ORTHOTOPIC LIVER TRANSPLANTATION AT GROOTE SCHUUR HOSPITAL : A SERIAL ANALYSIS OF BILIARY CYTOKINES AND BIOCHEMICAL PARAMETERS

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ABSTRACT

Orthotopic liver transplantation is the treatment of choice for many patients with end-stage liver disease. Despite advances in immunosuppression, acute rejection remains common (up to 70%) and results in significant patient morbidity.

It is frequently difficult to distinguish abnormal liver function due to rejection from that due to infection, biliary obstruction or ischaemic injury without performing invasive procedures such as a liver biopsy or angiography which may be hazardous. Clinically, the diagnosis of rejection is usually made late, once the immunological process is already established.

In this study, we evaluated standard biochemical parameters and cytokine concentrations (IL-1, IL-6 and TNF-alpha) in serial samples of bile obtained post-operatively via the T-tubes of patients following orthotopic liver transplantation in order to determine whether there are any biochemical or immunological pointers to the early diagnosis of rejection which would enable earlier administration of appropriate anti-rejection therapy.

Biliary cytokines did not prove to be useful and reliable markers of early rejection. Serial measurement of biliary bilirubin levels showed an early and significant decrease a few days prior to rejection, and were a more sensitive marker of graft function than serum bilirubin levels.

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CHAPTER ONE

Orthotopic Liver Transplantation At Groote Schuur Hospital

In the last decade, liver transplantation has evolved from an experimental procedure to its present status of being the treatment of choice for many patients with end-stage liver disease. The first human liver transplant was performed by T.E. Starzl in Denver in 1963. Initially, results were poor with only a few patients surviving for any significant length of time. However,with improved surgical techniques, intensive care and advances in immunosuppression, there has been a marked improvement in the results of liver transplantation. In June 1983, a Consensus Development Conference of the National Institutes of Health (USA) concluded that liver transplantation was no longer an experimental procedure but had become a therapeutic option[1].

Chronic liver disease accounts for approximately 5% of all admissions to the medical wards of South African hospitals. The medical management of complications related to liver failure is expensive and remains largely palliative. Most patients remain debilitated and unable to work. Orthotopic liver transplantation offers the patient the chance of cure and complete rehabilitation[2,3]. The quality of life for most survivors of liver transplantation appears similar to that of the normal population[4].

The liver transplantation programme was initiated at Groote Schuur Hospital in October 1988 and to date, 11 orthotopic liver transplantations have been performed. All patients referred to the MRC/UCT Liver Research Centre were assessed according to standard criteria for inclusion in or exclusion from the transplantation programme.

1.1 SELECTION PROTOCOL

Selection of suitable patients and the timing of transplantation are important factors in the initiation of a transplantation programme[5,6].

The indications for orthotopic liver transplantation[2] include chronic advanced liver disease, metabolic liver disease, hepatic malignancy and fulminant liver failure. Chronic liver disease may be subdivided into those conditions which are predominantly cholestatic (primary biliary cirrhosis, primary sclerosing cholangitis and biliary atresia) and those which predominantly affect the liver parenchyma. The latter include post-necrotic viral cirrhosis, chronic drug induced liver disease, alcoholic liver disease and autoimmune chronic active hepatitis. Patients with Budd-Chiari syndrome may also benefit from liver transplantation. Liver transplantation for hepatocellular carcinoma (HCC) is controversial because of the high recurrence rate. Cholangiocarcinoma is an absolute contraindication. However, other tumours such as the fibrolamellar variant and haemangioendotheliomas behave less

aggressively. Liver transplantation for fulminant liver failure is also controversial. Most of these patients rapidly deteriorate clinically and die before a suitable donor becomes available.

Various inborn errors of metabolism are now treatable by liver transplantation. These include processes where the liver is severely damaged (Wilson's disease, alpha-1 antitrypsin deficiency) or those where the liver is morphologically normal but contains a metabolic defect damaging another system (familial hypercholesterolaemia).

The timing of transplantation is difficult. Procrastination may allow a patient's condition to deteriorate to the extent that life support systems are required[2]. This may often preclude transplantation. Catastrophic complications are not always predictable and there is a tendency to underestimate the severity of the liver disease. Factors such as rapidly progressive jaundice, intractable ascites, spontaneous encephalopathy, repeated variceal bleeds and recurrent septicaemia are all indications for urgent transplantations. Other factors indicating the need for transplantation include coagulopathy with an INR > 2 unresponsive to vitamin K in chronic liver disease, estimated life expectancy of one year or less, or distressing symptoms such as intractable pruritus resulting in poor quality of life[7].

1.2 CONTRAINDICATIONS TO TRANSPLANTATION

Contraindications may be either absolute or relative and are continually being revised[2,7].

1.2.1 Absolute contraindications include:

1) Severe cardiorespiratory disease
2) Hypoxaemia (controversial)
3) Active biliary sepsis
4) Uncontrolled extrahepatic bacterial or fungal infections
5) AIDS
6) Metastatic liver disease
7) Extrahepatic malignancy
8) Active substance abuse or alcoholism

1.2.2 Relative contraindications include:

1) Portal vein thrombosis
2) Age greater than sixty years
3) Previous extensive hepatobiliary surgery
4) Active hepatitis B viral replication or Delta hepatitis virus infection
5) Advanced chronic renal disease except if renal transplantation is concurrently planned

1.3 PROCUREMENT AND OPERATIVE PROCEDURE

Procurement of livers for orthotopic transplantation is usually part of a multiple organ procurement programme. The most frequent combination at Groote Schuur Hospital is liver and kidneys, then liver, kidneys and heart and lastly liver, kidneys and heart-lungs.

The use of the Wisconsin preservation solution now enables graft storage for up to 12-24 hours, thus enabling liver transplantation to be performed as an elective procedure.

In orthotopic liver transplantation, the diseased native organ is removed and replaced with the new donor liver in the most anatomically normal way possible. The technical aspects of the transplant procedure have undergone major refinements over the years and have been described in detail elsewhere[8]. The introduction of the extra-corporeal veno-venous bypass in 1983 to decompress both splanchnic and systemic venous circulations has been an important refinement in surgical technique. Reconstruction of the biliary tract has become standardised and can be done either by connecting the donor and recipient common ducts end-to-end over a T-tube or the common duct of the donor is anastomosed to a jejunal limb in a Roux-en-Y anastomosis.

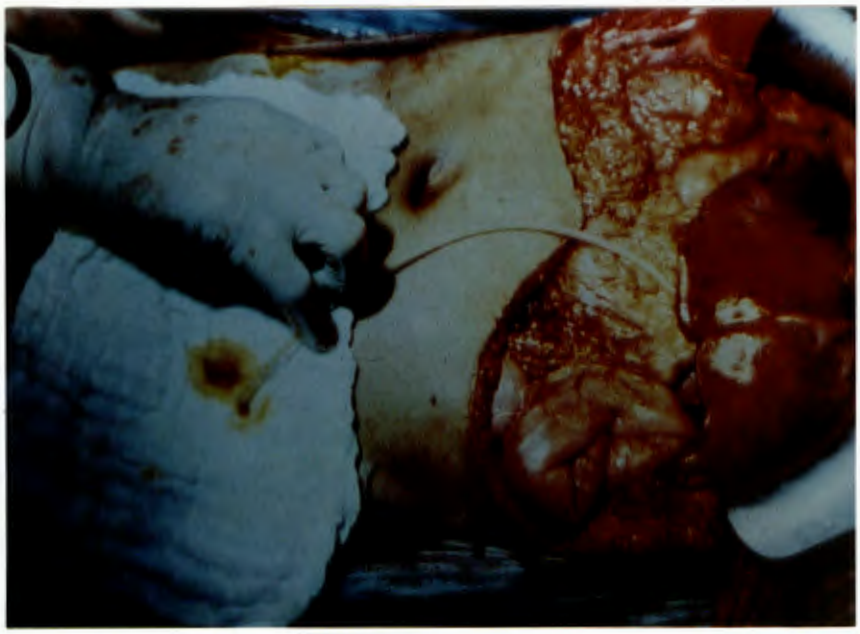


Figure 1.1: At orthotopic liver transplantation: T-tube in common bile duct with external drainage.

1.4 IMMUNOSUPPRESSION AND ANTIBIOTIC THERAPY

The introduction of the immunosuppressive agent cyclosporine in the late 1970s resulted in significant improvements in the results of solid organ transplantation. Currently, most patients receive a combination of steroids, cyclosporine and azathioprine as immunosuppression following transplantation. Intravenous cyclosporine has significant nephrotoxic properties. At Groote Schuur Hospital, we do not routinely use this drug immediately post-operatively until the patients

can take oral medication. OKT3, a monoclonal antibody is used 'prophylactically' for the first 5-7 days in place of intravenous cyclosporine. We do not have experience with FK506 and other monoclonal or polyclonal antilymphocyte antibodies. Rejection is treated by pulsing with medrol in as low a dose as possible.

All our patients receive routine prophylactic systemic antibiotics in the form of ampicillin or vancomycin and a cephalosporin on induction and at the time of biliary anastomosis. We do not routinely administer selective bowel decontamination therapy. Specific bacterial infections are treated with specific antibiotic therapy. All patients receive oral anti-fungal agents including nystatin drops and amphotericin lozenges. Patients also receive prophylactic cotrimoxazole 1 tablet bd for two days of the week for at least 90 days against pneumocystis infections. Acyclovir is also given prophylactically, initially intravenously and then orally for 10-14 days post-operatively.

1.5 PATIENT DATA

Eleven patients have undergone orthotopic liver transplantation at Groote Schuur Hospital since October 1988. They include 6 males and 5 females and their ages range between 21-56 years. Indications for liver transplantation included chronic active hepatitis progressing to cirrhosis (5); biliary cirrhosis (1); sclerosing cholangitis (2); alpha-1

antitrypsin deficiency (1); haemangioendothelioma (1) and cryptogenic cirrhosis (1). All patients with chronic liver disease had experienced at least one major complication relating to liver disease. These included intractable ascites, uncontrolled variceal bleeding, encephalopathy, recurrent bacterial peritonitis and septicaemia (See Table 1.1).

TABLE 1.1 PATIENT DATA

	Age	Sex	Diagnosis	Child's Stage*
OLTx 1	48	F	Autoimmune chronic active hepatitis	C
OLTx 2	42	M	Post-necrotic cirrhosis	C
OLTx 3	36	F	Biliary cirrhosis	B
OLTx 4	21	M	Chronic active hepatitis (HBV)	B
OLTx 5	56	M	Chronic active hepatitis	C
OLTx 6	39	F	Haemangioendothelioma	A
OLTx 7	36	M	Sclerosing cholangitis	B
OLTx 8	44	F	Chronic active hepatitis(?HBV)	C
OLTx 9	25	M	Alpha-1 antitrypsin deficiency	C
OLTx 10	30	F	Sclerosing cholangitis	B
OLTx 11	47	M	Cryptogenic cirrhosis	C

* Modified Child-Pugh classification Child C.G. III, Turcotte J. 1965. In: Child C.G. III (ed). The liver and portal hypertension. Philadelphia: W.B. Saunders Co.

Post-operative complications were mainly of a minor nature and included right lower lobe consolidation and effusions, seromas at sites of veno-venous bypass and one left medial cutaneous nerve palsy. More serious complications have included severe acute rejection (OLTx 2 and 5), acute tubular necrosis requiring dialysis (OLTx 2), delayed graft function (OLTx 7) which improved on conservative medical therapy, and

severe ischaemic injury with subsequent biliary strictures requiring revision of the biliary tract anastomosis (OLTx 11). One patient (OLTx 9) died on the eleventh day post-operatively with complications of a bleeding oesophageal ulcer, shock and candidaemia. Four patients (OLTx 3, 6, 8, 11) developed symptomatic cytomegalovirus infection. In two cases (OLTx 6, 8), this was mild and responded to a reduction in immunosuppressive therapy, but two patients (OLTx 3, 11) required treatment with specific anti-viral therapy (gancyclovir). One patient (OLTx 7) developed a late distal biliary stricture and has subsequently had a choledochojejunostomy performed 6 months post transplantation. At present, 10 patients are alive and well 2-32 months following transplantation and most have returned to work.

The post-operative period may be complicated by the development of infections (bacterial, viral or fungal), rejection, biliary obstruction and the consequences of ischaemic injury. As each of these complications requires specific therapy, it is important to make the correct diagnosis in order to institute appropriate therapy. Despite recent advances in immunosuppression, acute rejection is common and may result in significant patient morbidity. It is often difficult to distinguish abnormal liver function due to rejection from that due to infection, biliary obstruction or ischaemic injury without performing invasive procedures such

as liver biopsy or angiography. These invasive procedures are not without risk especially if there is an associated coagulopathy and the histology may often only show non-specific early changes.

Clinically, the diagnosis of rejection is usually made late and depends on the presence of progressive deterioration in liver function. The patient may experience fever, leukocytosis, graft tenderness and a change in the colour or quantity of bile. This is accompanied by a rise in serum bilirubin and parameters of liver injury as well as acute phase proteins and increased serum neopterin. These features imply an established immunological process and it is usually at this stage that the immunosuppressive therapy is boosted to combat acute rejection.

The cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-alpha) are pluri-potential cytokines which mediate immunological responses and acute phase reactions[9,10,11]. They are produced by activated mononuclear phagocytes which are central to cellular rejection processes. Urinary and serum IL-6 levels have been found to be high following surgery as part of the acute phase response and have been noted to be particularly elevated following episodes of renal transplant rejection[12].

Soluble interleukin-2 receptors have been shown to be elevated in the serum and bile[13,14] of liver transplant

patients at the time of rejection. The biliary levels IL-2R were more sensitive and specific than the serum levels for rejection[14]. T cell activation is associated with an increase in the expression of interleukin-2 receptors and this expression is closely regulated by the cytokines IL-1, TNF-alpha and IL-6. Measurement of these cytokines, particularly in bile, may provide a sensitive marker for early rejection.

Cholestasis occurs as a result of liver injury and rejection processes. Thus alterations in the biliary constituents as a result of liver injury due to rejection may be detectable prior to changes in the serum. Bile secretion is used as an index of hepatic recovery following liver transplantation. The absence of bile production following graft reperfusion is often an ominous sign and may be associated with primary graft failure[17]. Bile flow and bile salt secretion following liver transplantation has been measured[15], other studies have looked at lipid composition[16], while others have looked at water and electrolyte secretion following transplantation[17].

However, no-one has performed serial measurements of biliary biochemical parameters and correlated possible changes in these parameters with the development of rejection.

In this study we have evaluated standard biochemical parameters and cytokine concentrations (IL-1, IL-6 and TNF-alpha) in serial samples of bile obtained post-operatively via the T-tubes of patients following orthotopic liver transplantation in order to determine whether there are any

biochemical or immunological pointers to the early diagnosis of rejection which would enable earlier administration of appropriate anti-rejection therapy.

CHAPTER TWO

Immunological Reactions in Orthotopic Liver Transplantation

2.1 REJECTION

Despite recent advances in immunosuppression, acute rejection remains common and results in significant patient morbidity. It is important to recognise rejection as soon as possible so that appropriate immunosuppressive therapy can be instituted. It is vital that the diagnosis of rejection is correct. The unnecessary administration of large doses of steroids or anti-lymphocyte preparations may be extremely deleterious with the consequent failure of wound healing and the development of infections.

Acute rejection seldom occurs before the 4th day post transplantation and is more frequently seen between the 4th and 10th day. It is uncommon after 2 months, unless the patient has been inadequately immunosuppressed or has had a concurrent infection necessitating a reduction in immunosuppressive therapy.

The patient may experience fever, malaise, graft tenderness associated with lymphocytosis, eosinophilia together with a rise in bilirubin, alkaline phosphatase and transaminase levels.

However, none of the clinical signs and symptoms or the biochemistry are specific for rejection. Other non-immunological causes for early hepatic dysfunction must be systematically excluded. The differential diagnosis includes biliary obstruction, suboptimal revascularisation, viral or bacterial infections and drug toxicity. Frequently invasive procedures such as a liver biopsy are required to establish the diagnosis of rejection. In centres where regular protocol biopsies are taken, the incidence of acute rejection is up to 77%[18].

2.1.1 Pathology

a) Acute Rejection

Histological diagnosis of acute cellular rejection depends on the presence of a predominantly mononuclear inflammatory cell infiltrate in the portral areas associated with evidence of some parenchymal damage. The inflammatory infiltrate consists of a mixture of large blastic lymphocytes, plasma cells, macrophages, eosinophils, neutrophils and dendritic cells.

The infiltrate accumulates in the interstitium of the portal tracts and leads to portal expansion. The inflammatory infiltrate accumulates beneath hypertrophied portal vein endothelial cells and may be adherent to the endothelial surface, this appearance is called endothelialitis[19].

Similar endothelialitis may be seen in the terminal hepatic venules. The endothelial cells may be lifted up by the infiltrating cells. Medium-to-large vessel damage with arteritis and associated fibrinoid necrosis and thrombosis may occur[20]. The portal infiltrate may obscure small bile ducts and infiltrate the epithelium which shows a variety of abnormalities. Ductular cell hyperplasia and an increase in the nuclear to cytoplasmic ratio are the most frequent findings. Other degenerative changes in the biliary epithelium include subnuclear vacuolisation, nuclear pyknosis, eosinophilic degeneration or frank luminal disruption[20]. The portal mononuclear infiltrate may spill over and be associated with periportal hepatocyte necrosis and there may be some centrilobular hepatocyte necrosis associated with involvement of the central veins[20,21].

Canalicular cholestasis is usual but cellular cholestasis involving perivenular cells may also occur[18]. Following boosted immunosuppression, usually in the form of pulses of intravenous medrol, most patients show histological recovery, although minor residual portal scarring may persist[18].

Acute cellular rejection may be graded as mild, moderate or severe depending on the combination of the degree of exuberance of the monuclear portal infiltrate, the extent of tissue damage and the presence of arteritis and/or ischaemic necrosis[21].

