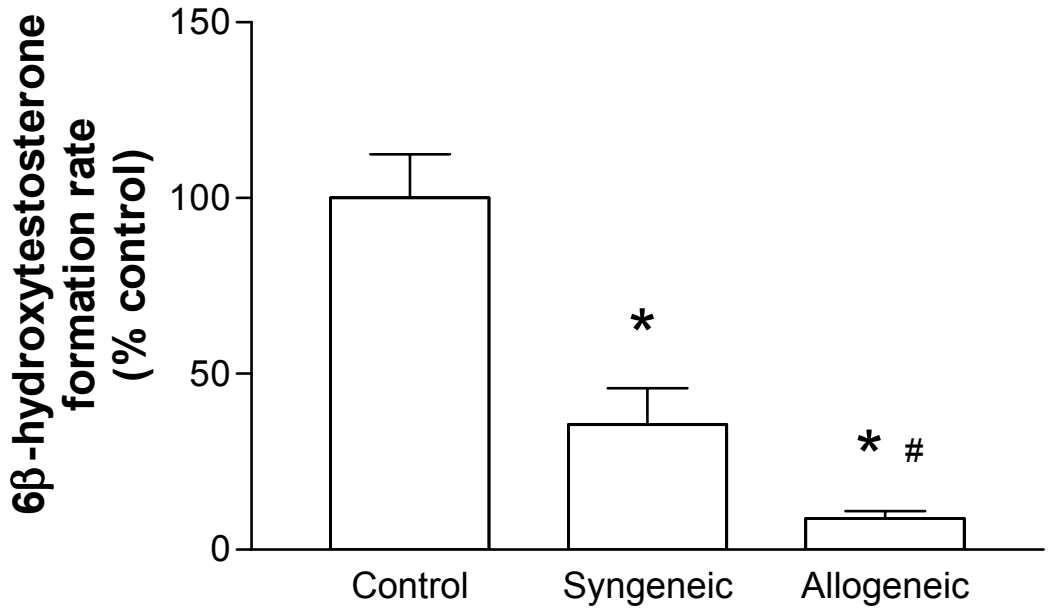

Figure 4. Vmax for CYP3A2 as measured by the formation of 6β-hydroxytestosterone (nmol/min/mg protein) from testosterone in liver microsomes from rats in control group, syngeneic transplant group and allogeneic transplant group. Control = 6.68 ± 0.83 nmol/min/mg protein (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

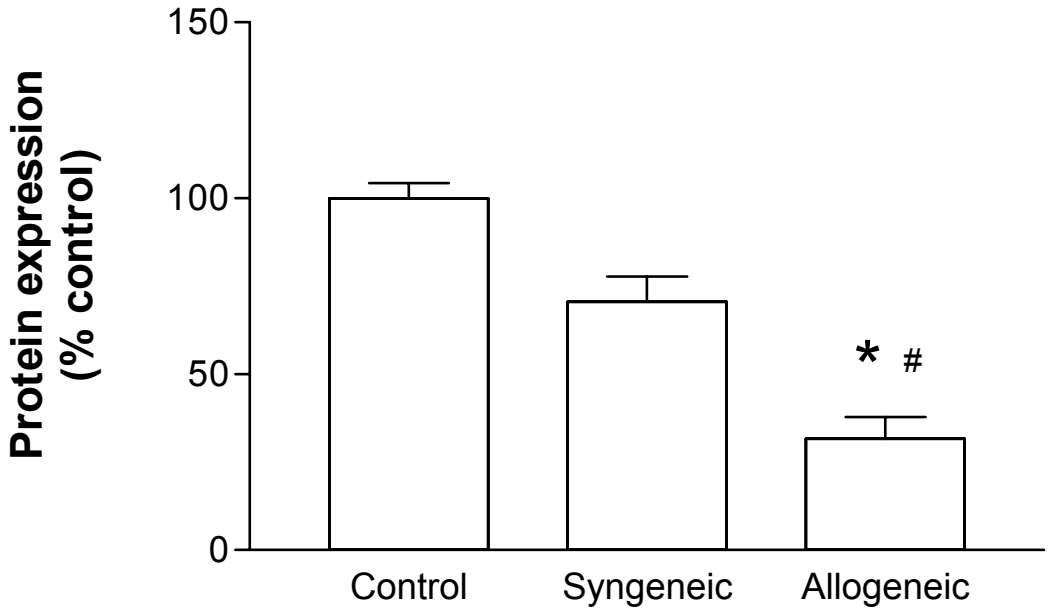

Figure 5. CYP3A2 protein expression in livers from rats in syngeneic transplant group and allogeneic transplant group expressed as a percentage of control group (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

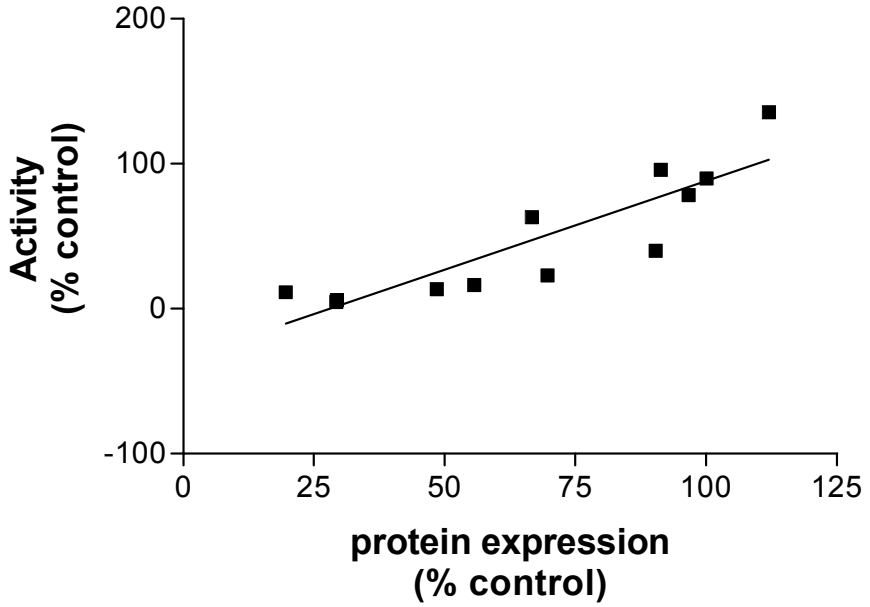

Figure 6. Correlation of CYP3A2 activity and CYP3A2 protein expression in livers from rats in control group, syngeneic transplant group and allogeneic transplant group (Pearson $r^2 = 0.77$, p = 0.0002 and Spearman r = 0.94, P ‹ 0.0001).

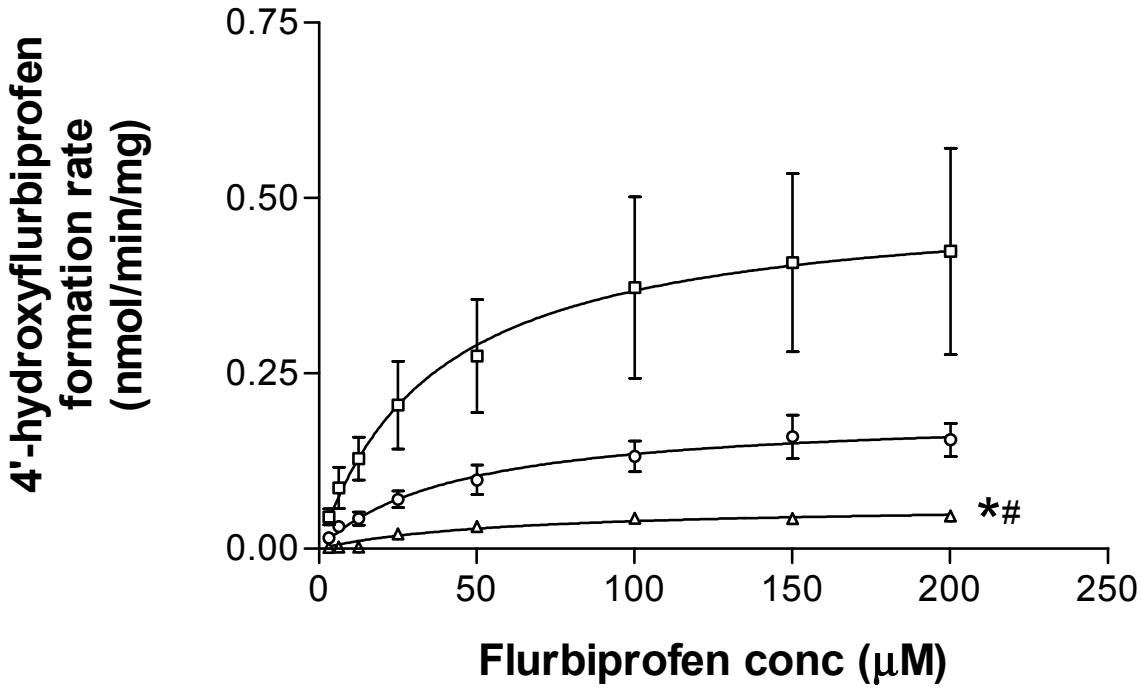

Figure 7. CYP2C11 Activity as measured by the formation of 4'-hydroxyflurbiprofen (nmol/min/mg protein) from flurbiprofen in liver microsomes from rats in control group (□), syngeneic transplant group (○) and allogeneic transplant group (Δ) (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group at the highest concentration used.**

 # Significantly different from syngeneic transplant group at the highest concentration used.

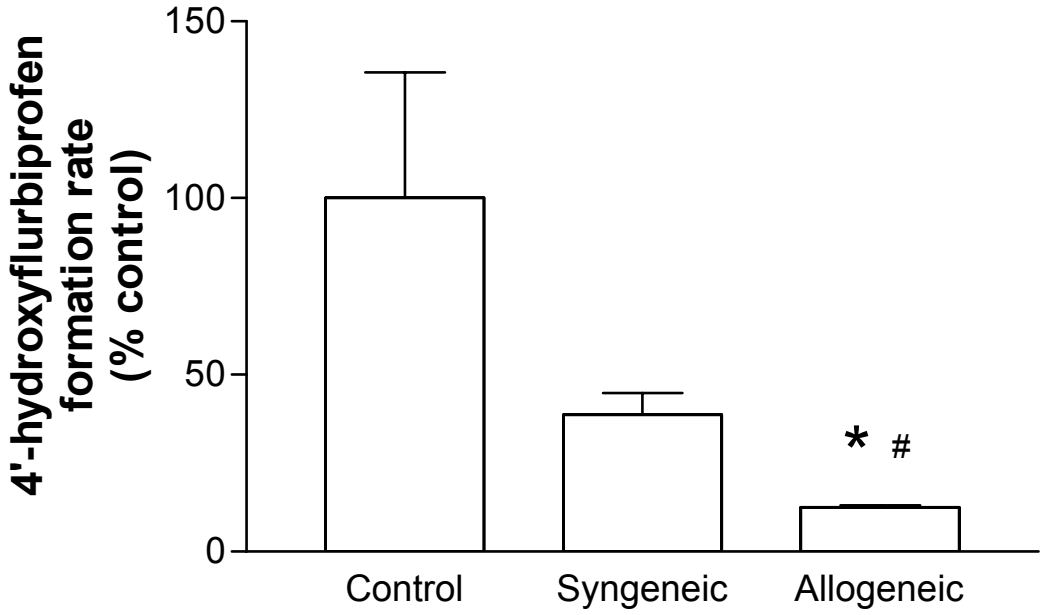

Figure 8. Vmax for CYP2C11 enzyme as measured by the formation of 4'-hydroxyflurbiprofen (nmol/min/mg protein) from flurbiprofen in liver microsomes from rats in control group, syngeneic transplant group and allogeneic transplant group. Control = 0.50±0.18 (nmol/min/mg protein) (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

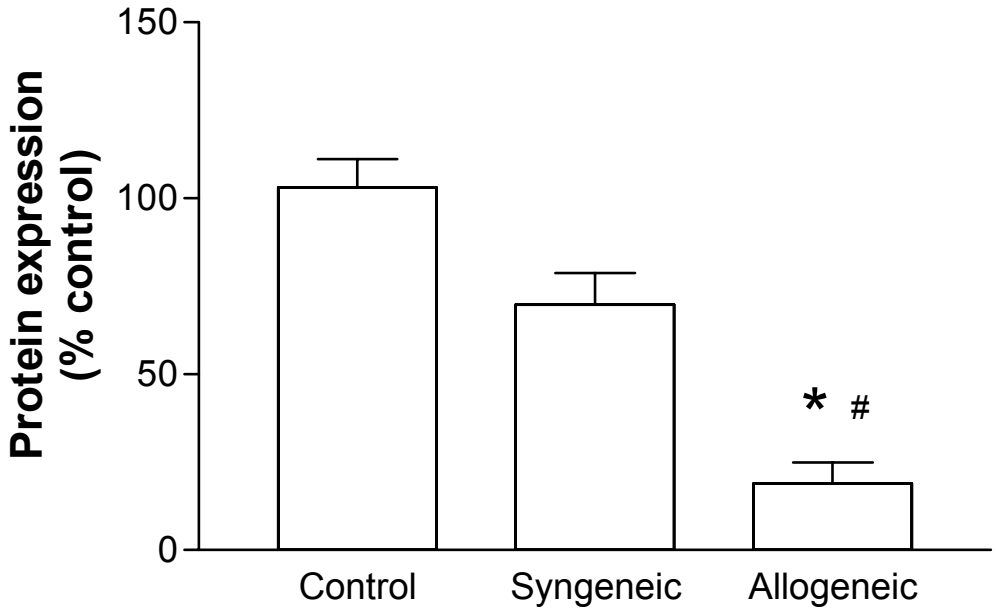

Figure 9. CYP2C11 protein expression in livers from rats in syngeneic transplant group and allogeneic transplant group expressed as a percentage of control group (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

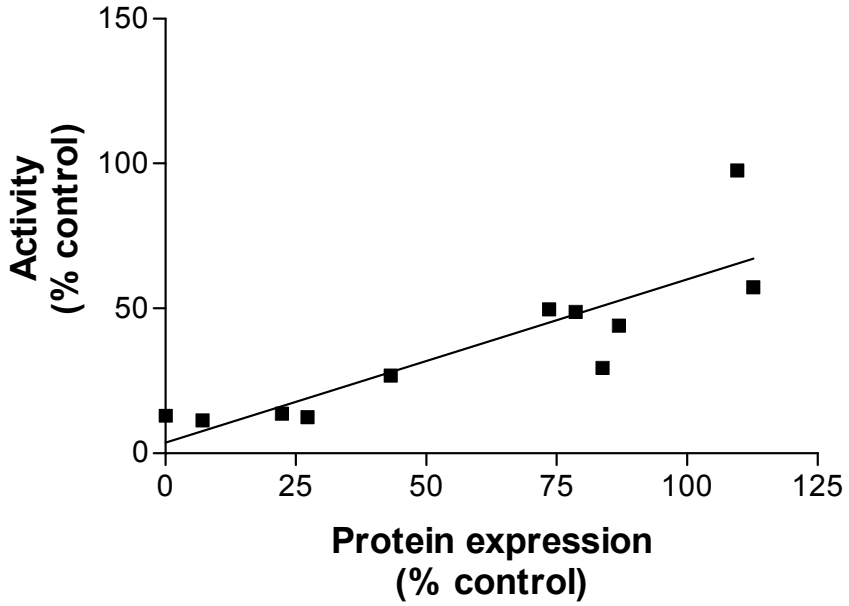

Figure 10. Correlation between CYP2C11 Activity and CYP2C11 protein expression in control group, syngeneic transplant group and allogeneic transplant group (Pearson r^2= 0.74, p = 0.0007 and Spearman r = 0.86, P = 0.0006).

CYP2E1 enzyme activity, protein expression and mRNA expression in rat livers:

Figure 11 shows CYP2E1 enzyme kinetics in the microsomes from the livers of rats in three different groups. The activity in the syngeneic group was significantly reduced to 28% of control. Acute rejection further reduced the CYP2E1 activity in the allogeneic group to 8%. There was a significant difference between the activity in the allogeneic and syngeneic groups (Figure 12). Km values were 157±12 μM for control, 204±16 μM for syngeneic transplant and 213±9 μM for the allogeneic transplant group. Km was significantly different between control and allogeneic groups. The Cl_{int} values were 27±1 for control, 6±1 for syngeneic transplant and 2±0 μl/min/mg protein for allogeneic transplant group. The Cl_{int} values were all significantly different from each other.

The CYP2E1 protein expression in the syngeneic transplant group was not significantly different from control group (87% of control) as shown in Figure 13. Acute rejection reduced the CYP2E1 protein expression to 51% of the control. Correlation between the activity and the protein expression was significant ($r^2 = 0.67$) as in Figure 14. The mRNA was reduced to 27 and 11% for syngeneic and allogeneic groups, respectively.

UGT1A9 enzyme activity, protein expression and mRNA expression in rat livers:

Figure 15 shows UGT1A9 enzyme kinetics in the microsomes from the livers of rats in three different groups. The syngeneic group was not significantly different from control (65% of control). Acute rejection significantly reduced the activity in the allogeneic group to 26% of the control as seen in Figure 16. Km values were 720±63 μM for control, 599±64 μM for syngeneic and 566±85 μM for allogeneic group. None of the values were significantly different from each

other. The Cl_{int} values were 32±2 for control, 26±4 for syngeneic transplant and 11±3 µl/min/mg protein for allogeneic transplant groups. The Cl_{int} values were significantly different between control and allogeneic and between allogeneic transplant and syngeneic transplant groups. Expression of UGT1A9 could not be evaluated since the antibodies that react with the rat UGT1A9 or even react with the whole UGT1A family are not readily available. However, mRNA expression for the whole UGT family was increased to 179 % of control in syngeneic and reduced to 71% of control in allogeneic group. Table 8 shows summary of the changes seen in both syngeneic and allogeneic groups in all the enzymes studied.

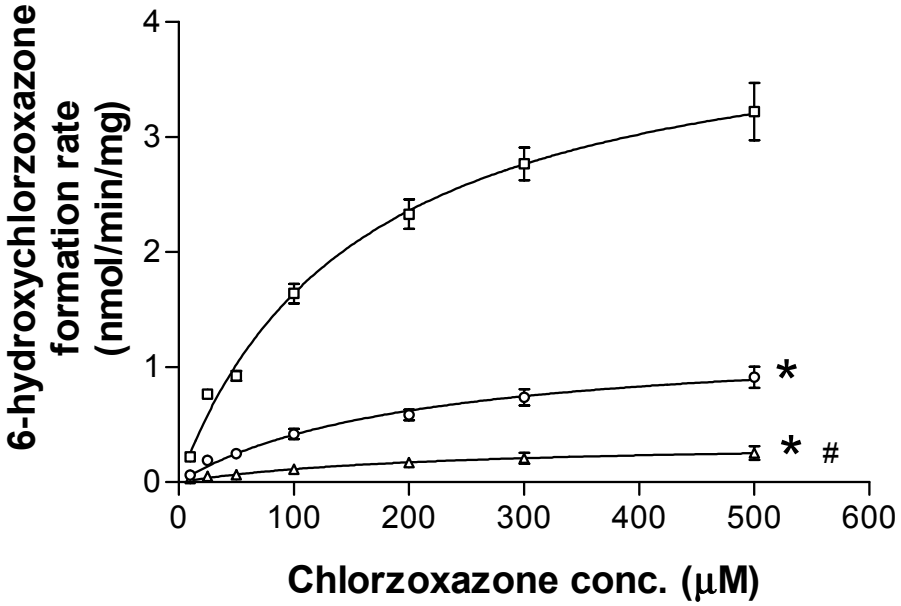

Figure 11. CYP2E1 enzyme activity as measured by the formation of 6-hydroxychlorzoxazone (nmol/min/mg protein) from chlorzoxazone in liver microsomes from rats in control group (□), syngeneic transplant group (○) and allogeneic transplant group (Δ) (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group at the highest concentration used.**

 # Significantly different from syngeneic transplant group at the highest concentration used.

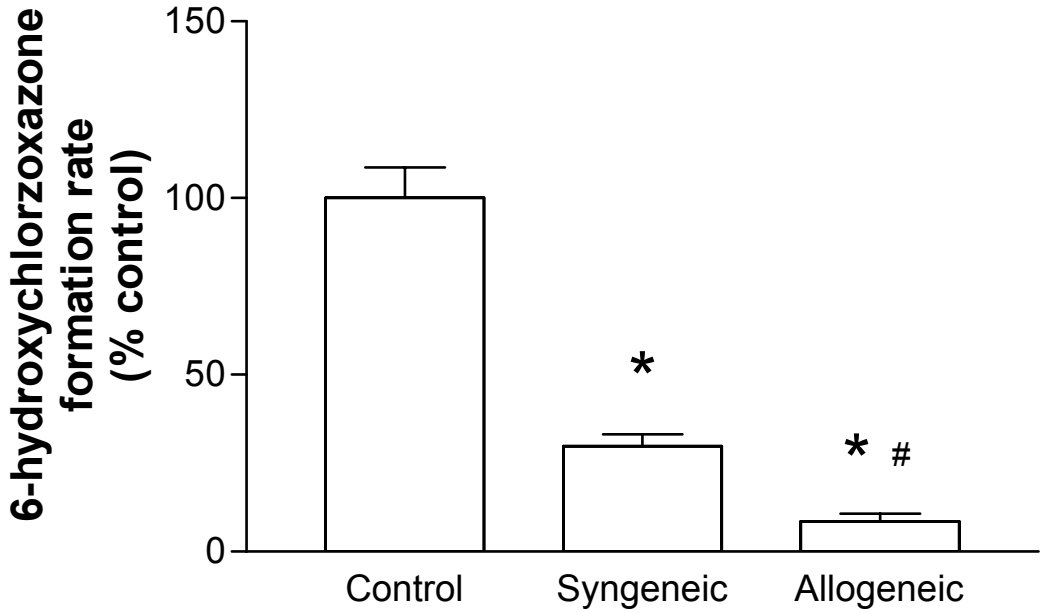

Figure 12. Vmax for CYP2E1 enzyme as measured by the formation of 6-hydroxychlorzoxazone (nmol/min/mg protein) from chlorzoxazone in liver microsomes from rats in control group, syngeneic transplant group and allogeneic transplant group. Control= 4.23±0.37 nmol/min/mg protein (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

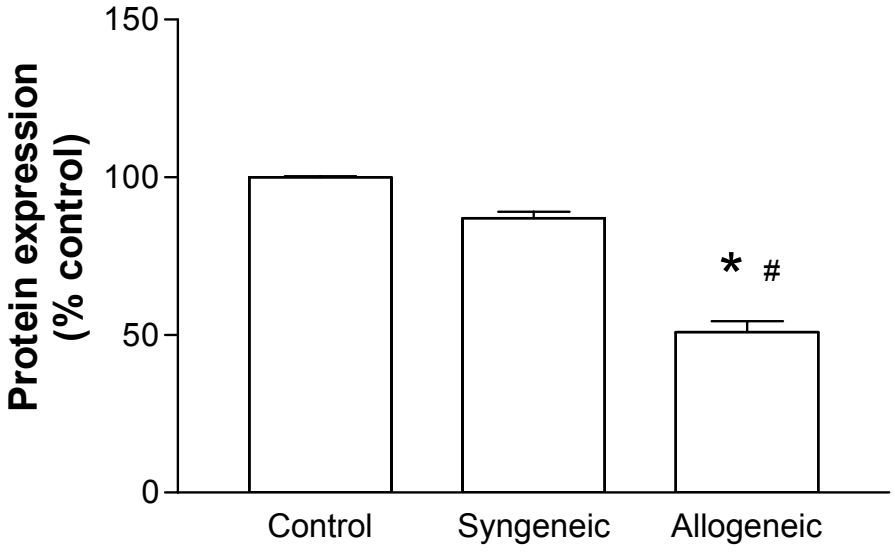

Figure 13. CYP2E1 protein expression in syngeneic transplant group and allogeneic transplant group expressed as a percentage of control (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

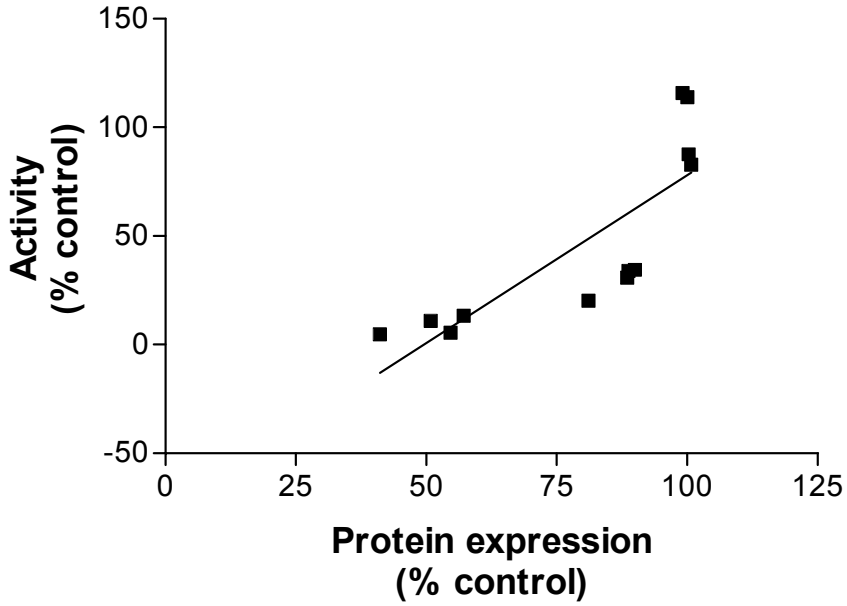

Figure 14. Correlation between CYP2E1 activity and CYP2E1 protein expression in livers from rats in control group, syngeneic transplant group and allogeneic transplant group (Pearson r^2=0.67, p = 0.0012 and Spearman r = 0.92, P ‹ 0.0001).

