ASSESSMENT AND MODULATION OF COMPLICATIONS FOLLOWING LIVER TRANSPLANTATION

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Dedicated to my mother

ABSTRACT

The clinico-pathogical characteristics, diagnostic protocols and management of events which cause graft or systemic complications following liver transplantation are inadequately defined. In this thesis, the principal categories of such post-transplant complications were determined from a systematic examination of 127 consecutive failed liver transplants and classified. Several clinical and experimental protocols for assessment and management of the defined major clinico-pathological categories were then examined.

Primary graft dysfunction, secondary to impaired donor organ viability, was an important but not easily predictable determinant of early post-transplant outcome. Measurement of liver cell enzyme activities in the preservation solution, washed-out at the end of graft storage, was found to be valuable in prediction of early graft function. Recipients with the highest AST enzyme activity in this fluid experienced the most impaired biochemical graft dysfunction. More significantly, rejection frequency was higher and one month graft survival lower in this group compared to patients with low levels. Markers of liver endothelial cell (CK-BB, PNP) damage did not correlate with early graft viability.

Vascular complications were also a common cause of severe graft dysfunction and failure. The value of hepatic angiography in defining the extent and pattern of arterial lesions and their significance in graft survival was examined. Intrahepatic attenuation of the arterial tree in early graft dysfunction was associated with an unfavourable outcome. Similarly, during chronic rejection the presence of arteriopathy, even if absent on histological assessment, was accompanied by a poor prognosis. Angiography was also found to be more sensitive than doppler ultrasonography in assessing hepatic arterial thrombosis or stenosis and is recommended as the preferred investigation.

Vascular compromise and systemic haemodynamic derangements are present in primary graft dysfunction. N-acetylcysteine was shown to improve systemic oxygen delivery, consumption and ICG extraction in post-transplant patients. These properties should be clinically useful in critical illnesses (especilly following transplantation) where the systemic and hepatic-splanchnic circulation is compromised.

Allograft rejection was found to be the single largest pathological process leading to transplant failure. Characterisation of clinical risk factors for rejection may provide insight into underlying mechanisms and development of therapeutic strategies. In renal transplantation the ethnic origin of graft recipients is recognised as an important determinant of graft survival. In this investigation, non-caucasoid liver recipients were found to have inferior patient and graft survival figures as compared to caucasoids with different frequencies of chronic allograft rejection contributing to the differences. Patients from ethnic minorities transplanted from a predominantly caucasian donor population should be considered at higher risk of rejection.

Immune monitoring may allow prediction of a rejection episode and the response to treatment. Stable end-products of the L-arginine:nitric oxide biosynthetic pathway were found to be increased during acute allograft rejection. Levels correlated with histological severity and declined following supplemental corticosteroids. Correlations between plasma NO_x levels and circulating TNF-α and IL-2R positive lymphocytes were present. NO_x was the most predictive parameter of a rejection episode. Measuring plasma levels of NO_x may be useful in the assessment of acute allograft rejection.

Improved pharmacological control of rejection should reduce graft failures. The efficacy and safety of FK506 immunosuppression was compared to conventional CyA based regimens in 50 recipients transplantated for fulminant hepatic failure. FK506 treated patients had an improved post-transplant outcome with higher survival figures, lower rejection and reduced intensive care stays. Toxicity was similar to conventional regimens.

Pre-transplant general clinical status plays a major role in determining early post-operative outcome and was given a separate category in the proposed classification. This effect may be especially important in patients transplanted for acute liver failure where systemic critical illness is severe. In patients transplanted for non-paracetamol induced ALF (n=79), no static variable on admission other than aetiology was predictive of 2 month patient survival. Fulminant Wilsons disease and idiosyncratic drug reactions had significantly better and worse outcomes respectively. At transplantation, of 4 dynamic variables significant univariately (encephalopathy grade, Apache 111 and organ system failure scores, and serum creatinine) only the last

was an independent variable in a stepwise logistic regression for 2 month survival. In patients transplanted for paracetamol hepatotoxicity (n=21), 'overdose to hepatectomy period' was the only significant static variable, with no parameter predictive of outcome present on admission. Of two dynamic variables which were significant at transplantation (serum bilirubin and Apache 111 score) only the latter parameter was an independent variable in the regression model. Selection for transplantation in ALF depends on assessment of both the prognosis of the primary illness on admission and the clinical status at the time of proposed transplantation.

Transplantation for HBsAg-positive patients is controversial in view of the frequency of disease recurrence following transplantation. The roles of passive immunoprophylaxis, a stricter patient selection policy and coexistent HDV infection on outcome was examined. HBsAg serological recurrence rate was halved by immunoprophylaxis and was especially valuable if the recipients are HBV DNA - negative or had co-existent HDV infection. Patient survival has improved with HBIg particularly in HDV-negative or actively replicating recipients. Co-existent HDV infection is protective with survival figures comparable to elective non-HBsAg recipients. Graft injury following recurrence appears to be diminished in recent patients with a reduction in the rate of fibrosing cholestatic hepatitis. Retransplantation for HBV-related graft injury is not justified without anti-viral treatment.

Clinical tolerance, with absence of pharmacological immunosuppression, may improve the natural history of liver transplant recipients. Identification of a marker of tolerance to select appropriate patients is required. The relationship between complete immunosuppression withdrawal and microchimerism (a recently proposed marker of tolerance) and the resulting clinical outcome in a group of long-term liver recipients was examined. Chimerism (determined by genotypic examination for donor-specific HLA-DRB1 alleles) was demonstrated in 40% of patients with skin the optimal tissue for testing. Approximately one-third of patients (all transplanted for non-immune mediated disorders) were successfully removed from immunosuppression. However, no difference in the frequency of tolerance was demonstrated between chimeric and non - chimeric recipients. Finally, the potential hazards of immunosuppresion, especially in primary immune transplant indications, are highlighted in the report of severe recurrence of auto-immune hepatitis in the context of low immunosuppression.

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AUTHOR'S DECLARATION

The projects involving clinical subjects were given approval by the research ethical committee of Camberwell Health Authority (now Kings Healthcare) under several protocol submissions.

All of the relevant data collection, biochemical, haemodynamic and molecular analyses presented in this thesis were performed by the author except where acknowledged. Data analysis and presentation has been the sole responsibility of the author. This work has not been submitted for any other degree.

signed:

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ABBREVIATIONS

AIH	autoimmune hepatitis
ALD	alcoholic liver disease
ALF	acute liver failure
AMA	antimitochondrial antibody
ANA	antinuclear antibody
ASGPR	asialoglycoprotein receptor
AST	amino aspartate transferase
AUC	area-under-curve
CBIR	cyclosporin A based immunosuppression regimen
CI	cardiac index
CT	computerised tomogram
CyA	cyclosporin A
DNA	deoxyribonucleic acid
DO_2	oxygen delivery
EDTA	ethylenediaminitetra-acetic acid
EGD	early graft dysfunction
ELISA	enzyme linked immunosorbent assay
ELTR	European liver transplant registry
EVLWI	extravascular lung water index
FCH	fibrosing cholestatic hepatitis
FHF	fulminant hepatic failure
FWD	fulminant Wilsons disease
GVHD	graft versus host disease
HAS	hepatic artery stenosis
HAT	hepatic artery thrombosis
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
HBIG	hepatitis B immunoglobulin
HLA	human leukocyte antigen