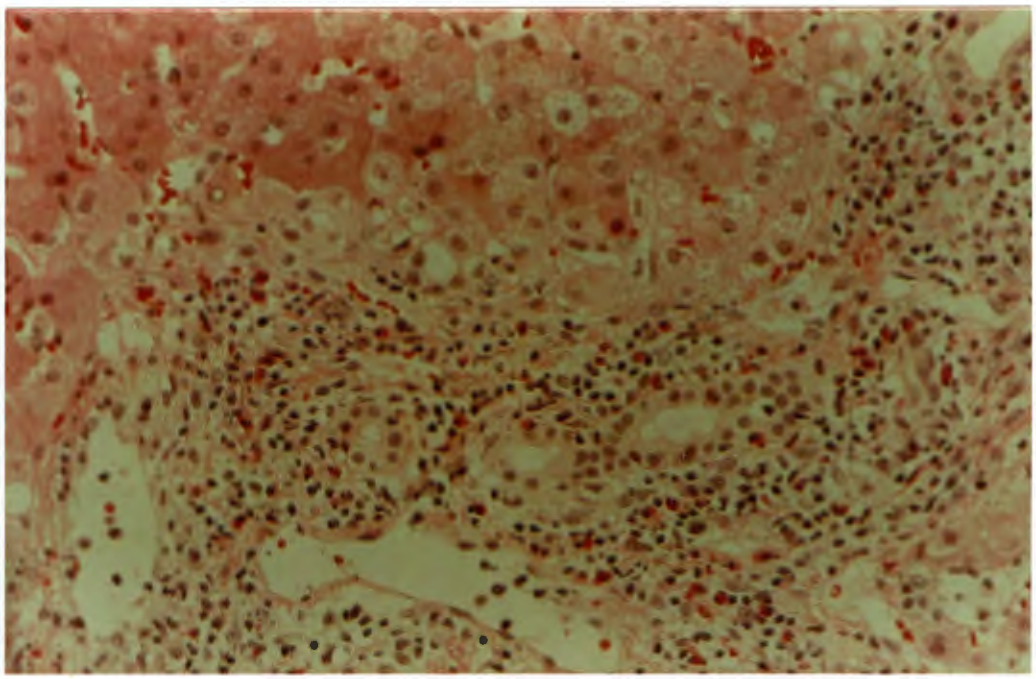


Figure 2.1: Florid cellular rejection. Hepatocellular necrosis, endothelial cell damage with mononuclear cell infiltration.

b) Chronic Rejection

This usually occurs after 2 months following transplantation. It is characterised by an indolent but progressive loss of bile ducts and obliterative arteriopathy[20,21]. Clinically patients become progressively jaundiced with a cholestatic liver enzyme pattern. Liver synthetic function remains intact until late in the course. Histological diagnosis of chronic rejection depends on the loss of small bile ducts and portal arteries or arterial mural thickening

and hyalinization[20,21]. There may only be a mild-to-moderate mononuclear portal infiltrate. The composition of the infiltrate differs from that in acute rejection in that it contains fewer blastoid lymphocytes, neutrophils and eosinophils with a possible increase in plasma cells[20]. With time, the bile ducts are completely destroyed and replaced by fibrous tissue. Portal tract expansion due to fibrosis and hyalinization of connective tissue occurs. Lobular changes include central canalicular cholestasis, intrasinusoidal foam cell clusters, mild acidophilic necrosis of hepatocytes and perivenular hepatocellular atrophy and sclerosis[21].

The obliterative arteriopathy involves the second and third order branches of the hepatic artery and is thus often not seen on biopsy. The usual pathology is subintimal foam cell deposition but an intimal lymphohistiocytic infiltrate together with smooth muscle proliferation, disruption of elastic lamina and intimal fibrosis may occur. Major bile ducts may be involved with epithelial sloughing, focal necrosis, mural fibrosis and features of both acute and chronic inflammation[21]. The histological findings of chronic rejection usually correlate well with the presence of a cholestatic liver enzyme pattern. Chronic rejection is usually unresponsive to increased immunosuppressive therapy. Graft failure is progressive and the only effective treatment is retransplantation.

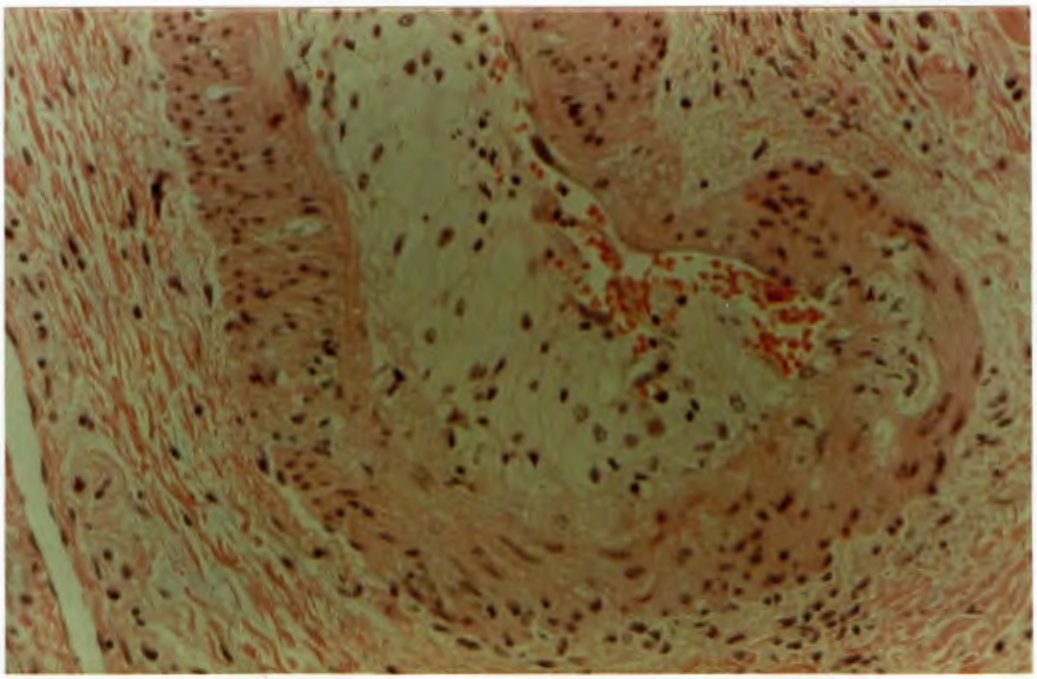


Figure 2.2: Obliterative arteriopathy with subintimal foam cell deposition.

2.1.2 Pathogenesis

Rejection is an immune reaction elicited by a genetic disparity between the donor and the recipient. It demonstrates both specificity and memory and is thought to be directed at the major histocompatibility complex (MHC), blood group and possibly at tissue specific antigens expressed on the surface of graft cells. It involves both cellular and humoral mediated immunity.

The morphological changes of acute rejection are comparable, in terms of the component cells and their relationship to small vessels, to the changes seen in cellular rejection of renal and other allografts. However, the liver differs from other organs in a number of important ways. Antibody mediated hyperacute rejection is uncommon even in the presence of circulating antibodies against graft antigens[18,22]. Also, the liver has the capacity to induce a specific immunological unresponsiveness to grafts of other organs from the same donor strain[18].

a) Hyperacute Rejection

Antibodies present in the recipient circulation prior to transplantation directed at antigens expressed on endothelial cells of the graft have the potential to cause an immediate form of vascularised allograft failure called hyperacute rejection. The major blood group isoagglutinins and warm T-lymphocytotoxic antibodies can both cause hyperacute rejection of kidney and heart grafts. Binding of the preformed antibodies to the graft vasculature results in complement fixation and activation, endothelial cell damage, vasospasm, platelet aggregation, subsequent activation of the clotting and fibrinolytic cascades and neutrophilic activation. The end result is diffuse intraorgan thrombosis, haemorrhagic necrosis and graft failure[21,32].

Liver allografts tend to be resistant to hyperacute rejection. Early graft failure is not usually observed when warm T-lymphocytotoxic antibodies are present at the time of transplantation. However, when major blood ABO group barriers are violated, then early graft failure due to haemorrhagic necrosis which is attributed to antibody-mediated rejection, is more frequently encountered.

The resistance of the liver to hyperacute rejection may be due to a number of factors which include:

1. Dual afferent blood supply: The liver receives afferent blood from both the hepatic artery and the portal vein. Compromise of either blood supply results in compensatory increased flow in the other and this may thus protect the liver from ischaemic injury[23].

2. Sinusoidal network lined with Kupffer cells: The majority of the microvasculature is sinusoidal, lined by fenestrated endothethial cells which lack an underlying basement membrane which plays a role in platelet aggregation. Also the Kupffer cells protect the liver by absorbing antibodies, immune complexes and platelet aggregates[24].

3. Secretion of soluble MHC antigens into the circulation: Soluble MHC antigens in the circulation may bind to and neutralise lymphocytotoxic antibodies thereby preventing binding to the endothelial and other graft cells. It has been shown that preformed lymphocytoxic antibodies disappear

from the serum soon after liver transplantation and that the liver protects other grafts eg. kidneys from the same donor against hyperacute rejection in highly sensitised recipients[21,25]. This may be due to Kupffer cell absorption and/or the neutralisation of the antibodies by solubilised antigens[21,26].

b) Acute Cellular Rejection

The major histocompatibility complex is localised in humans on the short arm of the sixth chromosome and it codes for the highly polymorphic glycoprotein HLA antigens[18].

Class I antigens comprise HLA - A, B and C, each of which has a separate locus within the MHC. Their products are transmembrane glycoproteins (MW 45 kD) which are associated at the cell surface with a smaller 12kD peptide, beta 2 microglobulin. Class I antigens were said to be expressed on the cell surfaces of all nucleated somatic cells[18]. However the liver shows considerable variability of expression of Class I antigens between different cell types. Sinusoidal lining cells and bile duct epithelium normally express the class I antigen[18,27,28] and hepatocytes are generally found to be negative. Arterial and venous endothelia have been found to express Class I antigen[27].

Class II antigens are encoded by at least three loci i.e. DP, DQ and DR. Their gene products include both alpha and beta glycoprotein chains with molecular weights of 34 and 28

kD respectively. Class II antigens are involved in antigen presentation and serve as the primary targets for helper T lymphocytes. Class II antigens have a more limited distribution and are expressed on monocyte/macrophage series whose function is to phagocytose and process foreign antigens. The processed foreign antigen is then recognised by other immunocompetent cells especially T-helper cells together with self HLA Class II antigen. Class II antigens are also expressed on B lymphocytes, activated T lymphocytes and dendritic cells[18,29].

In the liver, dendritic cells in the portal tracts sinusoidal cells and Kupffer cells all express Class II antigens[28,29]. Hepatocytes, bile duct epithelium and the vascular endothelia of the hepatic artery, portal and central venules are usually negative[18].

Changes occur in the expresssion of Class I and Class II major histocompatibility antigens by liver parenchymal and vascular endothelial cells of liver allografts during rejection, but also in grafts damaged by ischaemia, duct obstruction and hepatitis[3,30]. Class I antigens become expressed on hepatocytes; class II antigens are expressed on bile duct epithelium and on vascular endothelium[21].

Several cellular immune effector pathways have been implicated in acute rejection including:

(1) delayed type hypersensitivity

(2) allogeneic cytotoxic T lympholysis

(3) antibody - dependent cellular cytotoxicity mediated through killer cells

The histological diagnosis of acute cellular rejection depends on a predominantly mononuclear cell infiltration concentrated in the portal tracts together with oedema and some parenchymal necrosis. The bile ducts, veins and arteries are most commonly damaged.

The portal infiltrate consists of an admixture of large blastic lymphocytes, smaller lymphocytes, plasma cells, macrophages, neutrophils and eosinophils[19,20]. The T lymphocytes have been shown to be the predominant cells. T4 cells have predominated in some studies whereas T8 cells have predominated in others[18]. Classically, cytotoxic T8 cells were thought to be the cells which mediated graft destruction. It now appears that there is a complex interplay between cytotoxic T cells, helper T cells and macrophages. Helper T cells are thought to be central in rejection responses as they are able to activate cytotoxic T-cells, macrophages and B cells[18]. Helper T4 cells produce lymphokines including IL-2 which stimulate the cytotoxic T cells to proliferate and mature into effector cytotoxic T cells.

Allogeneic MHC antigens are capable of stimulating recipient T cells without needing to associate with host MHC antigens. Graft cells are capable of eliciting rejection reactions[31]. The graft cell expressing foreign MHC Class II antigens may stimulate host helper T cells which then induce cytotoxic T cells to destroy the target graft cell. T helper cells reacting to the graft release lymphokines which stimulate macrophages to enter the graft and destroy it[31]. In addition to the monocyte/macrophage series acting as antigen presenting cells, the dendritic cells are also thought to be important non-phagocytosing antigen presenting cells (APC). The dendritic cells constitutively express MHC Class II molecules and are potent stimulators of mixed lymphocyte reactivity[31]. After liver transplantation, these dendritic cells may leave the graft and enter the draining lymphatic system where they are particularly effective in sensitising the host[31].

Thus the relative distribution of Class I and Class II MHC antigens in the transplanted liver corresponds to the histological appearances of acute rejection with the concentration of immunocompetent cells in the portal areas and associated with the endothelium of central and hepatic venules.

Frequently there is a concurrent viral and/or bacterial infection in these patients which may lead to increased expression of MHC antigens and thus increased graft

damage[18,33]. The relative absence of Class I antigen and the complete absence of Class II antigen from hepatocytes, the main functional units of the liver, may explain why the liver appears to be rejected less aggressively than other organs and why histologically there is minimal hepatic necrosis. Class II antigens are strongly expressed in the Kupffer and dendritic cells of the graft and these donor cells are replaced by cells of host origin within 4 months of transplantation[18,34]. This may explain the tendency for acute rejection to occur in the first few months following transplantation. In addition to the interaction between T-cell receptor and the antigen-presenting cell together with MCH class II molecules, it is felt that the extracellular matrix also plays a role in T cell activation. Extracellular matrix may participate in T cell activation through binding to CD26 and VLA integrins. Moreover, the liver contains very little collagen and this may also explain why liver allograft rejection is less severe than other organ graft rejection[35].

c) Chronic Rejection

Many antigenic targets in the graft are similar since the bile ducts, endothelial cells and hepatocytes remain of donor origin and continue to express foreign MHC antigens. However donor Kupffer and dendritic cells are replaced by those of the recipient[18,34].

The loss of bile ducts may be attributed to immunological damage directed against Class I or Class II MHC antigens or both. This is supported by the evidence of similarity between the bile duct lesion in liver transplantation to that seen in graft vs host disease[20]. In end stage chronic rejection, the mononuclear portal inflammatory infiltrate is minimal and this may be because the target antigen in biliary epithelium is no longer present.

Bile ductopaenia may also relate to ischaemia. The portal tracts and their bile ducts receive most of their blood supply from the hepatic artery. Any compromise of that supply could result in damage and lead to the disappearance of bile ducts[18].

In the same way the obliterative arteriopathy is probably due to a combination of direct immunologic damage and ischaemic injury[36].

2.1.3 Patient data

The majority of patients transplanted at Groote Schuur Hospital experienced acute rejection at 4-6 days post transplantation. This usually manifested as fever accompanied by leukocytosis, an increase in serum bilirubin, alkaline phosphatase and gammaglutamyl transferase levels followed by a rise in the transaminase levels. Some patients experienced myalgia and arthralgia. In those patients who had T-tubes, the bile was noted to decrease in amount and

become pale and watery at the time of rejection. The rejection episodes were mild in all cases except in 2 patients (OLTx 2, 5). All responded to pulses of intravenous medrol, usually given as 500 mg or 250 mg boluses (depending on the severity) for 3 consecutive days and then 2 further pulses of medrol each following a day's break. No patients required OKT3, a monoclonal antibody for treatment of rejection. To date, none of our patients have experienced chronic rejection. The majority of our patients receive maintenance immunosuppression in the form of medrol, azathioprine and cyclosporine.

2.2 THE ACUTE PHASE RESPONSE

Tissue injury as a result of infection, physical and chemical trauma, malignancy and immunological disorders results in a complex series of local and systemic reactions collectively known as the acute phase response. Immediate local tissue responses include vasodilatation and the release of cellular contents such as lysosomal enzymes, prostaglandins and vasoactive amines.

Secondary local responses include chemotaxis, with the influx of leukocytes and the production of leukotrienes and arachidonate metabolites together with the activation of monocytes/macrophages and the production of cytokines. The systemic reaction is characterised by fever, leukocytosis, increased vascular permeability, a negative nitrogen

balance, alterations in plasma metal and hormonal concentrations and an increase in the synthesis of acute phase proteins. Serum iron and zinc levels decrease and increased caeruloplasmin levels result in increased serum copper levels. There is an increased production of insulin, glucagon, growth hormone, TSH and ACTH[37,38,39].

Most of the non-hepatic acute phase reactions are mediated by the cytokines Interleukin-1 (IL-1) and Tumour Necrosis Factor (TNF-alpha). Acute phase proteins are mainly hepatic in origin but some are also produced at extrahepatic sites. The principal mediators of the hepatic acute phase response are Interleukin 6 (IL-6), IL-1, TNF-alpha, Interferon (IFN) and the glucocorticosteroids. Although many different cell types produce these hormones, the most important sources during a local inflammation for all these hormones except the glucocorticoids are activated macrophages, monocytes, fibroblasts and endothelial cells. Glucocorticoids act syngergistically with the cytokines to cause the induction of acute phase protein genes[37].

IL-6 is the major direct regulator of the hepatic acute phase response and can induce the full spectrum of acute phase proteins[40]. IL-1 and TNF-alpha are important modulators of the hepatic acute phase response. They inhibit the induction of transcription of the genes for certain proteins known as the negative acute phase proteins[37]. Both IL-1 and TNF-alpha primarily released by activated

macrophages and monocytes can induce a potent release of IL-6 from stromal cells[41] and thus act indirectly on the liver. IFN exerts a regulatory action on the expression of several acute phase protein genes at extrahepatic sites. It induces the expression of complement C4 and factor B in monocytes as well as acting as a macrophage activating factor promoting the release of IL-1 and IL-6 with consequent indirect induction of the acute phase proteins[37].

2.2.1 Acute Phase Proteins

The acute phase proteins are structurally and functionally a diverse group but they all participate in the defence of the host against tissue damage and infection. They can be divided into 4 groups according to their concentration changes during the acute phase response. Group 1 (concentration increases 2-fold) includes C3 and caeruloplasmin; Group 2 (concentration increases 2-10 fold) includes fibrinogen, haptoglobin, alpha-1-antitrypsin and alpha-1-antichymotrypsin; Group 3 (concentration increases several hundred fold) includes C-reactive protein and serum amyloid A protein and Group 4 includes the negative acute phase proteins such as albumin and transferrin[37].

C3 and CRP opsonise bacteria, parasites, foreign particles and immune complexes and facilitate their clearance by phagocytic cells. CRP participates in complement activation and may also play a role as clearance factor for chromatin

fragments by binding to chromatin released from damaged cells in inflammation, thus facilitating tissue repair. CRP may also act as a modulator of the immune response during inflammation[42]. Fibrinogen participates in clotting, wound healing and tissue regeneration.