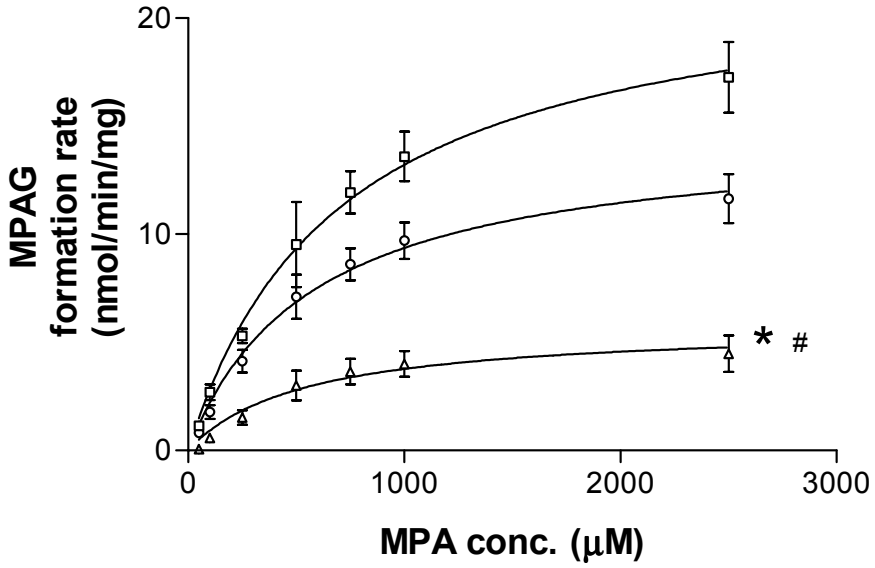

Figure 15. UGT1A9 enzyme activity as measured by the formation of MPAG (nmol/min/mg protein) from MPA in liver microsomes from rats in control group (□), syngeneic transplant group (○) and allogeneic transplant group (Δ) (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group at the highest concentration used.**

 # Significantly different from syngeneic transplant group at the highest concentration used.

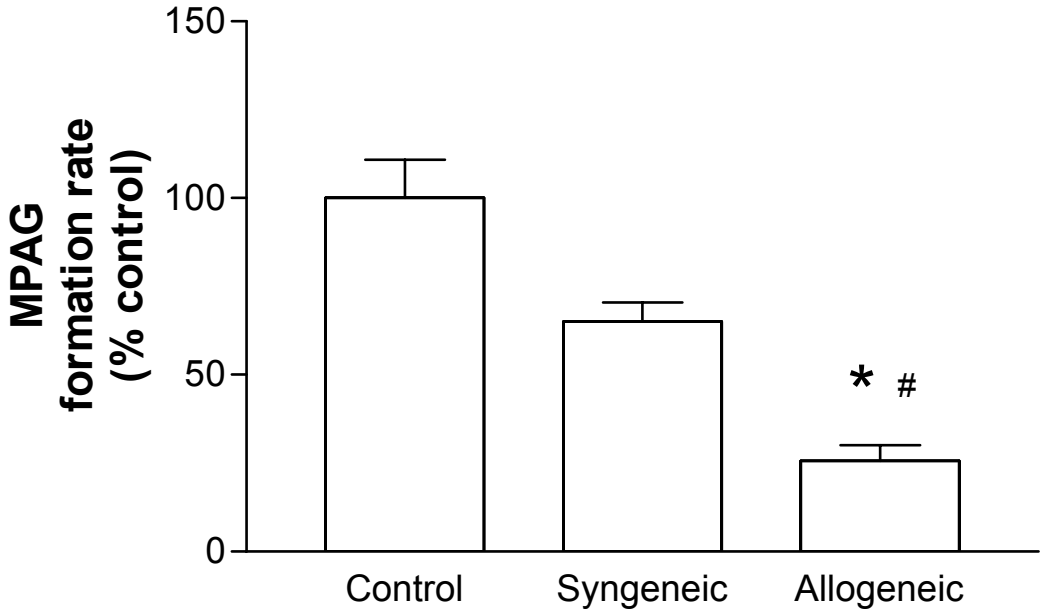

Figure 16. Vmax for UGT1A10 enzyme as measured by the formation of MPAG (nmol/min/mg protein) from MPA in liver microsomes from rats in control group, syngeneic transplant group and allogeneic transplant group. Control= 22.76±2.45 nmol/min/mg protein (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

Table 8. Summary of the changes in enzymes mRNA expression, protein expression and activity seen in syngeneic transplant and allogeneic transplant groups as compared to control group.

parameter	Group/enzyme	CYP3A2	CYP2C11	CYP2E1	UGT1A9
mRNA	Syngeneic	↔	↓	↓	↑
	Allogeneic	↓	↓	↓	↔
Protein content	Syngeneic	↔	↔	↔	NA
	Allogeneic	↓	↓	↓	NA
Activity (Vmax)	Syngeneic	↓	↔	↓	↔
	Allogeneic	↓↓	↓	↓↓	↓
Cl_{int}	Syngeneic	↓	↓	↓	↔
	Allogeneic	↓	↓↓	↓↓	↓

NA = no available antibody.

For Syngeneic group:

↔ indicates no difference from control.

↑ indicates significant increase from control.

↓ indicates significant decrease from control.

For Allogeneic group:

↔ indicates no difference from control.

↓ indicates significant decrease from control (in case syngeneic is also ↓) or from syngeneic (in case syngeneic is ↔) .

↓↓ indicates significant decrease from syngeneic and control.

Discussions:

Organ transplantation is an accepted therapy for diseases that result in chronic irreversible failure of organs such as liver, kidney, intestine, heart and lung. Organ transplant patients often receive multiple drug therapy that includes immunosuppressive drugs, antibiotics, antiviral agents, antifungal agents, antihypertensive agents and others. We have observed large variations in the pharmacokinetics of several drugs used in transplant patients. Following transplantation, patients undergo marked changes in their physiology, that may cause changes in absorption (due to changes in GI motility, altered splanchnic blood flow, change in bile composition and flow, changes in acid or enzymes secretion and presence of concomitant drugs), distribution (due to changes in organ blood flow, body fluid content, tissue partitioning and plasma protein binding) or elimination of drugs (due to changes in the intrinsic activity of the enzymes in the eliminating organs, the blood flow to the organ and the blood protein binding of the drug) (Venkataramanan *et al.* 1989).

During transplantation of an organ, the transplanted organ is subjected to two inflammatory processes that can potentially affect its function. Both of these processes involve the release of several cytokines. One of these processes is alloantigen-independent process, while the other is alloantigen-dependent process. The alloantigen-independent inflammatory process starts within the transplanted organ itself as a result of different conditions to which the organ is subjected such as surgery, cold ischemia, preservation, warm ischemia and reperfusion injury. The other, alloantigen-dependent process results from the recognition of the alloantigen by the host T-cells which can result in damage and eventual rejection of the transplanted organ (Bumgardner and Orosz 1999). As described in chapter 1, rejection which is categorized according to the time of

onset and the type of histological changes can be one of three types; hyperacute, acute or chronic rejection. During acute rejection of the liver, when several of the inflammatory mediators are released in the body, the clearance of antipyrine is significantly decreased in liver transplant patients (unpublished observations). In addition, in rats acute rejection of the liver also leads to impaired clearance of tacrolimus and reduced biliary excretion of BSP (unpublished observations). A systematic evaluation of the various factors that contribute to the alteration in the metabolism of drugs in transplant patients have not been carried out.

In the present study, rats in the syngeneic transplant group are subjected to surgery, altered blood flow to the liver (no hepatic arterial blood supply), ischemia and reperfusion injuries. Thus, differences observed between this group and the control group are due to the effect of all these processes together. On the other hand, rats in the allogeneic transplant groups are subjected to all processes mentioned in the syngeneic groups and in addition are subjected to acute rejection of the transplanted allograft. Thus, differences observed between the syngeneic and allogeneic groups are due to the process of graft rejection.

Ischemia and reperfusion injury are associated with the generation of several cytokines and other inflammatory mediators. During rejection, the immune system is activated to a higher degree and more cytokines are released in the body. Most of these cytokines and some of their receptors were primarily detectable in allogeneic transplant group and to a minor extent in syngeneic transplant group.

For CYP3A2, the reduction in the mRNA was only significant in the allogeneic transplant group. The reduction in mRNA expression resulted subsequently in a reduction in the protein expression in the allogeneic transplant group. The magnitude of this reduction in protein expression was less than the magnitude of reduction in the mRNA expression, perhaps related to the decreased stability of mRNA (or) increased stability of the protein expression. Finally, the CYP3A2 activity was reduced to 30 and 10% as compared to control in both syngeneic and allogeneic transplant groups. There was a significant correlation ($r^2 = 0.77$) between the protein expression and activity of CYP3A2 enzyme. Therefore, at least part of the reduction in CYP3A2 activity is due to reduction in the protein levels. Further decrease in the CYP3A2 activity may indicate involvement of posttranslational factors such as ROIs, which are known to be produced by cytokines and NO. ROIs may cause lipid peroxidation and conformational changes in the enzymes and may render some of the enzyme inactive (Ingelman-Sundberg 1977; Ingelman-Sundberg *et al.* 1981; Engelke *et al.* 1993; Minamiyama *et al.* 1997; Yukawa *et al.* 1999). Down regulation of different cytochrome P450s by the inflammatory cytokines can be a consequence of many processes, mainly the repression of gene transcription which is usually indicated by a reduction in the expression of mRNA. Other processes that can contribute to the down regulation of the metabolic capacity may involve an increase in the turnover of protein (Morgan 1989; Morgan *et al.* 1994) and mRNA (Morgan 1989; Barker *et al.* 1992). In addition, NO which is known to be produced in such conditions, has been shown to down regulate the metabolizing enzymes (Wink *et al.* 1993; Wink and Mitchell 1998). In our study, the mRNA expression of iNOS in liver tissues from both syngeneic and allogeneic transplant groups was not increased as compared to control group. This may exclude NO as a major contributor to the observed reduction in the mRNA of different CYP450 enzymes on the 6th day after transplantation. In the

present study, the mRNA expression of SOD, which converts superoxide radicals to hydrogen peroxides, the mRNA of CAT, which detoxifies the hydrogen and organic peroxides, and the mRNA of glutathione-s-transferase (GST) were all reduced in allogeneic transplant group. The mRNA expression of heme oxygenase (HO) was also increased in the allogeneic group. Heme oxygenase is also an antioxidant enzyme which breakdown the heme into iron, carbon monoxide and biliverdin. Degradation of cytochrome P450 increases the hepatic heme pool and induces the HO (Morgan 1989). When induced, HO can even attack the heme of the P450 enzymes and cause further damage to these enzymes. In addition, when the hepatic heme pool is low as a consequence of induced HO, inhibition of transcription of constitutive cytochrome P450 enzymes can occur (Dore 2002). Our observations are consistent with decreased levels of antioxidant and increased lipid peroxidation in liver transplant patients (Goode *et al.* 1994; Biasi *et al.* 1995).

For CYP2C11, the mRNA expression was reduced to 11 and 0% of control in syngeneic and allogeneic transplant groups, respectively. The protein content was reduced to 18% of control in allogeneic transplant group. But, it was not significantly reduced in the syngeneic transplant group. LPS treatment has been shown to rapidly reduce the CYP2C11 mRNA, without markedly decreasing the CYP2C11 protein (Morgan 1989). This may be a sign of protein stabilization or increase in the rate of translation of the protein from the available mRNA. This may also simply reflect a non-steady state condition of mRNA expression and subsequent protein synthesis. Cytokines such as IL-β, IL-2, IL-6, IFN- α and TNF-α have been shown to reduce the protein levels of CYP2C11 in rats. This reduction was parallel to the reduction in the mRNA level (Chen *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carlson and Billings 1996). Finally, the activity

was decreased to 11 and 37% of control in syngeneic and allogeneic transplant groups, respectively. There was a significant correlation ($r^2 = 0.74$) between the protein expression and the activity of CYP2C11, which indicates that part of the reduction in the activity is due to reduction in protein expression. However, other factors such as ROIs may also be involved in further reduction in CYP2C11 activity.

For CYP2E1, the mRNA expression was reduced to 27 and 11% as compared to control in both syngeneic and allogeneic transplant group, respectively. This reduction in mRNA expression did not result in a significant change in the protein expression in the syngeneic group and only reduced the protein expression in the allogeneic group to 51% as compared to control. This reduction in protein expression in allogeneic group suggests an involvement of pretranslational mechanism as a result of effect of cytokines on the transcription of this enzyme. In other studies, IL-1 and IL-6 have been shown to cause a reduction in mRNA levels of CYP2E1 (Morgan *et al.* 1994). In our study, protein expression of CYP2E1 was preserved more than all other P450 enzymes studied (CYP2C11 and CYP3A2). This finding is similar to what is known about regulation of CYP2E1, which is regulated to a high extent at the protein level (De Waziers *et al.* 1995; Hu *et al.* 1995; Lieber 1997). This may be a sign of protein stabilization in both groups. Increased rate of translation of the protein from the available mRNA can not be excluded. Finally, the reduction in activity was greater than the reduction in protein expression (28 and 8% for syngeneic and allogeneic groups, respectively) which may be indicative of protein inactivation by ROIs as described earlier for other enzymes. Correlation between protein expression and enzyme activity was significant ($r^2 = 0.67$), but r^2 was less than other enzymes studied.

The activity of UGT1A9 was only significantly reduced in the allogeneic group (26% of the control). In pig hepatocytes, IL-1, IL-6 and TNF-α has been shown to decrease the UGTs mediated metabolism of acetaminophen, morphine and 1-naphthol (Monshouwer *et al.* 1996). Protein expression of UGT1A9 could not be evaluated since the antibodies that react with the rat UGT1A9 or even the react with the whole UGT1A family are not readily available. The mRNA for the whole UGT family was increased in the syngeneic transplant group to 179% of control and it was reduced to 71% in the allogeneic transplant group. The reduction in the activity of phase II enzymes is less pronounced as compared to the phase I enzymes, which is consistent with what is known about phase II enzyme and its preservation as compared to phase I enzymes in different diseases of the liver (Shull *et al.* 1976; Kraus *et al.* 1978; Patwardhan *et al.* 1981; Ochs *et al.* 1986).

In summary and as shown in Table 8, alloantigen-independent inflammation and altered blood flow as studied in our syngeneic liver transplant group caused selective reduction in mRNA in the liver. However, this reduction did not significantly alter the protein levels of the CYP450 enzymes studied. On the other hand, occurrence of rejection (in addition to factors mentioned for the syngeneic transplant) resulted in a marked reduction in mRNA due to the presence of higher concentration of several cytokines in the allogeneic livers. This reduction in mRNA resulted in significant reduction in protein levels of all CYP450 enzymes studied. Finally, the enzymes in both syngeneic and allogeneic groups appear to be inactivated by the presence of perhaps ROIs. This inactivation was higher in case of allogeneic group due to the inhibition of several antioxidant enzymes in the allogeneic groups and the induction of HO in both groups. In case of

UGTs, the mRNA was reduced in the allogeneic transplant group but it was increased in the syngeneic transplant group, while the activity was only decreased in the allogeneic group. This differential effect between phase I and phase II, and even between different members of phase I enzymes exclude the mechanisms that considers decrease in mass of functional hepatocytes as the main reason for the observed effects of acute liver rejection on drug metabolism.

Including a sham group will add valuable information to the data we obtained. It will allow us to evaluate the effect of the surgery alone. Thus, the difference between our syngeneic transplant group and the proposed sham group will reflect the effect of other antigen-independent process together with the altered blood flow to the liver.

To further evaluate the involvement of various cytokines on down regulating the drug metabolizing capacity of the liver, we studied the effect of several cytokines on the activity of CYP3A4, the major enzyme responsible for hepatic drug metabolism of most of the drugs, using primary culture of human hepatocytes (Chapter 7).

Chapter 4

Effect of acute rejection of liver in rats on the extra-hepatic drug metabolizing enzymes activity and expression

Introduction:

Acute rejection of liver is an immune cell-mediated pathological inflammatory process which is characterized by the release of immune mediators such as cytokines. Many of these cytokines are known to inhibit drug metabolizing capacity of different enzymes. In chapter 3 of this dissertation we have shown that acute rejection of the liver inhibited hepatic drug metabolizing capacity of many enzymes mediating phase I and II metabolic pathways. The magnitude of the effect was different for different enzymes studied.

Recent observations indicate that disease or injury to an organ results not only in dysfunction of that organ, but can also lead to functional alterations in peripheral organs. This is illustrated by the effect of traumatic brain injury (TBI) and renal failure on hepatic drug metabolizing capacity (Chapter 1) (Heinemeyer *et al.* 1986; Wermeling *et al.* 1987; Boucher *et al.* 1991; Toler *et al.* 1993; O'Mara *et al.* 1995; Boucher and Hanes 1998; Poloyac *et al.* 2001). Our objective was to evaluate the effect of acute rejection of liver in rats on the extra-hepatic (kidneys and lungs) metabolic capacity, protein expression and mRNA expression of phase I (renal CYP3A2 and renal CYP2E1) and phase II (renal UGT1A10) drug metabolizing enzymes.

Experimental methods:

The study protocol was approved by the IACUC at the University of Pittsburgh. Male ACI and Lewis rats (170-230 g) were used in the study. ACI-Lewis combinations are strong responders to each other and are the animal model used to study acute rejection process.

Groups were designed as described in Chapter 2. Rats in the syngeneic and allogeneic groups were transplanted and monitored daily. On day 6, rats were sacrificed. Blood samples were obtained from these rats for serum biochemistry. The occurrence of rejection was monitored by general health and well being of these animals and by liver biochemistry and histopathology. Kidneys and lungs were harvested and stored at –80°C. Microsomes from those harvested organs were prepared as shown below and stored at –80°C.

Determination of enzyme activity:

Microsomal proteins were obtained by differential centrifugations. Microsomal proteins were incubated with different substrates (testosterone for CYP3A2, Chlorzoxazone for CYP2E1, and MPA for UGT1A10) as described in chapter 2.

Determination of enzyme expression:

Enzyme protein expression for each enzyme was evaluated by Western blotting as described in chapter 2.

Determination of mRNA expression:

Total RNA was isolated using Trizol reagent and mRNA expression was characterized by microarray analysis as described in chapter 2.

Statistical Analysis:

Sample size was chosen based on power calculation using Pass software version 6.0 (NCSS, Kaysville, UT). At $\alpha = 0.05$, the proposed sample size of $n = 4$ rats will allow the achievement of a power $\geq 80\%$ for all the studied enzymes. After log transformation of the data, one way ANOVA/Tukey test was used to evaluate the presence of significant differences between the groups at a p-value ≤ 0.05. Correlation between the enzyme activity and protein expression was determined using Pearson and Spearman correlation analysis using Prism software version 3.02 (GraphPad Software Inc., San Diego, CA).

Results:

Characterization of rejection (histopathology and biochemistry):

The presence of acute rejection was assessed by histopathology and biochemistry as shown in Figures 1, 2 and (Chapter 3).

mRNA expression of different cytokines in rat kidneys:

Table 9 shows the renal mRNA expression of different cytokines as measured by DNA microarrays.

mRNA expression of anti oxidant enzymes in rat kidneys:

The mRNA expression for glutathion-s-transferase (GST) was 106 and 39% for syngeneic and allogeneic transplant groups, respectively. The mRNA expression for Catalase (CAT) was 107 and 21% for syngeneic and allogeneic transplant groups, respectively. Finally, the mRNA expression for superoxide dismutase (SOD) was 102 and 45% for syngeneic and allogeneic transplant groups, respectively.

CYP3A2 enzyme activity, protein expression and mRNA expression in rat kidneys:

Figure 17 shows the activity of CYP3A2 enzyme in kidney microsomes from three different groups of rats. The activity in the syngeneic transplant group and allogeneic transplant group were significantly different from the control group. However, both activities in syngeneic and allogeneic groups were not different from each other and were reduced to 14% and 9% of control in syngeneic and allogeneic groups, respectively.

Protein expression as shown in Figure 18 was significantly reduced in the allogeneic group compared to control and syngeneic group. There was a significant but poor correlation ($r^2 = 0.44$) between CYP3A4 enzyme activity and protein expression in kidney microsomes (Figure 19). Finally, mRNA expression of this enzyme was also reduced to 81 and 25% of control for syngeneic and allogeneic transplant groups, respectively.

Table 9. Renal mRNA expression of different cytokines in syngeneic transplant group and allogeneic transplant group in comparison to control group.

Cytokine/group	Syngeneic	Allogeneic
• Pro-inflammatory: IL-1α, IL-2, IL-5, IL-12, IL-18, IFN-γ • Anti-inflammatory: 1L-4, IL-13, TGF-β	↔	↑↑
• Pro-inflammatory: IL-12	↑	↔

↔ indicates no difference from control.