ICG	indocyanine green
IFN-γ	interferon-gamma
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IL2(R)	interleukin 2 (receptor)
INR	international normalised ratio
IV	intravenous
IVC	inferior vena cava
LDH	lactate dehydrogenase
LKM	liver-kidney microsomal
LVSWI	left ventricular stroke work index
LOHF	late-onset hepatic failure
LSP	liver-specific membrane lipoprotein preparation
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MOF	multi-organ failure
n	number (of subjects)
NAC	n-acetylcysteine
NANB	non A-non B
NO	nitric oxide
OLT	orthotopic liver transplant
OSF	organ system failure
PBC	primary biliary cirrhosis
PCR	polymerase chain reaction
PEEP	positive-end-expiratory-pressure
PgI_2	prostacyclin
PGN	primary graft non-function
PNP	purine nucleoside phosphorylase
PSC	primary sclerosing cholangitis
PTLD	post-transplant lymphoproliferative disorder
RFLP	restriction-fragment-length-polymorphism
RIA	radio-immune-assay
RRT	renal replacement therapy
SD	standard deviation

SEM	standard error mean
SLC	sinusoidal lining cell
SSO	sequence-specific oligonucleotide
SSP	sequence-specific primer
TISS	therpaeutic intervention scoring system
TNF-α	tumour necrosis factor–α
UKTSSA	United Kingdom Transplant Support Service Authority
UNOS	United Network Organ Sharing
UW	University of Wisconsin
VBDS	vanishing bile duct syndrome
VO_2	oxygen consumption

SECTION A. BACKGROUND AND INTRODUCTION

CHAPTER 1

LIVER TRANSPLANTATION

1.1 HISTORY AND DEVELOPMENT

The first human liver transplants were performed by Starzl in 1963 (Starzl et al 1963). These whole organ orthotopically placed homografts were possible following the technical achievements in the animal laboratory pioneered by Cannon and Moore in Boston (Cannon et al 1956, Moore et al 1959). However despite the success achieved experimentally, it was a further 4 years before there was a long-term human survivor (Starzl et al 1968). These initial poor results predominantly reflected the limitations imposed by poor donor organ preservation with accompanying severe ischaemic injury, inadeqaute pharmacological control of rejection and the occurrence of systemic air embolism resulting from poor graft perfusion techniques (see sections 1.3 and 1.4). Following refinement of operative technique and anaesthetic care, initial survival post - operatively was regularly achieved which encouraged other centres in Cambridge, United Kingdom (UK), Hannover, Germany and Groningen, Netherlands to develop programmes in the late 1960's and early 70's (Calne et al 1968). Initial candidates were limited to those experiencing hepatic malignancy with later experience restricted to patients experiencing severe decompensation of chronic liver disease. Subsequent improvements in preservation and rejection control over the decade 1970-80, focused increasing attention onto selection of appropriate transplant candidates. The unacceptable results intially attained alerted transplant surgeons and physicians to the risks of operating on patients where the pretransplant clinical status was so unfavourable. Survival rates consistently less than 50% at one year were standard until the early 1980's.

During the decade 1980-90 following several advances discussed below, liver transplantation became established as the optimal treatment in a range of acute and chronic end-stage liver disorders. Patient survival rates of 80-90% at one year can now be achieved in low-risk elective recipients. Even in acute liver failure (ALF), despite the critical state of the patient, survival figures as high as 70-75% at one year are possible. As a result of these successes transplantation programmes have expanded rapidly such that annual totals now exceed those for heart recipients. In the United States, the annual increments have been the largest observed in solid-organ transplantation with the total number of liver transplants performed increasing from 62 in 1982 to approaching 3000 in 1992. Substantial increases in Europe have also occurred with an annual increase in transplants performed in the UK of between 15-20% per year. Such rapid expansion in liver transplantation is unlikely to continue over the next few years as the number of donor organs available is appearing to plateau. This is already stimulating debate on the appropriate allocation of organs, which have to be regarded as an increasingly scarce finite resource. Limitations in organ availability are particularly acute in the paediatric age group despite the recent innovative techniques of reduction and 'split' grafts. In the adult population, unless wider use is made of elective ventilation procedures or non-heart beating cadaveric organs - leaving aside possible changes in organ donation legislation - waiting lists for liver transplants can be anticipated to grow further (Roy First et al 1992). In the USA, reflecting the serious organ procurement problems, 23.2% of patients listed for liver transplantation died on the waiting-list in 1989 with the majority of these less than 45 years old (Annual report of UNOS 1991, UNOS Liver allocation data examined 1991). Latest reports indicate that the waiting-list mortality is increasing annually.

1.2 ORGAN VIABILITY AND PRESERVATION

In the pioneering days of clinical transplantation, the consequences which followed from the limitations imposed on donor organ retreival and preservation were soon evident. Organ procurement was only permitted following cardiac arrest i.e. from non-heart beating donors. As the risks of normothermic preservation (Goodrich and Welch 1956) were appreciated from animal experiments, hypothermic storage using rapid cooling of donor organs with a rudimentary electrolyte (lactated Ringers) solution was employed which limited viability to a matter of minutes rather than hours. Improvements, albeit marginal, to the preservation qualities of this solution followed from the introduction of dextran and then later buffered plasma. Of greater importance, the introduction of brain-stem death criteria (Diagnosis of brain death 1976, Definition of irreversible coma 1968) allowing organ retreival from an intact circulation, in addition to the use of either extracorporeal or ex-vivo organ perfusion with a modified heart-lung machine, extended viability significantly (Brettschneider et al 1968, Marchioro et al 1963).

It was later recognised that the use of hypothermic organ storage results in several detrimental biochemical and physiological effects to the liver graft which include development of intracellular acidosis, depletion of ATP and ADP and injury from reactive oxygen intermediates (for review see Blankensteijn et al 1991). The introduction of Collins solution was an attempt to counter these effects. However, perhaps the most significant present advance in liver preservation awaited the work of Belzer who, following documentation of the effects described above, systematically devised a new preservation solution addressing the specific biochemical injuries. The clinical introduction of the University of Wisconsin (UW) solution which followed his endeavours has extended the period of organ viability and improved early graft function (Belzer and Southard 1988). The wider significance of preservation injury has also come

to be recognised. In addition to the obvious insult to early graft function, increased rejection frequency, development of non-anastamotic bile duct strictures and vascular thrombosis have been implicated as consequences of preservation damage (Adam et al 1992, Campbell et al 1991, Howard et al 1990, Sanchez-Urdazpal et al 1992).

In contrast to programmes better knowledge and application of organ preservation techniques, there remains controversy over selection of suitable donor candidates. As Starzl pointed out in 1969 "an accurate picture of the quality of the donor liver may be difficult to obtain during the terminal phases of life". Certain absolute contraindications to donation such as HBsAg or HIV positivity, septicaemia and a recent history of malignancy are normally enforced. However, increasing relaxation over the donor clinical history with use of organs from donors with previously considered unfavourable characteristics (such as increasing age, obesity, vasopressor requirements, presence of renal failure, prolonged ITU admission) has apparently resulted in acceptable recipient clinical outcome in some centres (Mirze et al 1994). The use of *in-vivo* measurements of donor liver function employed prior to retrieval in an attempt to refine organ selection has not found widespread clinical application. Donor management, which may play an important part in organ viability, has not been properly addressed (Wijnen et al 1991).