Many acute phase proteins are proteinase inhibitors including alpha-1-antitrypsin which inhibits leukocyte elastase, alpha-1-antichymotrypsin and alpha-macroglobulins which are both able to inhibit the activity of natural killer cells and antibody-dependent cell mediated cytotoxicity[37]. Alpha-2 macroglobulins can suppress the chemotactic responses of monocytes and modulate the responses of lymphocytes to mitogenic and antigenic stimuli[43]. Alpha-2 macroglobulin also binds to cytokines including IL-1, TNF and IL-6[44]. Binding of these cytokines to the alpha-2 macroglobulin may affect the distribution, bio-availability, stability and clearance of cytokines[44]. Binding of proteinase inhibitor complexes to their macrophage receptors suppresses superoxide anion production, proteinase secretion, Ia antigen expression, tumour cell killing and possibly antigen presentation by macrophages. Thus, the imunosuppressive functions of these proteinase inhibitor complexes play an important role in dampening down inflammatory and immune responses and in appropriately terminating an inflammatory episode[37].

Acute phase proteins play a critical role in the defence mechanisms promoting the clearance of invading particles and modulating the immune responses against them. They form part of the non-specific first line of defence and are particularly effective because of their rapid inducibility, providing protection immediately after an insult when the specific immune response is still ineffective.

CHAPTER THREE

Physiology of Bile Secretion

Bile is an aqueous solution of bile acids, cholesterol, phospholipids, bilirubin, bile pigments and inorganic electrolytes. Bile is secreted by hepatocytes into bile canaliculi, transported and modified by the intrahepatic and extrahepatic biliary system and delivered to the duodenum through the ampulla of Vater. After entry into the duodenum some bile constituents eg. bile pigments are excreted while others such as the bile acids are reabsorbed by the intestine, return to the liver and are re-excreted into bile via the enterohepatic circulation.

The flow of bile is driven by the formation of osmotic gradients between the blood, the intercellular space and the hepatocyte on one hand and the lumen of the bile canaliculus on the other. These gradients are elaborated by the energy dependent transport of solutes from the hepatocyte into the canalicular lumen. Bile flows as these solutes are retained in the canalicular lumen and the gradients are dissipated by the passive movement of water and other solutes across the canalicular membrane and through tight junctions. The majority of solutes that make up these osmotic driving forces consist of organic anions, particularly bile acids and their conjugates, which are of sufficient size and

negative charge so as to be retained within the lumen by the tight junction barriers. Small amounts of fluid and solutes such as proteins, cholesterol and phospholipids are also transported into bile by the transcellular vesicular pathway. Small ions such as sodium diffuse through the tight junctions and maintain electroneutrality. The bile is then modified by secretory and absorptive processes along the bile duct epithelia[45,46,47].

3.1. BILE SECRETION AND FLOW

Three main processes have been postulated in the formulation of bile and subsequent bile flow.

3.1.1. Bile acid dependent bile flow (BADF)

Bile acids are the most concentrated organic solutes in bile and their excretion by hepatocytes is the major determinant of bile flow. There is a linear relationship between bile salt excretion and bile flow once bile acids exceed critical micellar concentrations[45]. Bile acids usually aggregate in micelles and their osmotic activity is thus reduced. Most of the osmotic activity of bile acids is provided by their accompanying counter-ions[46]. Water and electrolytes are drawn into the canaliculi by osmosis and convection via paracellular routes through tight junctions that separate blood from bile at the canalicular level[48]. A small amount of water may enter the canaliculus via the transcellular route. BADF accounts for 30-60% of spontaneous basal bile

flow[46].

3.1.2. Bile acid independent bile flow (BAIF)

Significant bile flow may also be generated in the presence of low bile acid output. Impermeant organic anions can be concentrated in bile by carrier mechanisms driven by the membrane potential. This could provide an osmotic driving force for secretion. Glutathione an organic anion, synthesised in the liver, is secreted into bile in sufficient concentration to exert an osmotic driving force for paracellular and transcellular water movement[49]. The inorganic transport mechanisms such as Na^+/K^+ -ATPase, Na^+/H^+ antiport, Cl^-/HCO_3^--antiport and Na^+/HCO_3^--symport have been implicated but their exact role is uncertain[45,46]. Bicarbonate excretion utilising the canalicular Cl^-/HCO_3^--antiport promotes BAIF[45,50].

3.1.3. Reabsorption and secretion of fluid and electrolytes by the biliary ductules and ducts

Finally bile secreted by the hepatocytes may be modified on its way through the ductules and ducts. The ductules are capable of absorbing water and NaCl and reabsorption is enhanced by somatostatin. Secretin increases duct secretion of water and this is accompanied by an increased secretion of bicarbonate[46].

3.2. BILE COMPOSITION

3.2.1 Water and electrolytes

TABLE 3.1. CONCENTRATIONS OF ELECTROLYTES IN HUMAN BILE[51]

	HEPATIC BILE Range (Mean)	GALLBLADDER BILE Range (Mean)
Na^+	132-158 mmol/l (146)	156-264 (209)
K^+	4.0-6.2 mmol/l (4.8)	8.2-19.6 mmol/l (12.8)
Ca^{2+}	1.05-5.6 mmol/l (2.6)	3.1-17.2 mmol/l (10.8)
Mg^{2+}	0.7-1.5 mmol/l	not known
Cl^-	83-117 mmol/l (105)	66 mmol/l

Other inorganic solutes include phosphorus and metallic ions (Fe,Cu, Zn, Mn).

3.2.2 Organic solutes

The main organic components of bile are conjugated bile acids, bilirubin, phospholipids, cholesterol and bile pigments.

TABLE 3.2. CONCENTRATIONS OF MAIN ORGANIC SOLUTES IN HUMAN BILE.

	HEPATIC BILE Range (Mean)	GALLBLADDER BILE Range (Mean)
Bile acids	16-35 mmol/l (25)	14-118 mmol/l (75.3)
Phospholipids	1.3-5.6 mmol/l (3.2)	12.5-67.5 mmol/l (33.5)
Cholesterol	2.1-5.4 mmol/l (3.6)	4.9-21 mmol/l (10.4)
Protein	1.4-2.7 mmol/l (1.8)	6.75 mmol/l
Bilirubin	0.21-2.31 mmol/l (1.11)	0.62-10.8 mmol/l (3.32)

Bile acids and their conjugates are the main organic solutes. The bile acids are conjugated with amino acids in the hepatocyte and exist mostly as taurine and glycine conjugates. Bile acids and salts form micelles above a critical micellar concentration[46]. The major bile acids are conjugates of the primary (cholic and chenodeoxycholic) and secondary (deoxycholic, lithocholic and ursodeoxycholic) bile acids. Secondary bile salts are formed by colonic bacterial 7 alpha - dehydroxylation of the primary bile acids[48]. Bile acids are the major determinant of phospholipid and cholesterol solubilisation in bile, incorporating them in mixed micelles.

Biliary proteins include transport proteins such as albumin, transferrin, caeruloplasmin, haptoglobin, apolipoproteins and immunoglobulins such as secretory IgA, IgM and IgG. Hormones such as insulin, epidermal growth factor and cholecystokinin as well as enzymes including lysosomal hydrolases, amylase, and enzymes associated with the hepatocyte plasma membrane, mitochondria and endoplasmic reticulum are also found[46,51]. Circulating plasma proteins are transported into bile by transhepatocyte or paracellular pathways. The transhepatocyte pathway is associated with vesicular transport and exocytosis, the proteins may go directly to the bile canaliculus or they may interact with lysosomes[52]. Biliary porphyrins exist mainly as copro-

porphyrin, 80-90% being coproporphyrin-I. Protoporphyrin is also found in small amounts[51].

3.3 MORPHOLOGY OF THE BILIARY SYSTEM RELATING TO BILE SECRETION

Bile is formed by the hepatocytes and is secreted into canaliculi. Bile canaliculi are 1μm in diameter, they branch and form a communicating network. Canaliculi are closed at one end situated near the central part of the hepatic lobule and at the other end situated near the portal space at the lobule periphery they drain into larger channels, the bile ductules which join to form bile ducts. The canaliculi have no walls of their own, they are bounded by the plasma membrane of the two adjoining hepatocytes. This area of the hepatocytes plasma membrane is known as the canalicular membrane and it has numerous microvilli which project into the lumen, thus providing a large surface area for secretion[46].

The canaliculus is surrounded by a narrow area of organelle-poor cytoplasm called the pericanalicular ectoplasm which contains actin microfilaments. These actin microfilaments insert into the area of the intercellular junction called the intermediate junction, form a network around the canaliculus and extend into the microvilli microfilaments. They may play a role in maintaining the shape of the canaliculi and microvilli[46]. The microfilaments undergo periodic contractions to facilitate propulsion of bile from

the central to the portal regions of the lobule[53]. Microtubules which are involved in vesicular transport are more randomly distributed in the cytoplasm but are also found in the pericanalicular ectoplasm[53].

The canaliculus is surrounded by two hepatocytes and tight junctions seal off the lumen from the intercellular space. The location of these junctions polarises the plasma membranes of the hepatocytes into an apical (canalicular) and a basolateral domain. The basolateral domain is formed by the sinusoidal plasma membrane which faces the space of Disse and the lateral domain which contains the desmosomes, intermediate junctions, tight junctions and gap junctions[46,54].

The desmosomes protect the membranes from deformation and damage due to distension. Intermediate junctions serve as an adhesive structure between the cells and provide attachment for the microfilaments. The permeability of the tight junction is important for the process of bile formation. Epithelial tight junctions vary in their permeability and those of the hepatocyte are of an intermediate type with numerous contact lines which are permeable to the flow of water and small solutes[45,46]. They prevent the diffusion of organic solules out of bile. The tight junction is associated with a specific cytoplasmic protein called ZO-1[54].

The ductules and ducts are lined by true epithelial cells and have microvilli projecting into the lumen. The ducts in the portal tracts are supplied by branches of the hepatic artery.

3.4. FACTORS DETERMINING THE BILE SECRETORY PROCESS

3.4.1. The translobular gradient[46,47]

There tends to be unidirectional blood flow through the hepatic lobule with blood flowing from the portal space through the sinusoids and then drained by a branch of the hepatic vein. Thus the periportal hepatocytes (Zone 1) are first exposed to afferent blood and receive blood with the highest solute concentration. The amount of solute removed by the hepatocytes as blood flows in the sinusoids towards the hepatic vein depends on the extraction efficiency for each solute. The periportal (Zone 1) hepatocytes are the primary sites of bile acid dependent secretion. Hepatocytes in Zone III which are located at distal sites in the hepatic lobule adjacent to the terminal hepatic veins are relatively bile acid deficient because of the efficient first pass clearance in the periportal cells and secretion here is determined mainly by bile acid independent mechanisms.

Blood flow through the sinusoids is not always unidirectional and pulsatile pressure changes within the sinusoids allow for mixing of solutes before hepatocyte uptake and thus reduce the steepness of the translobular

gradient. Bile flows in the opposite direction to the sinusoidal blood flow so that bile in the periportal canaliculi is more concentrated than that in canaliculi in the hepatic vein area. Bile flow is unidirectional and thus the concentrated bile in the periportal canaliculi does not come into contact with the Zone III hepatocytes which may contain lesser amounts of the same solute. Thus, back diffusion of solutes to the hepatocytes and sinusoidal blood is prevented. The translobular gradient favours the formation of bile with a high concentration of organic solutes.

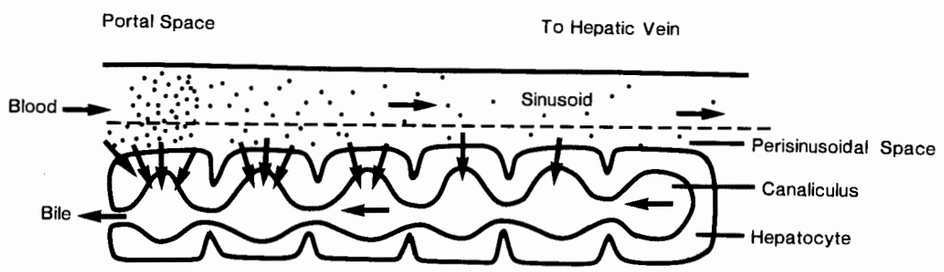


Figure 3.1: The translobular gradient modified from 'What causes cholestasis' by J. Boyer in The American Association for the study of Liver Disease: New Frontiers in Liver Disease, October 1989.

3.4.2. Membrane transport systems and their structural polarity

a) Na^+/K^+-ATPase

This is an integral membrane protein which is responsible for maintaining a low intracellular Na^+ and high K^+ concentration by utilising metabolic energy derived from hydrolysis of ATP. It thus provides for inwardly directed Na+ gradients and outwardly directed K^+ gradients. The ATPase membrane pump exchanges $3Na^+$ for $2K^+$ and is thus electrogenic resulting in a negative intracellular electric potential. K^+ exits from the cell through K^+ channels thereby increasing the negative intracellular potential (usually - 35mV). The inwardly directed transmembrane sodium gradient provides the energy for driving a number of secondary active transport systems[50].

The Na^+/K^+-ATPase is situated on the sinusoidal membrane of hepatocytes where it provides the major driving force for secondary active solute transport in both directions across the sinusoidal plasma membrane as well as potential-driven anion excretion across the canalicular membrane[50].

b) Other ion transport systems

These include the Na^+/H^+ antiport, HCO_3^-/Cl^- exchanger and the Na^+/HCO_3^--symport. The Na^+/H^+ exchangers and the Na^+/HCO_3^- symporters are present on the sinusoidal membranes whereas the HCO_3^-/Cl^- exchangers together with a chloride

conducive channel are found on the canalicular membrane. These ion transporters are important in maintaining cell homeostasis including intracellular pH, cell volume and ion composition. These ion transporters affect bile excretion since they are interrelated through effects on the transmembrane potential differences and ion gradients which in turn influence organic ion transport[47].

In addition to the above primary and secondary active transport mechanisms, there are two tertiary active transport mechanisms, the sinusoidal OH^-/SO_4^- antiport and the canalicular (OH^- or HCO_3^-)/SO_4^- antiport which are driven by ionic gradients established by secondary active transport mechanisms. The sinusoidal OH^-/SO_4^- antiport can move a variety of organic anion co-substrates in either direction across the sinusoidal membrane and the canalicular (OH^- or HCO_3^-)/SO_4^- antiport can move similar anions into bile.

c) Bile acid transporters

Bile acids are the most concentrated organic solutes in bile and their excretion is the major determinant of bile flow.

Only small amounts of biliary bile acids are derived from de novo synthesis from cholesterol. Thus most bile acids must be taken up across the sinusoidal membrane, transported transcellularly to the canalicular domain and then secreted into the canaliculi. Bile acid uptake into the hepatocyte

occurs against a concentration gradient and requires energy. Bile acid uptake may be sodium dependent and mediated by a sodium co-transport system[45]. This transport system is thus a secondary active mechanism[46,50] as it uses the sodium gradient as its energy source which is maintained by the primary active transporter, Na^+/K^+-ATPase. A 48kD protein[45,50] has been identified in the sinusoidal membrane which acts as a Na^+ coupled carrier for bile acids such as taurocholate, using the sodium gradient as the driving force to transport the anions against opposing chemical and electrical gradients.

Bile acid uptake is also sodium independent and a sinusoidal transmembrane protein with molecular weight of 54kD has been identified. Unconjugated bile acids such as cholic acid may exchange with hydroxyl or sulphate ions as well as with other organic anions released by the liver suggesting that uptake of unconjugated bile acids may be mediated by a multispecific anion exchanger which might be identical to the 54kD bile acid transporting protein[47,53].

The intracellular transport of bile acids from the sinusoidal to the canalicular pole of the hepatocyte depends mainly on the diffusion of free or protein-bound bile acids. Several cytoplasmic proteins including the Y protein (ligandin or glutathione transferase), Z protein (fatty acid binding protein) and Y′ protein (alpha hydroxysteroid dehydrogenase) exist which bind bile acids and other organic

anions[45,47]. Some bile acids are transported via microtubule dependent vesicular pathways into the canaliculus[53].

Bile acids are excreted into bile against a concentration gradient. A 100kD carrier protein has been identified in the canalicular membrane[45,50]. This carrier associated transport of bile acids into the canaliculus may be driven by the membrane potential in which the cytoplasm is negatively charged with respect to the canaliculus lumen, thus allowing the movement of negatively charged anions out of the cell and into the canaliculus. The formation of micelles, thus decreasing the concentration of free bile acids in the canaliculus also facilitates the movement of bile acids into the lumen.

d) Other organic solutes

Bile acid independent flow is thought to be generated by the biliary excretion of other osmotically active organic solutes.

These organic solutes include anions (bilirubin, free fatty acids), cations and neutral compounds which are conjugated by the liver to increase their water solubility and facilitate excretion into bile[45]. Uptake of the organic anions may involve the sinusoidal 54kD multispecific organic anion exchanger.

Glutathione is an organic anion which is synthesised by the hepatocyte and secreted into plasma and bile by carrier mediated mechanisms. Biliary excretion is active and is driven by the transmembrane electrical potential. Biliary excretion of glutathione is one of the major determinants of bile acid independent flow[49].

3.4.3 Intracellular organelles

The transcellular vesicular pathway is responsible for the movement of large molecules such as IgA and transferrin. It involves endocytosis and exocytosis and is dependent on intact microtubules. Hepatic microsomes are involved in conjugating lipid soluble organic solutes into water soluble compounds for excretion into bile. Golgi vesicles may also be involved in excretory processes. Lysosomes may fuse with the canalicular membrane and release their contents into bile[47,53].

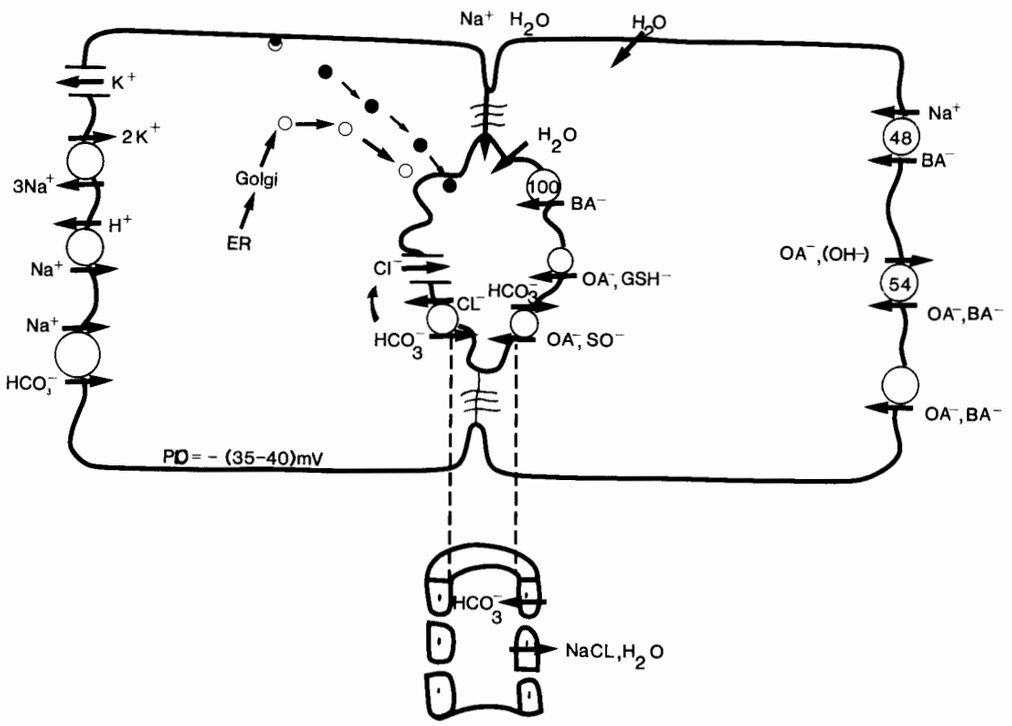


Figure 3.2: Location of membrane transport systems in hepatocytes and bile ducts. Modified from 'Physiology of bile secretion and cholestasis' by M. Sellinger and J. Boyer. In: Progress in Liver Diseases. Vol. IX. Eds. H. Popper and F. Schaffner

CHAPTER FOUR

Cholestasis

Cholestasis occurs when the determinants of bile formation (as discussed in the previous chapter) are no longer able to maintain standing osmotic gradients between bile and the hepatocyte and the intercellular spaces. As the cholestatic liver injury progresses, these osmotic gradients dissipate and the driving force for osmotic filtration of bile is diminished. In many cholestatic disorders it is not certain whether these disturbances in the determinants of bile secretion are primary effects or secondary consequences of cholestatic injury.