↑ indicates at least 30% difference from control

↑↑ indicates at least 30% from syngeneic and 60% difference from control

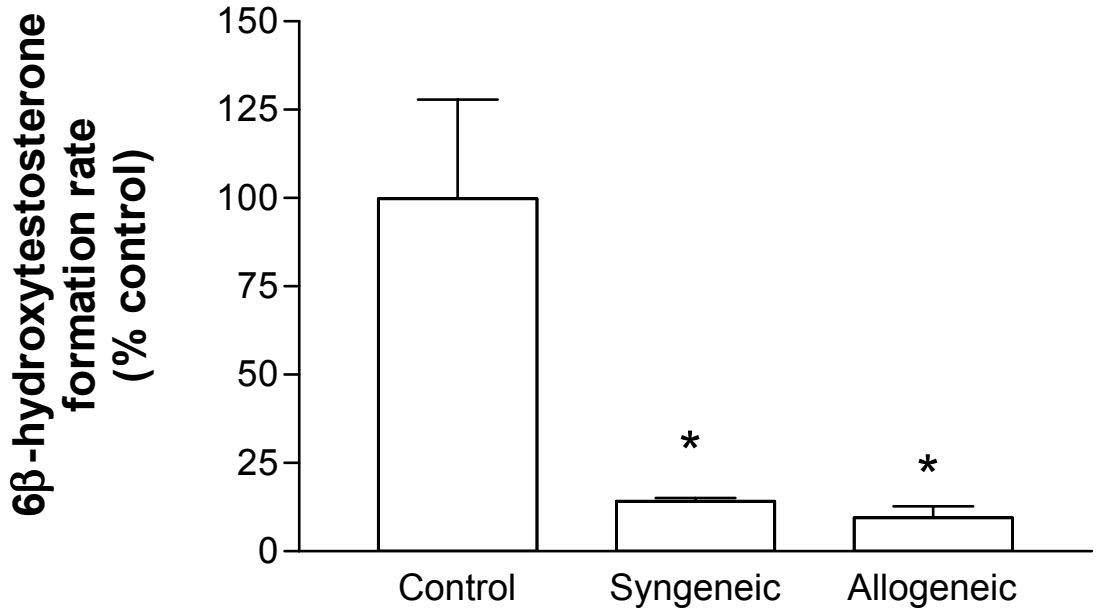

Figure 17. CYP3A2 enzyme activity as measured by the formation of 6β-hydroxytestosterone (nmol/min/mg protein) from testosterone in kidney microsomes from rats in control group, syngeneic transplant group and allogeneic transplant group expressed as a percentage of control group. Control= 0.116±0.065 nmol/min/mg protein (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

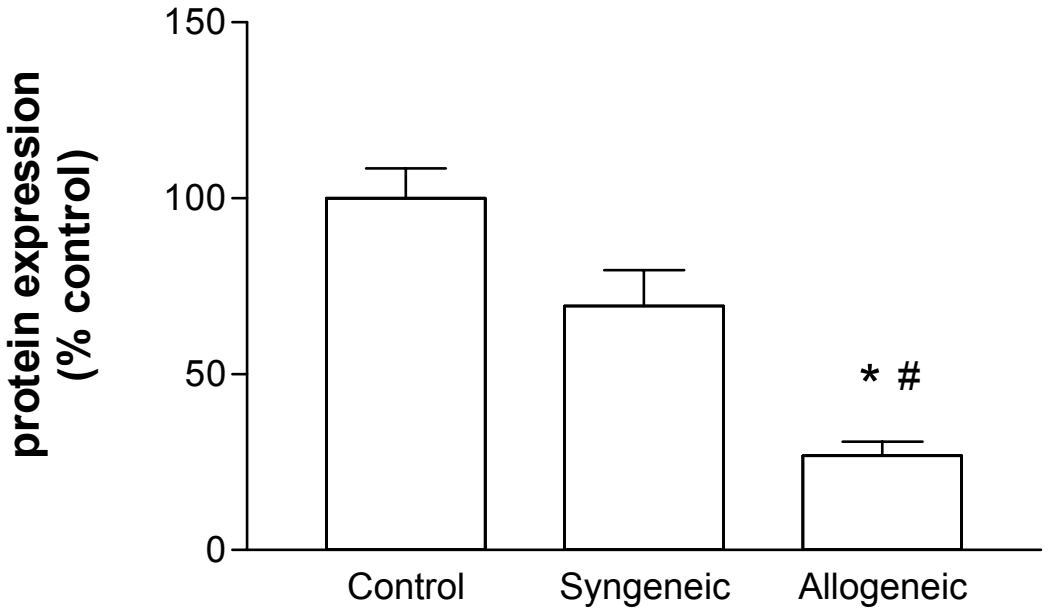

Figure 18. CYP3A2 protein expression in kidneys from rats in syngeneic transplant group, allogeneic transplant group expressed as a percentage of control group.

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

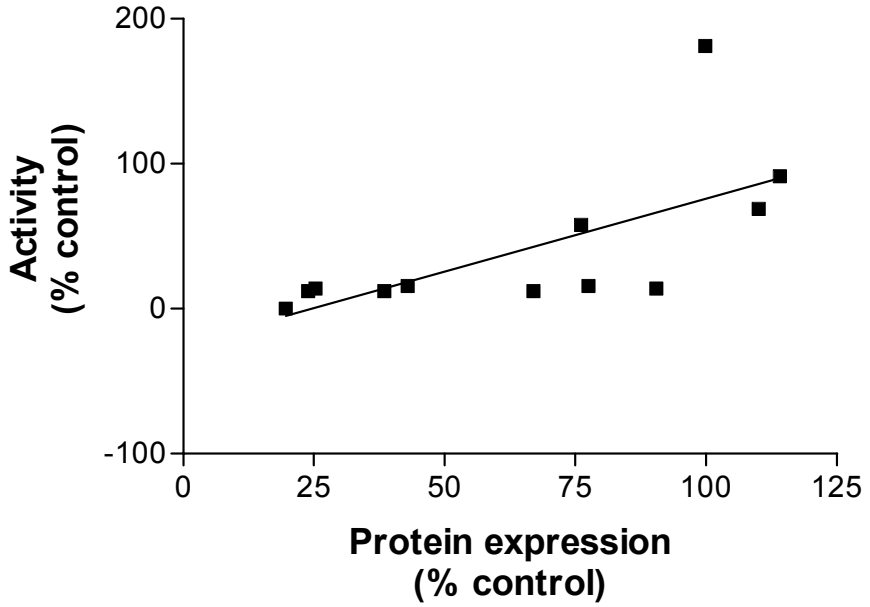

Figure 19. Correlation of CYP3A2 activity and CYP3A2 protein expression in kidneys from rats in control group, syngeneic transplant group and allogeneic transplant group (Pearson $r^2 = 0.44$, $p = 0.0187$ and Spearman $r = 0.84$, $P = 0.0007$).

CYP2E1 enzyme activity, protein expression and mRNA expression in rat kidneys:

Figure 20 shows the activity of CYP2E1 enzyme in kidney microsomes from three different groups of rats. It was only significantly reduced in allogeneic transplant groups as compared control.

Protein expression as shown in Figure 21 was reduced significantly only in allogeneic group (40% of control). There was no significant correlation ($r^2 = 0.25$) between enzyme activity and protein expression (Figure 22). The mRNA expression of this enzyme was not different from control for syngeneic transplant groups while the allogeneic transplant groups showed a significant reduction in mRNA expression to 25% of the control level.

UGT1A10 enzyme activity, protein expression and mRNA expression in rat kidneys:

Figure 23 shows the UGT1A10 enzyme activity in kidney microsomes from three different groups of rats. It was higher in the syngeneic transplant group than the control group (185% of control). On the other hand, activity in the allogeneic transplant group was not significantly different from control.

Antibody was not available to evaluate the protein expression of this enzyme. Also, the microarray method that was used did not include the mRNA for UGT1A10. However, the mRNA for the whole UGT family was not significantly different in syngeneic group but was reduced to 18% in the allogeneic group. Table 10 shows summary of the changes seen in both syngeneic and allogeneic transplant groups in all the enzymes studied.

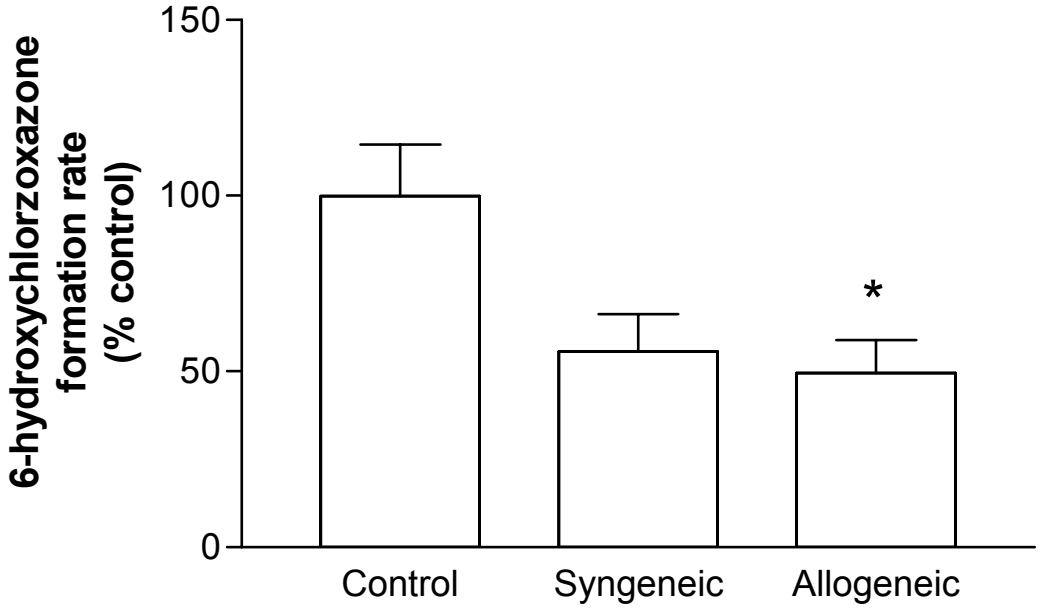

Figure 20. CYP2E1 enzyme activity as measured by the formation of 6-hydroxychlorzoxazone (nmol/min/mg protein) from chlorzoxazone in kidney microsomes from rats in control group, syngeneic transplant group and allogeneic transplant group expressed as a percentage of control group. Control= 0.31±0.05 nmol/min/mg protein (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

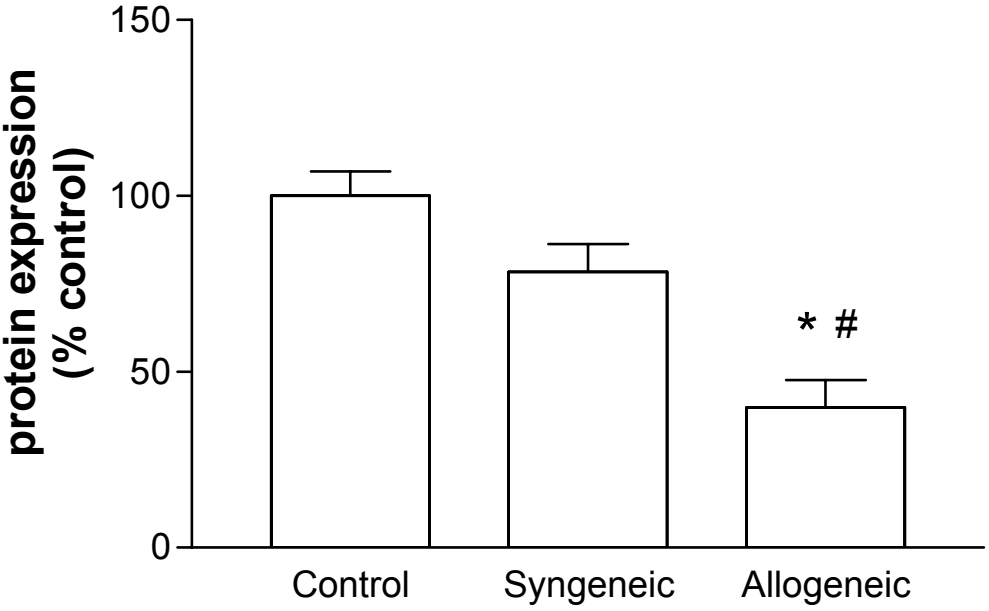

Figure 21. CYP2E1 protein expression in kidneys from rats in syngeneic transplant group, allogeneic transplant group expressed as a percentage of control group (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

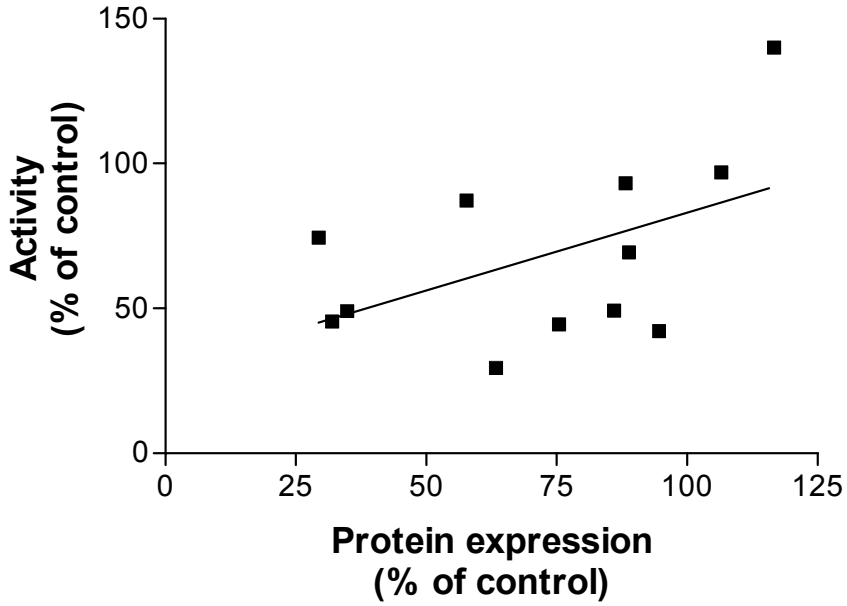

Figure 22. Correlation of CYP2E1 activity and CYP2E1 protein expression in kidneys from rats in control group, syngeneic transplant group and allogeneic transplant group (Pearson $r^2 = 0.25$, p = 0.0989, Spearman r = 0.37, P = 0.2356).

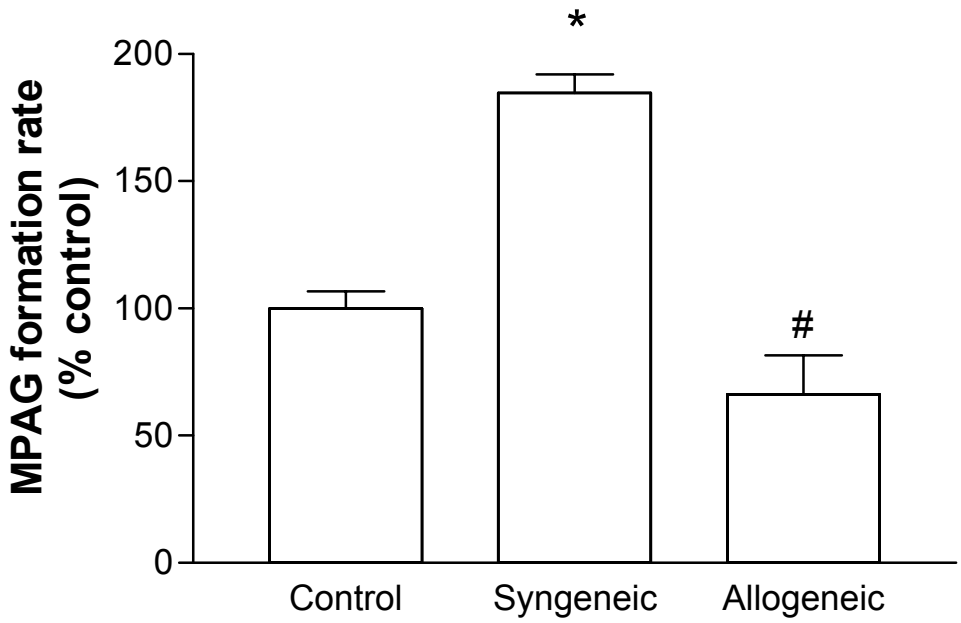

Figure 23. UGT1A10 enzyme activity as measured by the formation of MPAG (nmol/min/mg protein) from MPA in kidney microsomes from rats in control group, syngeneic transplant group and allogeneic transplant group expressed as a percentage of control group. Control= 2.88±0.19 nmol/min/mg protein (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group.**

 # Significantly different from syngeneic transplant group.

Table 10. Summary of the changes in enzymes mRNA expression, protein expression and activity seen in syngeneic transplant and allogeneic transplant groups as compared to control group.

parameter	Group/enzyme	CYP3A2	CYP2E1	UGT1A10
mRNA	**Syngeneic**	↔	↔	↔
	Allogeneic	↓	↓	↓
Protein	**Syngeneic**	↔	↔	NA
	Allogeneic	↓	↓	NA
Activity	**Syngeneic**	↓	↔	↑
	Allogeneic	↓	↓	↔

NA= no available antibody

For Syngeneic group:

↔ indicates no difference from control.

↑ indicates significant increase from control.

↓ indicates significant decrease from control.

For Allogeneic group:

↔ indicates no difference from control.

↓ indicates significant decrease from control (in case syngeneic is also ↓) or from syngeneic (in case syngeneic is ↔) .

Other enzymes:

The mRNA expression of other enzymes that are involved in drug metabolism was also modified in both syngeneic and allogeneic groups as shown in Table 11. A change of 30% or more was considered significant.

mRNA expression of different cytokines in rat lungs:

Table 12 shows the pulmonary mRNA expression of different cytokines as measured by DNA microarrays.

mRNA expression of anti oxidant enzymes in rat lungs:

The mRNA expression for glutathion-s-transferase (GST) was 170 and 145% for syngeneic and allogeneic transplant groups, respectively. The mRNA expression for Catalase (CAT) was 465 and 798% for syngeneic and allogeneic transplant groups, respectively. Finally, the mRNA expression for superoxide dismutase (SOD) was 210 and 189% for syngeneic and allogeneic transplant groups, respectively.

Other enzymes:

The mRNA expression of other enzymes was also modified in both syngeneic and allogeneic groups as shown in Table 13. A change of 30% or more was considered significant.

Table 11. Renal mRNA expression of different enzymes in syngeneic transplant group and allogeneic transplant group in comparison to control group.

Enzyme/group	Syngeneic	Allogeneic
CYP1A1, CYP2A1, CYP2A3, UGT1A7	↑	↑
CYP1B1	↑	↓
CYP2B1	↓	↔
CYP4A10	↔	↓
UGT1A1	↔	↔

↔ indicates no difference from control.

↑ indicates significant increase from control.

↓ indicates significant decrease from control.

Table 12. Pulmonary mRNA expression of different cytokines in syngeneic transplant group and allogeneic transplant group in comparison to control group.

Cytokine/group	Syngeneic	Allogeneic
• Pro-inflammatory: IL-1β, IL-6, 1L-12, IL-15, IFN-α • Anti-inflammatory: IL-13	↔	↑↑
• Anti-inflammatory: TGF-α	↑↑	↑
• Anti-inflammatory: IL-4	↑	↑
• Pro-inflammatory: IL-18,TNF-α	↑	↔
• Pro-inflammatory: IFN-γ • Anti-inflammatory: TGF-β	↑	↑↑

↔ indicates no difference from control.

↑ indicates at least 30% difference from control

↑↑ indicates at least 30% from the other group (syngeneic or allogeneic) and 60% difference from control

Table 13 Pulmonary mRNA expression of different enzymes in syngeneic transplant group and allogeneic transplant group in comparison to control group.

Enzyme/group	Syngeneic	Allogeneic
CYP2A3, CYP2C11, CYP2E1, CYP3A2, CYP4A10	↑	↑
CYP1B1	↑	↔
UGT, UGT1A7	↔	↑
CYP1A1	↑	↓
UGT1A1	↓	↔
CYP2D18	↓	↓

↔ indicates no difference from control.

↑ indicates significant increase from control.

↓ indicates significant decrease from control.

Discussions:

The impact of damage to an organ on the activity of other organs in the body is documented by the effect of traumatic brain injury (TBI) and renal failure on hepatic drug metabolism. In patients suffering from TBI, the pharmacokinetics of several hepatically eliminated drugs are altered (Boucher and Hanes 1998). For example, the clearance of antipyrine (Boucher *et al.* 1991), phenytoin (O'Mara *et al.* 1995) and phenobarbital (Heinemeyer *et al.* 1986; Wermeling *et al.* 1987) in humans was increased following TBI. Traumatic brain injury in rats was shown to decrease the mRNA expression of both CYP2C11 and CYP3A in the liver by 50% as compared to control without any significant changes in the activity or the protein expression of these enzymes (Toler *et al.* 1993). TBI in rats has been shown to significantly reduce hepatic cytochrome P-450 activity after 24 hours and reduce the activity of CYP2E1 after 48 hours of the injury, while the activity of CYP2E1 in kidneys at 24 hours was increased (Poloyac *et al.* 2001). Recently, cytochrome P450 activity, protein contents and mRNA expression of different CYP enzymes have been shown to be significantly reduced in liver of rats with CRF as compared to normal rats. CYP3A2 activity was reduced by 50% as a result of chronic renal failure (CRF) (Leblond *et al.* 2001). The concentration of blood urea nitrogen was correlated with the content of cytochrome P450 and its activity in the liver of rats with CRF (Uchida *et al.* 1995).

Liver is the major organ that is involved in drug metabolism. In addition to the liver, intestine, kidneys, lungs and brain also play an important role in the metabolism of many endogenous and exogenous compounds. The kidney expresses many of the CYP450 enzymes such as CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, CYP3A and CYP4A. It also has many of the conjugative enzymes, especially the UGTs (Lohr *et al.* 1998). The intestine contains CYP3A4 as the major

CYP450 isoform. It also expresses CYP2E1 in addition to many of the phase II enzymes. The lung expresses CYP1A and CYP4A isoforms and the brain expresses CYP4A and CYP2E isoforms. Stimulation of immune system during acute rejection of liver is associated with the release of many inflammatory mediators such as cytokines, NO and ROIs, which inhibit hepatic drug metabolism (Chapters 1 and 3). Since these mediators distribute throughout the body, they have the potential to inhibit metabolism in other organs in the body as well. In this study we evaluated the effects of acute rejection of liver on the metabolic ability of the kidneys and lungs.

In our study, rats in the syngeneic transplant group were subjected to surgery, alteration in hepatic flow blood flow, ischemia and reperfusion injuries. Therefore, differences observed between this group and the control group are due to the effect of all these process together. On the other hand, rats in the allogeneic transplant groups are subjected to all processes mentioned for the syngeneic groups, in addition to rejection of the liver. Thus, differences observed between the syngeneic and allogeneic groups are solely due to the process of liver rejection.

The renal mRNA expression of CYP3A2 was only reduced significantly in the allogeneic group possibly as a result of high level of cytokines in that group. This reduction in mRNA expression resulted in a significant reduction in the protein expression for this enzyme (27% of control) in the allogeneic group. The CYP3A2 activity was reduced to 14% and 9% of control in syngeneic transplant and allogeneic transplant groups, respectively despite the presence of high protein levels (69 and 27% as compared to control). The significant, but low, correlation ($r^2 = 0.44$) between CYP3A2 enzyme activity and CYP3A2 protein expression indicates that at least part of the reduction in CYP3A2 activity can be explained by reduction in protein expression. Down

regulation of different cytochrome P450s during inflammation can be a consequence of many processes, mainly the repression of gene transcription which is usually indicated by a reduction in the expression of mRNA. Other processes that can contribute to the reduction in the metabolic capacity may involve increasing the turnover of protein (Morgan 1989; Morgan *et al.* 1994) and mRNA (Morgan 1989; Barker *et al.* 1992) of these enzymes. In addition, NO which is known to be produced in such conditions, has been shown to down regulate the metabolizing enzymes (Chapter 1) (Wink *et al.* 1993; Wink and Mitchell 1998). However, in our study, the mRNA expression of iNOS in kidney tissues from both syngeneic and allogeneic groups was not increased as compared to control. This may exclude NO as a major player in any alteration in the syngeneic and allogeneic transplant groups in the 6th day after transplantation. The production of cytokines and NO is also known to produce many ROIs that are known to down regulate drug metabolism. Therefore, even though the mRNA levels of most cytokines and NO were normal in kidney tissues, the effects of their produced mediators (ROIs) may still contribute to the reduction in drug metabolizing enzymes. However, we found alteration in some of the antioxidant enzymatic defense systems. The mRNA of superoxide dismutase (SOD), which converts superoxide radicals to hydrogen peroxides, was decreased to 45% of control in syngeneic transplant group. The mRNA of catalase (CAT), which detoxifies the hydrogen and organic peroxides, was decreased in the allogeneic transplant group to 21% of control. The mRNA of GST (glutathione-s-transferase) was reduced in allogeneic transplant group to 39% of control group. The mRNA expression for heme oxygenase (HO) was not induced in the kidneys from both syngeneic and allogeneic transplant groups.

The mRNA expression of CYP2E1 was only reduced in the allogeneic transplant group to 25% of control. This led to a significant reduction in the protein expression in the allogeneic transplant group (40% of control). Even though CYP2E1 protein was reduced, the reduction in mRNA for the allogeneic transplant group did not reflect on the protein expression to the same magnitude. This may involve post translational mechanisms such as protein stabilization. The enzyme activity of syngeneic and allogeneic transplant groups was 56 and 50% of control, respectively. This resulted in a non significant correlation between the activity and the protein expression ($r^2 = 0.25$) of this enzyme.

Renal UGT1A10 activity was reduced in allogeneic transplant group to 51% of control and induced in syngeneic transplant group to 185% of control value. Also, mRNA for whole UGT family was increased to 113% of control for the syngeneic group and reduced to 18% of control in the allogeneic transplant group. Same as in case of CYP2E1, the magnitude of the reduction in the activity is similar to the reduction in the mRNA expression which may be indicative of a minimal involvement of ROIs.