Despite these advances, a note of caution is required in view of recent data from the European liver transplant registry (ELTR). As previously referred to, annual increments in both transplant numbers and patient survival in the registry reports have come to be expected. However the most recent report from the ELTR has for the first time not only demonstrated a minimal increase in the annual total but additionally a small reduction in 1 - year patient survival in elective recipients (1992 [80.5%, n=1782] versus 1991 [81.7%, n=1764]) (ELTR 1992). The latter may reflect the recently perceived greater usage of inferior donor organs as demand for grafts outstrips supply.

1.3 SURGICAL AND TECHNICAL CONSIDERATIONS

The present surgical procedures which form the basis of modern clinical transplantation reflect the distillation of considerable experimental and clinical experience since the first documented liver transplant performed on a dog by Welch in 1955 (Welch et al 1955). This initial heterotopic approach, which foundered because of portal venous delivery and drainage problems, was quickly superceded by orthotopic graft placement in 1956 by Cannon and in 1958 by Moore (Cannon et al 1956, Moore et al 1959 and 1960). Progression from these canine to porcine models quickly occurred reflecting the unique venous drainage problems of canine grafts and the closer anatomical (and as it turned out immunological) similarity of the pig liver and vasculature to humans (Starzl et al 1965). Within a decade of these innovations, the first human liver graft had been transplanted (see section 1.1).

Several major advances improving the operative procedure have evolved since that era. Splenectomy at the time of transplant, which in retrospect was not only unnecessary but indeed further predisposed to post-operative infection, has been abandoned in routine procedures. Severe intra-abdominal haemorrhage also resulted in a large proportion of technical failures in the early series. The introduction of veno-venous bypass, introduced to offset the circulatory derangements in the systemic and portal circulations present during the anhepatic phase, has also probably reduced intestinal congestion, abdominal infections, post-operative renal failure and bleeding secondary to portal hypertension (Papas et al 1971). This condition and associated hypersplenism alongside the coagulopathy of poor hepatic synthetic function, contributed to the severity of peri-operative bleeding. The more recent use of rapid transfusion lines and cell-savers (reducing the harmful effects from massive transfusion of stored blood) has also made a significant contribution to the management of intra-operative bleeding. Vascular graft

complications were also identfied early in the clinical transplant series as potentially catastrophic events. Devascularisation of the graft from hepatic artery thrombosis (HAT) in the early post-operative period often progressed to liver failure as was predicted from the experimental experience. The increased prevalence of this complication in paediatric recipients was recognised in the Denver experience. Biliary tract complications were also recognised as a common cause of major graft complications (Martineau et al 1972). Reconstruction of the biliary system has evolved from a cholecystoduodenostomy to gallbladder conduits and is presently standardised (in the absence of extra-heptic biliary disease) on a direct choledocho-choledochostomy. The major historical causes of biliary pathology were due not to surgical limitations (these were rare in dog and pig transplants) but to a combination of the tenuous vascular supply of the human biliary system promoting breakdown of the anastamosis and the corrosive effects of retained bile salts after storage resulting in sludge and obstruction. Shortening the donor common bile duct, improved storage techniques and irrigation of the biliary system at retrieval with preservation solution have abrogated both these problems (McMaster et al 1979a).

Other causes of technical transplant failures in the early series included overwhelming sepsis, air embolism and gastro-intestinal (particularly oesophageal) bleeding. Administration of anti-bacterial and anti-fungal prophylaxis and the introduction of improved immunosuppression regimens (see section 1.5) has reduced to a more acceptable level the early morbidity and mortality from sepsis. In contrast, the latter two problems have now been virtually removed by improvements in organ perfusion methodology and gastric antacid prophylaxis.

Following these advances in intra- and post-operative care and the consistent achieval of early survival, surgical innovation has more recently centred on strategies to either increase organ allocation or avoid continued immunosuppression. In particular, the

imbalance between the potential population of paediatric recipients and the pool of optimally sized donor organs has directed development to increase organ supply in this age-group. Reduced-size grafts including 'splits' are now regularly employed in experienced centres. The use of auxiliary grafts has also recently undergone renewed vogue. First employed in 1964, this approach had been attempted with consistently unsuccessful results until a 5 year survivor was reported in 1977. The attractiveness of this approach which can offer temporary liver support and thereby allow a period of native liver regeneration which may finally permit withdrawal of maintenance immunosuppression and atrophy of the donor graft is considerable (Moritz et al 1990). Initial attempts at heterotopic placement of auxiliary grafts have on the whole yielded unsatisfactory results due to vascular drainage and portal vein supply problems. Presently, there is increasingly widespread use of auxiliary partial liver orthotopic liver transplants (APOLT) which are technically more simple and have been successfully employed in correcting liver-specific metabolic defects and in patients experiencing acute liver failure.

1.4 DISEASE INDICATIONS AND PATIENT SELECTION

Patient selection is crucial in determining the outcome following transplantation. Selection of appropriate candidates is based on a careful clinical judgement of both the individual's prognosis and life expectancy with regard to the primary illness, as well as the effects of the illness on their quality of life; additionally and more controversially there is a difficulty of balancing the former with the optimal timing of the procedure which will bring a successful outcome. These principles were learned by trial and error in the early transplant series where the choice of candidates (see section 1.1) was so unfavourable to a satisfactory long-term outcome.

The need for accurate assessment of prognosis in liver disorders - which can be the basis in evaluating the need for transplantation or other therapy - has led to number of landmark studies in the development of clinical prognostic models (Powell and Klatskin 1968, Dickson et al 1989, Wiesner et al 1989, Farrant et al 1991, Weissberg et al 1984). Of these the natural history of primary biliary cirrhosis (PBC) is the best validated and the model has also been used to examine the important principle of disease severity at the tme of transplantation in relation to outcome. Studies have examined the effect of referral patterns, disease severity at transplantation and subsequent outcome in terms of survival, morbidity and financial cost (Neuberger et al 1990, Markus et al 1989, Cooper et al 1990). In the UK, late referrals associated with higher serum bilirubin levels and a raised prognostic index were reflected in lower post-transplant survival. Stratification for risk scores, using the Mayo Clinic model, in PBC patients makes it possible to delineate outcome in different severity categories in relation to morbidity and costs (Wiesner et al 1992). Reduced hospitalisation and costs were demonstrated in those transplanted with lower risk scores i.e. earlier in the natural history. This sort of clinically relevant data, which is poorly characterised in other chronic progressive parenchymal disease categories which proceed to transplantation such as primary sclerosing cholangitis (PSC) or alcoholic liver disease (ALD), cannot presently be applied to malignant or viral disorders.