Cholestasis is associated with several functional disturbances including the following defects:

1. Impaired transport of organic anions resulting in raised plasma and tissue bile salt levels associated with pruritus, retention of bilirubin causing jaundice.

2. Defects in lipid excretion resulting in hypercholesterolaemia and associated xanthelasma, hyperphospholipidaemia and alterations in red cell membranes (target cells, burr cells).

3. Decreased intestinal bile salt concentrations resulting in steatorrhoea and consequent malabsorption of fat soluble vitamins (vitamins A, D, K, E).

4. Increased synthesis or release of enzymes from liver plasma membranes manifested as raised serum alkaline phosphatase and gammaglutamyl transferase levels.

Cholestasis is caused by a number of intrahepatic and extrahepatic disorders in which the disturbances in the determinants of bile secretion are often similar.

4.1 PATHOPHYSIOLOGY OF CHOLESTASIS AS APPLICABLE TO ORTHOTOPIC LIVER TRANSPLANTATION

4.1.1. Alterations in morphology

a) Bile duct obstruction and anastomosis problems

Morphological changes include progressive dilatation of the bile canaliculi and loss of canalicular membrane microvilli[56]. In the hepatocytes, the number of Golgi vesicles decrease in the canalicular region and the smooth and rough endoplasmic reticulum is generally decreased. Intracellular vesicles, often containing bile pigment, lipids and secretory protein such as IgA increase in number near the secretory pole[45,59]. The cytoskeleton is important in maintaining the structural polarity of the hepatocyte. During cholestasis, changes occur in the peri-canalicular cytoplasm which include an increase in number and disarray

of the actin microfilaments resulting in thickening of the peri-canalicular cytoplasm. These changes are associated with decreased canalicular contractility[45]. Within the tight junction the number and density of contact lines decrease and are no longer arranged in parallel to the lumen of the canaliculus[45,57]. The cytoplasmic protein ZO-1 which is associated with the tight junction becomes discontinuous along the submembranous region of the junction[58]. As a result of these structural changes junctional permeability is increased, resulting in a loss of the osmotic driving forces due to reflux of the bile acids and other osmotically active compounds into the intercellular space and then into the sinusoid. Loss of integrity of the tight junction also results in a loss of the structural and functional polarity of the hepatocyte leading to a redistribution of canalicular proteins (alkaline phosphatase, bile acid 100 kD carrier protein) on the sinusoidal domain[45,54].

b) Intrahepatic cholestasis (rejection, graft injury and hepatitis)

Many similar morphological changes occur to those seen in extrahepatic bile duct obstruction.These changes include distortion of the cytoskeleton involving the microfilaments and impairment of the tight junctions with morphologic disruptions of the junctional strands and random cytoplasmic distribution of ZO-I proteins. Interleukin-6, a pro-inflammatory cytokine has been shown to decrease expression

of intermediate junctions and desmosomes with consequent decrease in cell-cell contact[60]. The resulting decrease in canalicular contractility and increased paracellular permeability to organic solutes is similar to that seen in extrahepatic cholestasis.

4.1.2 Loss of the translobular gradient

The translobular gradient is lost in cholestatic injury and the bile acids and other solutes which are normally predominantly cleared by the periportal cells accumulate in the circulation. Canalicular bile stasis tends to predominate in Zone III of the hepatic lobule[47].

The maintenance of osmotic driving forces for bile formation is critical for sustaining this secretion. Thus factors which may increase the osmolarity of sinusoidal blood such as total parenteral nutrition may reduce the osmotic gradient between plasma and bile at the hepatocyte level and thus contribute to cholestasis[61].

4.1.3. Changes in membrane permeability

Cellular function is dependent on the ability to maintain ion concentration gradients between intra and extracellular spaces. The hepatocyte membranes are relatively impermeable to Na^{+} and Ca^{2+} whilst readily permeable to Cl^{-} and CO2. Steep concentration gradients between the cell and the extracellular space are maintained by plasma membrane ATP-

dependent pumps (Na^+/K^+ATPase and Ca^{2+} ATPase). Membrane injury may lead to an increase in Na^+ and Ca^{2+} permeability leading to the dissipation of these ion gradients with resulting loss of Na^+ coupled solute transport and increases in cytosolic calcium[47]. The increase in cytosolic calcium may be due to failure of sequestration in the endoplasmic reticulum. Depletion of an essential regulatory calcium pool in the endoplasmic reticulum could impair a number of intracellular transport processes[45,53]. Also, the increased cytosolic calcium levels may result in sustained tonic contraction of the canaliculi thus interfering with the bile flow[47]. Monohydroxy bile acids such as lithocholate and its conjugates have been shown to increase the permeability of smooth endoplasmic reticulum to calcium ions, resulting in an increase in cytosolic calcium[53]. Thus cholestasis associated with lithocholate may be due to impairment of intracellular transport processes associated with increased cytosolic calcium[53].

Decreases in canalicular membrane permeability to water could also affect bile flow as that is one of the determinants of transcellular water flux which is independent of the osmotic driving forces[45].

However, whether the changes in membrane permeability to ions and water are the cause or the consequence of cholestasis is not known.

4.1.4. Changes in membrane fluidity

Membrane fluidity relates to the specific composition of lipids and influences the activities of many membrane-bound enzymes including Na^+/K^+-ATPase. Both increases and decreases in membrane fluidity may be associated with inhibition of bile secretion suggesting that there is a narrow physiological range in membrane viscosity for optimal secretory function[45,47].

4.1.5. Alteration of membrane carriers, ion pumps and intracellular transport mechanisms for:

a) Bile Acids: Inhibition of bile acid transport which results in a decrease in BADF may be due to a number of causes including:

i) **Alterations of the sinusoidal transport mechanisms:** The sodium dependent uptake of bile acids may be impaired as a result of dissipation of the Na^+/K^+-ATPase dependent sodium gradient which may occur as a result of membrane injury. Membrane injury may also directly affect the sinusoidal transport carriers, i.e. 48 and 54 kD carrier proteins[45,47]. Moreover, competition for the Na^+ dependent transport proteins as may occur between bile acids and amino acids may also result in decreased uptake[45]. This competition may play a role in cholestasis seen in total parenteral nutrition.

ii) **Alterations in transcellular transport:** Rapid transport to the canalicular domain usually occurs as a result of diffusion of free or protein bound bile acids. Competitive binding by other substances to these cytosolic binding proteins may decrease bile acid excretory rate[47]. Moreover, bile acids are also transported by microtubule dependent (ATPase Kinesin) vesicular pathways and are secreted by exocytosis[53]. This pathway becomes increasingly important as the transcellular flux of bile acid increases and the intracellular organelles such as microsomes and Golgi vesicles may accumulate bile acids[53].

This may act as a protective mechanism during liver injury when bile acid excretion is impaired and intracellular bile acid concentrations reach detergent levels[47]. This retention of bile acids results in a vicious cycle with increasing membrane damage and accelerated impairment in the bile secretory process[45,47,62,63].

iii) **Alterations in canalicular excretion:** Canalicular excretion is the rate limiting step in bile acid excretion. Injury to the 100 kD canalicular membrane carrier or depolarisation of the hepatocyte with resultant loss of the major driving force, i.e. transmembrane electrical potential difference, for excretion diminishes the ability of the hepatocyte to

excrete bile acids and generate bile flow. Also loss of functional hepatocyte polarity with relocalisation of the 100 kD canalicular protein to the sinusoidal domain as is seen in bile duct obstruction results in decreased bile acid excretion[45,47].

b) Organic anions: Excretion of organic ions especially glutathione contribute to BAIF. Thus inhibition of uptake or excretion of these solutes may decrease bile flow[45,49].

c) Inorganic ions: Ion transport mechanisms including Na^+/K^+ antiport, HCO^{3-}/Cl^- antiport and Na^+/HCO_3^- symport are thought to be impaired in cholestasis[47]. This may be the result of membrane injury with consequent changes in membrane viscosity. Whether this is a contributory cause or a consequence of cholestasis is uncertain. Loss or inhibition of sinusoidal Na^+/K^+ATPase activity results in alterations in ion gradients across the cell membrane which may inhibit bile acid uptake and other sodium dependent carrier mechanisms and thus inhibit bile secretion[45].

4.1.6 Changes in bile duct function

Bile secreted by hepatocytes is modified during its passage through the bile ductules and ducts[64]. Imbalances in duct secretion and absorption may play a role in cholestasis with a decrease in secretion resulting in inspissated bile[45]. Increase in bile secretion is associated with bile ductular cell hyperplasia seen in extrahepatic cholestasis.

4.2. Cholestasis post orthotopic liver transplantation

Jaundice is frequently seen in the early post operative period, the pathogenesis is often uncertain and it may be multifactorial. Although rejection is always considered, a number of other aetiologies must be considered and excluded and if necessary a liver biopsy is performed before boosting immunosuppression.

Common causes of cholestasis include:

4.2.1. Biliary tract obstruction:

Biliary tract complications are frequent as there is a high risk of breakdown of biliary anastomoses and/or biliary obstruction[65,66,67] both of which are frequently associated with ascending infection. The frequency of biliary tract complications is thought to be related to the tenuous blood supply of the reconstructed common bile duct. The major blood supply to the bile duct is by small arterial branches that arise from the gastroduodenal artery (retroduodenal and retroportal arteries) and ascend along the bile duct[68]. These arterioles are usually transected during donor hepatectomy and the allograft bile duct depends on arterial branches coming from the hilum of the liver[65].

Early postoperative biliary tract complications are usually due to technical surgical problems or ischaemic injury resulting in obstruction at the anastomotic site, anastomotic breakdown or stricture formation. Multiple

intrahepatic strictures are usually associated with arterial thrombosis[65]. Patients with biliary obstruction may manifest with septicaemia with acute cholangitis or with relapsing episodes of fever and fluctuating liver injury parameters. The diagnosis is usually made radiologically on ultrasound or cholangiogram. Histologically, there may be little to find if the diagnosis is made early. Otherwise typical findings include bile lakes and dilated, proliferative, tortuous ducts with periductal oedema. Perivenular cholestasis is seen but the presence of perivenular fibrosis usually suggests rejection. If cholangitis is present, there is a predominantly polymorph portal infiltrate present in contrast to the predominantly mononuclear portal infiltrate of rejection[20]. Late biliary tract complications manifesting as multiple intrahepatic and extrahepatic strictures are usually associated with arterial compromise or are the consequence of cholangitis[20]. Chronic rejection manifests as the vanishing bile duct syndrome and angiography may be necessary to confirm the associated obliterative arteriopathy.

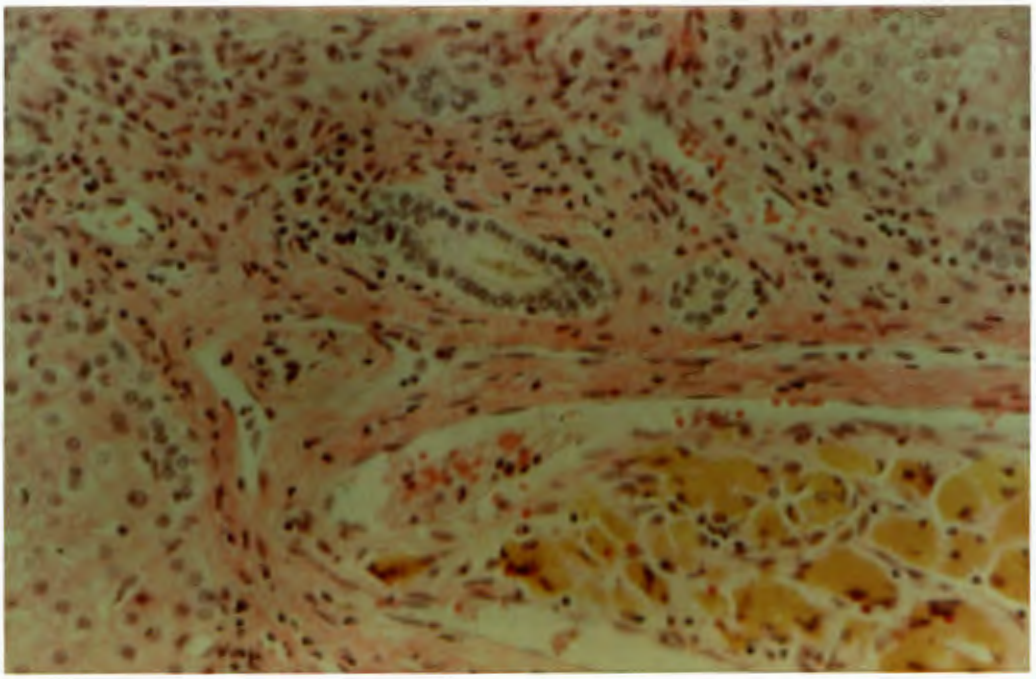


Figure 4.1: Cholestasis with ductular proliferation

4.2.2 Drug toxicity:

Most patients receive a combination of steroids, cyclosporine and azathioprine as immunosuppression post transplantation. Immunosuppressive therapy including cyclosporine and azathioprine can be hepatotoxic. In the case of cyclosporine this tends to be dose related and is associated with a modest hyperbilirubinaemia and a mild elevation in transaminases which settle on decreasing the cyclosporine dosage. Documented histological changes include

cholestasis and random acidophilic degeneration of hepatocytes[20].

Azathioprine has been associated with venosclerosis within the liver and glucocorticoids have been shown to induce hepatic steatosis[20].

4.2.3 Hyperalimentation

A complicated post-operative course following transplantation may necessitate prolonged total parenteral nutrition which may produce structural alterations. These changes include hepatocanalicular cholestasis, steatosis, cholangiolar proliferation with ductal cholestasis, steatosis, pigment deposition and sinusoidal fibrosis[20].

4.2.4 Infections

a) Bacterial infections such as acute cholangitis and ascending infection. Ductular cholestasis, where dilated ductules contain inspissated concretions of bile, is often associated with systemic infections or septicaemia[18].

b) Viral infections:

Cytomegalovirus hepatitis is common and is usually due to reactivation of a latent virus. It occurs most frequently 4 to 5 weeks after initiation of the immunosuppressive regime. Patients present with pyrexia, myalgia, arthralgia and mucosal ulceration and have prolonged abnormal liver

function tests. It is often difficult to distinguish this clinically from rejection. Serology usually reveals reactivation of a latent infection and cultures including throat gargles and urine cultures may be positive. Liver biopsy confirms the nuclear or cytoplasmic cytomegalovirus inclusion bodies together with microgranulomas[20].

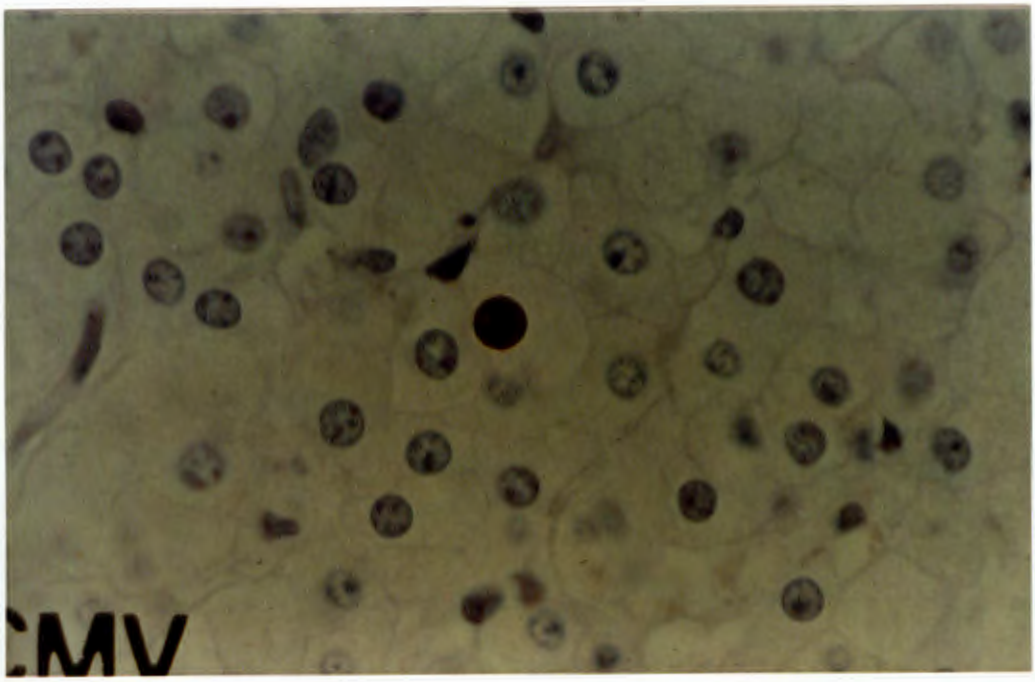


Figure 4.2: CMV Hepatitis

Hepatitis B: Patients with chronic active hepatitis B prior to transplantation usually have a recurrence of hepatitis B in the graft[3]. They present with malaise, jaundice and an

increase in transaminase levels coincident with the reappearance of HBeAg in the serum and HBcAg in tissue. Histologically there is a preferential lobular insult with little or no damage to structures targeted by rejection, i.e. bile ducts and venous endothelium[20].

Non A, Non B hepatitis and Hepatitis C tend to recur less frequently post liver transplantation than hepatitis B[3].

4.2.5. Vascular thrombosis

Portal vein and hepatic artery thrombosis may occur in the first four months following transplantation and usually within the first two weeks. Thrombosis is often associated with sepsis complicated by a coagulopathy requiring multiple transfusions. The clinical picture varies from an almost asymptomatic patient to that of a rapidly progressive deterioration in liver function necessitating retransplantation.