We also evaluated the mRNA expression for other phase I and phase II enzymes. The mRNA expression of CYP1A1, CYP2A1, CYP2A3 and UGT1A7 in both groups was increased. The process of rejection caused a significant reduction in the mRNA expression of CYP4A10 as compared to the syngeneic group. The mRNA expression of CYP1B1 was increased in syngeneic transplant group. However, the occurrence of rejection reversed the situation and caused the expression to decrease. The mRNA of CYP2B1 was only decreased in the syngeneic group. Finally, the expression of the phase II enzyme, UGT1A1, was not changed in both groups

as compared to the control. These findings are important since these enzymes are involved in metabolism of several drugs. Therefore a decrease or increase in their expression will affect transformation of exogenous and endogenous compounds in the kidneys.

In lung tissues, we found an increase in the mRNA expression of many cytokines in both syngeneic and allogeneic groups. The mRNA expression of CYP2A3, CYP2C11, CYP2E1, CYP3A2 and CYP4A10 was increased in both syngeneic and allogeneic transplant groups. The mRNA expression of CYP1B1 and CYP1A1 was only increased in syngeneic transplant group. However, occurrence of rejection caused a reduction in the mRNA expression of CYP1A1 and did not change the expression of CYP1B1. The rejection caused an increase in the mRNA expression of the whole UGT family and UGT1A7. The mRNA of UGT1A1 was only decreased in the syngeneic transplant group. Finally, CYP2D18 was decreased in both groups. These observations show the important of practicing caution when administering drugs that are targeting the lungs and are substrates for these enzymes.

In conclusion, liver rejection altered the activity and expression of different drug metabolizing enzymes in extra-hepatic organs. However, the magnitude of change in metabolizing enzymes was not as high as seen in the rejecting liver (local effect) and this is expected since the kidneys are not directly targeted by the immune system. In addition, the effects of syngeneic and allogeneic transplant peripherally were not the same as we found in the local organ (liver). For example CYP3A2, CYP2E1 and UGT1A was decreased in both liver and kidneys, but increased in the lungs.

Despite the low involvement of the extra-hepatic tissues in drug metabolism as compared to the liver, changes in the activity of these enzymes in extra-hepatic tissues may be of important toxicological consequence to that organ.

Chapter 5

Effect of chronic rejection of liver in humans on the hepatic drug metabolizing enzymes activity and expression

Introduction:

Chronic rejection of liver usually occurs slowly over months or years after transplantation. Histologically, it is characterized by loss of the bile ducts (and hence the name ductopenic rejection). It may be also characterized by interstitial fibrosis, hepatocellular ballooning, and dropout, and finally obliterative endarteritis. In addition, there is evidence for the involvement of alloantibodies in causing chronic rejection. However, the precise mechanism of contribution of these alloantibodies to chronic rejection is not completely understood. Currently, there is neither a test to predict the development of chronic rejection nor a drug to reverse this condition. Patients who chronically reject their livers are maintained on high dose of immunosuppressive drugs. Many of the immunosuppressive drugs are metabolized by enzymes of the phase I (e.g. tacrolimus and cyclosporine are metabolized by CYP3A) and phase II (e.g. MPA is metabolized by UGT1A9/10) pathways. Therefore, it's crucial to understand how the metabolizing enzymes are affected during chronic rejection of the liver.

We hypothesized that the protein expression and activity will be uniformly reduced for all drug metabolizing enzymes during chronic rejection. The objective of this study was to evaluate the effect of chronic liver rejection in humans on the hepatic metabolic capacity and protein expression of phase I (CYP3A4, CYP2C9 and CYP2E1) and phase II (UGT1A9) enzymes.

Experimental methods:

Human liver tissues were obtained from the tissue bank. These samples were from normal individuals (organ donors) and from patients with chronic rejection. The occurrence of rejection was monitored by liver biochemistry and histopathology. The medications list for these patients is shown in Table 14.

Groups were designed as described in Chapter 2. Microsomes from these liver samples were prepared as described in chapter 2 and stored at –80°C.

Determination of enzyme activity:

Microsomal proteins were obtained by differential centrifugations. Microsomal proteins were incubated with different substrates (testosterone for CYP3A2, flurbiprofen for CYP2C11, Chlorzoxazone for CYP2E1 and MPA for UGT1A9) as described in chapter 2.

Determination of enzyme expression:

Enzyme protein expression of each enzyme was evaluated by Western blotting as described in chapter 2.

Statistical Analysis:

After log transformation of the data, t-test was used to evaluate the presence of significant differences between the groups at a p-value ≤ 0.05. Correlation between the enzyme activity and protein expression was determined using Pearson and Spearman correlation analysis using Prism software version 3.02 (GraphPad Software Inc., San Diego, CA). Sample size was chosen based

on power calculation using Pass software version 6.0 (NCSS, Kaysville, UT). At $\alpha = 0.05$, the proposed sample size of $n = 4$ will allow the achievement of a power $\geq 80\%$ for all the studied enzymes.

Table 14. List of medications taken by normal individuals and patients with chronic rejection from whom tissues were obtained for this study.

Patient number	Medications
Normal # 1	Dopamine prior to harvest.
Normal # 2	Dopamine prior to harvest.
Normal # 3	Unknown
Normal # 4	Dopamine prior to harvest.
Chronic rejection # 1	Unknown
Chronic rejection # 2	Prednisone, Trimethoprim sulfamethoxazole, Vitamin K, Hydroxyzine HCL, Ursodiol, Nystatin, Nifedine, Tacrolimus, Aluminum and magnesium hydroxide and $FeSO_4$
Chronic rejection # 3	Cyclosporin A, Prednisone, $MgSO_4$, $ZnSO_4$, Vitamin K, Calcitrol, Folate, Calcium, Tobramysin, Ciprofloxacin HCL, Heparin, Clotrimazole, Aluminum and magnesium hydroxide
Chronic rejection # 4	Unknown

Results:

Characterization of rejection (histopathology and biochemistry):

The presence of chronic rejection was assessed by histopathology and biochemistry. In the normal group, the structure of the liver was normal. In the chronic rejection group, loss of the bile ducts, which is the primary sign of chronic rejection, was seen in all samples. Table 15 shows the results of serum biochemistry in both groups.

Effect of chronic liver rejection on CYP3A4:

Figure 24 shows the effect of chronic liver rejection on the enzyme kinetics of CYP3A4. The activity in the chronic rejection group was not significantly different from the normal group as shown in Figure 25. The Km values were 69 ± 13 μM for normal and 66 ± 10 μM for rejecting individuals. There were no significant differences in Km values between the groups. On the other hand, the CYP3A4 protein expression (Figure 26) was significantly higher (154% of normals) in the chronic rejection group as compared to the normal group. The protein expression was poorly correlated ($r^2 = 0.17$) with the activity (Figure 27). The Cl_{int} values were 35.0±1.3 for normal and 31.5±0.4 μl/min/mg protein for the rejection group, was not significantly different from each other.

Effect of chronic liver rejection on CYP2C9:

Figure 28 shows the effect of chronic liver rejection on the enzyme kinetics of CYP2C9. The activity of the chronic rejection group was significantly higher than the normal group (209% of normals) (Figure 29). The Km values were 9 ± 4 μM for normal and 13 ± 8 μM for chronic rejection group. There were no significant differences in Km values between the groups. On the

other hand, the protein expression (Figure 30) was higher in chronic rejection group (118% of normals). The protein expression was poorly correlated (r^2 = 0.34) with the activity results (Figure 31). The Cl_{int} values were 59.9±13.5 for normal and 92.9±14.4 µl/min/mg protein for the rejection group.

Table 15. Serum biochemistry in normal and subjects with chronic rejection of liver (n=4 for each group).

Parameter	Normal liver tissues	Liver tissues undergoing chronic rejection
Total bilirubin (mg/dl)	0.78 ± 0.14	21.15 ± 4.51*
AST (u/l)	135.00 ± 50.91	303.50 ± 57.71*
Alk. Phos. (u/l)	62.50 ± 21.10	2521.50 ±740.24*

T-test was used to evaluate the presence of significant difference between groups.

* significantly different from control.

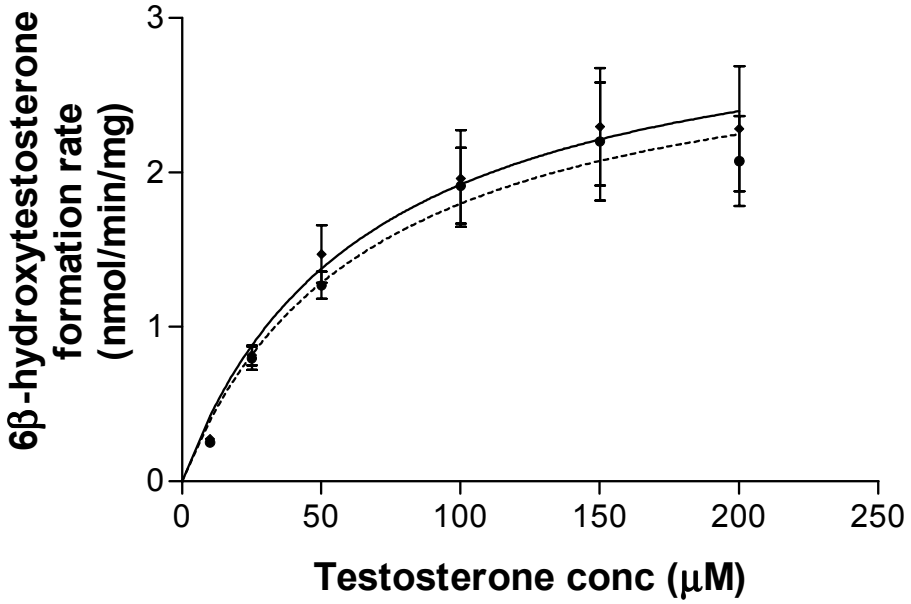

Figure 24. CYP3A4 activity as measured by the formation of 6β-hydroxytestosterone (nmol/min/mg protein) from testosterone in liver microsomes from normal subjects (—) and liver transplant patients (…) with chronic rejection (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

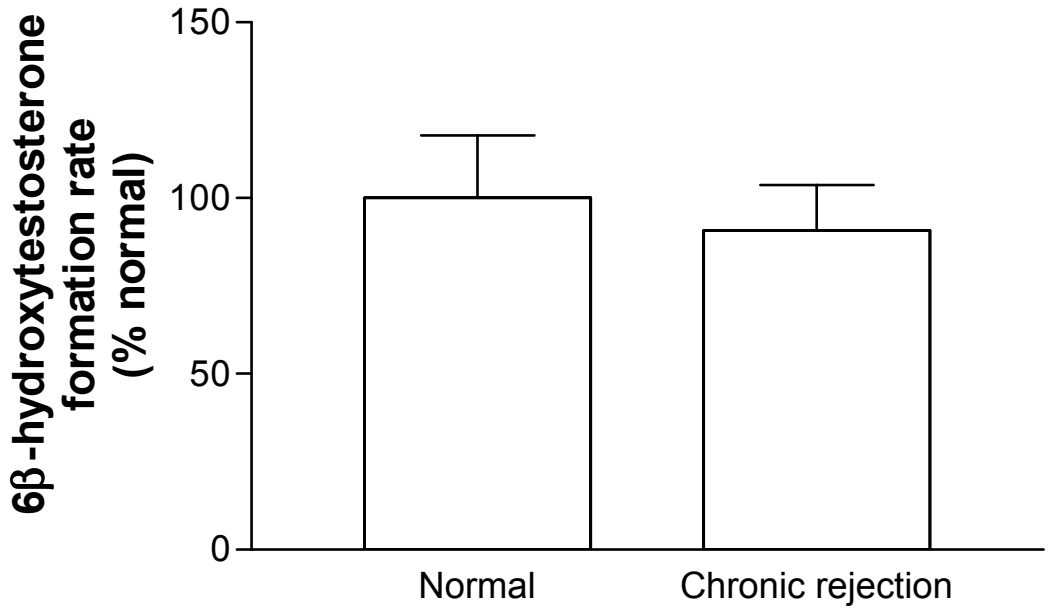

Figure 25. Vmax for CYP3A4 as measured by the formation of 6β-hydroxytestosterone (nmol/min/mg protein) from testosterone in liver microsomes from normal subjects and liver transplant patients with chronic rejection expressed as percentage of control. Normal = 3.47±1.53 nmol/min/mg protein (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

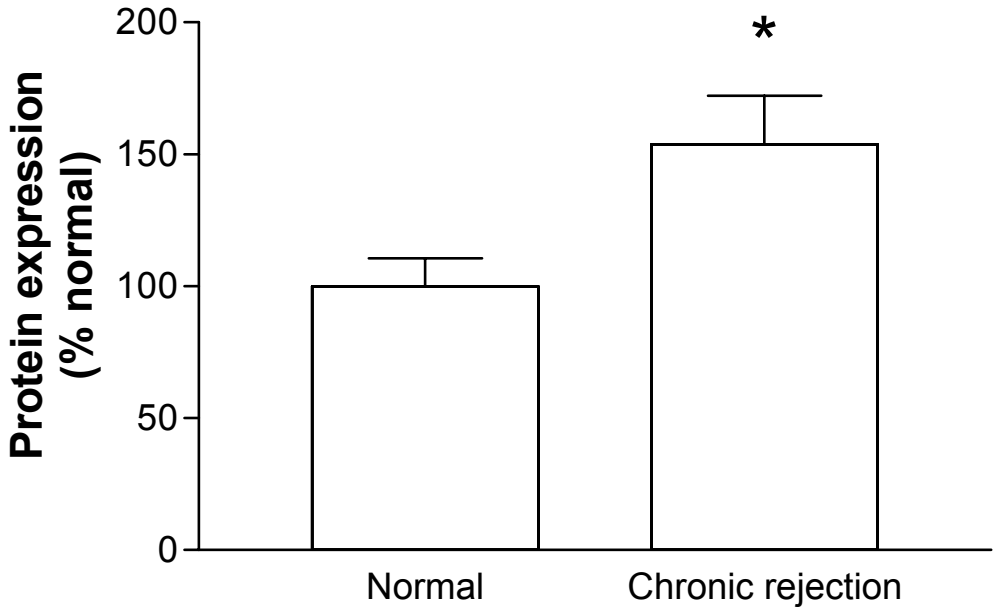

Figure 26. CYP3A4 protein expression in livers from normal subjects and transplant patients expressed as percentage of normal (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

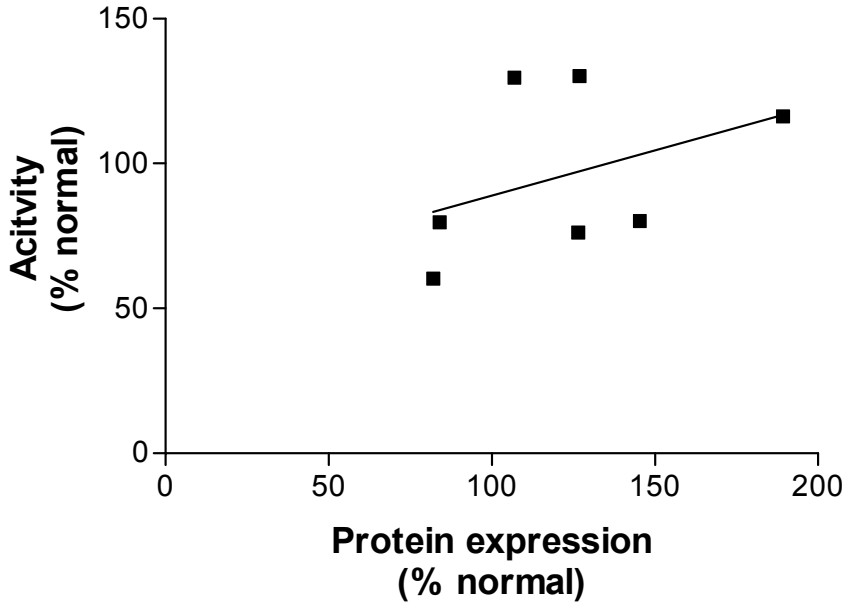

Figure 27. Correlation of CYP3A4 activity and CYP3A4 protein expression in livers from normal subjects and livers from transplant patients who had chronic rejection (Pearson r^2 = 0.17, p = 0.3645 and Spearman r = 0.54, P ‹ 0.2357).

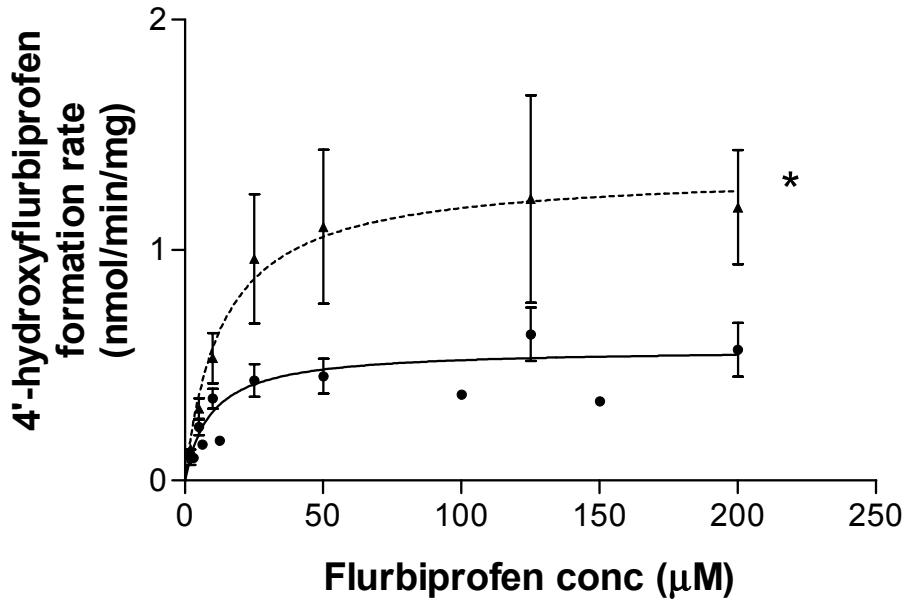

Figure 28. CYP2C9 activity as measured by the formation of 4'-hydroxyflurbiprofen (nmol/min/mg protein) from flurbiprofen in liver microsomes from normal subjects (—) and liver transplant patients (…) with chronic rejection (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group at the highest concentration used.**

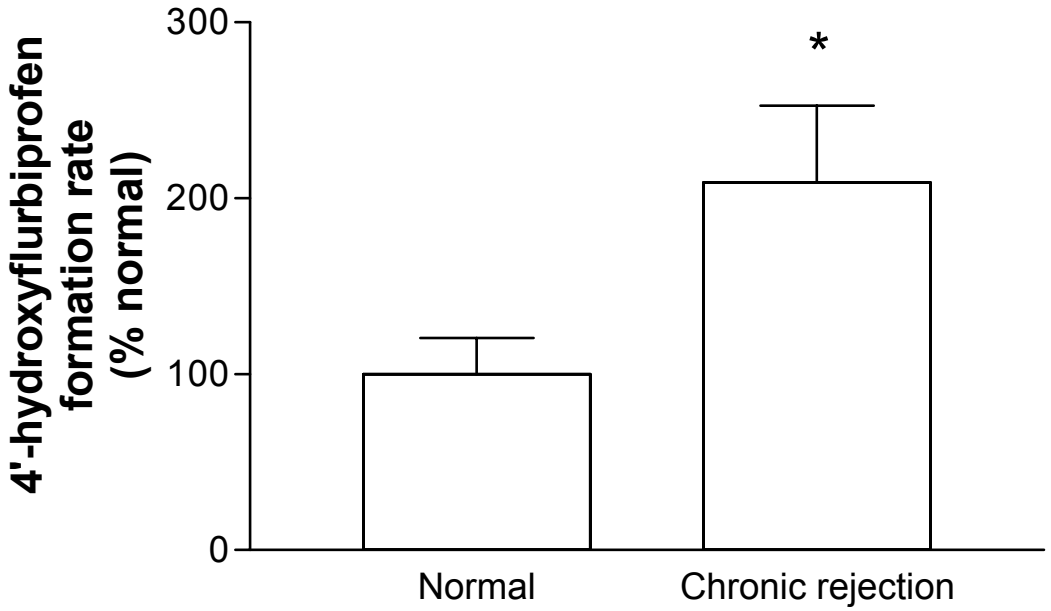

Figure 29. Vmax for CYP2C9 as measured by the formation of 4'-hydroxyflurbiprofen (nmol/min/mg protein) from flurbiprofen in liver microsomes from normal subjects and liver transplant patients with chronic rejection expressed as percentage of control. Normal = 0.57±0.05 nmol/min/mg protein (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

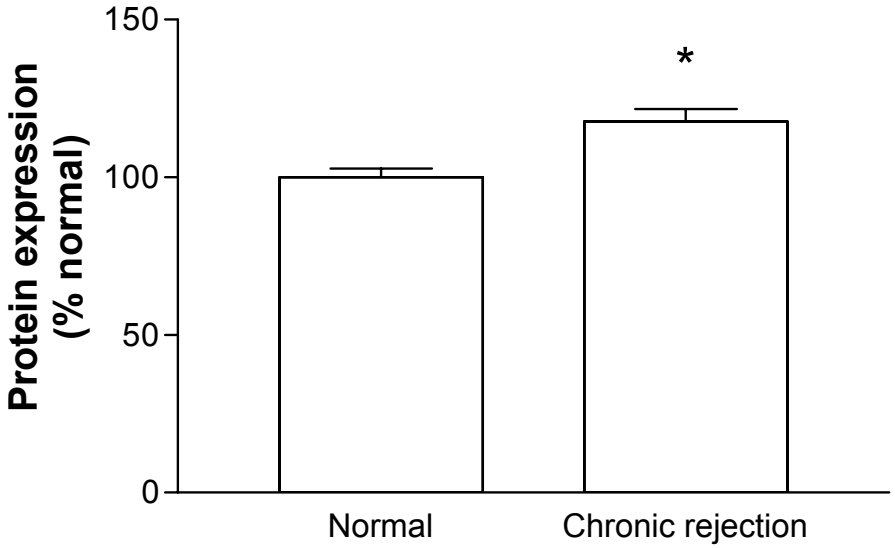

Figure 30. CYP2C9 protein expression in livers from normal subjects and transplant patients expressed as percentage of normal (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

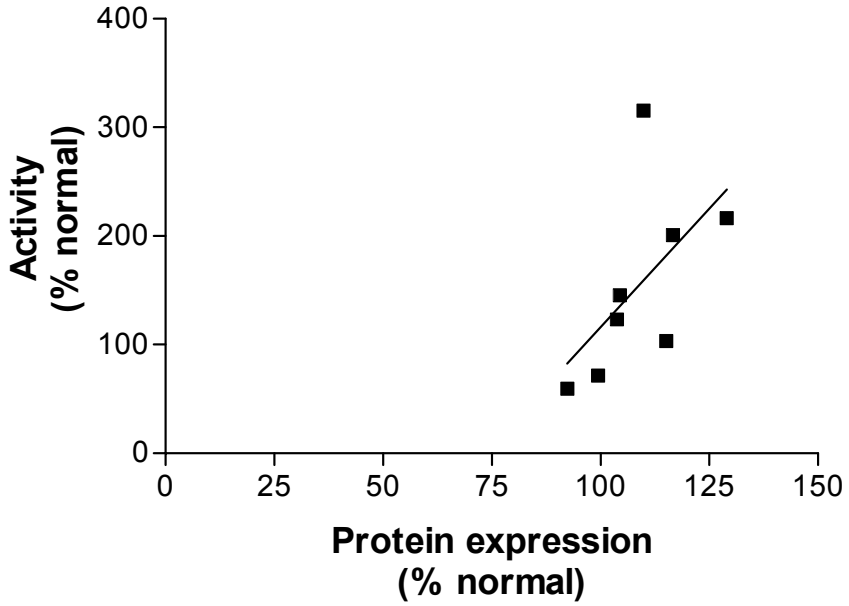

Figure 31. Correlation of CYP2C9 activity and CYP2C9 protein expression in livers from normal subjects and livers from transplant patients who had chronic rejection (Pearson r^2 = 0.34, p = 0.1296 and Spearman r = 0.74, P = 0.0458).

Effect of chronic liver rejection on CYP2E1:

Figure 32 shows the effect of chronic rejection of liver on the enzyme kinetics of CYP2E1. The activity of the chronic rejection group was significantly lower than the normal group (52% of normals) as shown in Figure 33. The Km values were 111 ± 16 μM for normal and 82 ± 36 μM for chronic rejection group. There were no significant differences in Km values between the groups. The protein expression (Figure 34) was significantly lower in chronic rejection group. The CYP2E1 protein expression was poorly correlated (r^2 = 0.51) with the CYP2E1 activity (Figure 35). The Cl_{int} values were 14.5±0.6 for normal and 10.0±2.3 μl/min/mg protein for the rejection group. However, the values were not significantly different from each other.