Transplantation for hepatic malignancy results in survival rates at least 20-30% lower than other disease groups. These unfavourable results in liver tumours were recognised in the early transplant series where a large proportion of the recipients where patients experiencing unresectable hepatocellular carcinomas (HCC). Transplantation for cholangiocarcinoma (either central or peripheral) and large, multifocal HCC is associated with inevitable disease recurrence (Iwatsuki et al 1985). Detection of extra-hepatic malignancy, with the possible exception of haemangioendothelioma and carcinoid / apudomas, remains an absolute contraindication to proceeding with a transplant.

Worldwide, hepatitis B virus (HBV) and hepatitis C virus (HCV) -related liver disease represent the commonest potential indications for elective transplantation. The natural history of unmodified HBV infection after transplantation is one of rapid serological recurrence and graft injury (O'Grady et al 1992). Following isolation, cloning and development of serological assays for the detection of HCV, the patterns of reinfection and graft damage from this virus after transplantation are being quickly elucidated.

It was probably not anticipated in the early years of developing liver transplantation that ALD would quickly rise to be the largest single indication accounting for 15.4% of recipients in the US and increasing yearly. The surprisingly good results currently being obtained in ALD is dependent in most centres upon a very careful psychosocial assessment of the alcoholism quite apart from other important medical aspects which can affect the results of transplantation.

Given the success achieved with elective transplantation for chronic liver disorders, it was inevitable that emergency liver replacement with restoration of hepatic function would be attempted as a management option in ALF. Although renewed attempts are being made to develop artificial liver support with extra-corporeal circuits / columns and hepatocyte transplantation which may radically change physicians approach, at present transplantation has to be regarded as the optimal procedure for selected patients. Current surgical methods are centred on whole organ orthotopic transplantation with the role of APOLT and of hepatectomy prior to organ retrieval for earlier and very late cases respectively remaining to be defined. With survival rates reported for some centres in Europe as low as 46%, the need for adequate back-up in liver intensive care and medical aftercare in obtaining good results with transplantation is increasingly evident. Clinical and biochemical indices have been developed and are now available on which to reliably base the selection of patients whose prognosis is otherwise very poor (O'Grady et al 1989, Bernuau et al 1990).

Selection of transplant candidates must take into account measures to prevent disease recurrence. In addition to the viral and malignancy related liver disorders already referred to, recurrence of ALD, PBC, PSC, Budd-Chiari syndrome and autoimmune hepatitis (AIH) has been reported. The extent of graft injury following recurrence is variable and may be modified by the anti-rejection regimen used, for instance cyclosporin A (CyA) may affect recurrence of PBC whereas corticosteroids will promote replication in HBV and probably HCV viral reinfection.

1.5 REJECTION AND IMMUNOSUPPRESSION

The liver allograft is immunologically priviledged. This fundamental tenet went unrecognised in the initial canine transplants which normally underwent brisk rejection, although there were occasional long-term survivors in pharmacologically unmodified graft animals. It was not until the observations of Garnier in porcine grafts, which were confirmed later by the Bristol and Cambridge groups, that rejection was often abrogated or even spontaneously reversed that the unique nature of the liver allograft was suggested (Garnier et al 1965, Calne et al 1967, Peacock and Terblanche 1967).

When it came to human transplantation, the expediency of allocating liver grafts to transplant recipients on the basis of clinical need, irrespective of donor-recipient tissue matches, allowed the observations that human liver grafts could successfully transcend the normal allogeneic barriers of the human leukocyte antigen (HLA) and even occasionally the ABO antigen systems (Markus et al 1988, Donaldson et al 1993, Fischel et al 1989). Immunological liver graft failures are lower than other solid organ transplants reflecting the reduced frequency and severity of all forms of rejection. Why the liver is resistant to the "unrelenting incompatibility of the homograft" as described by Billingham still remains

speculative (for review see Wood et al 1993).

Despite this lower host-versus-graft response to liver transplants, inadequate control of rejection contributed to the poor outcome in early human transplantation. Pharmacological modification of the liver allograft response relied upon the immunosuppression regimens employed in renal transplantation by 1963 which comprised schedules of corticosteroids and azathioprine. Corticosteroids had been shown to possess immunosupppressive qualities by Billingham and Medwar in 1951 (Billingham et al 1951). The immunosuppressive qualities of purine analogues had previously been demonstrated by Calne in canine renal homografts in 1961 with clinical introduction a year later in view of a valuable synergistic action when administered alongside corticosteroids (Calne et al 1961, Starzl et al 1963). The clinical introduction of azathioprine undoubtedly contributed to the early attempts at human liver transplantation. Despite the promising results in human renal grafts, all the early liver recipients who were administered this dual therapy regimen under Starzl died from a variety of causes including irreversible rejection and as he noted "*overwhelming bacterial, fungal and viral infection*". Problems with azathioprine overdosage and accompanying marrow suppression further contributed to these infectious problems. These early results, with non-specific immunosuppression resulting in global inhibition of natural immunity, highlighted the marginal balance between rejection and infection which has remained the Achilles tendon of liver transplantation.

Further attempts at clinical transplantation only proceeded following the introduction of anti-lymphocyte serum (ALS) or globulin (ALG) as an adjunct to the immunosuppression armamentarium alongside corticosteroid and reduced azathioprine schedules (Abaza et al 1966). However, this regimen also proved inadequate and even the use of ancillary measures to treat presumed rejection employing local graft irradiation, cyclophosphamide, actinomycin and thoracic duct drainage were unsuccessful. It was not until the clinical

introduction of CyA by Calne,, following elucidation of its immunosuppressive properties by Borel, that adequate control of rejection was regularly achieved (Borel et al 1977, Calne et al 1978, McMaster et al 1979b). This agent has considerably improved patient and graft survival rates allowing significant reductions in corticosteroid and azathioprine dosages. Refinement of the use of CyA in the early post-operative period and tailored as part of a triple therapy regimen alongside azathioprine and prednisolone to reduce toxicity has been the preferred maintenance immunosuppression regimen employed in most liver transplant programmes over the last decade.

1.6 RESULTS AND LATE OUTCOME

The establishment of liver transplant central registries in the USA (Pitt-UNOS Liver transplant registry) and the ELTR in Europe provide the best documentation on the current status of liver transplantation, allowing accurate determination of outcome in large numbers of subjects in relation to a range of clinical and demographic characteristics (Belle et al 1991, Belle and Detre 1993, ELTR 1992). In addition to the primary disease (published survival rates vary by up to 50% in different liver disorders as discussed in section 1.2) and the technical considerations outlined above, other factors such as general medical condition, nutritional status, presence of renal failure, age, race and retransplantation can significantly influence outcome (Williams et al 1987, Belle and Detre 1993, Cuervas-Mons et al 1986a).