4.2.6. Functional cholestasis

Pure parenchymal cholestasis without associated rejection or bile duct inflammation may be seen in the early postoperative period, particularly in patients in a poor condition pre-operatively with associated coagulopathy postoperatively requiring multiple transfusions[69].

4.2.7 Preservation injury

This may be associated with a cholestatic syndrome which resolves spontaneously. Histologically there is ballooning degeneration of hepatocytes together with centrilobular cholestasis[70].

4.3 TRANSPLANTATION EXPERIENCE AT GROOTE SCHUUR HOSPITAL

As can be seen, cholestasis may be a common problem post transplantation and there may be numerous causes. It is important to make the correct diagnosis in order to institute appropriate treatment. It is often difficult to clinically distinguish rejection from ischaemia or infections especially viral without performing a liver biopsy. If a liver biopsy is required, histological interpretation is much easier if it is performed prior to boosting anti-rejection therapy.

Five of our transplant patients have experienced prolonged cholestasis following orthotopic liver transplantation. The management of cholestasis included establishing the aetiological diagnosis. This involved taking regular specimens of T-tube bile for culture to exclude biliary sepsis; T-tube cholangiograms and ultrasound of the liver were performed to exclude biliary obstruction. If hepatic artery thrombosis was suspected, angiography was performed. Liver biopsies were performed as indicated. Cyclosporine

levels were regularly monitored to maintain optimum therapeutic levels.

In two patients (OLTx 2 & 5), rejection was the only aetiological factor as infection and biliary tract obstruction were excluded and there was no evidence for any ischaemic or preservation injury. One patient (OLTx 7) experienced prolonged cholestasis associated with delayed graft function as a result of a severe preservation injury. One patient (OLTx 8) had cholestasis associated with a prolonged coagulopathy requiring multiple transfusions and infection in the form of bilateral lower lobe pneumonia and a mild CMV hepatitis. Another patient (OLTx 11) had prolonged cholestasis associated with a severe ischaemic injury with the subsequent development of an obstruction at the biliary tract anastomosis requiring reconstruction of the biliary tract with the formation of a choledochojejunostomy. However, despite the refashioning of the biliary tract anastomosis, this patient remained jaundiced and T-tube cholangiogram revealed the development of intrahepatic biliary strictures and a biliary leak at the site of the anastomosis (biloma) as a result of the previous ischaemic injury. This required insertion of two percutaneous biliary drainage catheters, one placed across a significant stricture at the origin of the left hepatic duct and the other draining the biloma. These catheters established good biliary drainage and the biloma decreased

in size. His post-operative course was further complicated by the development of a significant CMV hepatitis. At present, he still has an external biliary drainage catheter and may subsequently require internal stenting. If the intrahepatic strictures progress, in association with chronic hepatic ischaemia, retransplantation may be necessary.

All of the above patients experienced acute rejection but this was only severe in two patients (OLTx 2 & 5). These patients all received total parenteral nutrition usually only for a period of 3 days, but two cases (OLTx 7 & 11) received TPN for 7-10 days.

The medical treatment consisted of treating rejection with pulses of medrol and maintaining cyclosporin blood levels. Infections were treated appropriately. Bile salts in the form of ursodeoxycholic acid are administered not only to improve absorption of lipid, fat soluble vitamins and cyclosporin, but also as a protective mechanism. Ursodeoxycholic acid is hydrophilic and thus less toxic than other bile acids in cholestasis and it also promotes bile secretion in addition to blocking biological effects of endotoxin.

CHAPTER FIVE

Cytokines

Cytokines are a group of glycosylated or non-glycosylated polypeptides which are produced by activated lymphocytes and monocytes/macrophages as well as by a number of other cells, e.g. endothelial cells. Their biological activity is not dependent on their glycosylation[71]. They act as 'messenger molecules', by which cells communicate and modulate each other's functions. Cytokines can be active in a local environment in an autocrine or paracrine manner or may disseminate widely and act as classical hormones[72].

The effects of cytokines may be due to:

i) A single cytokine working alone;

ii) Quantitative effects increased or decreased according to the status of second signals;

iii) Synergistic where one cytokine alone has no effect in the absence of a second one[73]. Synergistic effects of cytokines may be as a result of **1) cascade activation** in which a T cell is activated to divide by a signal which includes IL-2 and the activated cell releases more IL-2 which acts on both the original cell and on other responsive cells; **2) Dual activation** in which some cells such as macrophages require triggers from two different

cytokines together, before developing their full range of functions; 3) **Receptor induction**[73].

Unrelated cell types can produce the same cytokine and a single cytokine can affect numerous other cell types. Most cytokines are not secreted constitutively but stimulation or activation of the cell is necessary to induce cytokine gene transcription and production. The activity of each cytokine is determined by the presence of specific high-affinity receptors on responding cells. There tends to be no cross-reactivity at receptor level and receptor expression is regulated. Post receptor signal transduction is poorly delineated as yet, but activation of various protein kinases with subsequent protein phosphorylation appears to play a role[74].

Cytokines participate in a number of cellular responses including the regulation of the immune system where they are involved in 5 major areas, namely T Cell activation, B Cell activation, haematopoeisis, toxicity and inflammation.

In this chapter, the roles of Tumour necrosis factor-alpha (TNF-alpha), Interleukin I (IL-1) and Interleukin 6 (IL-6) will be discussed in terms of the immune response and the hepatic acute phase reaction.

5.1. INTERLEUKIN I (IL-1)

There are two structurally related IL-I namely alpha and beta, both of which are encoded by genes on chromosome 2. IL-1 alpha and IL-1 beta have 26% amino acid homology and 45% homology at nucleic acid level. Despite this lack of homology they both bind to the same receptor and have similar biologic activities[75].

Interleukin I is produced by a wide variety of cells including T and B lymphocytes, natural killer (NK) cells, macrophages, skin keratinocytes, brain astrocytes, microglia, mesangial cells and the endothelium[72]. IL-1 beta has a molecular weight of 17kD and is the predominant form synthesised by macrophages. The production of IL-1 is induced by antigens, toxins, injury and inflammatory processes. Monocytes and macrophages produce IL-1 in response to bacterial products such as endotoxin, immune complexes, complement cleavage product C5a and many cytokines including TNF-alpha, macrophage colony-stimulating factor (M-CSF) and gamma-interferon (IFN). The principal target cells for IL-1 are T and B lymphocytes, macrophages, endothelium and tissue cells[73].

IL-1 receptors are found on many cells including leukocytes, haemopoietic cells, smooth muscle cells, endothelium, fibroblasts, chrondrocytes, hepatocytes, epidermal cells and pituitary cells[76]. The IL-1 receptor is a single chain molecule with molecular weight of 80 kD. The exact mode of

post receptor signal transduction is not certain. It has been suggested that IL-1 transiently increases cAMP and that the activation of adenyl cyclase involves a GTP-binding protein[77,78]. Other reports suggest that IL-1 binding induces the transient production of diacylglycerol generated from phosphatidyl turnover ultimately leading to activation of a protein kinase[79]. During IL-1 signal transduction, the binding of IL-1 to its receptor not only results in phosphoregulation of the receptor, but also leads to the translocation of the ligand-receptor complex into the nucleus[82]. IL-1 may also induce increases in prostaglandin E2 levels in target cells[74,80]. Native IL-1 inhibitors exist and distinct factors with molecular weights of 8kD, 20-25kD and 95kD have been identified[83]. These inhibitors prevent the deleterious effects of unbalanced IL-1 secretion. Autoantibodies to IL-1 also exist[88] and may serve as a carrier for IL-1, thereby retarding clearance and protecting the cytokine from proteolytic degradation. These autoantibodies may also have a protective function by controlling potentially dangerous stimulation of circulating lymphocytes.

5.1.1 Involvement in the immune system

IL-1 acts like an endogenous adjuvant serving as a cofactor during lymphocyte activation, primarily by inducing the synthesis of other lymphokines and the activation of resting T lymphocytes. Resting T helper lymphocytes recognise

antigens once they are presented on the surface of a macrophage together with MHC Class II molecules. The processed antigen is presented to the resting T cell together with IL-1. The resting T helper cell is activated and secretes a number of cytokines including IL-2, 3, 4 and 6 and gamma-IFN[72]. IL-1 increases T cell production of IL-2[74,71] and increases IL-2 receptor expression[72]. IL-1 enhances the initial T-helper cell activation in the presence of IL-2.

B cells are activated in a multistep cascade that begins with a small resting B cell and ends with a plasma cell committed to producing one type of antibody. The activation involves specific activation of the virgin or memory B cell, proliferation and clonal expansion and differentiation into antibody producing cells. Initial activation involves binding of the antigen to the immunoglobulin on the surface of the small resting B cell which is then converted to a large proliferating cell and further activation then requires B cell growth or stimulating factors. IL-2 plays a central role in regulating B cell activation, proliferation and differentiation into immunoglobulin producing plasma cells[74]. A number of cytokines including IL-6 can amplify antibody production but only in the presence of IL-2.

IL-1 which plays a role in B-cell activation, stimulates the synthesis of IL-6 and enhances its action[41]. IL-1 synergises with IL-6 to enhance immunoglobulin production in the presence of glucocorticoids[81].

5.1.2. Acute phase response

IL-1 together with TNF-alpha and IL-6 are involved in the acute phase response of the liver[37,86]. IL-1 inhibits the synthesis of the negative acute phase proteins such as albumin and transferrin and also inhibits the synthesis of the positive acute phase protein, fibrinogen[84,85]. It inhibits the induction of transcription of these genes[86]. It also stimulates the hepatic synthesis of complement factor B and C3. IL-1 can indirectly affect the hepatic acute phase response by stimulating the synthesis of IL-6 in fibroblasts and other stromal cells[37]. IL-1 also acts as a pyrogen and can stimulate enzyme synthesis in osteoclasts and chrondrocytes[73,86].

5.2 TUMOUR NECROSIS FACTOR ALPHA (TNF-ALPHA)

TNF-alpha exists as a secreted form (17 kD) and as a cell associated transmembrane form (26kD). The 26 kD transmembrane molecule is a precursor of the 17 kD secretory component but may also have inherent bioactivity as a paracrine mediator. The gene for TNF-alpha is situated on the short arm of chromosome 6[87].

TNF-alpha is synthesised by activated T cells, macrophages, monocytes, natural killer cells and the Kupffer cells of the liver. A wide variety of infections or inflammatory stimuli are capable of triggering TNF-alpha biosynthesis including bacterial endotoxin, enterotoxin, viruses, C5a and fungal or parasite antigens. IL-1 and Interferon-gamma enhance TNF-alpha biosynthesis[87].

The TNF-alpha receptors are found on activated T and B cells, natural killer cells, macrophages, neutrophils, haematopoietic stem cells, endothelial cells, fibroblasts, hepatocytes, adipocytes and osteoclasts. TNF-alpha receptors can be upregulated on some cells by gamma-1FN[74]. The TNF-alpha receptor has a molecular weight of 300kD and consists of dissimilar subunits. The binding subunit of the receptor is thought to have a molecular weight of 75kD[11]. The TNF-alpha receptors are a high affinity type and the receptor-ligand complex is internalised but the mode of post receptor signal transduction is not clear[11,87]. However, TNF-alpha has been shown to induce phosphorylation of a 28kD stress protein and induce the synthesis and myristoylation of a specific protein kinase C substrate[85].

TNF inhibitors exist which appear to be a soluble form of membrane associated TNF receptor[89,90]. Autoantibodies to TNF also exist[88].

TNF-alpha acts in synergy with IL-1 with which it shares many systemic properties and with IFN-gamma. Depending on the concentration of TNF-alpha , duration of exposure and presence of other mediators (IL-1 and gamma-IFN) in the cellular environment, the net biological effects may either benefit or injure the host. Acute systemic release results in septic shock and tissue injury; persistent TNF-release provokes cachexia and if lesser amounts are released into tissues, the beneficial effects may predominate and mediate enhanced host defence against pathogens and coordinate tissue remodelling[73,87].

5.2.1 Involvement in the immune system

TNF-alpha is a primary mediator in the pathogenesis of infection, injury and inflammation and in the beneficial processes of host defence and tissue homeostasis.

TNF-alpha induced tissue injury is partly mediated by enhanced endothelial procoagulant activity which promotes fibrin deposition and diffuse intravascular coagulation[73,87]. TNF also promotes adherence of neutrophils to the endothelium as a result of increased expression of endothelium - leukocyte adhesion molecules (ELAMS)[11,87]. Leucostasis occurs as a result of increased expression of intercellular leukocyte adhesion molecules (ICAMS)[87]. The adherent neutrophils are stimulated by TNF-alpha to degranulate and form reactive oxygen intermediates such as

superoxide anions and hydrogen peroxide. TNF-alpha is chemotactic for macrophages and neutrophils and it increases their phagocytic and cytotoxic activity[73,87]. TNF-alpha activates natural killer and killer cells.

Many of the harmful effects of TNF-alpha are caused by the induction of mediators including peptide regulating factors such as IL-1, IL-6, granulocyte macrophage-colony stimulating factor (GM-CSF), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor beta (TGF-beta) and eicosanoids such as prostaglandins, leukotrienes and platelet-activating factor[87].

Septic shock appears to be the result of immunological over-responsiveness to an invasive stimulus with the lethal toxicity of TNF-alpha synergistically influenced by IL-1, INF-gamma and LPS[87].

TNF-alpha acts as a pyrogen both as a result of a direct effect on the hypothalamus and by inducing IL-1 synthesis[87]. It can also regulate the biosynthesis of several metabolic enzymes. The catabolic rate of adipocytes and skeletal muscle increases with resulting increased lipolysis and glycogenolysis. Glucagon mediated amino acid uptake increases and there is decreased lipoprotein lipase activity with consequent hypertriglyceridaemia. The derangements of lipid and protein metabolism induced by TNF-alpha resemble the metabolic alterations associated with injury, infection

or chronic illness which include increased whole-body energy expenditure, lipolysis and protein turnover[11,87].

TNF-alpha is also involved in tissue remodelling. TGF-beta which is a TNF-alpha inducible cytokine,may suppress TNF-alpha induced inflammation by suppressing the hydrogen peroxide releasing capacity of neutrophils and decreasing leucocyte adherence to endothelium[87,91]. TNF-alpha may act as a growth factor, stimulating fibroblasts and mesenchymal proliferation directly. Together with TGF-beta it can promote angiogenesis[87]. As a destructive mediator TNF-alpha induces the biosynthesis of collagenases and proteases. Phagocytosis of senescent glycosylated tissue proteins triggers macrophages to secrete TNF-alpha and IL-1 which then amplify the degradative and reparative mechanisms of normal tissue remodelling[87].

5.2.2 Acute phase response

TNF-alpha is less potent than IL-1 but it also indirectly affects the hepatic acute phase response by stimulating IL-6 synthesis in fibroblasts and other stromal cells[37]. TNF-alpha also causes significant downregulation of negative acute phase proteins including albumin, transferrin, and to a lesser extent, fibronectin[84]. It may also stimulate hepatic synthesis of complement factor B and C3[37,85].

5.3 INTERLEUKIN 6

IL-6 is a glycopeptide derived from a precursor protein of 212 amino acids by removal of a N-terminal hydrophobic leader peptide. It has two potential N-glycosylation sites and has a molecular mass ranging from 21-28kD depending on the cellular source[92]. The gene for IL-6 has been localised to chromosome 7 (7p21)[92]. IL-6 is produced by a variety of cells including fibroblasts, keratinocytes, endothelial cells, monocytes/macrophages, bone marrow stromal cells, T and B cells and the folliculostellate cells of the anterior pituitary[92]. A number of cell lines including T cell lines (HTLV-I transformed), monocyte lines (U937), T24 bladder carcinoma line as well as Tumour cells such as myeloma and cardiac myxoma cells also produce IL-6[10]. Hepatocytes do not produce IL-6 although certain hepatoma cell lines do produce IL-6[37].

In the steady state, IL-6 is not usually constitutively produced by normal cells, but its expression is readily induced in response to injury such as viral or bacterial infections, and physical or chemical trauma[92]. A number of cytokines including IL-1 and TNF-alpha either alone or in combination with IFN-alpha, platelet derived growth factor, IL-3 and GM-CSF also induce IL-6 production[92,93,94,95]. Compounds which activate protein kinase C have been shown to stimulate IL-6 gene expression in fibroblasts[92], suggesting that the protein-kinase C dependent signal transduction

pathway may trigger IL-6 gene expression. The production of IL-6 in fibroblasts in response to IL-1 is cAMP dependent and protein kinase C independent[92]. Glucocorticoids negatively regulate IL-6 gene expression[10,37,85]. The existence of multiple mechanisms of IL-6 gene regulation is consistent with the presence of several transcriptional enhancer elements (glucocorticoid responsive element, AP-1 binding element, cyclic AMP responsive element, C-fos serum responsive element and NF-kB binding element) in the 5' flanking region of the IL-6 gene[92,96,97,98].

IL-6 receptors may be of a high or low affinity variety[92,99,100]. The IL-6 receptor is a glycoprotein with a molecular weight of 80 kD consisting of 449 amino acids. The extracellular portion of the molecule contains 340 amino acids with a 90-residue N-terminal loop characteristic of the Ig superfamily. There is a transmembrane domain which consists of 28 residues and is followed by an intracytoplasmic segment of 82 amino acids. The intracellular segment of the IL-6 receptor lacks a tyrosine-kinase domain[101]. Moreover, it is thought that the intracellular domain of the IL-6 receptor plays no role in the transduction of the IL-6 signal as a soluble form of the receptor lacking the transmembrane and intracellular domains is still responsive to IL-6. After binding of IL-6, the 80 kD receptor associates with a 130 kD glycoprotein and it is thought that the IL-6 signal may be transduced by the 130 kD

glycoprotein rather than by the IL-6 receptor itself. The 130 kD glycoprotein may also stabilise the complex between IL-6 and the 80 kD receptor and thus increases the binding affinity[102].

IL-6 circulates in serum as a complex with alpha 2-macroglobulin[105]. The liver is the major target organ[104,106] of IL-6 where it is taken up by both hepatocytes and Kupffer cells, degraded and excreted into bile[104]. IL-6 is also cleared by the kidneys[106]. It has also been shown that after binding, IL-6 is released from the hepatocyte membrane and transported to skin where it is degraded[107].

However, IL-6 is a pleiotropic cytokine and thus the IL-6 receptor is found on many cell lines including those of epithelial, fibroblastic, haematopoietic and neural origin[10,92,98]. Dexamethasone increases the expression of the IL-6 receptor[103].