Effect of chronic liver rejection on UGT1A9:

Figure 36 shows the effect of chronic liver rejection on the enzyme kinetics of UGT1A9. The UGT1A9 activity of the chronic rejection group was significantly lower than the normal group (46% of normals) as shown in Figure 37. The Km values were 333 ± 44 μM for normal and 268± 13 μM for chronic rejection group were not significantly different. The protein expression (Figure 38) was significantly lower in the chronic rejection group (52% of normals). The UGT1A9 protein expression was significantly correlated (r^2 = 0.82) with the UGT1A9 activity (Figure 39). The Cl_{int} for rejecting group (40.9±8.1 μl/min/mg protein) was significantly lower than normal group (73.6±8.3 μl/min/mg protein).

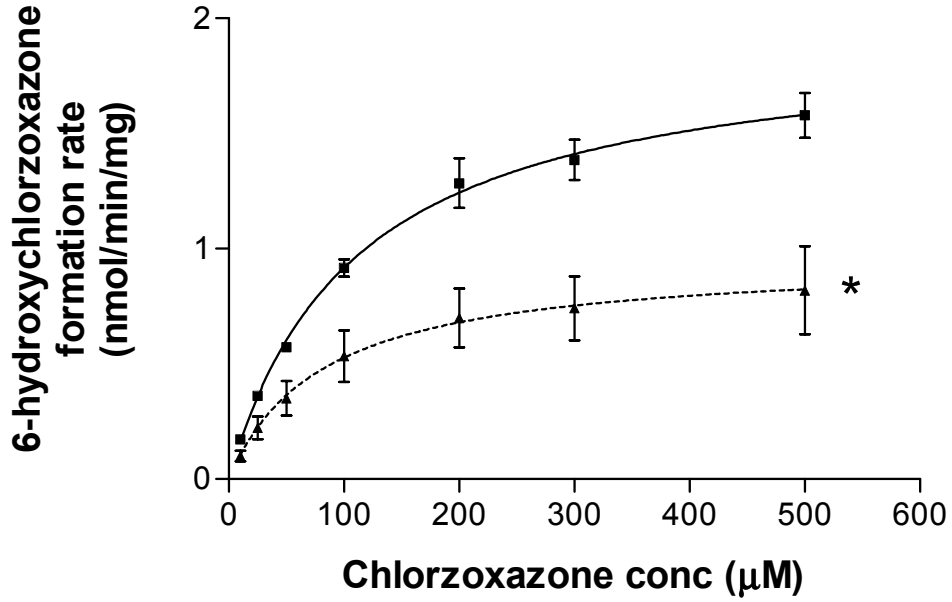

Figure 32. CYP2E1 activity as measured by the formation of 6-hydroxychlorzoxazone (nmol/min/mg protein) from chlorzoxazone in liver microsomes from normal subjects (—) and liver transplant patients (…) with chronic rejection (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group at the highest concentration used.**

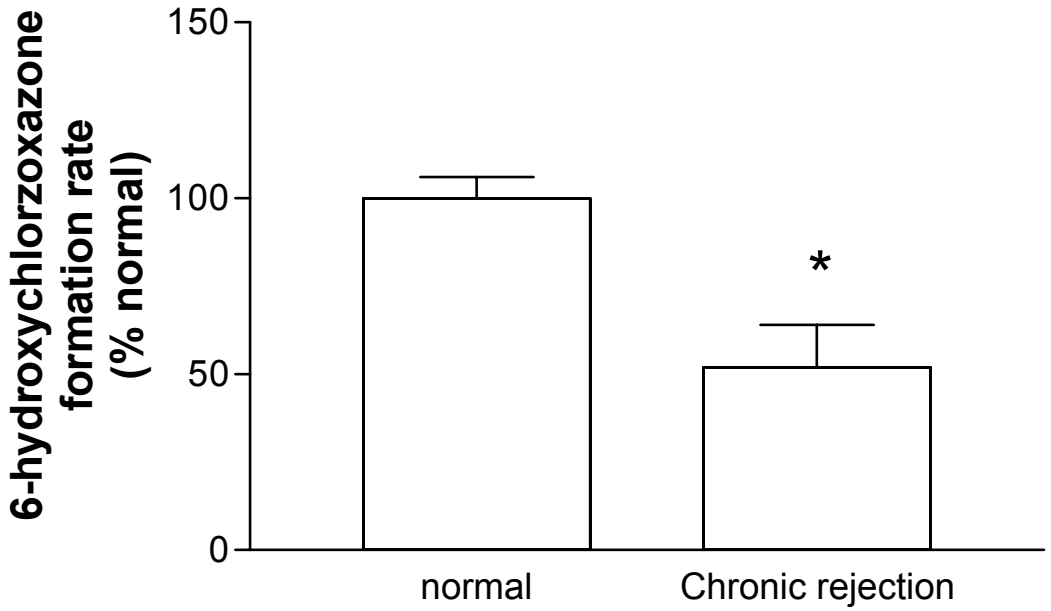

Figure 33. Vmax for CYP2E1 as measured by the formation of 6-hydroxychlorzoxazone (nmol/min/mg protein) from chlorzoxazone in liver microsomes from normal subjects and liver transplant patients with chronic rejection expressed as percentage of control. Normal = 1.93±0.09 nmol/min/mg protein (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

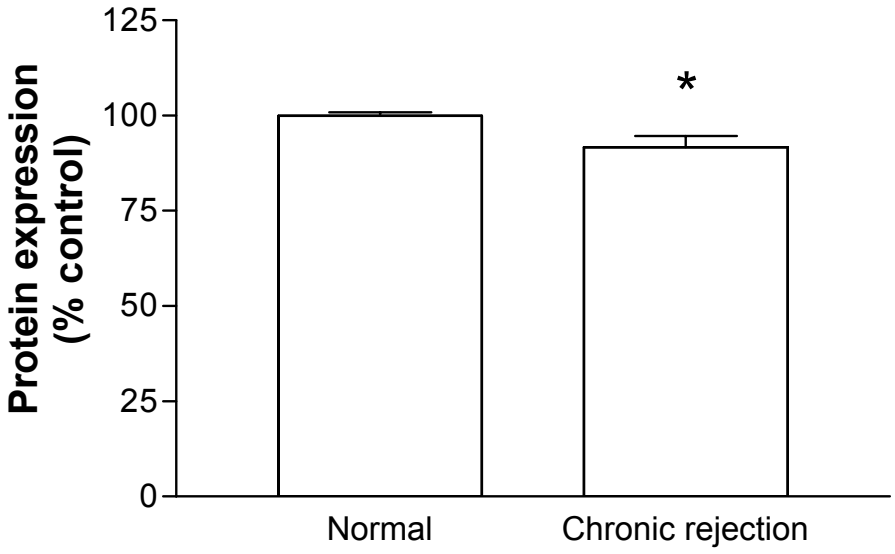

Figure 34. CYP2E1 protein expression in livers from normal subjects and transplant patients expressed as percentage of normal (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

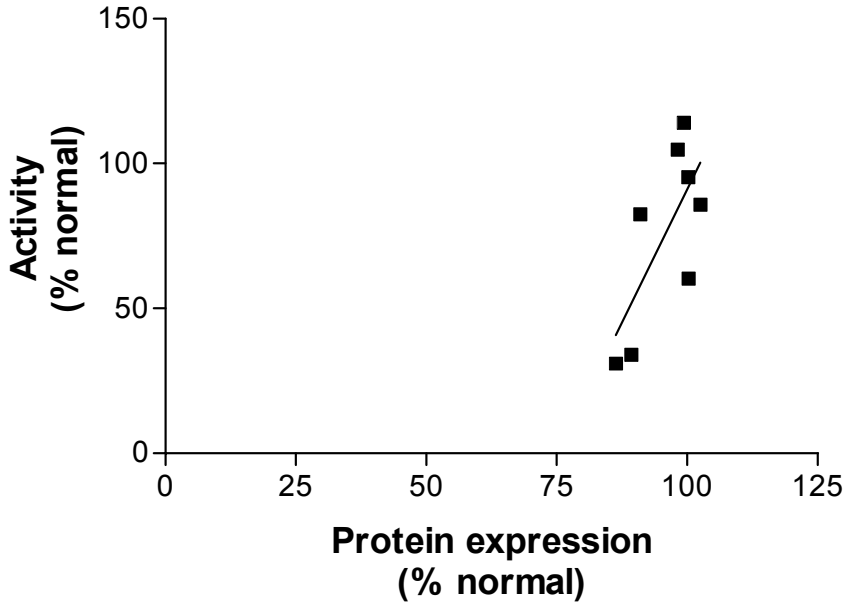

Figure 35. Correlation of CYP2E1 activity and CYP2E1 protein expression in livers from normal subjects and livers from transplant patients who had chronic rejection (Pearson r^2 = 0.51, p = 0.0455 and Spearman r = 0.48, P = 0.2431).

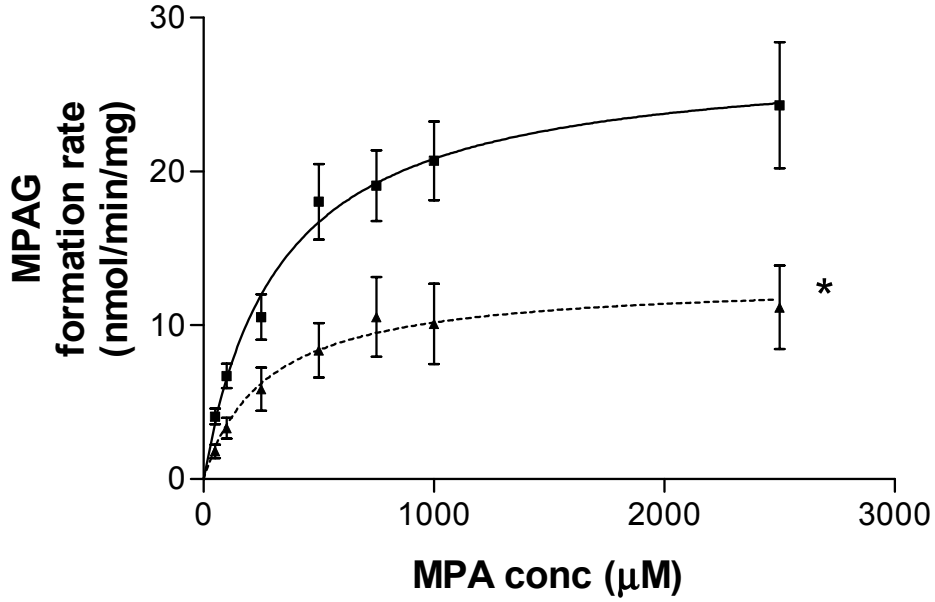

Figure 36. UGT1A9 activity as measured by the formation of MPAG (nmol/min/mg protein) from MPA in liver microsomes from normal subjects (—) and liver transplant patients (…) with chronic rejection (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** Significantly different from control group at the highest concentration used.**

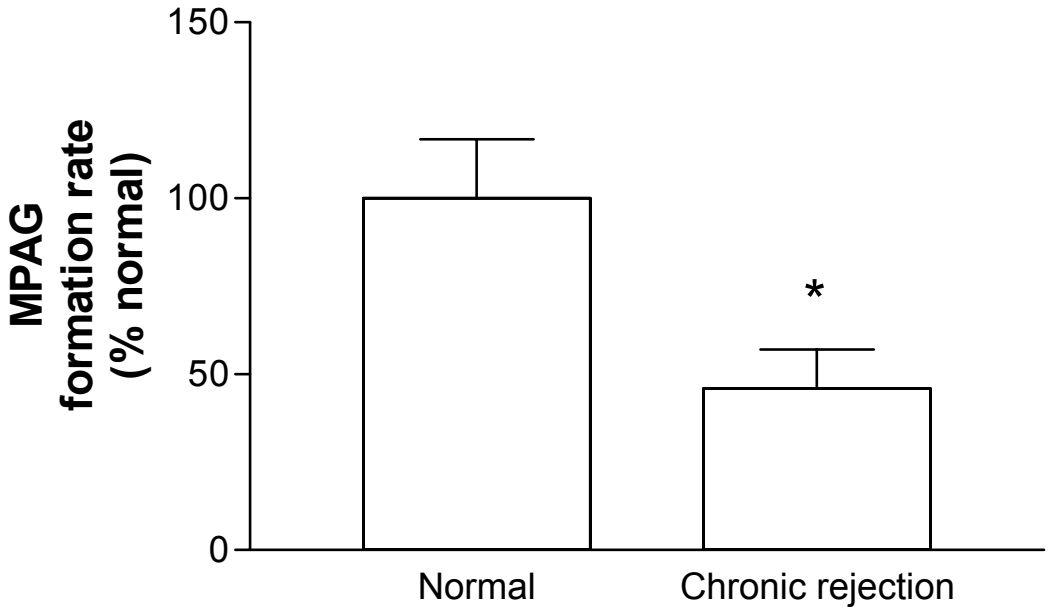

Figure 37. Vmax for UGT1A9 as measured by the formation of MPAG (nmol/min/mg protein) from MPA in liver microsomes from normal subjects and liver transplant patients with chronic rejection expressed as percentage of control. Normal = 27.96±4.54 nmol/min/mg protein (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

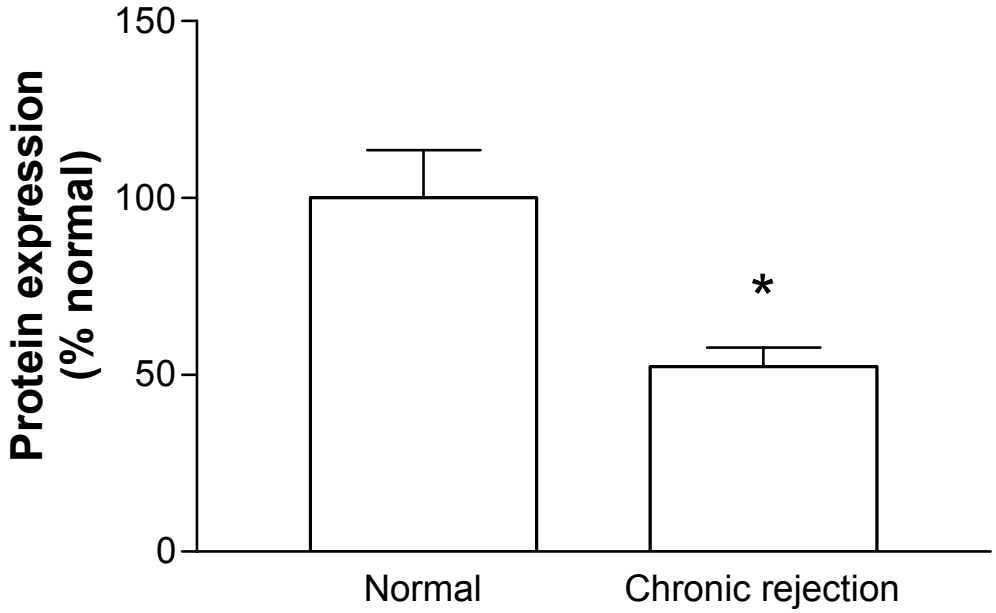

Figure 38. UGT1A protein expression in livers from normal subjects and transplant patients expressed as percentage of normal (n=4 for each group).

T-test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

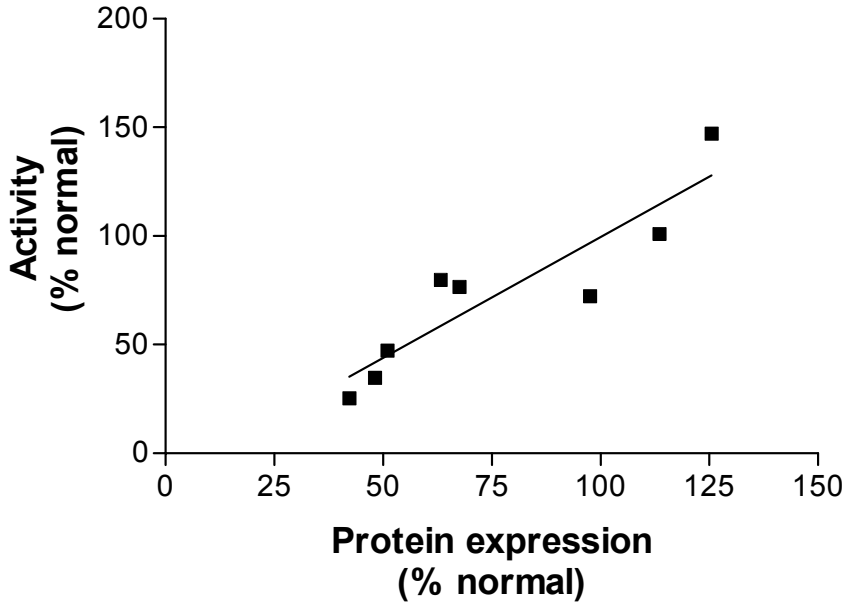

Figure 39. Correlation of UGT1A9 activity and UGT1A protein expression in livers from normal subjects and livers from transplant patients who had chronic rejection (Pearson r^2 = 0.82, p = 0.002 and Spearman r = 0.90, P = 0.0046).

Discussions:

It is well known that diseases of an organ will lead to impairment in the function of that organ. In renal failure, elimination of drugs by the kidneys is impaired and in cases of liver diseases, hepatic metabolism is impaired. In liver disease, the total content of CYPs is decreased but the magnitude of reduction is selective for individual enzymes. Moreover, the effect was related to the type of liver disease (with or without cholestasis) (George *et al.* 1995a; George *et al.* 1995b). The activity of CYP1A2, CYP2A6, CYP2E1, CYP3A4 and CYP2C19 (S-mephenytoin) was decreased in patients with liver disease (Howden *et al.* 1989; Kraul *et al.* 1991; Murray 1992; Wilkinson 1996; Adedoyin *et al.* 1998; Marino *et al.* 1998). The reduction in CYP2A6 and CYP3A4 activity in liver biopsies shown to be proportional to the degree of liver damage (Kraul *et al.* 1991; Huang *et al.* 1993). On the other hand, liver diseases didn't affect the activity of CYP2D6, CYP2C8, CYP2C9 and CYP2C19 (R-mephenytoin) (Howden *et al.* 1989; Adedoyin *et al.* 1998; Marino *et al.* 1998).

Earlier, we have shown that acute rejection of liver leads to differential reduction in the activity of several drug metabolizing enzymes (Chapter 3). In this study, the activity of CYP3A4 was not significantly different between control group and chronic rejection group. However, the protein expression was significantly higher in the rejecting group in comparison to the normal group (154% of normal group). This is not consistent with what is known about this enzyme in patients with liver disease. Most of the previous studies on liver diseases reported reduction of CYP3A4 activity and protein expression (George *et al.* 1995a; George *et al.* 1995b). On the other hand, a small number of studies have reported induction of the expression of CYP3A4 protein. In a study that involved 21 patients with liver disease (twelve of them are alcoholic and nine are non-

alcoholic patients), the protein expression of CYP3A4 was increased in both groups of patients with the induction being higher in the alcoholic patients. In another study, alcohol-induced liver disease in rats increased the expression of CYP3A4 protein (Niemela *et al.* 1998). In the present study, this induction is most likely through induction of mRNA as this is the most common way of induction of CYP3A4. This could be due to the high levels of a cytokine (IL-4) which is known to induce some of the metabolizing enzymes (Lagadic-Gossmann *et al.* 2000). This cytokine was shown in some studies to be increased during chronic rejection. In a study that evaluated liver biopsies from patient with acute and chronic rejection, IL-4 mRNA and protein expression increased during rejection (Conti *et al.* 1999). In the same study, no increase in the expression of this enzyme was observed in stable liver transplant patients. The other possible explanation is that patients in our study were on drugs that induced the CYP3A4. The patients in this study were not on any drugs (other than steroids) known to induce CYP enzymes. However, medication history of some of the individuals in this study was not fully known. Steroids are known to induce CYP3A4 (Pichard *et al.* 1992). Finally, the difference between the activity and protein expression indicate potential involvement of ROIs and NO which may inactivate the available protein by producing lipid peroxidation and consequent conformational changes in the enzymes. (Ingelman-Sundberg 1977; Ingelman-Sundberg *et al.* 1981; Engelke *et al.* 1993; Minamiyama *et al.* 1997; Yukawa *et al.* 1999). In support of this was the observation that in twelve liver transplant patients, the antioxidant levels were low. Also, lipid peroxidation increased in these patients (Goode *et al.* 1994). Similar results were found in 19 other patients who received liver transplants (Biasi *et al.* 1995). This may be the reason that we did not observe a significant correlation between the activity and the protein expression of CYP3A4 ($r^2 = 0.17$).

In conclusion, this enzyme may be induced as a result of cytokines, such as IL-4 or due to drug therapy. However, the protein was inactivated by possible ROIs and NO.

The activity of CYP2C9 was higher in the livers from patients with chronic rejection as compared to normal group (209% of normals). Also, the protein expression was significantly higher in the livers from patients with chronic rejection (118% of normal group). The increase in protein expression was not the same as the increase in the activity. This may be at least theoretically due to inability of the primary antibody that we used to detect all CYP2C9 proteins. Induction of CYP2C9 by steroids is not likely in this case as this enzyme has not been reported to be induced by steroids. There was no significant correlation between the activity and protein expression ($r^2 = 0.34$) for this enzyme.

The activity of CYP2E1 was significantly reduced in the livers from patients with chronic rejection (52% of normal group). The reduction in the protein expression (92% of normal group) was not as much as the activity. This maybe indicative of involvement of post translational mechanisms (Morgan *et al.* 1994). In this case protein expression of CYP2E1 was essentially preserved. This finding is consistent with the fact that CYP2E1 is regulated to a major extent at protein level (De Waziers *et al.* 1995; Hu *et al.* 1995; Lieber 1997). Also, steroids have not been shown to affect the expression of this enzyme (Pichard *et al.* 1992). As in the case for other studied enzymes, the reduction in activity was higher than reduction in protein expression as result of protein inactivation by ROIs and NO production during the course of chronic rejection. There was a poor correlation ($r^2 = 0.51$) between the activity and protein expression of this enzyme.

The activity of UGT1A9 was significantly reduced in the liver from patient with chronic rejection (46% of normal group). The protein expression for the UGT1A (whole family) was also reduced to about the same extent (52% of normal group) as the activity. It indicates that the reduction may be mediated mainly by a reduction in mRNA expression that subsequently reduced the protein expression. Since the activity was reduced to the same extent as the protein expression, we may conclude that the involvement of NO and ROIs are not a major factor in case of UGT1A. Thus, there was a significant correlation (r^2 = 0.82) between the activity and the protein expression. It is important here to notice that the protein expression was measured by a non-specific antibody that recognizes the whole UGT1A subfamily and not just the UGT1A9 alone.

In conclusion, our result showed the selectivity of the chronic rejection in affecting the different drug metabolizing enzymes. Some of the enzymes activity and protein expression were increased (CYP2C9), while others were decreased (CYP2E1 and UGT1A9). Thus, caution should be exercised when drugs that are metabolized by these enzymes are administered to such patient population. Unexpectedly, both protein and expression of UGT1A9 was decreased markedly in this study. In comparison to effects of acute rejection of liver in Chapter 3 (despite the different model, rat as opposed to human), the magnitude of the effect of chronic rejection on the enzymes activity and expression was lower. In addition, during chronic rejection the activity and expression of some of the enzymes were increased. Finally, it should be noted that despite the presence of no significant changes in the intrinsic clearance of these enzymes (except UGT1A9), the in vivo metabolism of drugs in these patients can still be altered by the changes in unbound

fraction and the blood flow to the liver. Future studies addressing these issues are necessary to make specific guidelines for optimal drug therapy in transplant patients. It is also important to point out that we were not able to find information about previous medication history for some of the patients as mentioned in the result section. Also, we did not have any information regarding the patients' nutritional status. The nutritional status may be important in affecting the levels of co-factors which are important in many of the enzymatic reaction (e.g. UDPGA). However, our studies were done in vitro and we provided these co-factors in excess.