The impact of pretransplant functional clinical status (as assessed by the six-point United Network for Organ Sharing [UNOS] score which can be applied to all liver candidates) has also been shown to correlate with post-transplant outcome. This pattern was also found in the earlier study by Williams et al 1987 on hospital length of stay and total costs

of transplantation using a similar scale of pretransplant functional clinical status. The latest Pitt-UNOS registry data demonstrates a one-year patient survival of 84.4% versus 60.1% between recipients in the best and worst categories of this scale respectively. Significant differences in patient survival have also been shown by the registry in relation to demographic characteristics. Inferior survival rates were noted with respect to age (young paediatric and older adult recipients), race (black and oriental recipients fared worse) in addition to nature of primary liver disease. Extremes of age were until recently considered relative contraindications to liver transplantation. In many programmes however, older patients are now being transplanted with the mean age of all recipients rising consecutively in the ELTR from 35 years in 1985 to 43 years in 1992. An early report from Pittsburg encouragingly reported no significant difference in survival in a cohort of 92 patients between 50 to 77 years compared to a group of adults 18-49 years old as well as a similar return to domestic activities (Starzl et al 1987). More recent data from both the Pitt-Unos and the European registries are less optimistic with reductions in survival noted for patients greater than 60 years old. The differences are comparatively modest in the first few years post-transplant (approximately 5% or less) although they increase with time perhaps reflecting the contribution from natural mortality in this elderly population. Overall, children have survival rates marginally higher than adults although those less than 1 years old fare less favourably due mainly to a higher rate of technical complications.

Considerable experience and data is now available documenting the early outcome of liver transplant recipients with the clinical factors which influence patient and graft survival in the post-operative period and the initial years following transplantation well described (see above). In contrast, limited experience has been reported concerning the clinical status and natural history of long term liver transplant survivors with no detailed clinicopathological reports. In a recent analysis, the prevalence of systemic and graft complications in recipients beyond 5 years post-transplant was described from this unit (Slapak et al 1995).

Histological and biochemical abnormalities of the graft were unexpectedly common and could be broadly classified pathologically into seven main groups: a hepatitis-like picture, rejection, recurrent disease, sinusoidal damage / nodular regenerative hyperplasia, chronic cholangitis, scarring or cirrhosis. Normal biopsies were present in a minority of patients only.

Despite the potential pitfalls awaiting liver recipients at all stages following transplantation, as outlined above, the present populations are experiencing an enhancement of quality of life and often a return to employment (Lowe et al 1990, Tarter et al 1988).

1.7 OBJECTIVES OF THESIS

1. Identification of the present major aetiological categories of graft failure and death following liver transplantation.

2. Examination of the influence of novel epidemiological, clinical, biochemical and immunological factors in identifying and monitoring recipients at risk of these disorders.

3. Assessment of new therapeutic strategies in the prevention and management of selected graft and systemic complications.

CHAPTER 2

AETIOLOGY OF LIVER TRANSPLANT FAILURE

2.1 BACKGROUND AND INTRODUCTION

Over 6000 liver transplantations are presently performed annually with approximately 20 - 30% of procedures failing within the first post-operative year. A liver transplant can either fail from inadequate graft function necessitating regrafting or a graft or systemic complications which results in death. Unlike renal or cardiac transplantation, where rejection of the graft is the predominant pathological process leading to transplant failure, the circumstances which result in graft loss following liver transplantation are much more varied. This heterogeneity in the absence of a recognised system for classifying the various processses hinders proper analysis of factors involved as well as clinical and scientific communication in this field.

The two major studies on early mortality and graft failure in the current literature were reported from the Pittsburgh programme and are based on historical experience between 1981-84 and 1984-88 respectively (Cuervas-Mons et al 1986b, Quiroga et al 1991). The latter analysis performed in graft failures included only those transplant events which proceeded to retransplantation and accordingly only identified those pathological mechanisms which primarily affected the graft and resulted in loss of function. A classification scheme based around such 'allograft failures' would provide only limited and selected information on the total pathological events which liver recipients are exposed to, since the majority of transplant failures are in the context of patient death. A further study, from the Nebraska group, attempted to stratify the causes of post-transplant

mortality on the basis of an analysis of 44 deaths between 1985 and 1988 (Shaw et al 1989). In that systematic examination, the authors divided the causes of all deaths into four categories - deaths related to pre-transplant condition, failure of the allograft to provide adequate early function, failures of immunosuppression and finally a miscellaneous group. However, similar to above, a classification based on the clinico - pathological events which led to a death or graft loss is more informative than one centred around the immediate cause of death. The terminal event is often in the context of sepsis and multi-organ failure and such terms provide no information on the major underlying pathology.

A standardised classification of the clinico-pathological causes of liver transplant failures is required. Such a scheme would promote interpretation and comparison of the results emerging from both individual transplant programmes, registries and large multi-centre trials. A consensus use of nomenclature should also facilitate internal and external audit and permit easier identification of changing clinical patterns which accordingly could serve as a basis for directing clinical and research resources to those areas of particular need.

In this investigation, a prospective analysis of clinico-pathological events recorded in 126 consecutive primary transplant failures over a recent three year period in the Kings College Hosptial liver transplantation programme was performed. On the basis of the precise clinical syndromes which were considered the primary and predominant morbid event leading to transplant failure, a classification encompassing the observed clinico - pathological processes was proposed. This investigation, by identifying the present major causes of transplant failure, serves as a basis for the subsequent studies in this thesis which examines strategies in the identification, assessment and modulation of serious graft injury.

2.2 SUBJECTS

2.2.1 Patients

126 consecutive primary graft losses either requiring retransplantation or leading to death which occurred in liver transplant recipients between 1/1/90-31/12/93 were determined and examined. This study cohort was derived from the population of primary liver recipients who were transplanted in this centre since its inception as an independent transplant centre in 1988. All patients transplanted within the combined Cambridge-Kings College transplant programme were excluded from this analysis (data incomplete in a significant proportion).

2.2.2 Methods

The primary and predominant aetiological syndrome which contributed to the transplant failure was determined following a combined clinicopathological examination. Specifically, a review of the clinical events leading up to the graft loss and histopathological examination of the explanted graft (where retransplanted) or perimortem percutaneous biopsy specimens was performed. Post-mortem reports were available in the majority of deaths. All laboratory and imaging investigations were taken into account in allocating the major cause of failure.

2.3 RESULTS

2.3.1 Clinical description

The study population comprised 65 males and 62 females with a mean age of 36.8 ± 1.8 years (range 0.1 - 67 years). The aetiological categories of the primary transplant indications were PBC 19, PSC 8, HBV 11, HCV 6, non A non B (NANB) 9, biliary atresia 9, ALF 23, AIH 8, ALD 6, hepatic malignancy 4 (HCC 1, metastatic carcinoid 1, cholangiocarcinoma 1, haemangioendothelioma 1), cryptogenic cirrhosis 2, disorders with a co-existing hepatic malignancy 9 (cirrhosis and HCC [8], PSC and cholangiocarcinoma [1]), and miscellaneous causes (13).

From the 127 transplant failures, 100 resulted in patient death and 27 from retransplantation. The majority of these losses occurred within the first post-operative month with greater than 50% by the 60th post-operative day. Transplant failures beyond one year were rare in this cohort (figure 2.1).