5.3.1 Involvement in the immune system

IL-6 plays a major role in the terminal differentiation of B lymphocytes into immunoglobulin producing plasma cells particularly in the presence of IL-2. IL-6 receptors are expressed on activated but not on resting B cells indicating that IL-6 acts only on the final maturation stage of activated B cells[108,109]. IL-6 is not involved in the growth of activated B-cells[74]. Synergy exists between IL-1 and IL-6

in presence of glucocorticoids to enhance immunoglobulin production[81].

IL-6 acts as an essential competence factor which synergises with IL-1 to control the initial steps of T cell activation. This synergy relates partly to the fact that IL-6 acts predominantly by enhancing IL-2 responsiveness as a result of IL-2 receptor induction whereas IL-1 increases IL-2 production[92]. IL-6 also stimulates proliferation of activated lymphocytes[92] and TNF-alpha may act synergistically with IL-6 in inducing T cell growth[110]. IL-6 together with IL-1 can function as a killer helper factor in the induction and proliferation of cytotoxic T lymphocytes[10,92].

IL-6 also stimulates the proliferation of mature $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymocytes[111] and the differentiation of cytolytic T cells from thymic precursors[112,113]. IL-6 induces serine esterases which are required for the expression of cytotoxic function[112]. IL-1 induces IL-6 production in thymocytes and increases their sensitivity to IL-6[114].

IL-6 receptors are expressed on resting T lymphocytes and the expression of IL-6 receptor is downregulated upon T-cell activation[108]. IL-6 is important in the initial stages of T cell activation and its main role is to move T cells from Go to an early G_1 phase where they can become responsive to IL-

2 and further progression into the cycle is then controlled by IL-1 which is required for IL-2 induction[115].

5.3.2 Acute phase response

IL-6 is the major direct regulator of the hepatic acute phase response[84,116,117,118,119]. Both IL-1 and TNF-alpha released primarily by activated monocytes-macrophages induce a potent release of IL-6 from stromal cells[37] and thus indirectly affect the hepatic acute phase response[92]. It induces the full spectrum of acute phase proteins including serum amyloid A, C-reactive protein, haptoglobin, alpha 1-antichymotrypsin, alpha 1-antitrypsin, fibrinogen, alpha 1-acid glycoprotein, caeruloplasmin and complement factor B; whereas the synthesis of the negative acute phase proteins including albumin, transferrin, fibronectin and C3 is considerably decreased[116,117,118,119]. IL-6 exerts its control on acute phase proteins partly at the transcriptional level[92]. It induces the interaction of specific nuclear factors (i.e. DNA binding proteins) with IL-6 responsive promoter elements of several acute phase protein genes[85].

IL-6 also contributes to the host's defences by inducing fever and stimulating the release of ACTH[92].

was felt that measurement of biliary cytokines might be more specific for liver rejection than serum levels.

CHAPTER SIX

Patients, Methods and Results

6.1 PATIENTS

Eleven patients have undergone orthotopic liver transplantation at Groote Schuur Hospital since October 1988 (see Chapter 1). All experienced episodes of acute rejection and in two patients this was severe (OLTx 2 and 5). Diagnosing the rejection clinically is not always straightforward as there may be other contributing factors to liver dysfunction such as infection, drug toxicity, previous ischaemic or preservation injuries. Diagnosis must often be confirmed histologically. Liver biopsy may however be contraindicated because of coagulation abnormalities, ascites or biliary dilatation. Analysis of the biochemistry and cytokine concentrations of bile obtained from the T-tubes of transplant patients may provide an early non-invasive diagnostic aid. Of the 11 patients who have undergone orthotopic liver transplantation, nine had external biliary drainage, i.e. the common bile ducts of donor and recipient were anastomosed end-to-end over a T-tube. The T-tube is usually clamped at 14 days but remains in situ for 3 months before it is removed under antibiotic cover. This allows easy access to the biliary tract should a cholangiogram be necessary to exclude obstruction. The bile usually flows via

the T-tube into the external biliary drainage bag as well as into the duodenum. Occasionally, however, there may be preferential drainage into the duodenum. In these cases there may be little or no external drainage of bile.

Although 9 of our patients had T-tubes, we were only able to collect bile from 7 patients (OLTx 2, 3, 4, 6, 7, 8, 11). Serial bile samples were obtained from 4 patients (OLTx 6, 7, 8, 11). Bile samples were obtained at time of clinical rejection, viral infection or cholestasis in the remainder of the patients. Bile was also obtained from organ donors and used to set baseline values.

6.2 METHODS

6.2.1 Collection of bile samples

Bile samples were collected daily where possible from the external biliary drainage tubing and reservoir of the patients and stored in aliquots of 2 ml at -70°C. The donor bile was obtained from the gallbladder at the time of harvesting and prior to flushing with Wisconsin preservation solution.

6.2.2 Evaluation of hepatic function

Liver function tests including serum biochemistry (bilirubin, transaminases, gammaglutamyl transferase and alkaline

phosphatase levels) and coagulation profiles (platelets, INR, partial thromboplastin time, fibrinogen levels) were performed daily. If the clinical findings together with abnormal liver function tests suggested either allograft rejection or cholestasis due to another cause, then further investigations such as abdominal ultrasound, T-tube cholangiogram and liver biopsy were performed.

6.2.3 Biliary biochemical parameters, cytokines and porphyrin measurements

a) Biochemical parameters

Measurement of the biliary biochemistry was performed by the Department of Chemical Pathology, Groote Schuur Hospital. Once the appropriate bile dilutions were made, all measurements were automated using the Boehringer Mannheim Hitachi System 704 machine and the Beckman ASTRA™ machine. The principles of measurement are presented below.

The biochemical parameters measured included:

(i) Total bilirubin: This was measured on the HITACHI 704 and the method used is the modified Jandrassik and Grof method. Bile is reacted with diazotized sulphanilic acid in a solution of acetate buffered caffeine benzoate which accelerates the coupling of bilirubin to the diazotized sulphanilic acid.

(ii) Gammaglutamyl transferase (GGT): This was measured on the HITACHI 704 and depends on the ability of GGT to catalyse the cleavage and transfer of gammaglutamyl residues from peptides or other compounds to an acceptor.

i.e. L-y-glutamyl-3-carboxy-4-nitroanilide + glycylglycine $\xrightarrow{GGT}$ L-y-glutamyl-glycylglycine + 5-amino-2-nitrobenzoate.

(iii) Alkaline phosphatase (ALP): This was also measured on the HITACHI 704. This measures the ability of alkaline phosphatase to cleave phosphate groups from a wide variety of substrates.

i.e. p-Nitrophenylphosphate + H_2O $\xrightarrow{ALP}$ Phosphate + p-nitrophenol.

(iv) Calcium and magnesium: These were both measured on the HITACHI 704. The measurement of calcium depends on the ability of Ca^{2+} to form a violet complex with o-cresolphthalein complexone in an alkaline medium.

Magnesium ions form a purple-red complex with xylidyl blue in an alkaline solution. The magnesium concentration is measured bichromatically in terms of decrease in absorbance of xylidyl blue at 660/700 nm. Interference by calcium is prevented by EGTA present in the buffer.

(v) Sodium, potassium and chloride: These were all measured on the ASTRA™. The sodium-potassium chemistry module uses ion-selective electrodes to determine the presence of these ions in a solution. When the mixture of sample in buffer comes into contact with the ion-selective electrode an ion exchange (sodium) or ion complexing (potassium) occurs, resulting in a change in voltage potential at the electrode. This potential follows the Nernst equation allowing the calculation of sodium or potassium concentration in solution.

Measurement of chloride depends on the formation of insoluble silver chloride (AgCl) from chloride (Cl^-) and silver (Ag^+) ions. The total number of silver ions required to titrate the chloride is equivalent to the quantity of chloride present in a sample. The production of Ag^+ ions at the silver electrodes generates an electrical current which is measured.

b) Cytokines

Tumour necrosis factor-alpha (TNF-alpha), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) levels were determined by a solid phase ELISA (Quantikine human IL-6, IL-1 beta and TNF-alpha immunoassays by Research and Diagnostics systems purchased from British Bio-technology Limited).

These assays employ the quantitative 'sandwich' enzyme immunoassay technique. All the samples were tested in

duplicate and a series of wells was prepared using known concentrations of IL-6, IL-1 and TNF-alpha standards. The range of concentrations for the IL-6 and IL-1 standards was 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.25 pg/ml, and 0 pg/ml. The range of concentrations for the TNF-alpha standards was 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.7 pg/ml and 0 pg/ml. The appropriate diluent was used as the zero standard.

Assay procedure

(i) The microtiter plates were coated with a monoclonal antibody specific for IL-6, IL-1 or TNF-alpha.

(ii) 200 µl of bile or standard was added per well and incubated for 2 hours at room temperature for IL-6 and IL-1 and at 37°c for TNF. If the samples contained any of the cytokines, they were bound by the immobilised antibody.

(iii) Each well was then washed with 400 µl of wash buffer (Buffered Surfactant) to remove any unbound sample proteins. This washing procedure was repeated three times.

(iv) Then 200 µl of 'conjugate' (polyclonal antibody against IL-6, IL-1 or TNF conjugated to horseradish peroxidase) was added to each well and the plate incubated for 2 hours at room temperature for IL-6 and

IL-1 and at 37°C for TNF. This allowed binding of the conjugate to the cytokine which was bound during the first incubation.

(v) The plate was then washed three times as in step (iii).

(vi) 200 µl of 'substrate' solution (made from equal volumes of stabilised hydrogen peroxide and stabilised chromogen) was added to each well and the plate incubated for 20 minutes at room temperature. A colour developed in proportion to the amount of cytokine bound in the initial step.

(vii) 50 µl of 'stop' solution (2 N sulphuric acid) was added to each well and mixed well.

(viii) The optical density of each well was determined within 30 minutes using a spectrophotometer set to 450 nm and using dual wavelength correction (correction wavelength was 540 nm). This corrected for optical imperfections in the polystyrene microtiter plate.

Sensitivity and specificity of the assay: Minimum detectable levels are 3.5 pg/ml for IL-6; 4.5 pg/ml for IL-1 and 4.8 pg/ml for TNF-alpha. The assay is highly specific showing no cross reactivity with other cytokines.

Bio-assays were not used because of the cytotoxicity of

the bile salts and variable concentrations of cyclosporine present in bile samples. Bioassays are also not specific for individual cytokines.

c) Porphyrin levels

Porphyrin levels were measured by the Porphyria Laboratory, Medical School, UCT. Porphyrins were esterified with sulphuric acid in methanol, extracted with chloroform and then separated by thin layer chromatography. Amounts present were quantified by fluoroscanning and comparison with standard porphyrin solutions.

6.3 RESULTS

6.3.1 Biliary biochemistry

The biochemistry results are expressed as medians and ranges except where the biochemistry of the individual patients is presented, in which case actual values are documented.

a) Donor (controls) data

TABLE 6.1. BIOCHEMICAL PARAMETERS IN DONOR BILE

	Median	Range
Total bilirubin	7200	3100 - 21200 µmol/l
Alkaline phosphatase	520	200 - 1260 units/l
Gammaglutamyl transferase	1070	340 - 6320 units/l
Calcium	13.3	4.7 - 20.4 mmol/l
Magnesium	9	2.7 - 10.7 mmol/l
Sodium	346	208 - 408 mmol/l
Potassium	14.4	10.2 - 21.4 mmol/l
Chloride	108	0 - 246 mmol/l

Biliary biochemistry of 11 donors was analysed. The donor bile was obtained from the gallbladder and the levels of electrolytes (except for chloride which is lower) are normally higher than in T-tube bile (see Chapter 3). However, in these donors the slightly higher than usual Na^+, K^+, Ca^{2+} and Mg^{2+} levels together with the high Cl^- levels suggest possible cell membrane injury with alterations in membrane ion transport systems. The usual ranges for ALP and GGT are not known.

The donor biliary biochemical parameters were then compared with those of T-tube bile obtained from the transplant patients. The values were compared at various time periods post transplantation (Day 1, Days 2-4, 5-8, 9-10 and 11-14) (see figures 6.1, 6.2, 6.3 and Table 6.2).

Figure 6.1 BILIARY BILIRUBIN & ALP LEVELS POST TRANSPLANTATION

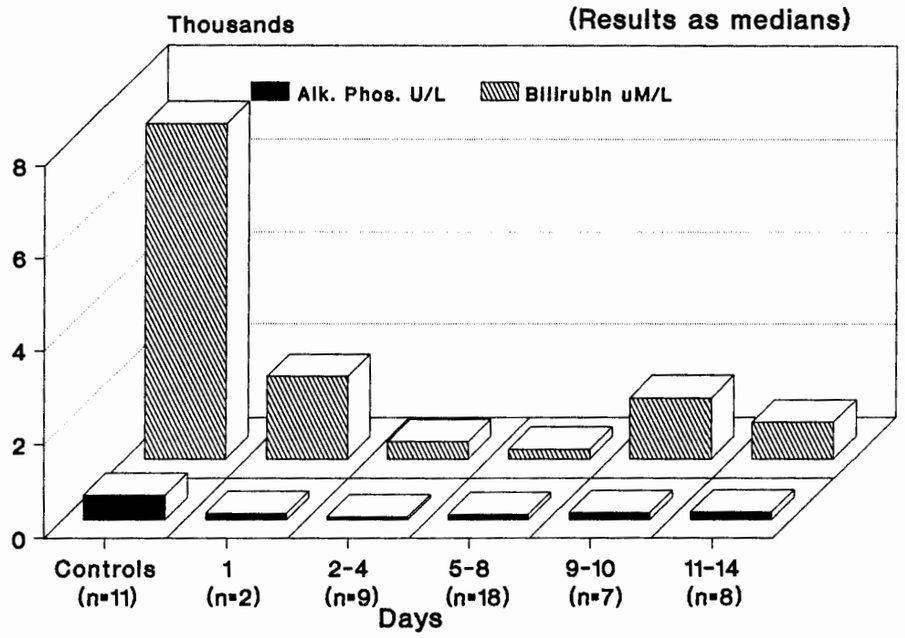


Figure 6.2 BILIARY CALCIUM & MAGNESIUM LEVELS POST TRANSPLANTATION

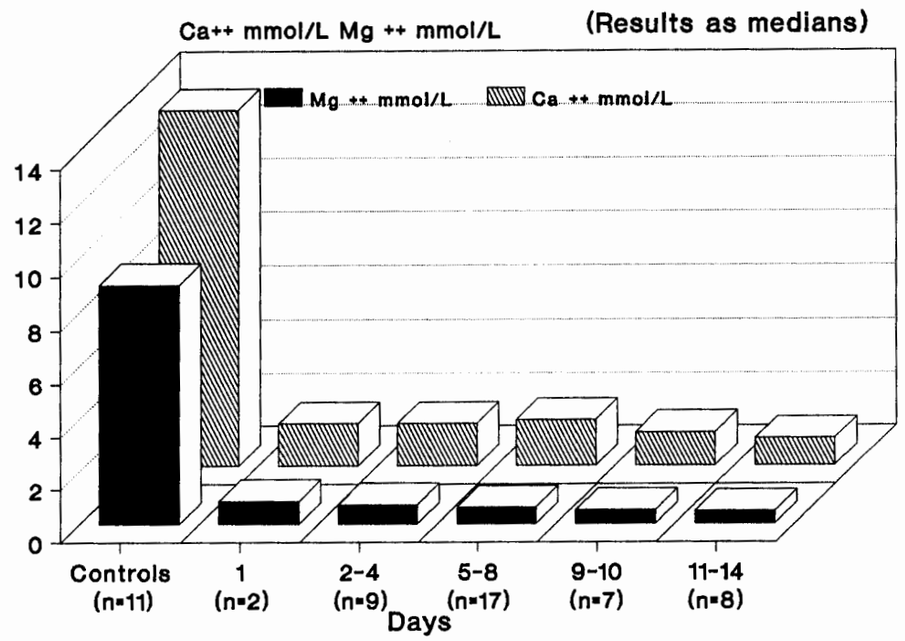


Figure 6.3 BILIARY SODIUM, POTASSIUM & CHLORIDE POST TRANSPLANTATION

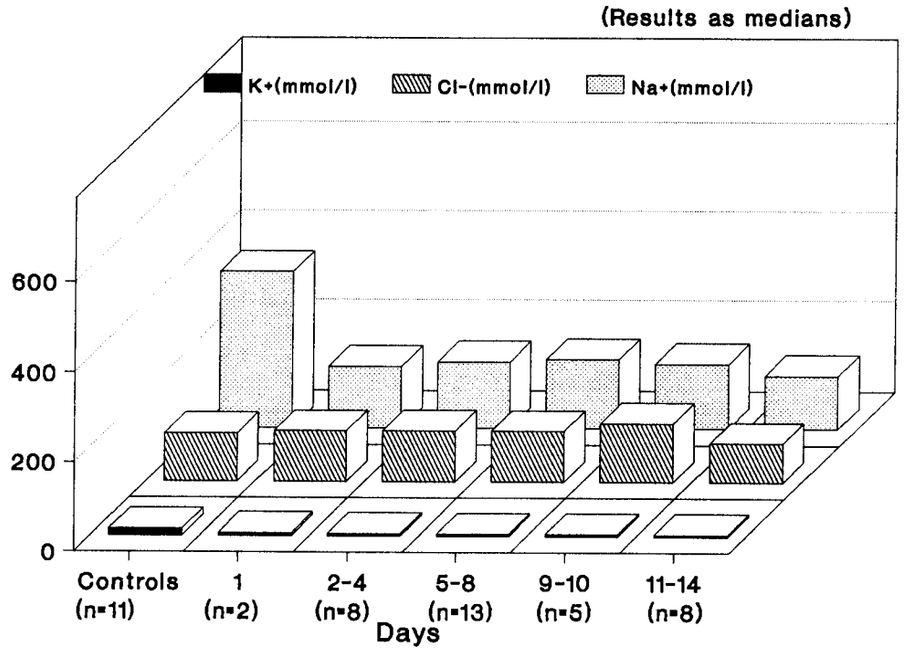


TABLE 6.2. BILIARY GGT AND ALP LEVELS POST TRANSPLANTATION

	Controls (n=11)	Day 1 (n=2)	Days 2-4 (n=9)	Days 5-8 (n=18)	Days 9-10 (n=7)	Days 11-14 (n=8)
GGT units/l						
Median	1070	551	194	268	480	566
Range	340-6320	242-860	0-4530	6-1480	68-1242	264-2000
ALP units/l						
Median	520	129	54	112	140	158
Range	0-3000	68-190	0-650	20-1180	18-960	36-1118

As can be seen from the previous figures, the donor biliary biochemical parameters are higher than those of the transplant patients. Although gallbladder bile is normally more concentrated than T-tube bile (higher Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} levels), cell membrane injury may also have contributed to the higher levels.