Chapter 6

Effect of rejection of liver in rats and humans on the hepatic and extra-hepatic expression of drug transporters

Introduction:

Acute liver rejection is an immune cell-mediated pathological inflammatory response that takes place within the allograft. It usually occurs within days to weeks after transplantation. It is characterized histologically by the infiltration of the graft with lymphocytes. It is also characterized by the presence of bile duct damage and endothelialitis of the blood veins and arteries. Chronic liver rejection usually occurs slowly within months or years after transplantation. Histologically, it is characterized mainly by loss of the bile ducts (and hence the name, ductopenic rejection). It may be also characterized by interstitial fibrosis, hepatocellular ballooning, and dropout, and finally obliterative endarteritis. In addition, there is evidence for the involvement of alloantibodies in causing chronic rejection. Acute liver rejection is reversible upon achieving optimal immunosuppressive therapy in the transplant patient. However, until today, there is neither a test to predict the development of chronic rejection nor a drug to reverse this condition. Since transplant patients often experience acute and chronic rejection and use drugs such as tacrolimus, cyclosporine and sirolimus that are transported by P-gp, it is important to study the effect of rejection on P-gp expression in the liver. Based on the observations that disease of a specific organ impairs not only the functions of that organ but also the function of other organs, we also evaluated the expression of P-gp in kidneys and lungs during acute and chronic rejection of the liver.

The specific aims of this study were to evaluate the effect of acute and chronic rejection of the liver in rats and human, respectively on the hepatic and extra-hepatic (kidneys and lungs) protein and mRNA expression of P-gp. In addition, mRNA expression of other transporters (mrp1, mrp2, mdr3, OCT, BSEP) was evaluated.

Experimental methods:

The animal study protocol was approved by the IACUC at the University of Pittsburgh. Male ACI and Lewis rats (170-230 g) were used in the study.

Groups design:

For acute rejection the groups were design as described in Chapter 2. Rats in the syngeneic and allogeneic groups were transplanted and monitored daily. On day 6, rats were sacrificed by decapitation. Blood samples were obtained from these rats for serum biochemistry. The occurrence of rejection was monitored by general health and well being of these animals and by liver biochemistry and histopathology. Livers were harvested on day 6 and stored at –80°C.

For the effect of acute rejection on the extra-hepatic P-gp expression, groups were design as described in Chapter 2. Rats in the syngeneic and allogeneic groups were transplanted (by Dr. Noriko Murase's group in the Thomas E. Starzl Transplantation Institute) and monitored daily. On day 6, rats were sacrificed. Blood samples were obtained from these rats for serum biochemistry. The occurrence of rejection was monitored by general health and well being of these animals and by liver biochemistry and histopathology. Kidneys and lungs were harvested and stored at –80°C.

The effect of chronic rejection on hepatic P-gp expression was evaluated in human liver tissues obtained from liver transplant patients who were diagnosed to have chronic rejection based on histopathological and biochemical evidences. These livers and normal healthy livers were

obtained from a tissue bank (University of Minnesota tissue bank). Groups were designed as described in Chapter 2.

Determination of P-gp expression:

Protein expression for P-gp was evaluated by Western blotting as described in chapter 2.

Determination of mRNA expression:

Total RNA was isolated using Trizol reagent and mRNA expression was characterized by microarray analysis as described in chapter 2.

Statistical Analysis:

After log transformation of the data, one way ANOVA/Tukey (for rat acute rejection studies) or t-test (for human chronic rejection studies) were used as appropriate to evaluate the presence of significant differences between the groups at a p-value ≤ 0.05. Sample size was chosen based on power calculation using Pass software version 6.0 (NCSS, Kaysville, UT).

Results:

Characterization of rejection (histopathology and biochemistry):

The presence of acute and chronic rejection was assessed by histopathology and biochemistry as shown in chapter 3 (for acute rejection) and chapter 5 (for chronic rejection) of this document.

mRNA expression of different cytokines in rat liver:

Table 16 shows the effect of acute rejection of the liver on the hepatic mRNA expression of different cytokines as measured by DNA microarrays. Most of the proinflammatory cytokines were mildly increased in syngeneic transplant group and markedly increased in the allogeneic transplant group. We did not evaluate the expression of cytokines in our chronic rejection samples.

mRNA expression of different cytokines in rat kidney:

Table 17 shows the effect of acute rejection of the liver on the renal mRNA expression of different cytokines as measured by DNA microarrays. Most of the proinflammatory cytokines were mildly increased in syngeneic transplant group and markedly increased in the allogeneic transplant group.

mRNA expression of different cytokines in rat lung:

Table 18 shows the effect of acute rejection of the liver on the pulmonary mRNA expression of different cytokines as measured by DNA microarrays. Most of the proinflammatory cytokines were mildly increased in syngeneic transplant group and markedly increased in the allogeneic transplant group.

P-glycoprotein:

Effect of acute liver rejection on hepatic P-gp expression:

During acute rejection of the liver, the protein expression of P-gp in rat livers in syngeneic and allogeneic transplant groups were 177% and 222%, respectively as compared to the control group (Figure 40). There was a significant increase in the mRNA expression of mdr1 gene in the liver in the allogeneic group (159% of control), whereas the mRNA in the syngeneic group was not significantly different from the control group.

Table 16. Hepatic mRNA expression of different cytokines in rats from syngeneic or allogeneic liver transplant groups in comparison to rats from control group.

Cytokine/group	Syngeneic	Allogeneic
• Pro-inflammatory: IL-1α, IL-1β, 1L-12, IL-15, IL-18, TNF-α • Anti-inflammatory: IL-13	↔	↑↑
• Pro-inflammatory: IFN-γ • Anti-inflammatory: TGF-β	↑	↑↑
• Pro-inflammatory: IL-2	↑	↑
• Pro-inflammatory: IL-5, IFN-α • Anti-inflammatory: IL-4, TGF-α	↑	↔

↔ indicates no difference from control.

↑ indicates at least 30% difference from control

↑↑ indicates at least 30% from syngeneic and 60% difference from control

Table 17. Renal mRNA expression of different cytokines in rats from syngeneic or allogeneic liver transplant group in comparison to rats from control group.

Cytokine/group	Syngeneic	Allogeneic
• Pro-inflammatory: IL-1α, IL-2, IL-5, IL-12, IL-18, IFN-γ • Anti-inflammatory: 1L-4, IL-13, TGF-β	↔	↑↑
• Pro-inflammatory: IL-12	↑	↔

↔ indicates no difference from control.

↑ indicates at least 30% difference from control

↑↑ indicates at least 30% from syngeneic and 60% difference from control

Table 18. Pulmonary mRNA expression of different cytokines in rats from syngeneic or allogeneic liver transplant group in comparison to rats from control group.

Cytokine/group	Syngeneic	Allogeneic
• Pro-inflammatory: IL-1β, IL-6, 1L-12, IL-15, IFN-α • Anti-inflammatory: IL-13	↔	↑↑
• Anti-inflammatory: TGF-α	↑↑	↑
• Anti-inflammatory: IL-4	↑	↑
• Pro-inflammatory: IL-18,TNF-α	↑	↔
• Pro-inflammatory: IFN-γ • Anti-inflammatory: TGF-β	↑	↑↑

↔ indicates no difference from control.

↑ indicates at least 30% difference from control

↑↑ indicates at least 30% from the other group (syngeneic or allogeneic) and 60% difference from control

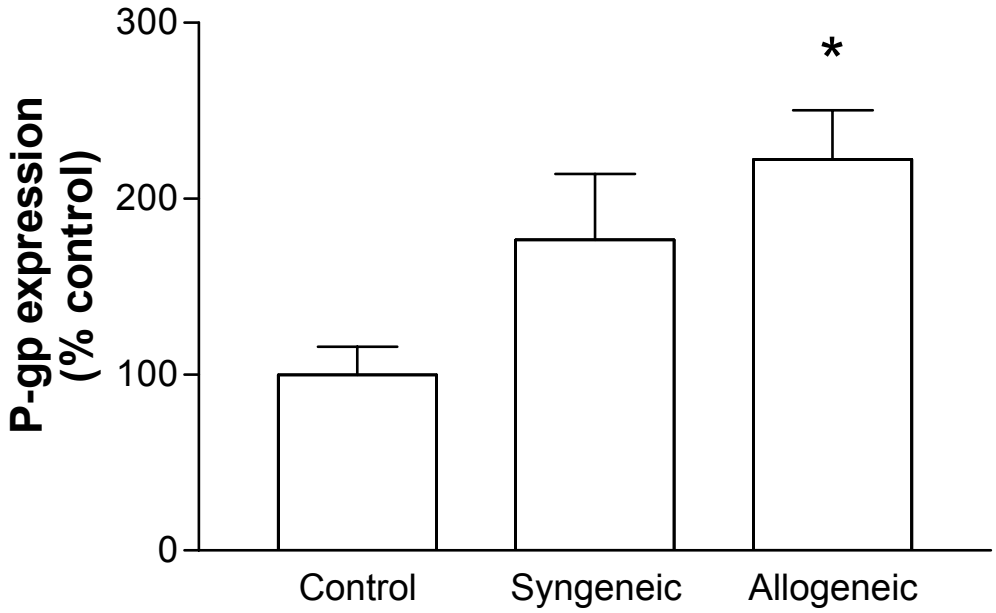

Figure 40. Protein expression of P-gp in livers of rats after syngeneic or allogeneic liver transplantation expressed as percentage of control (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

Effect of acute liver rejection on renal P-gp expression:

Acute rejection of the liver did not affect to a significant level the protein expression of P-gp in rat kidneys in both syngeneic and allogeneic groups (Figure 41). The mRNA expression of mdr1 gene in the both syngeneic and allogeneic groups was reduced to 47 and 28% as compared to control, respectively.

Effect of acute liver rejection on pulmonary P-gp expression:

Acute rejection of the liver caused increase in the protein expression of P-gp in rat lungs only in the allogeneic group (147% as compared to the control group) (Figure 42). However, it was not significant. The mRNA expression of mdr1 gene in the both syngeneic and allogeneic groups was increased to 169 and 133% as compared to control, respectively.

Effect of chronic liver rejection on hepatic P-gp expression:

Chronic rejection of the liver in transplant patients significantly increased the protein expression of P-gp in livers by about three folds compared to normal individuals (284% of normal group) as shown in Figure 43.

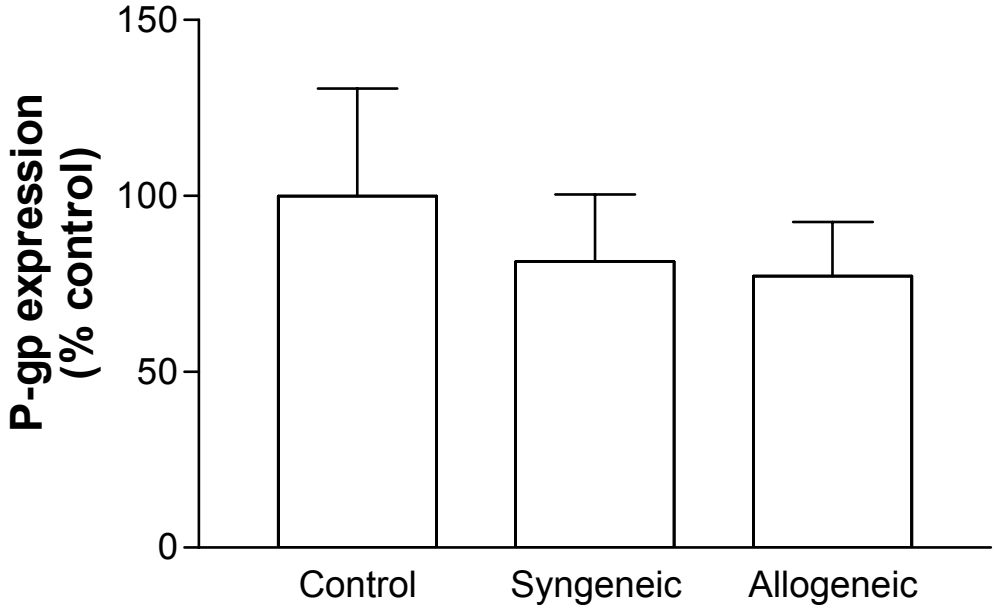

Figure 41. Protein expression of P-gp in kidneys of rats after syngeneic or allogeneic liver transplantation expressed as percentage of control (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

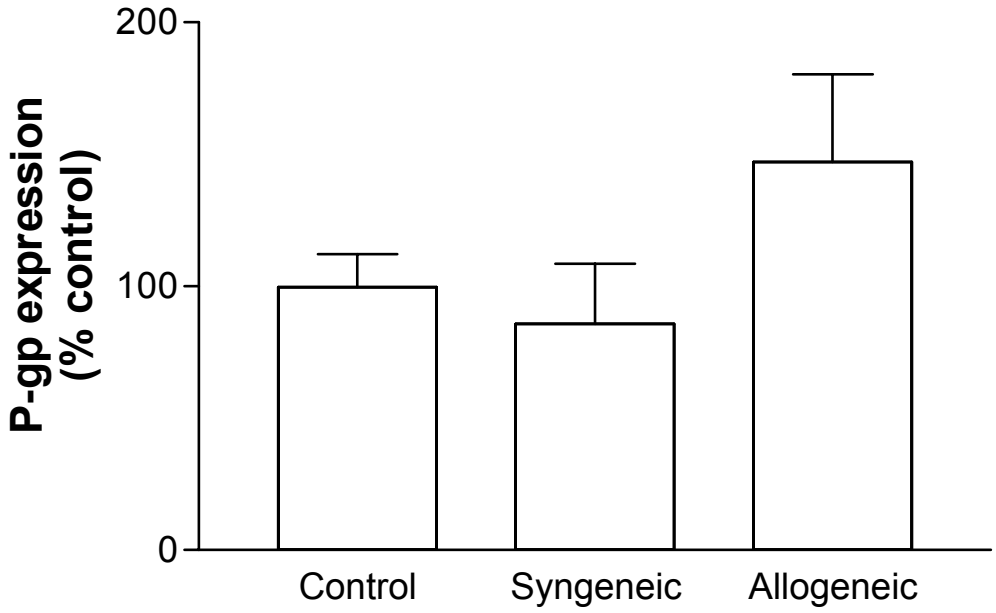

Figure 42. Protein expression of P-gp in lungs of rats after syngeneic or allogeneic liver transplantation expressed as percentage of control (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

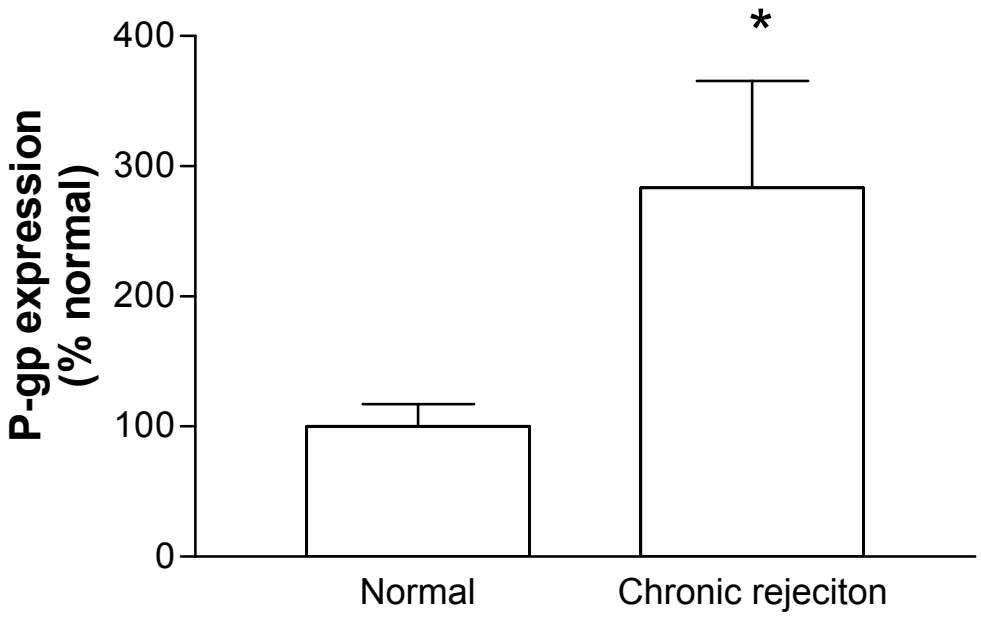

Figure 43. Protein expression of P-gp in livers obtained from patients who had chronic liver rejection expressed as percentage of control (n=4 for each group).

One way ANOVA/Tukey test was used to evaluate the presence of significant difference between groups at a $p \leq 0.05$.

*** significantly different from control.**

Other transporters:

mRNA expression of hepatic transporters:

The mRNA expression of different transporters was altered differentially in livers from rats in the syngeneic and allogeneic transplant groups (Table 19). Rejection per se increased the mRNA expression of mdr3 and decreased the mRNA expression of BSEP (SPGP).

mRNA expression of renal transporters:

The mRNA expression of different transporters was generally decreased in kidneys from rats in the syngeneic and allogeneic transplant groups (Table 20).

mRNA expression of pulmonary transporters:

The mRNA expression of different transporters was altered differentially in lungs from rats in the syngeneic and allogeneic transplant groups (Table 21). Rejection per se increased the mRNA expression of mrp2 (cMOAT).

Table 19. Hepatic mRNA expression of different transporters in livers of rats from syngeneic or allogeneic liver transplant group in comparison to rats from control group.

Transporter	Syngeneic	Allogeneic
MRP1	↑↑	↑
OCT	↑	↔
BSEP (SPGP)	↔	↓
MDR3	↔	↑
MRP2 (cMOAT)	↓	↔

↔ indicates no difference from control.

↑ indicates at least 30% difference from control.

↓ indicates at least 30% difference from control.

↑↑ indicates at least 30% from the allogeneic group.

Table 20. Renal mRNA expression of different transporters in kidneys of rats from syngeneic or allogeneic liver transplant group in comparison to rats from control group.

Transporter	Syngeneic	Allogeneic
MRP1, OCT	↑	↓
MDR3	↔	↓
BSEP (SPGP), MRP2 (cMOAT)	↓	↓↓

↔ indicates no difference from control.

↑ indicates at least 30% difference from control.

↓ indicates at least 30% difference from control.

↓↓ indicates at least 30% from the syngeneic group.

Table 21. Pulmonary mRNA expression of different transporters in lungs of rats from syngeneic or allogeneic liver transplant group in comparison to rats from control group.

Transporter	Syngeneic	Allogeneic
MRP2 (cMOAT)	↑	↑↑
MRP1, OCT	↑	↔
BSEP (SPGP)	↓	↔
MDR3	↔	↔

↔ indicates no difference from control.

↑ indicates at least 30% difference from control.

↓ indicates at least 30% difference from control.

↑↑ indicates at least 30% from the syngeneic group.

Discussions:

Transporters are important in facilitating the movement of drugs from one part of the body to another and from an organ to outside. These transporters are expressed in the liver, intestine, kidney, lung and brain and they regulate the absorption, distribution and elimination of several endogenous and exogenous chemicals. Some of the transporters are also expressed in lymphocytes and are involved in regulating the concentration of drugs in the lymphocytes and hence response to the drugs at this site. The transporters of importance include multi-drug resistance protein (MDR, which encode for P-Glycoprotein), multi-drug resistance associated proteins (MRPs), multi-specific organic anion transporter (cMOAT), organic cation transporter (OCT) and bile salt export pump (BSEP). Of all these transporters, P-gp appears to be the most important transporter that is involved in modulating the bioavailability and disposition of many drugs.

Despite the large volume of research that has been carried out on P-gp, the regulation of its expression is still not fully understood. It is known that the expression and activity of P-gp (mdr gene) is controlled by many mechanisms including gene transcription and amplification, mRNA stabilization and processing, protein stabilization and plasma membrane incorporation. Of these mechanisms, those that involve the effects on the mRNA level of mdr gene are reported the most in the literature (Sukhai and Piquette-Miller 2000).

Conditions of inflammation which can be either localized or systemic are known to alter the activity and expression of P-gp. Different models of inflammation have been shown to alter both the activity and protein expression of the hepatic P-gp. Localized and systematic inflammation,

using turpentine and endotoxin respectively, caused reduction in the activity of P-gp and its mRNA expression in rat livers (Piquette-Miller *et al.* 1998). This reduction was attributed to the release of different cytokines in such conditions (IL-1β, IL-6, TNF-α, IFN-γ) in both rodents and man (Walther and Stein 1994; Stein *et al.* 1996; Sukhai and Piquette-Miller 2000; Sukhai *et al.* 2000; Sukhai *et al.* 2001). Other studies have found that inflammation caused up-regulation of the mRNA expression of mdr1b and at the same time had no effect on the mRNA expression of mdr1a or mdr2 (Vos *et al.* 1998). The inflammatory cytokine IFN-α has been shown to induce the P-gp activity and expression in multi-drug resistant Chinese hamster ovary cells (Kang and Perry 1994).

In our model system, syngeneic and allogeneic transplantation caused an increase in the release of cytokines both locally and peripherally as measured by the mRNA expression of these cytokines in the tissues taken from the livers, kidneys and lungs (Tables 15, 16 and 17). The P-gp protein expression in the livers was significantly increased to 222% of control in the allogeneic group. This increase in the allogeneic protein expression of P-gp was associated with an increase in the mRNA content of mdr1 to 159% of the control group. The increase in hepatic P-gp expression in our study may indicate different profiles of cytokines and ROIs in different models of inflammation. In human livers that were subjected to chronic rejection, the protein expression of the P-gp was also significantly increased to 284% as compared to samples from normal individuals. This observation is similar to a recent report indicating an increase in the renal P-glycoprotein expression during rejection of kidney in a kidney transplant patient. This increase in expression was proportional to the severity of rejection (Vergara *et al.* 1998). Also, it has been

shown that P-gp is up-regulated in peripheral T-Cell subsets from solid organ transplant patients (Donnenberg *et al.* 2001).

In the kidneys, there were no significant differences in P-gp protein expression. There was however a decrease in the mRNA expression of mdr1 to 47 and 28% for both syngeneic and allogeneic groups, respectively. In the lungs, we found higher but not significant expression of P-gp protein (147% of control) along with a higher expression of mRNA in the allogeneic transplant group.

In addition to P-gp, the mRNA expression of other transporters in liver, kidneys and lungs were also altered by the inflammatory processes in our model system. In the liver, the mRNA expression of mdr3 was only increased in the allogeneic groups as in the case of mdr1 which may indicate that both mdr1 and mdr3 are regulated by similar pathway in the liver. The mRNA expression of BSEP (SPGP) was reduced by process of rejection in the allogeneic group. This is consistent with the observed decreased bile secretions after liver transplantation. Also, rejection did not change the mRNA expression of OCT. These observations on the mRNA expression of these transporters in the allogeneic group are probably due to the presence higher levels cytokines. Also, high levels of ROIs are expected since the mRNA expression of different antioxidant enzymes such as GST, SOD and CAT were decreased in allogeneic transplant group.

In the kidneys, the mRNA expression of all the studied transporters was decreased as a result of the process of rejection in the allogeneic group. However, syngeneic transplant also reduced the mRNA expression of BSEP (SPGP) and mrp2 (cMOAT) but to a lower extent as compared to

the allogeneic group. The only exception in the kidney is the increase in the mRNA expression of mrp1 and OCT in the syngeneic group. These effects suggest that kidneys may be very sensitive to effect of cytokines and ROIs. The mRNA expression of antioxidant enzymes (GST, SOD and CAT) was only reduced in the allogeneic group and not changed in the syngeneic group.

In the lungs, the mRNA expression of mrp2 (cMOAT) was increased in syngeneic group and the occurrence of liver rejection caused further increase in the expression of this transporter. Finally, the mRNA expression of mdr3 was not changed in both groups. The involvement of ROIs in the lungs is not expected due to the presence of induced antioxidant enzymes (GST, SOD and CAT) in both syngeneic and allogeneic groups thus accumulation of ROIs is not likely in this tissue.

Similar observations in the expression of transporters where some transporters are increased while other are decreased have also been reported in the literature. For example, mrp1 expression was increased while in the same study P-gp expression was decreased in human colon carcinoma cell lines after treatment with TNF-α (Stein *et al.* 1997).

In conclusion, we found an increase in the expression of P-gp in liver in acute and chronic rejection. However, chronic rejection did not cause a significant change in the expression of P-gp in kidneys and lungs. This point to differential expression of P-gp in different organs in response to an inflammatory process. The processes of transplantation and rejection are associated with the release of different mediators that cause induction of the expression of different transporters. Changes in expression and activity of different transporters are expected to contribute to the

variability in the pharmacokinetics of drugs used in transplant patients. Future studies must address the effect of individual cytokines on the expression of different transporters in different organs.