On the basis of the clinical syndrome and accompanying pathological features of the graft (where appropriate), the antecedent disorder(s) which were directly related to the graft losses are summarised in table 2.1. Allograft rejection was the largest single aetiological category with significant variations in the contributions from the different rejection syndromes. Hyperacute rejection did not account for a single case of graft failure with the proportion of losses attributable to intractable acute cellular rejection also low at 4 cases only. All of these latter cases occurred in the first month post-transplant. In contrast, graft failure secondary to development of chronic allograft rejection was experienced by 26 (20%) patients. The mean post-transplant period for chronic rejection resulting in graft

loss was 251 days (range: 20-854 days). Technical complications resulted in transplant failure much earlier in the post-transplant phase with hepatic artery thrombosis / stenosis a common preceeding event to graft failure accounting for 18 (14%) cases (12 of whom died subsequently) at a median of 53 post-operative days (range:1-288 days). The other major technical complication present in this series was abdominal bleeding which led to death in 9 patients. Systemic or graft sepsis leading to death or retransplantation as a primary event was present in 16 patients, with this complication particularly common in those recipients transplanted for acute liver failure. Disease recurrence leading to death or regrafting was, in this present series, restricted to viral or malignant primary transplant indications. The other major causes of transplant failure arose unexpectedly in specific organ systems. Cardiovascular events (myocardial infarction 2, cardiac failure 3, pulmonary hypertension, pulmonary embolism and tamponade) led to death in 8 patients. Fatal cerebral complications comprised cerebral coning in 2 patients, cerebrovascular accident 2, intracerebral haemorrhage 3 (mycotic embolism 1), accounting for death in all 7 cases in the early post-operative phase. Gastrointestinal bleeding and bowel perforation was a comparatively rare cause of death (2 cases each). The development of any of these organ-system complications precluded retransplantation. Miscellaneous events which caused transplant failure included acute graft-versus-host-disease in 2 patients, non - thrombotic graft infarction (massive haemorrhagic necrosis) 2, pulmonary heamorrhage and Stevens-Johnson syndrome in 1 case each. De novo malignancy was a rare complication accounting for 2 deaths only (both post-transplant lymphoproliferative disorders).

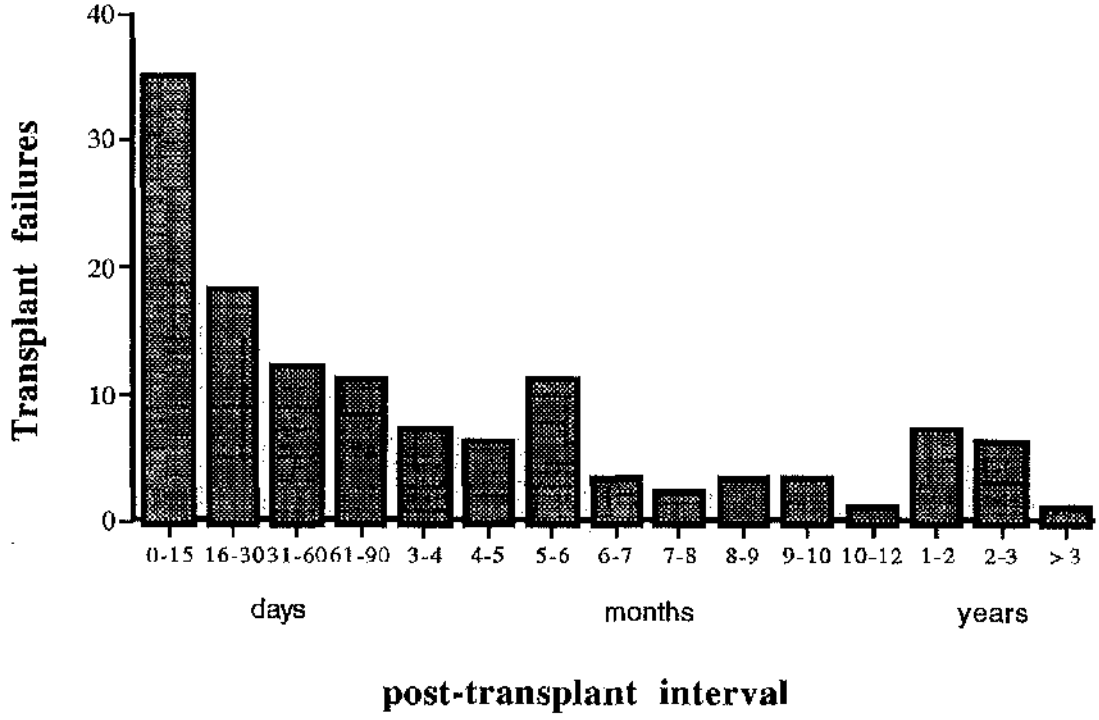


Figure 2.1 Timing of transplant failures. Majority occurring in the early post-operative period usually before the first hospital discharge.

Table 2.1 Clinical diagnoses leading to death or retransplantation (transplant failures) in 127 consecutive cases

Diagnoses	Transplant failure		
	Deaths	Retransplants	**Total**
Rejection	14	16	30
intractable	2	2	4
chronic	12	14	26
Primary graft non-function	8	3	11
Sepsis	13	3	16
Hepatic artery thrombosis/stenosis	15	3	18
Haemorrhage	9	0	9
Gastrointestinal	5	0	5
bleeding	2	–	2
perforation	2	–	2
torsion	1	–	1
HBV recurrence	4	1	5
Malignancy recurrence	8	0	8
hepatocellular carcinoma	3	–	3
cholangiocarcinoma	3	–	3
haemangioendothelioma	1	–	1
carcinoid	1	–	1
De novo malignancy	2	0	2
Cardiovascular	8	0	8
myocardial infarction	2	–	2
cardiac failure	3	–	3
pulmonary hypertension	1	–	1
tamponade	1	–	1
pulmonary embolism	1	–	1
Cerebral	7	0	7
cerebral oedema	2	–	2
cerebrovascular accident	2	–	2
intracerebral haemorrhage	3	–	3
Graft-versus-host disease	2	0	2
Massive haemorrhagic necrosis	1	1	2
Miscellaneous	4	0	4
Stevens-Johnson syndrome	1	–	1
pulmonary haemorrhage	1	–	1
de novo HCV	1	–	1
unclassified	1	–	1

2.3.2 Stratification of Transplant failure (Graft failure and death)

From the description of the underlying causes of transplant failures described above, a classification on the basis of the predominant underlying clinico-pathogenic mechanism can be proposed: This classification has 8 categories: 1) Pretransplant clinical status of the recipient n=14, 2) Primary graft dysfunction n=12, 3) Technical complications n=25, 4) Immunological n=34, 5) Disease recurrence n=13, 6) Immunosuppression-related n=12, 7) Primary organ system failure n=11 and 8) Miscellaneous n=6 (figure 2.2).

The general medical status of the recipient immediately prior to transplantation was considered to have predisposed directly to the graft loss in a significant proportion (11%) of recipients. This category essentially comprised 3 separate patient groups. Those in whom there was unrecognised cardiovascular or cerebral pathology (coronary artery disease 2, pulmonary hypertension 1, inadequate cardiac reserve 2, aortic dissection 1, cerebral oedema 2). A second group was made up of patients where there was unrecognised infection at the point of transplantation (typically patients undergoing emergency transplantation for ALF). A final group were characterised by malnutrition and general debility who typically had prolonged intensive care admissions terminating in death. The second category of primary graft dysfunction is a term which could be employed to encompass those recipients in whom impaired donor organ viability leads to either primary graft non-function (PGN) or early graft dysfunction and which proceeds eventually to graft failure. Technical graft failures is a third category which comprises those events directly attributable to the surgical procedure and would predominantly be made up of vascular events or post-operative haemorrhage. Immunological graft failure would be a term to encompass all forms of transplant failure attributable to rejection and graft-versus-host-disease processes. Recurrence of the primary transplant indication

remains a major cause of graft damage and loss and merits a separate category. Those primary events which are either initiated or aggravated significantly by immunosuppression and which would include de novo infection and malignancy and direct toxicity are categorised as immunosupression-related transplant failures. Events not directly attributable to either graft pathology or any of the other categories listed above and which primarily affect systemic organs could be classified under the umbrella term 'organ - system' transplant failure. Finally, there are causes of graft failure or death which cannot be readily placed in any of the above categories and require a final 'miscellaneous' grouping. The time following transplantation when these categories accounted for the transplant failures are shown in figure 2.3.