Biochemical parameters for the transplant patients were evaluated at the different time intervals previously stated in order to see whether any significant trends in changes of biochemical parameters could be related to clinical events such as rejection and infection. Day 1 values were separated from the rest as these values might still reflect the donor bile values. Rejection usually occurs between 4-10 days, therefore the Day 2-4 period was selected to detect pre-rejection changes in biochemical parameters. The Day 5-8 and 9-10 periods were chosen for early or late immune/rejection events. The Day 11-14 period might reflect the changes following administration of boosted immunosuppressive therapy or reflect changes relating to development of infection or cholestasis post-liver injury.

At this stage, our number of transplant patients is still small and thus we were unable to group patients according to early or late rejection episodes. Thus evaluating changes in biochemical parameters at these time periods does not always accurately reflect changes relating to rejection as all patients (whether early or late rejection occurred) were

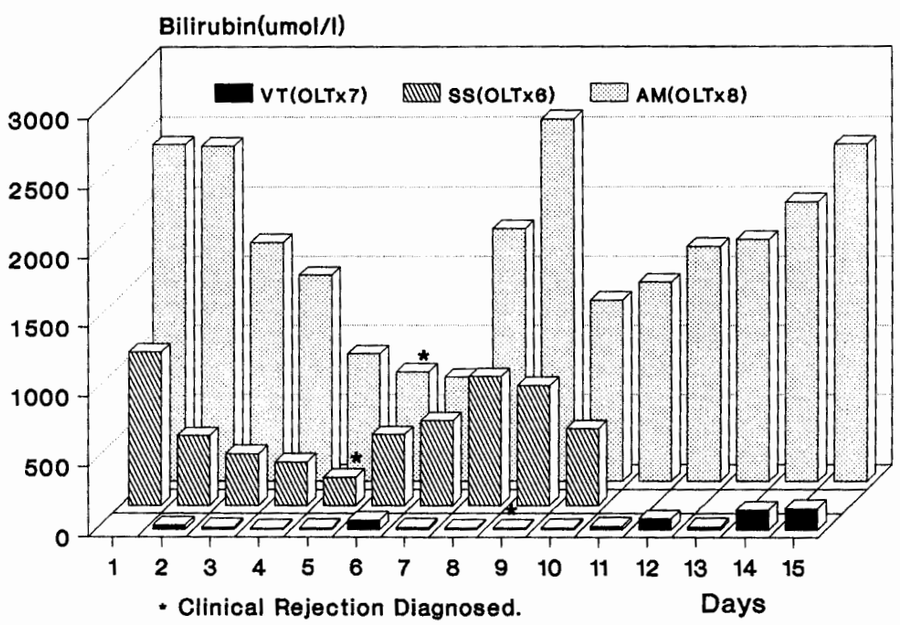
grouped together. However, there was a marked decrease in the biliary bilirubin levels prior to the usual time period for rejection, i.e. 4-10 days post-transplantation. The biliary ALP and GGT levels initially decreased in the first few days post-transplantation followed by an increase during the usual time period for rejection.

It proved more useful to look at the biliary biochemical parameters of individual patients especially in those cases where serial bile samples were obtained.

b) Individual patient data

Serial bile samples were obtained from patients SS (OLTx 6), VT (OLTx 7) and AM (OLTx 8) and the biliary bilirubin parameters for these individual patients are presented in Figure 6.4.

Figure 6.4 BILIARY BILIRUBIN LEVELS POST TRANSPLANTATION

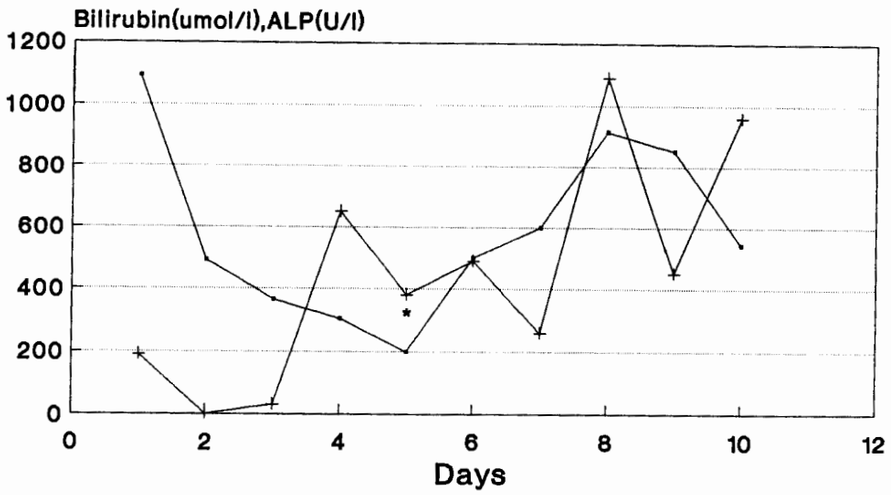


When assessing individual patients, the biliary bilirubin levels proved a sensitive method of evaluating liver injury. The biliary bilirubin levels decreased in patients AM (OLTx 8) and SS (OLTx 6) some time before clinical rejection was diagnosed. However, patient VT (OLTx 7) who had suffered a severe preservation injury, initially produced very little watery bile and only at Day 14 post transplantation did the biliary bilirubin levels start rising. Ultrasound of the liver and T-tube cholangiography excluded biliary obstruction in all three patients. A hepatic angiogram was performed on VT (OLTx 7) and excluded hepatic artery thrombosis.

In the following graphs these patients' individual biochemical data is presented in more detail.

Patient SS (OLTx 6)

Figure 6.5 BILIARY BILIRUBIN AND ALP LEVELS POST TRANSPLANTATION

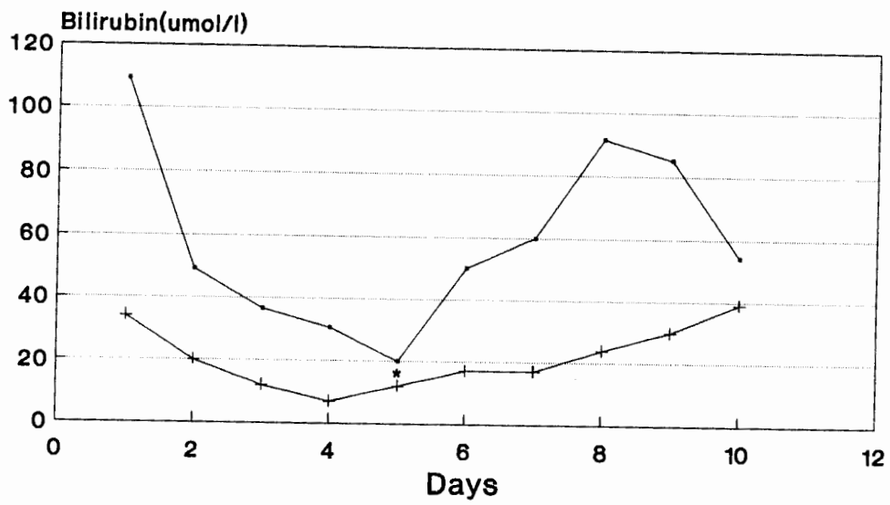


* = Clinical Rejection Diagnosed. Pulsed Medrol on Days 5,6,7 and 10.

SS(OLTx6)

The biliary bilirubin levels decreased 3 days prior to the clinical diagnosis of rejection followed by a later rise in ALP. Within 24 hours of administering pulsed medrol, the biliary bilirubin levels begin to rise, reaching a plateau at Day 8 and then decreasing.

Figure 6.6 BILIARY AND SERUM BILIRUBIN LEVELS POST TRANSPLANTATION

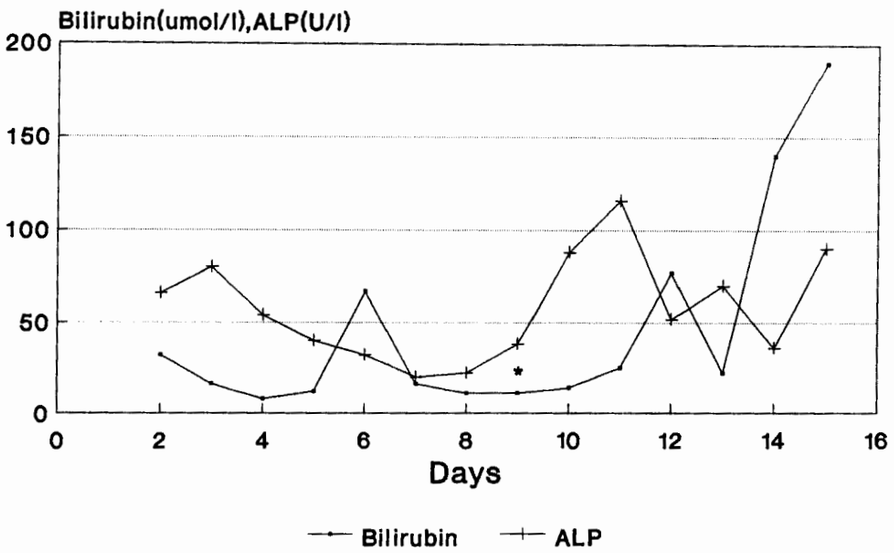


SS(OLTx6)

When comparing the biliary and serum bilirubin levels in relation to rejection, it can be seen that there is no significant change in the serum bilirubin levels prior to clinical rejection as opposed to the obvious marked decrease in biliary bilirubin levels (Fig. 6.6).

Patient VT (OLTx 7)

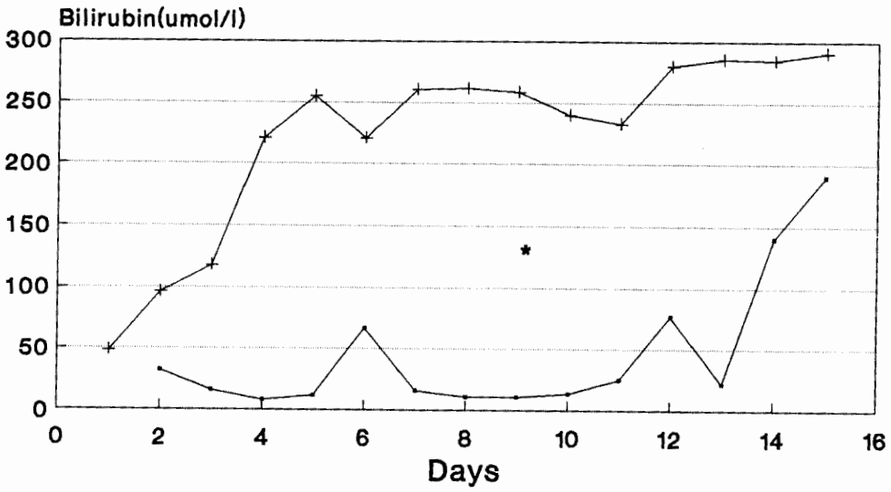
Figure 6.7 BILIARY BILIRUBIN AND ALP LEVELS POST TRANSPLANTATION



VT(OLTx7)

VT (OLTx 7) suffered a severe preservation injury resulting in delayed graft function with prolonged cholestasis. Clinical rejection was diagnosed on Day 9 and pulsed medrol commenced. As can be seen from figure 7, following commencement of medrol, the biliary bilirubin levels begin to rise with a significant increase occurring at Day 14 (22-140 µmol/l). There is a delayed rise in ALP associated with the diagnosis of rejection followed by a decrease in levels after administration of medrol. Liver biopsy was not possible because of haemostatic considerations.

Figure 6.8 BILIARY AND SERUM BILIRUBIN LEVELS POST TRANSPLANTATION



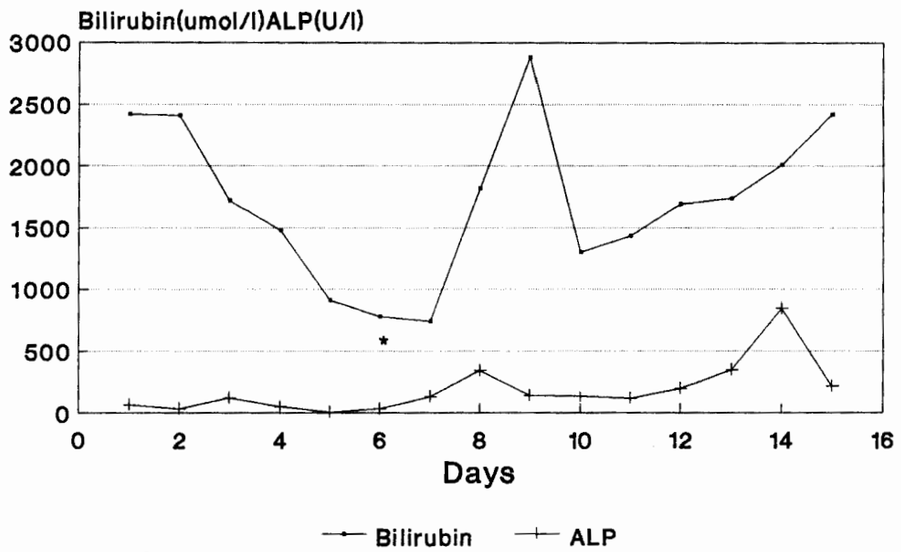
VT(OLTx7)

Figure 8 compares the biliary and serum bilirubin levels in VT (OLTx 7). The serum bilirubin levels rise following the preservation injury and remain elevated, reflecting the consequent prolonged cholestasis. However, the biliary bilirubin levels remain low in the first 9 days in keeping with delayed graft function but following the administration of medrol for clinical rejection, there is a progressive rise in biliary bilirubin levels suggesting improvement in graft function. However, because of prolonged cholestasis and possible binding of bilirubin to albumin, there may be slow

clearance of serum bilirubin. The serum bilirubin levels thus do not reflect the possible early improvement in graft function.

Patient AM (OLTx 8)

Figure 6.9 BILIARY BILIRUBIN AND ALP LEVELS POST TRANSPLANTATION

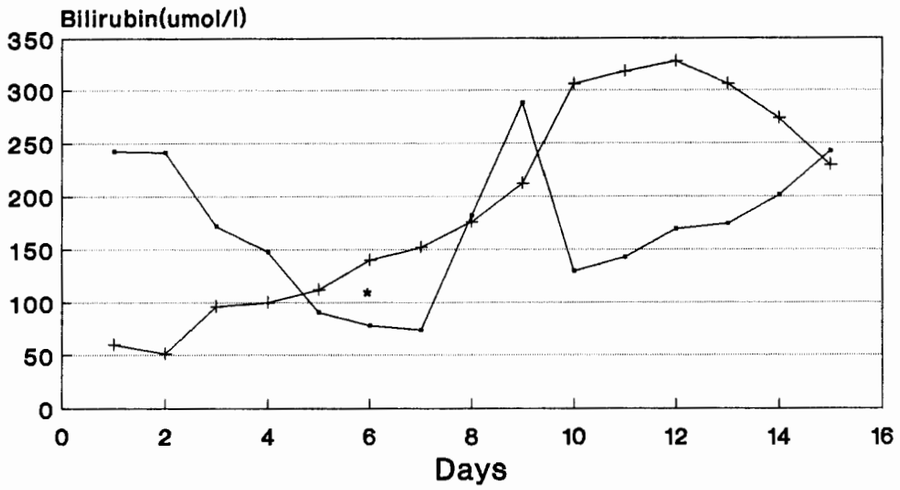


* = Clinical Rejection Diagnosed. Pulsed Medrol on Days 6,7,8,10 and 11.

AM(OLTx8)

The biliary bilirubin levels once again decrease 3 days prior to the clinical diagnosis of rejection followed by a delayed but less marked rise in ALP levels (Fig. 6.9).

Figure 6.10 BILIARY AND SERUM BILIRUBIN LEVELS POST TRANSPLANTATION



* = Clinical Rejection Diagnosed. Pulsed Medrol on AM(OLTx8) Days 6,7,8,10 and 11.

In Figure 10, it can be seen that there is no significant change in the serum bilirubin levels in contrast to the marked drop in biliary bilirubin levels prior to rejection followed by a rebound rise in levels after administration of pulsed medrol. Liver biopsy taken at Day 30 revealed no evidence for further rejection.

OLTx 11 suffered severe ischaemic injury and developed marked cholestasis. Serial bile samples were only obtainable 1 week post-transplantation and bilirubin levels in these samples

remained low (200-300 μmol/l). Liver biopsy confirmed extensive preservation injury with cholestasis.

Biliary GGT levels initially appeared to decrease in the first few days post-transplantation followed by a rise during the usual time period for rejection (see Table 6.2). However, serial analysis of biliary GGT levels in individual patients (OLTx 6, 7, 8) did not show any consistent change in relation to rejection.

6.3.2 Cytokines

a) TNF-alpha and IL-1

Biliary TNF-alpha levels were assayed in 5 donors and in the transplant patients (OLTx 2, 3, 4 and 6) and were undetectable. The IL-1 assay was performed on the same bile samples. IL-1 was detectable in three of the donor bile samples (median 56, range 0-73 pg/ml). One patient (OLTx 4) had an elevated level of 250 pg/ml on Day 8 post transplantation at the time of reactivation of Hepatitis B. IL-1 was undetectable in the other transplant patients even at times of rejection.

As the biliary TNF-alpha and IL-1 levels did not appear to increase at times of rejection and in view of the expense of

these assays, these cytokines were not measured in subsequent donor and transplant patient bile.

b) IL-6

IL-6 is the major direct mediator of the hepatic acute phase response. IL-6 estimations were performed on all bile samples obtained from donors and transplant patients.

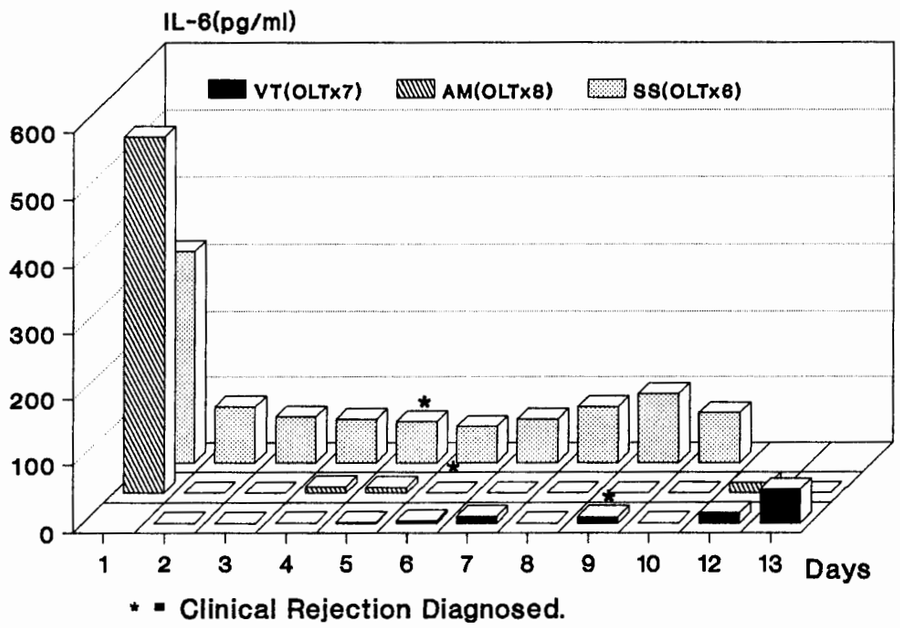
Calculation of results: An average of the duplicate readings was taken. The zero standard optical density reading was subtracted from the sample optical density readings. The optical density for the standards versus the concentration of the standards was plotted. The data was linearised by log transformation and regression analysis was applied (the r value was 0.997 for the IL-6 standard concentration curve, similar r values were obtained for the TNF and IL-1 standard concentration curve).