Chapter 7

Effect of cytokines on hepatic CYP3A4 activity and expression in human hepatocytes

Introduction:

The effects of inflammation and infection on cytochrome P450 are largely due to stimulation of the cellular immune response (Morgan 1997). As early as in 1966, various agents that stimulate the immune system were shown to prolong barbiturate sleeping time (Wooles and Borzelleca 1966; Morgan 1997). Also, infection with influenza virus (Chang *et al.* 1978) or injection of influenza vaccines (Renton *et al.* 1980) caused elevation in plasma concentrations and increase in half-lives of theophylline and other drugs (Morgan 1997). Most of the effect of inflammation and infection is mediated by cytokines. Cytokines are soluble hormone-like proteins which are produced by different cells following stimulation with different inducers. In contrast to hormones, which are synthesized by endocrine tissues, cytokines are produced by a variety of cells. They can be classified broadly into interleukins (IL1-18), interferons (IFN-α,β and γ), growth factors (HGF, CSF, EGF, etc), chemokines (CXC, CCβ and Cγ) and tumor necrosis factors (TNF-α and β) (Simpson *et al.* 1997). IL-1, IL-2, IL-6, TNF and IFN are some of the important mediators that can cause changes in the metabolic capacity of an organ.

The aim of this study was to evaluate the effect of IL-1β, IL-2, IL-6 and TNF-α on the constitutive and inducible hepatic CYP3A4 activity and protein expression using human hepatocytes. In particular, we evaluated the effect of different treatment orders corresponding to different scenarios observed in clinical settings on CYP3A4 activity and protein expression.

Experimental methods:

Isolation of human hepatocytes:

Hepatocytes were isolated from donor livers by a three-step collagenase perfusion technique. The viability of the hepatocytes was assested by Trypan Blue exclusion test as described in chapter 2.

Hepatocytes culture:

Hepatocytes were suspended in Williams E medium (HMM) in the presence of 0.1μM insulin, 0.1 μM dexamethasone, 0.05% gentamicin and 10% BCS (bovine calf serum). This hepatocyte suspension was plated on six well plates which were covered with collagen. These hepatocytes were then kept for 4-6 hours to attach to the collagen. The medium was then replaced with fresh medium with out the BCS. A fresh medium was added every 24 hours and hepatocytes were maintained at 37°C at all times in the presence of 5% CO_2 and 95% O_2.

Hepatocytes incubation for dose response studies:

Forty eight hours after plating the hepatocytes, the hepatocytes were maintained in fresh medium. After that hepatocytes were treated with a range of concentrations (10-300 pg/ml) of each cytokine (IL-1β, IL-2, IL-6 and TNF-α) alone for another forty eight hours. At the end of the last day of treatment, the hepatocytes were washed with fresh blank medium for one hour to wash out the cytokines. Then, hepatocytes were incubated with a fresh blank medium containing testosterone. After 30 minutes, the medium was collected and frozen at -20°C until analysis. The hepatocytes were collected from the plates using scrapers in the presence of 150μl phosphate

buffer (0.1 M, pH 7.4) for protein measurement. Samples were frozen until further protein determination and western blotting.

Hepatocytes incubation for treatment order studies:

Twenty four hours after plating the hepatocytes, the hepatocytes were maintained in fresh medium. After that the hepatocytes were treated for four days (each two days with different treatment) as seen in Table 12 using 300 pg/ml of each cytokine (IL-1β, IL-6 and TNF-α). At the end of the fourth day of treatment, the hepatocytes were washed with fresh blank medium for one hour to wash out the cytokines. Then, hepatocytes were incubated with a fresh blank medium containing testosterone. After 30 minutes, the medium was collected and frozen at -20°C until analysis. The hepatocytes (protein) were collected from the plates using scrapers in the presence of 150µl phosphate buffer (0.1 M, pH 7.4) for protein measurement. Samples were frozen until further protein determination and western blotting.

Table 22. Different treatment orders used in the human hepatocyte culture studies.

days	1	2	3	4	5	6
A	Plate	HMM	HMM	HMM	HMM	test with testosterone
B	Plate	HMM	HMM	Rif	Rif	test with testosterone
C	Plate	Cyto	Cyto	Cyto	Cyto	test with testosterone
D	Plate	Cyto	Cyto	Rif	Rif	test with testosterone
E	Plate	Cyto	Cyto	Cyto+Rif	Cyto+Rif	test with testosterone

HMM=hepatocytes maintenance media, Cyto=cytokine, Rif=Rifampicin

Total protein estimation:

The hepatocytes protein contents were determined according to the procedure of Lowry as described in chapter 2.

Determination of enzyme protein expression:

Protein expression for CYP3A4 enzyme was evaluated by Western blotting as described in chapter 2.

Statistical Analysis:

After log transformation of the data, one way ANOVA/Tukey test was used to evaluate the presence of significant differences between the groups at a p-value ≤ 0.05. This was carried out using Prism software version 3.02 (GraphPad Software Inc., San Diego, CA).

Results:

Dose response studies:

Pre treatment of the hepatocytes with cytokines (IL-1β, IL-6 and TNF-α) (10-300 pg/ml) for two days resulted in inhibition of CYP3A4 activity. IL-1β was the most powerful inhibitor of CYP3A4 activity followed by TNF- α and IL-6. Treatment with IL-2 did not alter the activity of CYP3A4 at all the concentration tested. Therefore, we did not use IL-2 in any further studies.

Treatment order studies:

Treatment A in all the Figures (44-47) is the control and shows CYP3A4 activity and protein expression when the cells were not exposed to any treatment (only medium). Treatment B shows the activity and protein expression of CYP3A4 after induction with rifampicin. In two of the cultures we studied only treatment A, B, C and E while in the other two cultures we evaluated treatment A, B, D and E (Table 22). In all the cultures studied, IL-1β, TNF-α and to a lesser extent IL-6 reduced the constitutive activity and expression of the CYP3A4 enzyme as shown for the treatment "C" in Figures 44 and 45. In case of treatment "E" in Figures 46-47, the presence of both the cytokine and rifampicin after two days of pretreatment with cytokines alone blocked the induction by rifampicin mainly by IL-1β followed by TNF-α. IL-6 on the other hand blocked the induction in some cases (Figures 45 and 47) but showed no blockage in others (Figures 44 and 46). When the hepatocytes were treated with cytokines for two days and followed by rifampicin alone for another two days (treatment D), there were blockage in induction but this blockage was not as strong as in case of the continues presence of cytokines together with the rifampicin. IL-1β and TNF-α blocked the induction in both cases studied (Figures 46 and 47). IL-6 did not block the induction in both cases (Figures 46-47).

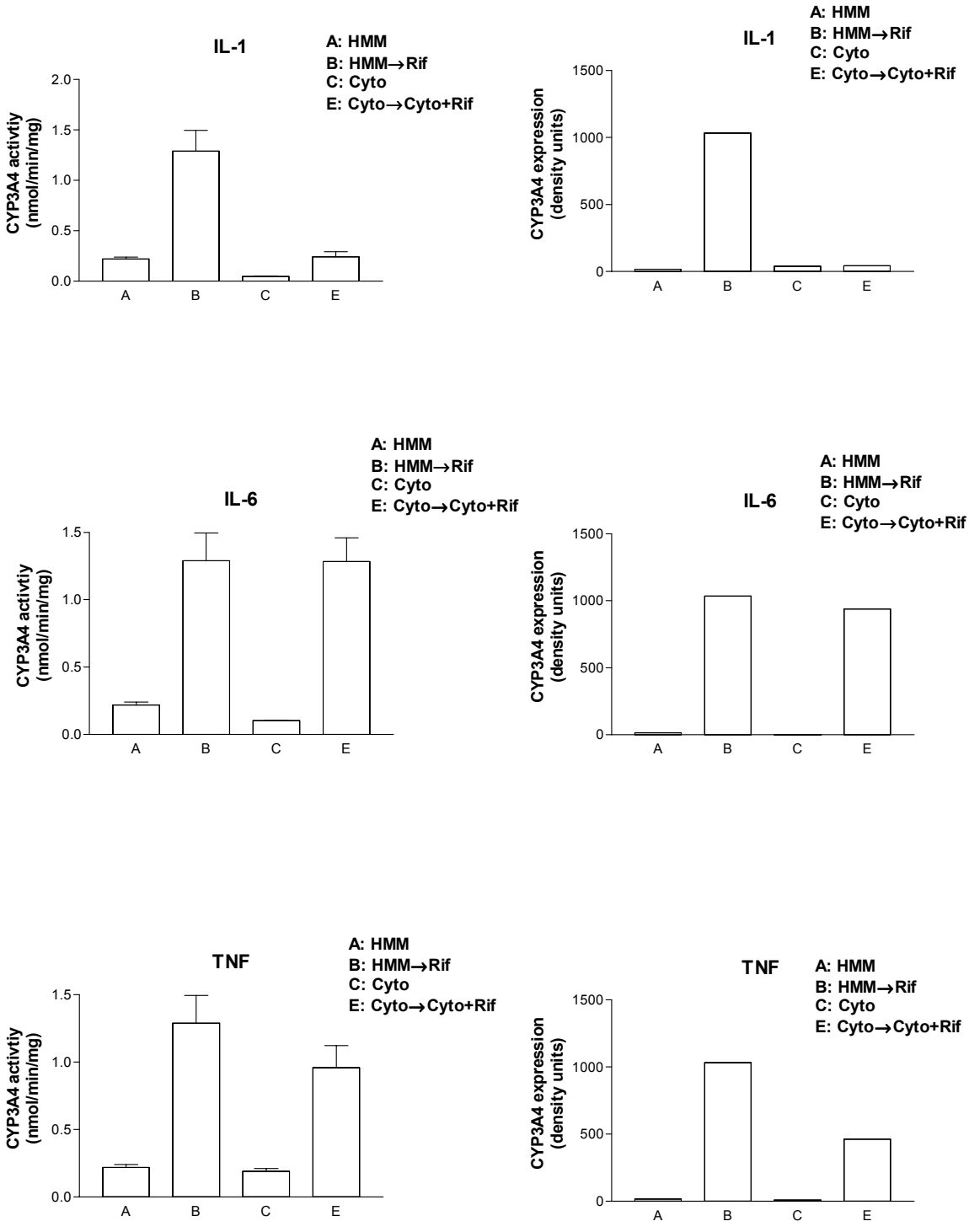

Figure 44. Effect of different cytokines on CYP3A4 enzyme activity and protein expression following different treatment orders in human hepatocyte cultures (HH975). The concentration used for each cytokine was 300 pg/ml.

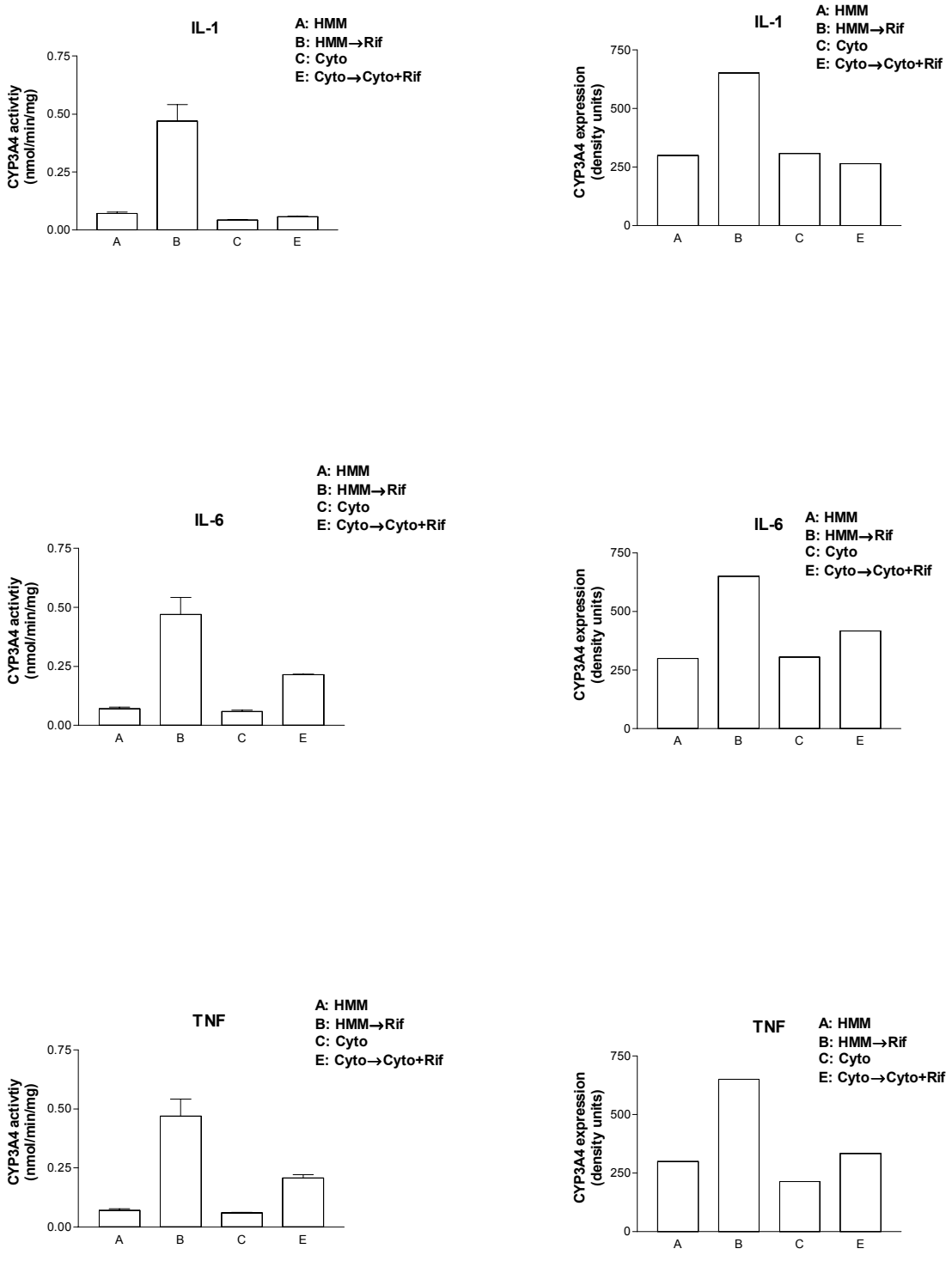

Figure 45. Effect of different cytokines on CYP3A4 enzyme activity and protein expression following different treatment orders in human hepatocyte cultures (HH976). The concentration used for each cytokine was 300 pg/ml.

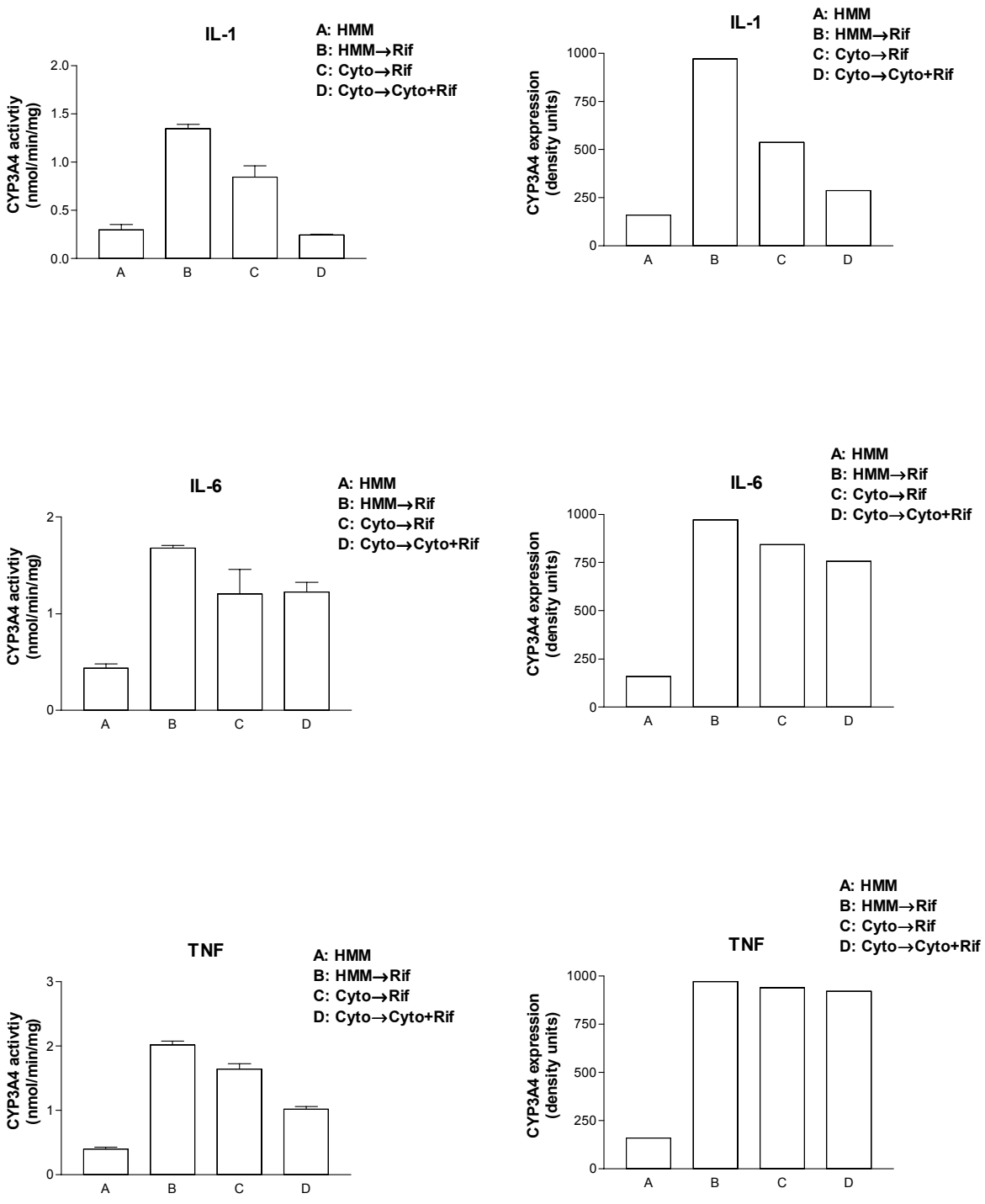

Figure 46. Effect of different cytokines on CYP3A4 enzyme activity and protein expression following different treatment orders in human hepatocyte cultures (HH959). The concentration used for each cytokine was 300 pg/ml.

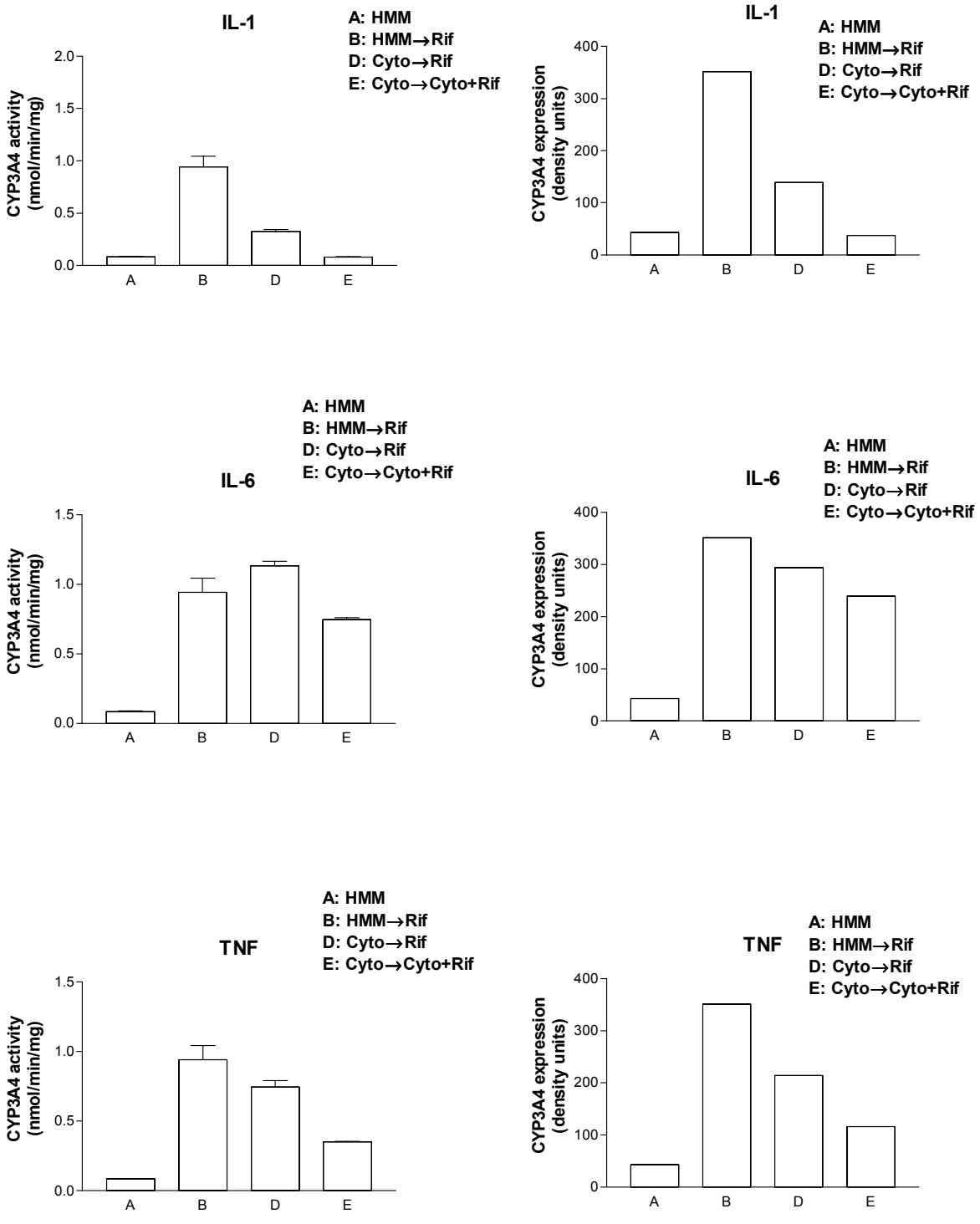

Figure 47. Effect of different cytokines on CYP3A4 enzyme activity and protein expression following different treatment orders in human hepatocyte cultures (HH993). The concentration used for each cytokine was 300 pg/ml.

Effect of combination of cytokines:

When using combination of cytokines we saw a similar trend in activity and protein expression as in case of individual cytokines (Figures 48 and 49). Treatment of hepatocytes with combination of cytokines during the four pretreatment days (treatment "C" in Figures 48 and 49), caused inhibition of both CYP3A4 activity and protein expression in both cases studied. In treatment "D", the induction by rifampicin after two days of treatment with cytokines combination was blocked in the first culture. However, in the other culture, the cytokines did not cause any blockage of the induction (for both CYP3A4 activity and protein expression) produced by rifampicin (Figure 49). On other hand, when treating the hepatocytes for two days with the combination of cytokines for two days and then treat them with both cytokines and rifampicin (treatment "E"), the induction was blocked in both cases.

Effect of co-administration of cytokines and testosterone:

When we co-administered the cytokines and the testosterone without pre treatment of the hepatocytes, we did not see any direct effect of cytokines on CYP3A4 enzyme activity (Figure 50).