2.4 DISCUSSION

This investigation reports a large and modern series of consecutive liver transplant recipients in whom the primary transplant failed resulting in either death or regrafting. As such, it identifies and provides data on the contributing roles of the disparate pathological processes which can result in failure of a liver transplant. The transplant registries although providing accurate data on the influence of demographic variables on outcome are less valuable when determining the precise causes which lead to a transplant failing. In this regard, single-centre data and experience should be more accurate with documentation of all losses based on careful assessment of the clinico-pathological course.

From the data, it is clear that the early post-transplant period remains the most hazardous

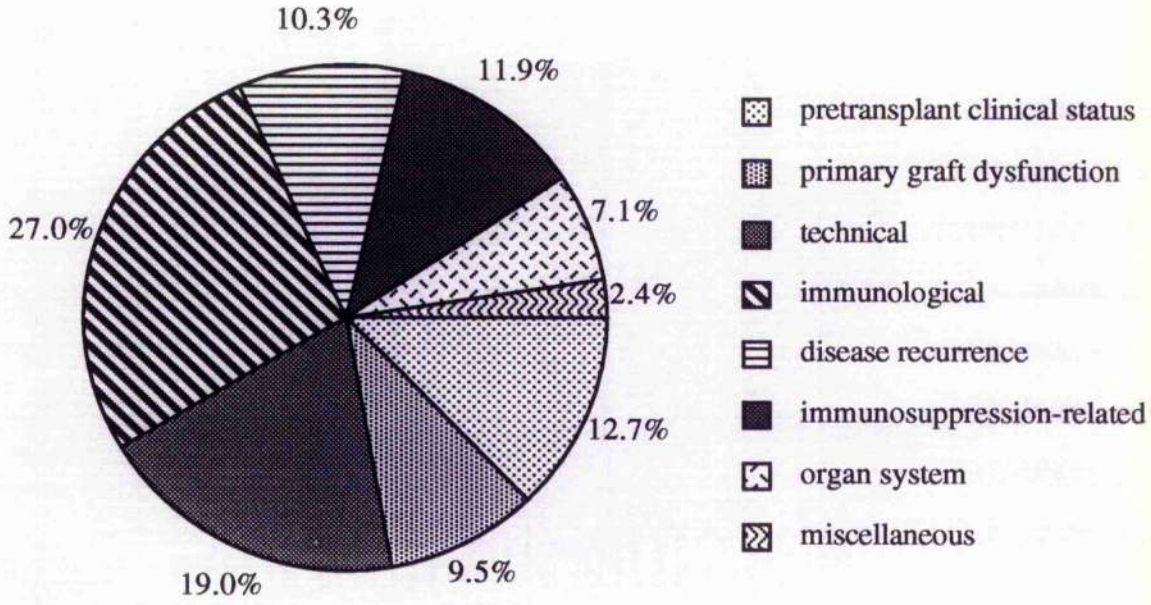


Figure 2.2. Proportions of the 8 clinico-pathological categories which are associated with transplant failure

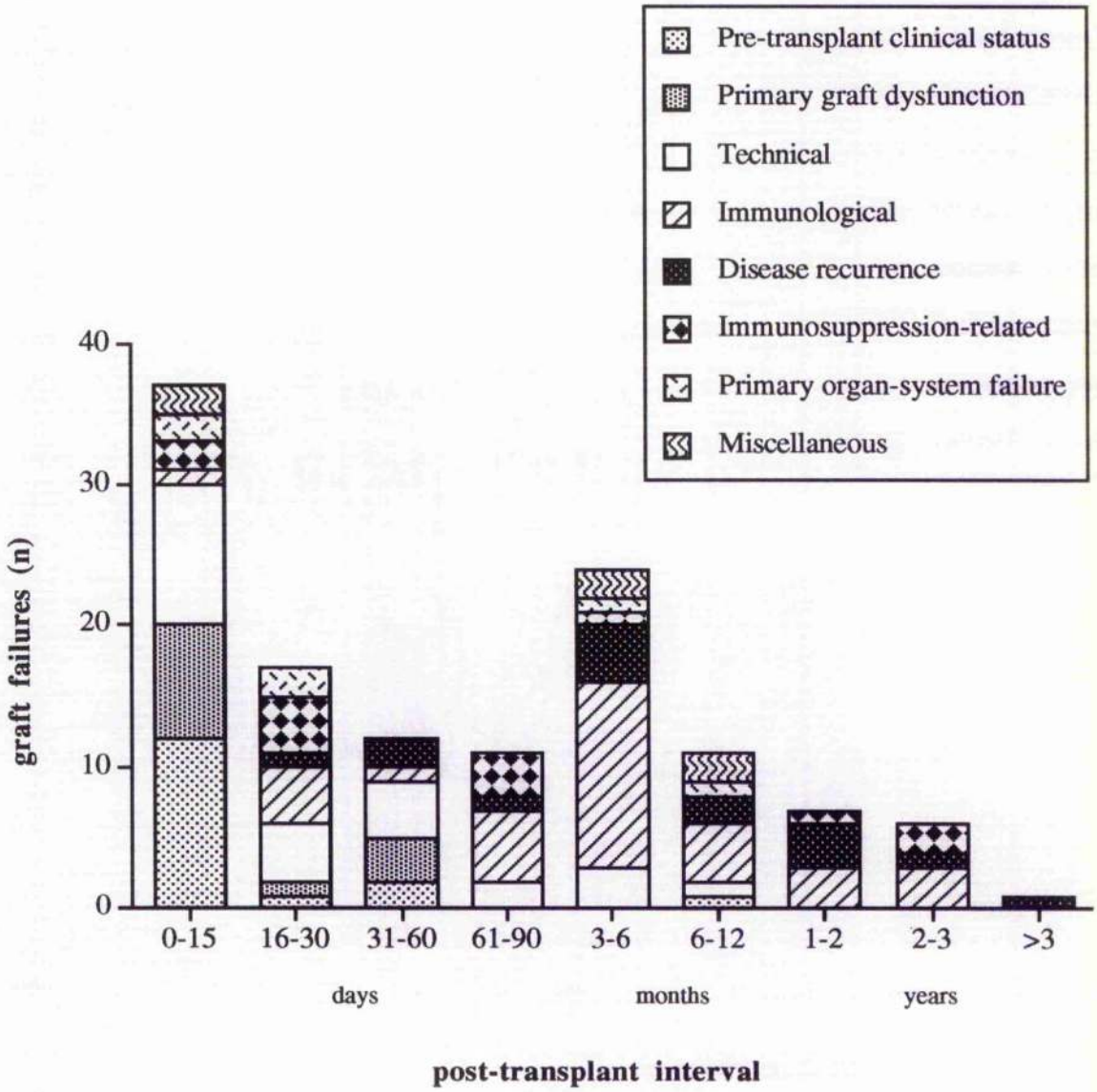


Figure 2.3 Relationship between the proposed categories and timing of the associated transplant failure.