TABLE 6.3. BILIARY IL-6 LEVELS POST TRANSPLANTATION

	IL-6 (range pg/ml)	Median
Controls (n=11)	0-360	60
OLTx		
Days 1 (n=2)	320-535	427
2-4 (n=9)	0- 85	0
5-8 (n=18)	0-158	44
9-10 (n=6)	0-105	45
11-14 (n=6)	0-192	14

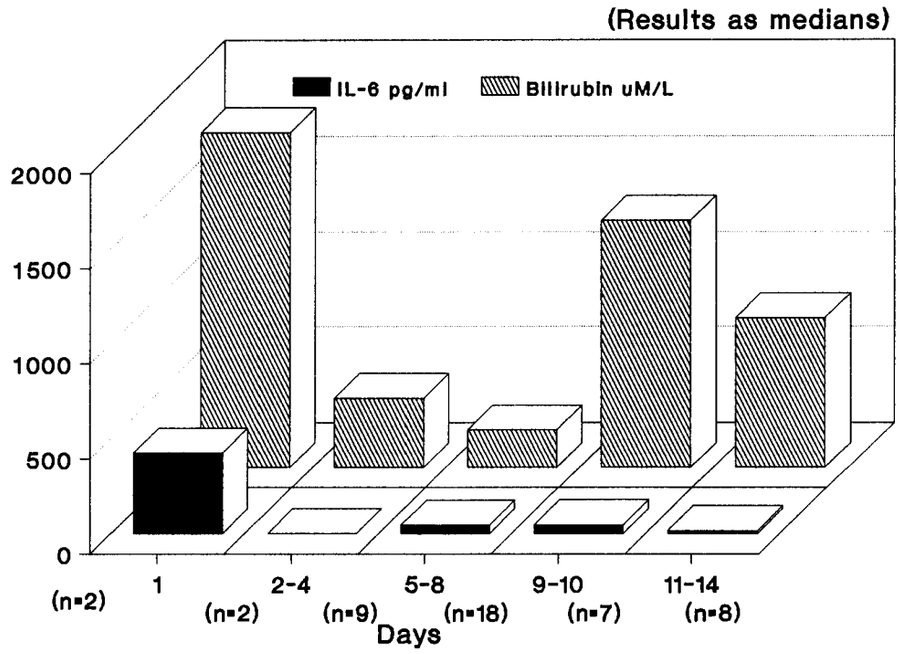
IL-6 levels were high in donor bile and on Day 1 post-transplantation. Although IL-6 levels appeared slightly elevated during the usual period of rejection (days 4-10), this was not significant and there was no increase in IL-6 levels during the Day 2-4 period (see Table 6.3). Thus, IL-6 did not appear to be a sensitive marker of rejection and this was confirmed when serial IL-6 levels were analysed in the individual patients SS (OLTx 6), VT (OLTx 7) and AM (OLTx 8) (see Fig. 6.11).

Figure 6.11 **BILIARY INTERLEUKIN-6 LEVELS POST TRANSPLANTATION**



Elevated IL-6 levels were noted in OLTx 11 at times of biliary sepsis (192 pg/ml) and cytomegalovirus infection (1820 pg/ml). OLTx 3 had an elevated IL-6 level of 110 pg/ml associated with a cytomegalovirus infection.

Figure 6.12 BILIARY BILIRUBIN & IL-6 LEVELS POST TRANSPLANTATION



Biliary bilirubin and IL-6 levels at the stipulated time periods were then compared (Fig. 6.12). As can be seen, there is a marked decrease in bilirubin levels but apart from the initial decrease in IL-6 levels after Day 1 (this probably still reflects the high donor IL-6 levels). There are no significant changes in the IL-6 levels.

6.3.3 Porphyrins

Porphyrins were measured in 7 donors and 5 transplant patients (OLTx 2, 3, 4, 6 and 7). Biliary porphyrin profiles were similar in donors and in the transplant patients (see Table 6.4).

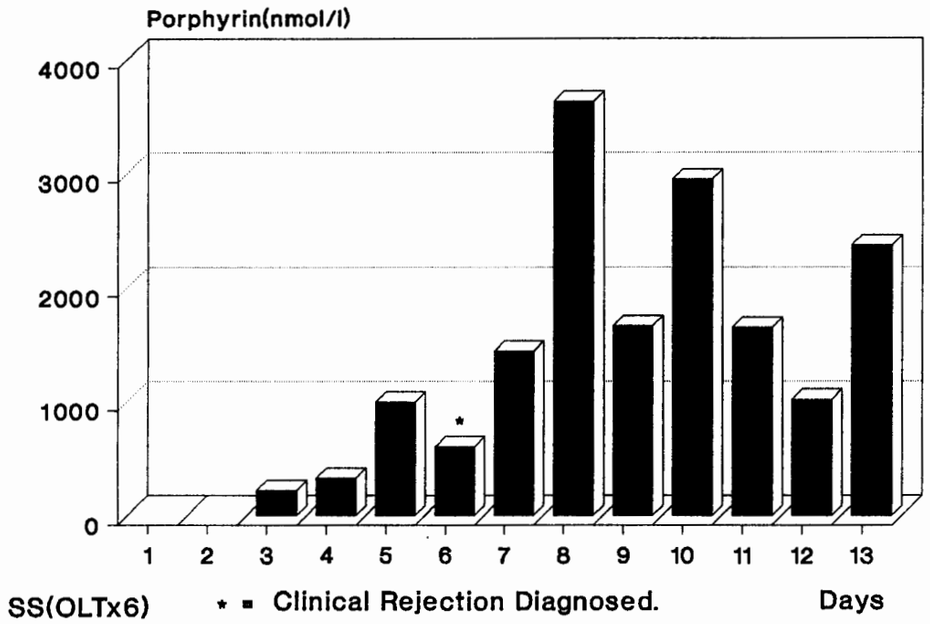
TABLE 6.4. BILIARY PORPHYRIN PROFILE

	Donors	OLTx
Coproporphyrin	52%	62%
3-COOH	17%	10%
Protoporphyrin	31%	28%

Bile contains large amounts of coproporphyrin which predominates over protoporphyrin. There is also a significant amount of 3-COOH porphyrin present in bile. This is the reverse of the normal faecal pattern.

Serial biliary porphyrin analysis was only performed on two transplant patients (OLTx 6, 7). Interestingly, in OLTx 7 (VT) who had experienced a severe preservation injury, the total porphyrin concentration was 219.5 nmol/l on Day 1 and thereafter fell to undetectable levels and remained undetectable for the next 2 weeks. Patient SS (OLTx 6) had initially low levels following transplantation but after administration of medrol for clinical rejection, the porphyrin levels rose and then levelled off (see Fig. 6.13).

Figure 6.13 BILIARY PORPHYRIN LEVELS POST TRANSPLANTATION



6.4 SUMMARY

It was thought that serial measurement of pro-inflammatory cytokines IL-1, IL-6 and TNF-alpha in bile might provide an early and sensitive marker for liver allograft rejection. However, this was not confirmed in our study. The measurement of biliary cytokines did not appear to predict rejection episodes. IL-6 levels were high in donor bile and then decreased rapidly. Isolated elevated IL-6 and IL-1 levels were associated with episodes of viral or bacterial infection.

However, serial biliary bilirubin measurements appeared to be a sensitive marker for liver injury. The biliary bilirubin levels decrease significantly prior to rejection and were very low in transplant patient OLTx 7, who experienced a severe ischaemic injury.

These results, together with an overview, are discussed in more detail in Chapter 7.

CHAPTER SEVEN

Discussion

7.1 INTRODUCTION

Orthotopic liver transplantation has become established at Groote Schuur Hospital as the treatment of choice for selected patients with end-stage chronic liver disease. In some cases (OLTx 6 and OLTx 10), the post-operative course was uncomplicated, the patients being mobile within a few days and leaving hospital within 2.5 weeks of the operation. Others, however, had a stormy post-operative course complicated by severe rejection (OLTx 2, 5); infections (OLTx 9, 11); the consequences of preservation injury (OLTx 7) or ischaemic injury with the development of biliary strictures and leak (OLTx 11).

Cholestasis is a common post-operative problem and major aetiological factors include ischaemic/preservation injury, rejection, sepsis (bacterial, viral or fungal infections) and biliary anastomotic complications. It is not always easy to clinically distinguish rejection from other aetiological factors. The cause of cholestasis may be multifactorial. Rejection is often diagnosed once immunological processes have already become established. Thus, readily measurable biochemical or immunological parameters which could act as early specific and sensitive markers for rejection would be

a valuable clinical tool in the post-operative management of transplant patients.

7.2 CYTOKINES

These post-operative complications are associated with and influenced by the pro-inflammatory cytokines IL-1, TNF-alpha and IL-6. These pluripotential cytokines are produced by activated macrophages and mediate immunological responses and the acute phase reactions as discussed in Chapters 2 and 5. IL-6 is of particular importance as a major and direct mediator of hepatic acute phase protein synthesis. Lymphocyte activation is an important early step in allograft rejection and the cytokines IL-L and TNF as well as IL-6 could play important roles in this regard.

IL-6 has also been shown to have effects on cell-cell association and cell mobility in ductal breast carcinoma cell lines[60]. IL-6 decreases expression of the intermediate junctions which provide anchorage for actin microfilaments. These are responsible for maintaining the shape of microvilli and canaliculi[46]. Bile flow is facilitated by periodic contractions mediated by these microfilaments[53]. The intermediate junctions also serve as adhesive structures between cells. IL-6 also decreases the number of desmosomes which are responsible for protecting the cell membrane from deformation and damage due to distension. The loss of these 'adherens' type junctions, i.e. intermediate junctions and

desmosomes results in decreased cell-cell contact[60], possible breakdown of tight junctions with loss of cell polarity.

The loss of cell polarity may be associated with the redistribution of canalicular membrane transport carriers to the sinusoidal domain[45,47,54].

Thus, IL-6 may contribute to the development of cholestasis with associated cytoskeletal changes. These may be central to intrahepatic (graft injury, rejection or hepatitis) or even extrahepatic (biliary tract strictures, anastomotic obstruction) cholestasis with ascending infection.

IL-6 may through the alteration of cell contacts play a modulatory role in liver regeneration which may occur following hepatocyte necrosis in graft rejection or viral hepatitis.

Serum soluble IL-2 receptor levels have been shown to rise prior to the clinical diagnosis of liver allograft rejection. Raised serum IL-2 receptor levels were not specific for rejection as they were also elevated in viral infections[13]. Biliary soluble IL-2 receptor levels were also found to be elevated in liver allograft rejection and to be more specific and sensitive than elevated serum IL-2 receptor levels[14].

IL-6 levels have been shown to be elevated in the serum and urine of renal transplant patients immediately post-operatively. The levels then slowly decrease but increased again in association with acute rejection. The increase in IL-6 levels is more marked in urine[12].

Serum TNF-alpha, IL-1 and interferon-gamma levels were found to be elevated in rejection but were not specific as they were also elevated in bacterial or viral infections[120].

It was thought that measurement of the pro-inflammatory cytokines IL-1, TNF-alpha and IL-6, which are involved in T cell activation and the acute phase response might be sensitive markers for acute rejection. Moreover it was thought that biliary levels of these cytokines (the Kupffer cells of the liver are a source of cytokine production) might be more specific for rejection than serum levels which might also be elevated in systemic infection.

In our study, the biliary IL-1 levels were slightly elevated in 3 of the 5 donors (73, 57 and 56 pg/ml) tested. Acute rejection was not associated with an elevation of IL-1 (OLTx 2, 3, 4, 6). The only significantly elevated IL-1 level (250 pg/ml) was associated with reactivation of hepatitis B viral replication on day 8 (OLTx 4).

No detectable TNF levels were measured. This may be due to very low levels which were not detectable (unlikely in view of the sensitivity of the assay) or due to biliary TNF being

labile and therefore not measurable. Biliary IL-6 levels were markedly elevated in 4 of the 11 donors tested (360, 359, 265 and 247 pg/ml). In the remaining 7 donors, the IL-6 levels ranged between 0-89 pg/ml. These differences in the IL-6 levels in donor bile could relate to differences in cause of organic brain death (trauma, head injury or subarachnoid haemorrhage).

Amongst the transplant patients, elevated biliary IL-6 levels of 320 and 535 pg/ml were noted on day 1 in two patients (OLTx 6 and OLTx 8). Elevated IL-6 levels were also noted in association with biliary sepsis (192 pg/ml in OLTx 11) and CMV hepatitis (110 pg/ml in OLTx 3 and 1820 pg/ml in OLTx 11). There were no significant reproducible increases in biliary IL-6 levels in association with early or established acute rejection.

Thus, the elevated biliary IL-1 and IL-6 levels appear to reflect non-specific acute phase responses seen in organic brain death (i.e. the donors) as well as in viral and bacterial infections. The elevated IL-6 levels seen on day 1 in two transplant patients probably still reflect the values of the donors as neither of these two patients had experienced significant preservation or ischaemic injuries and the levels had dropped to 85 pg/ml in one patient and were undetectable in the other patient on day 2.

There may be a number of reasons why the biliary cytokines particularly IL-6 were not elevated in acute cellular rejection. These could relate to:

i) The presence of acute phase proteins which serve as serum carrier proteins: Alpha2-macroglobulin is one of the major plasma proteins[43,44] and levels may rise during acute phase responses. Alpha-2 macroglobulin binds to a number of cytokines including TNF-alpha[44,121], IL-1 and IL-6[44,105]. This complex formation exerts effects on the distribution, bio-availability, stability and clearance of cytokines[44]. The binding of alpha-2 macroglobulin with IL-6 may protect IL-6 from the action of proteases, regulates the release of, and slows the rate of clearance of IL-6 from the circulation. Thus less IL-6 may be detectable in bile and urine during acute phase responses in which alpha-2 macroglobulin is induced.

Alpha-globulins have also been shown to decrease secretion of TNF by peripheral blood lymphocytes[12]. Increased hepatic alpha-globulin production during acute phase response may suppress the cells which are secreting TNF, thus limiting lymphocyte activation[121].

ii) Cytokine catabolism: The liver not only acts as a source of production but also as a scavenger of the cytokines IL-1, TNF-alpha and IL-6[85]. The liver is the major target organ of IL-6[106]. It has been shown that IL-6 binds to parenchymal

and non-parenchymal (mainly Kupffer cells) liver cells. IL-6 is endocytosed, degraded, excreted into bile as degradation products in a rat model[104]. There is also significant clearance of IL-6 by the kidneys. Interestingly, it has been shown in rats, that most of IL-6 after binding to the hepatocytes and inducing acute phase protein synthesis, is released from the hepatocyte membrane and transported to the skin where it is degraded[107]. The situation in man is speculative.

IL-1 is also cleared by the kidneys and TNF-alpha is cleared mainly by the liver, kidneys and spleen[104]. Thus the liver is not the only site of clearance and degradation of cytokines. Biliary levels of these cytokines might not always reflect the increased levels of the cytokines associated with acute phase responses.

iii) Immunosuppression and Glucocorticoids: Glucocorticoids act synergistically with the cytokines IL-1, IL-6 and TNF to cause induction of acute phase proteins. However glucocorticoids may also negatively downregulate IL-6 gene expression in inflammatory cells as well as inhibiting IL-1 and TNF-alpha synthesis, thus helping to terminate the acute phase response by a negative feedback mechanism[10,37,85]. Glucocorticoids also enhance IL-6 receptor expression[103] and may thus enhance IL-6 binding to hepatocyte, uptake and degradation.

In addition to the endogenous glucocorticoids secreted during the acute phase response, transplant patients all receive maintenance immunosuppressive medrol as well as pulses of medrol at times of rejection. The exogenous together with endogenous glucocorticoids may thus enhance the negative feedback on cytokine synthesis, with the result that high biliary levels of cytokines might not be detected in rejection.

Thus, in contrast to the study in rats where biliary IL-6 levels were shown to rise with the progression of liver allograft rejection and then fall in the terminal stages of rejection[122], our study failed to show any significant rise in any of the biliary cytokines at times of rejection. This may relate to the fact that our patients are receiving high dose immunosuppressive therapy. Acute phase reactants and alpha-2 macroglobulin kinetics may be different in rats. It has been shown that alpha-2 macroglobulin mRNA induction by IL-6 may be 'sex-specific' in Wistar rats with only the male rats showing an acute phase response[117].

7.3 BIOCHEMICAL PARAMETERS

There was a significant, progressive diminution of bilirubin levels from 3 days before the clinical diagnosis of rejection. This was accompanied by a slightly later rise in the ALP. Moreover, the administration of medrol with clinical improvement of symptoms was associated with a

prompt rise (often within 24 hours) of biliary bilirubin levels. Measurement of the electrolytes, calcium and magnesium did not show any particular trends in association with rejection. The levels of these parameters in donor bile was slightly higher than the usual gallbladder values, suggesting possible membrane injury associated with brain death in these donors.

Interestingly, the porphyrins, obligate intermediates of the haem synthetic pathway, were found to be undetectable in patient OLTx 7. This patient had delayed graft function due to preservation injury. In another patient (OLTx 6), who was tested serially, porphyrin levels were shown to rise after treatment for rejection. The liver is the major source of haem synthesis and the production of porphyrins. Thus the low levels of porphyrin associated with graft dysfunction are quite compatible. Porphyrin levels in bile could be used as a marker of hepatic function. We are evaluating this possibility in more depth.

7.4 SUMMARY

Biliary cytokines did not prove to be useful and reliable markers of early rejection. The evaluation of these parameters has given valuable information and resulted in further avenues for research. The serial measurement of biliary bilirubin levels show an early and significant decrease a few days prior to rejection. The biliary

bilirubin levels were also a more sensitive marker of graft function than the serum bilirubin levels which did not vary significantly prior to rejection nor following the administration of medrol.

Measurement of biliary bilirubin levels is a simple, inexpensive and non-invasive test. Serial measurements of biliary bilirubin although not specific, should prove useful in the early diagnosis of liver rejection in the correct clinical context.

The data on the biliary cytokines in orthotopic liver transplantation has been accepted for presentation at the Ares Serono International Symposium on IL-6 : Physiology and Clinical Potentials. Montreux, Switzerland - October 1991.

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