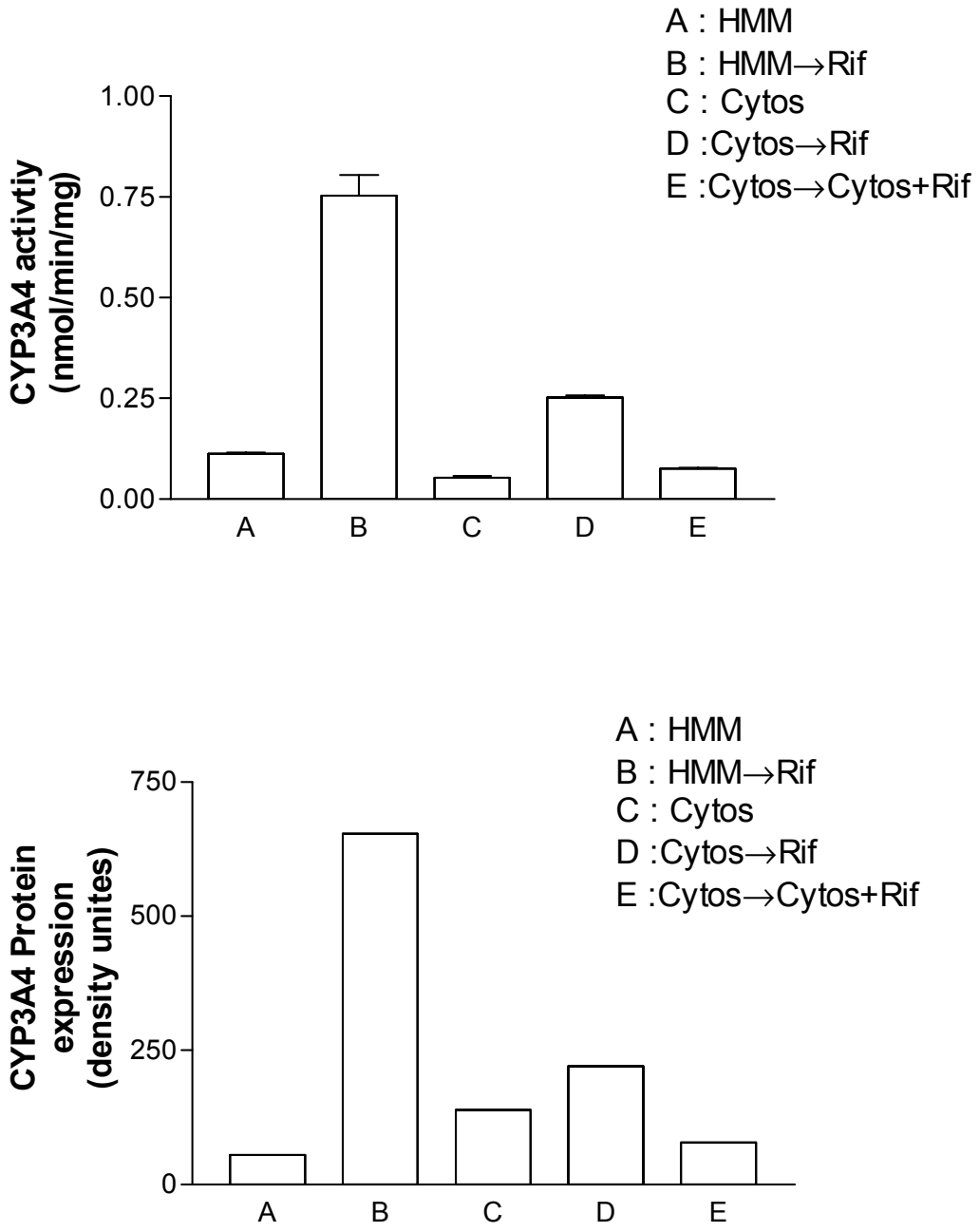

Figure 48. Effect of different cytokines on CYP3A4 enzyme activity and protein expression following different treatment orders in human hepatocyte cultures (HH995) using combination of cytokines (300 pg/ml of each cytokine).

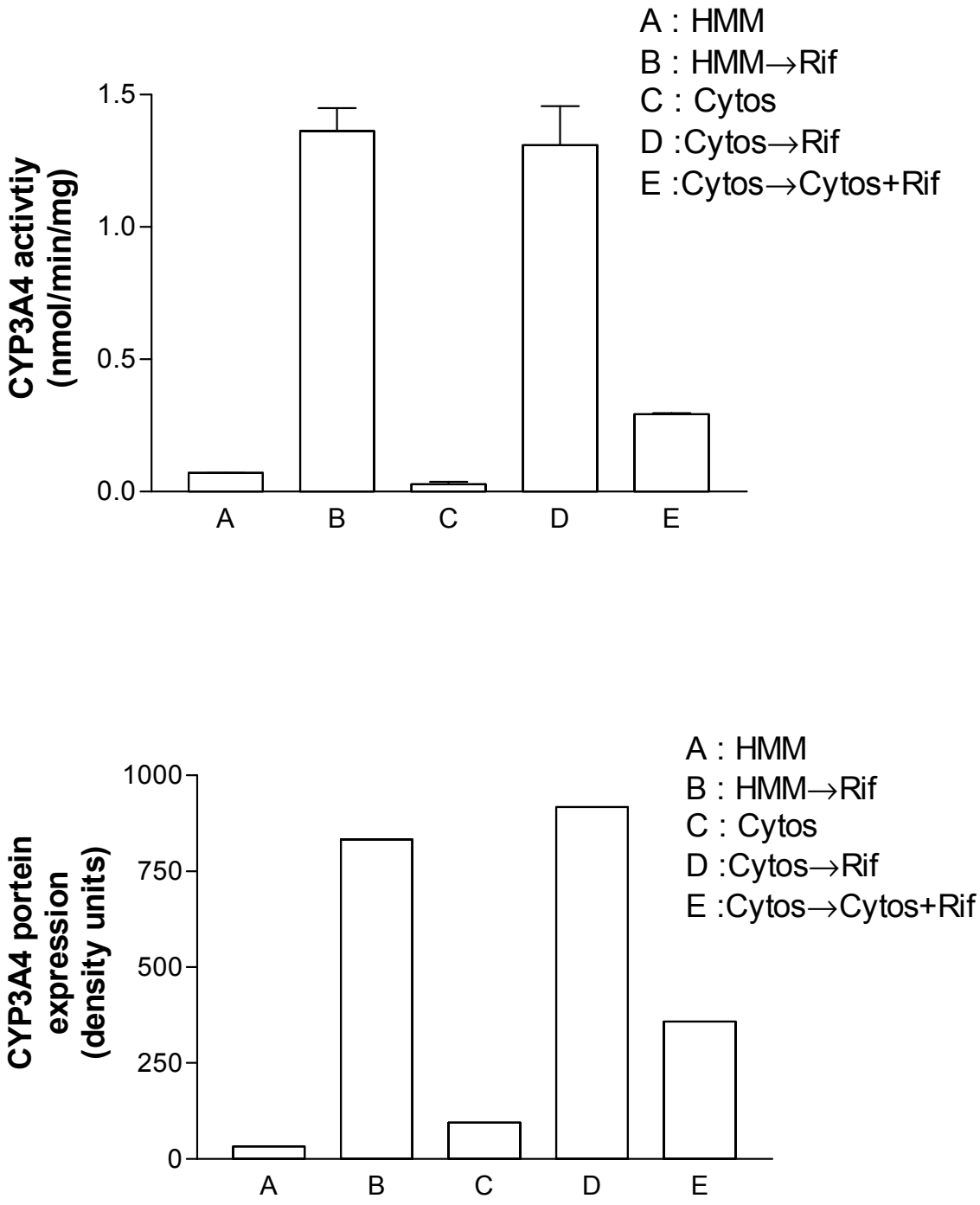

Figure 49. Effect of different cytokines on CYP3A4 enzyme activity and protein expression following different treatment orders in human hepatocyte cultures (HH996) using combination of cytokines (300 pg/ml of each cytokine).

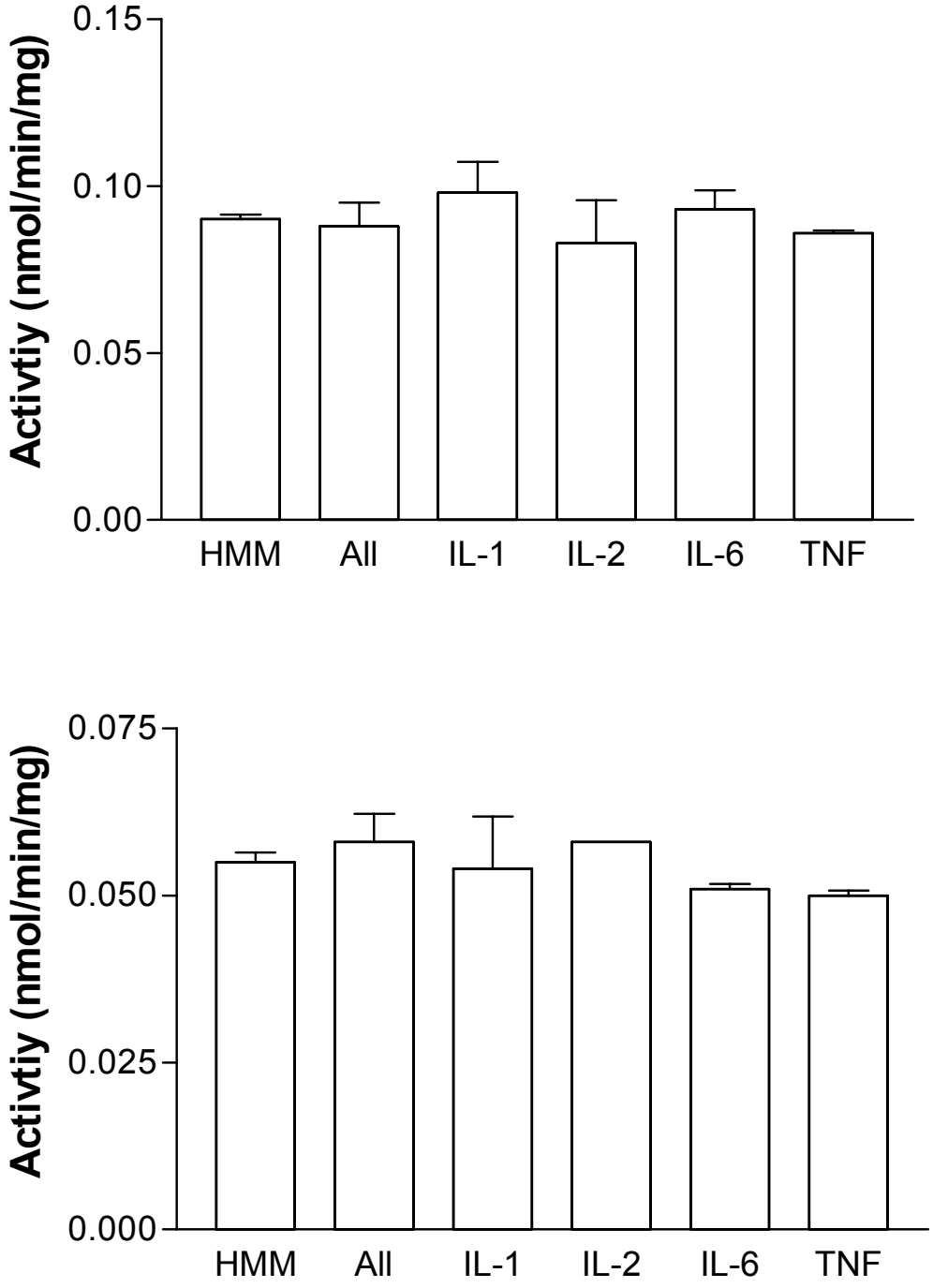

Figure 50. Effect of co-treatment with cytokines on CYP3A4 activity as measured by metabolism of testosterone (HH995 and HH996). Cytokines were added to the culture either separately (300 pg/ml) or together (300 pg/ml of each cytokine).

Discussions:

It is well known in the literatures that cytokines impair the metabolic capacity of drug metabolizing enzymes and this explains the changes seen in conditions such as inflammation. Many researchers have studied the direct effect of cytokines on drug metabolism both in vivo and in vitro using microsomal systems. However, the use of human hepatocytes in such studies is limited. Among the studies carried out using hepatocytes, human liver hepatocytes were not commonly used and if used, most of the studies evaluated only the protein and/or mRNA expression of these enzymes and not the activity. Furthermore, non-physiologic concentrations of cytokines were evaluated in previous studies. In our studies, we utilized the human hepatocytes as a model for studying the direct effect of different cytokines on CYP3A4 activity and protein expression. We also studied different orders of pre treatment which correspond to different clinical situations. These different scenarios were not investigated in the studies reported in the literature. Hepatocytes within the "A" treatment were not treated with anything other than the hepatocytes maintenance media. Treatment "A" in our studies serves as a control that represents the constitutive or basal levels of CYP3A4 activity and protein expression. Treatment "B" serves as positive control for induction and these hepatocytes were induced with rifampicin. Treatment "C" shows the effect of cytokines on the constitutive levels of CYP3A4. Treatment "D" shows the potential of induction after a previous exposure to cytokines as in case of patients recovering from an inflammatory condition or infection. Finally, Condition "E" represents the induction potential during an inflammatory condition or infection.

The effect of cytokines on the activity and expression of CYP3A was studied by many researchers. The activity of CYP3A2 in rats was reduced by IL-1β and TNF-α (Ferrari *et al.*

1993; Nadin *et al.* 1995). Its protein expression was also down regulated by IL-1α, IL-1β, IL-2, TNF-α, IFN-α, IFN-β and IFN-γ in both human and rodents (Craig *et al.* 1990; Craig *et al.* 1993; Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carelli *et al.* 1996; Carlson and Billings 1996). The mRNA expression for this enzyme was also down regulated by IL-1α, IL-1β, IL-2, TNF-α and IFN-α in both human and rodents (Craig *et al.* 1990; Craig *et al.* 1993; Chen *et al.* 1995; Muntane-Relat *et al.* 1995; Nadin *et al.* 1995; Tinel *et al.* 1995; Carlson and Billings 1996).

We first conducted a study using various concentrations of cytokines (10-300 pg/ml) observed in patients undergoing rejection of transplanted organs in order to select the concentration that we can use for the next part of the study (treatment order). We subsequently used a concentration of 300 pg/ml in further studies. Our results were consistent with what is published in the literature about effect of different cytokines (IL-1β, IL-2, IL-6 and TNF-α) on drug metabolism.

Based on the result obtained from dose response studies, we found that IL-1β, TNF-α and to a lesser extent IL-6 reduced the constitutive activity and protein expression of the CYP3A4 enzyme. In the treatment orders studies, induction by rifampicin (B) caused an increase in the activity and protein expression of CYP3A4 enzyme as compared to basal levels (A). Similar to the result seen during evaluating the dose response curve, IL-1β, IL-6 and TNF-α caused reduction in the constitutive activity and protein expression of CYP3A4 in the two cases studied. In cases when the hepatocytes were induced following a previous exposure to cytokines (D) as in situations when patients just recovered from inflammation or infection and then given a medication that has the potential for inducing CYP3A4, the induction was blocked by the

presence of cytokines mainly by IL-1β and TNF-α. However, IL-6 did not block the induction in both cases. In a previous study performed using human hepatocytes, IL-6 and TNF-α blocked the induction of CYP3A4 protein and mRNA expression by rifampicin (Muntane-Relat *et al.* 1995). However, most of the studies used a non-physiological concentration of cytokines. In treatment "E", the presence of both the cytokine and rifampicin after two days of pretreatment with cytokines as in condition when patients are already undergoing a condition of inflammation or infection and then given an inducer for CYP3A4, caused blockage for the induction by rifampicin mainly by IL-1β followed by TNF-α. The blockage was stronger than seen in treatment "D". Effect of IL-6 was different between cases since it only caused blockage of induction in some case.

Similar trend for CYP3A4 activity and protein expression was seen when we did the treatment order studies using a combination of cytokines. In treatment "C", treating hepatocytes with cytokine combination during the four pretreatment days, the protein and activity were inhibited in both cases studied. The induction by rifampicin after two days of treatment with cytokines combination (D) was blocked in one culture while the second culture did not show any blockage. On the other hand, when treating the hepatocytes for two days with the combination of cytokines for two days and then treat them with both cytokines and rifampicin (E), the induction was blocked in both cases. The changes observed in CYP3A4 activity and protein expression by cytokines were not due to a direct effect of the cytokines on the CYP3A4 enzyme since we found that co-incubating the cytokines with the testosterone for 30 min did not change the activity of the CYP3A4 enzyme.

In conclusion, clinically relevant concentrations of IL-1β, IL-6 and TNF-α caused a reduction in both constitutive and induced CYP3A4 activity and protein expression. Previous exposure to cytokines decreased the magnitude of induction with Rifampicin. Continued presence of cytokines abolished the inductive effect of rifampicin. These results may explain the variability observed in the pharmacokinetics of several CYP3A4 substrates in patients with different inflammatory conditions or in patients recovering from infections.

Chapter 8

Conclusions and future directions

Conclusions:

Organ transplantation is an accepted therapy for diseases that result in chronic irreversible failure of organs such as liver, kidney, intestine, heart and lung. Organ transplant patients often receive multiple drug therapy that includes immunosuppressive drugs, antibiotics, antiviral agents, antifungal agents, antihypertensive agents and others. Following transplantation, patients undergo marked changes in their physiology, that may cause changes in absorption (due to changes in GI motility, altered splanchnic blood flow, change in bile composition and flow, changes in acid or enzymes secretion and presence of concomitant drugs), distribution (due to changes in organ blood flow, body fluid content, tissue partitioning and plasma protein binding) or elimination of drugs (due to changes in the intrinsic activity of the enzymes in the eliminating organs, the blood flow to the organ and the blood protein binding of the drug). We have observed large variations in the pharmacokinetics of several drugs used in transplant patients.

After transplantation of an organ, the transplanted organ is subjected to two inflammatory processes that can potentially alter its function. Both of these processes involve release of several cytokines. One of these processes is alloantigen-dependent while the other is alloantigen-independent. The alloantigen-independent inflammatory process starts within the transplanted organ itself as a result of different process including surgery, cold ischemia, preservation, warm ischemia and reperfusion. The other, alloantigen-dependent process results from the recognition of the alloantigen by the host T-cells which can result in the damage and eventually the rejection of the transplanted organ. Rejection can be one of three types which are categorized according to the time of onset and the type of histological changes to hyperacute, acute or chronic rejection. During acute rejection of the liver, when several of the inflammatory mediators are released in

the body, the clearance of antipyrine is significantly decreased in liver transplant patients (unpublished observations). In addition, in rats acute rejection of the liver also leads to impaired clearance of tacrolimus and reduced biliary excretion of BSP (unpublished observations). Systematic evaluations of the various factors that contribute to the alteration of metabolism of drugs in transplant patients have not been carried out.

The objectives of this dissertation were to evaluate the effect of acute rejection of liver in rats on the hepatic (Chapter 3) and extra-hepatic (Chapter 4) drug metabolizing capacity, protein expression and mRNA expression for phase I and phase II enzymes, to evaluate the effect of chronic rejection of liver in humans on the hepatic metabolic capacity and protein expression of phase I and phase II enzymes (Chapter 5), to evaluate the effect of acute and chronic rejection of liver in rats and human, respectively on the hepatic and extra-hepatic protein and mRNA expression of P-gp and the mRNA expression of other transporters (Chapter 6) and finally, to evaluate the effect of different cytokines on the constitutive and inducible hepatic CYP3A4 activity and protein expression using human hepatocytes (Chapter 7).

Alloantigen-independent inflammation and altered blood flow as studied in our syngeneic transplant group caused a reduction in the activity of drug metabolizing enzymes. This indicates the importance of properly preserving the organ during the surgery, preservation and transportation from the donor to the recipient sites. This may be achieved by methods such as adding antioxidants to the preservation solutions in order to minimize the enzymes inactivation by the free radicals.

In addition, occurrence of rejection (in addition to the presence of all factors mentioned for the syngeneic transplant) resulted in a further reduction in the activity of the metabolizing enzymes probably due to presence of higher levels of different cytokines and ROIs. The effect of cytokines can be minimized by optimizing the immunosuppressive therapy by closely monitoring the concentrations of immunosuppressive drugs in transplant patients. Also, reducing the concentration of cytokines in these patients can help to minimize the variation on the metabolism of certain drugs. This can be achieved by the use of soluble cytokines receptors. Our observation also points out that use of cytokine receptor antagonists are likely to increase the levels of circulating cytokines and subsequently decrease the hepatic drug metabolism.

We also observed that chronic rejection of the liver in humans selectively altered the activity and protein expression of different phase I and phase II enzymes with UGT1A9 being reduced to a higher magnitude as compared to other CYP450 enzymes. This result was not expected and it was different from what is known about the preservation of the UGT enzymes in patients with liver diseases. The concentration of MPA is likely to increase and lead to toxicity in transplant patients when they are chronically rejecting their livers.

We also observed alteration in the metabolism in extra-hepatic tissues (kidneys and lungs). Despite the low involvement of the extra-hepatic tissues in drug metabolism as compared to the liver, changes in the activity of these enzymes in extra-hepatic tissues may be of important toxicological consequences to that organ.

We also observed increased expression of P-gp in the rejecting liver and alteration of expression of other transporters. These finding may be of importance since these transporters regulate the concentrations of different drugs inside different organs and cells.

In our hepatocytes work, we found that IL-1β and TNF-α decreased the activity and protein expression of constitutive and induced CYP3A4 enzyme. This finding indicates that patients are likely to have less magnitude of induction due to drugs like rifampicin compared to normal subjects and as a consequence, one should exercise caution when extrapolating data from normal healthy subjects to patients who are subjected to inflammatory process. It also indicates that depending on the patient's inflammatory status, CYP3A4 activity and protein expression will be different. Thus, its clear that the status of immune system of the patient contributes to the observed variability in the pharmacokinetics and perhaps the pharmacodynamics of drugs in transplant patients.

Future directions:

The studies presented in this dissertation have evaluated the effect of the process of liver transplantation and the subsequent inflammatory processes (alloantigen-independent and alloantigen-dependent) that are associated with it on drug metabolism and transport using syngeneic and allogeneic liver transplantation in rat. We also evaluated the effects of chronic rejection using human liver samples, and finally effects of direct additions of different cytokines using human hepatocytes. These studies are a first step toward understanding the effect of rejection on drug metabolism and transport in transplant patients. However, these are in vitro studies. Our observations point to additional studies that are needed in order to further our understanding of the effects of the process of rejection on drug metabolism in man. In our studies we addressed changes in the activity, protein expression and mRNA expression of different enzymes and expressions of transporters. We also evaluated changes in the intrinsic clearance. However, in vivo, pharmacokinetics of a drug depends not only on the intrinsic clearance but also on the blood flow to the organ and the unbound fraction of drug in blood. Thus, future studies should also evaluate these parameters. In addition to elimination, pharmacokinetics of drugs are also dependent on absorption and distribution which are not addressed here, thus future work should also evaluate these components.

In the rat studies, we observed significant change in the activity and expression of several drug metabolizing enzymes in the syngeneic transplant group. In order to limit the magnitude of such a "syngeneic effect" future studies can be designed differently. For example, rats can be treated with immunosuppressive drug therapy directly after the transplantation of the organ for a period of time (to allow enough time for the cytokines and other mediators released as a result of the

surgery itself to return to normal levels) and then allow the organ to reject and evaluate the activity of various drug metabolizing enzymes.

For some enzymes studies (other than CYP3A, CYP2C, CYP2E and UGT1A), we only evaluated mRNA expression of these enzymes. However, the protein expression and activity of these enzymes may not be affected in a similar manner. Thus, future work should also include markers for measuring the activity of these enzymes and also western blotting for these enzymes need to be evaluated. Similarly, when we evaluated different transporters, we measured mRNA and protein expressions (in case of P-gp) or only mRNA (in case of other transporters). These expressions do not necessarily indicate that the transporter is active despite the presence of high protein and mRNA levels. Therefore, evaluating the functional aspects of these transporters is important in the future work. Cytokines were evaluated at the mRNA levels in the present study. Future studies should also measure the plasma concentrations of individual cytokines and relate their concentrations to observed changes in activity of drug metabolizing enzymes.

It will be of interest to study the effect of the rejection of other organs (such as kidney, heart and intestine) on the hepatic metabolic and transport capacity. The effect of intestinal transplantation on the hepatic drug metabolism is especially important since all the cytokines produced locally will be delivered to the liver directly through the portal vein.

While we studied effect of cytokines on the activity and protein expression of CYP3A4 in human hepatocytes, other enzymes (CYP1A2, CYP2E1 and UGTs) need to be evaluated similarly since patients undergoing inflammation and infection conditions will be taking medications that are

not only substrates for CYP3A4. Furthermore, it will be interesting to evaluate the effect of cytokines on P-gp and other transporters expression and activity using human hepatocytes cultures. Currently, we are in the process of evaluating the mRNA expression of CYP3A4 as well as the protein and mRNA expression of P-gp and other transporters.

Finally, we expect the process of rejection and the associated inflammatory condition to have an effect on the pharmacodynamics of drugs due to possible increase in P-gp expression in the lymphocytes which are target of most of the immunosuppressive drugs. This will subsequently lead to lower drugs accumulation inside the lymphocyte and thus reduce the effect of these drugs on lymphocyte proliferation.

Chapter 9

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