with this phase being exposed especially to the major risks of either inadequate organ viability or technical complications. Primary graft dysfunction remains a difficult event to predict and thereby avoid. Clinical and histological evaluation of the donor liver is not sufficiently sensitive to exclude those organs which will not provide adequate function. Although several dynamic measurements of hepatic function prior to organ retrieval have been proposed as adjuncts in improving organ selection (see section 3.1), these have not been widely employed in current clinical practice. The increasing waiting lists and plateauing of donor numbers (see section 1.2) is enforcing increased use of what were previously considered inferior livers. Despite this expediency, the rates of this complication are presently not increasing in this unit. These results contrast distinctly from the previous report of allograft failures from the University of Pittsburgh. In that report (experience from 1984-1988), PGN accounted for 53 (30%) of 177 primary graft losses. Although these studies are not directly comparable (since the present investigation examined all transplant failures), PGN accounted for only 12 cases from the total of 126 transplant failures. In the Pitt-UNOS registry, primary graft failure accounted for 13.5% of deaths within 1 week of transplantation (Belle and Detre 1993). Technical complications resulting in transplant failure were in this series predominantly related to graft ischaemia secondary to hepatic artery thrombosis or post-operative bleeding and predictably were associated with transplant failure when occurring in the early post - transplant phase. Several of the patients in the above 2 groups ultimately died from a terminal infection episode which was considered secondary to the initial complication. Systemic or graft sepsis, as a primary cause of transplant failure (normally death), was also a common event in this period and most likely reflects the combined immunodeficiency which results from impaired liver function and introduction of high - dose induction pharmacological immunosuppression. Finally, organ failures (the majority of which were unexpected) arising from the cerebral, cardiovascular and gastrointestinal

systems led to a signficant proportion of early transplant failures. These complications caused mortality rather than primary graft failure with liver function usually intact at the time of death. In the later phases following transplantation, the major pathological event causing graft failure was rejection with smaller contributions from primary disease recurrence and complications secondary to immunosuppression. The sharp decline in transplant failures beyond the first 2-3 months is in keeping with the data from the transplant registries. They also show that chronic rejection (which was amenable to regrafting in 50% of cases in this series) accounts for the largest proportions of second transplants (Belle et Detre 1993).

No formal and standardised classfication scheme exists for stratifying liver transplant failures. In this study, an attempt to devise a scheme based around the recent experience of this unit has been performed. This 8-point classification takes into account all the clinico-pathological factors identified from this investigation which predisposed to ultimate transplant failure. The placement of a transplant failure in a specific category of this classification should be based on all available investigations and await the post - mortem findings where performed. Specifically, all 4 of the categories in the classification proposed by Byers-Shaw for post-transplant mortality have been included in this new scheme. i.e: a category reflecting the pre-transplant clinical status, surgical / technical complications, rejection (under immunological graft failure) and also a miscellaneous group. Of these, the pre-transplant clinical status is perhaps the most subjective category to place individual cases. The importance of the general medical status with respect to nutritional and functional status, age and the presence of specific conditions such as renal failure is well-recognised (Belle and Detre 1993, Cuervas-Mons et al 1986). If these or other parameters are considered to represent the predominant underlying cause of death or retransplantation, then this should be the preferred category. Allocating transplant failures

to this group is easier if investigations reveal specific pre-transplant pathology which led directly to death (e.g. presence of sepsis or cerebral oedema present immediately pretransplant causing death post-operatively). Technical events and immunological failure (rejection or graft-versus-host disease) are self-explanatory categories. In addition, 4 new categories are proposed which take into account the larger and more recent experience now available and the recognition of new post-transplant syndromes since the early report from Nebraska. A separate category of primary graft dysfunction is proposed which would include the spectrum of impaired initial hepatic function secondary to organ viability and storage. At one extreme this would result in PGN or in less severe cases an impairment in function which ultimately and directly leads to either a requirement for graft replacement or in death (the latter event often in the context of multi-organ failure and sepsis within the first 2 post-operative weeks). Diagnosing and placing transplant failures under disease recurrence should be comparatively simple for viral and malignant disorders but more problematic for immune or biliary disorders where such a diagnosis may be contentious. A new category for immunosuppression-related complications is justified to reflect the considerable morbidity and occasional mortality secondary to de novo infection episodes and late malignancies (especially post-transplant lymphoproliferative disorders and others) which are attributable to the pharmacological inhibition of natural immunity.

In this classification, no separate category for sepsis as a cause of transplant failure is included. Systemic infection episodes normally either reflect impaired graft function (and accordingly would be classified under an appropriate category) or, especially in the presence of normal liver function, are directly attributable to immunosuppression. Similarly, isolated sepsis of the graft which is a rare clinical finding in this unit's experience is restricted to either grafts which either are ischaemic or have biliary drainage disorders or finally affects recipients transplanted for acute liver failure (i.e reflects pre-

transplant clinical status). All of these predisposing pathologies can be placed in categories within the proposed classification. A separate category of transplant failures secondary to primary organ-system failures is proposed. In this series, many of these complications were attributable to disorders present at the time of transplantation and accordingly should be classified under pre-transplant clinical status (e.g. coronary artery disease leading to myocardial infarction). Certain transplant failures cannot be placed in the other proposed categories and are of sufficient number to justify this individual category. A final and potentially small category is proposed to place those patients who experience a transplant failure from miscellaneous aetiologies and which are not readily placed in those listed above. For example, the cases of non-thrombotic graft infarction have been placed in this category. This complication, which was a common cause of retransplantation in the Pittsburgh series (termed ischaemic graft injury), is characterised by initially adequate early graft function followed by severe deterioration without major vesssel insufficiency around the 4-10 post-transplant day. As such it appears not to represent an organ viability / preservation problem and it is also presently not possible to attribute the graft failure to rejection or possibly disease recurrence in those recipients transplanted for NANB hepatitis. We also placed a case of de novo HCV infection which progressed to graft failure in this final category.

As with any proposal for a classification with respect to clinicopathological events, a potential for overlap between the different categories exists e.g. a patient with chronic rejection is managed with increased immunosuppression and eventually dies of CMV pnuemonia. In such an instance the cause of the transplant failure would primarily be rejection (an immunological transplant failure). If programmes wish to record the aetiology of the terminal cause of death, then the same categories could be used for stratifying causes of mortality rather than as transplant failure (so in the above instance the

transplant failure would be immunological but the cause of immediate death would be immunosuppression-related).

The proposed terminology and classification should enhance clinical data management and communication as well as directing programmes to areas of clinical deficiencies. Investigative strategies and protocols to reduce liver transplant failures should standardise on nomenclature in the design of the study and subsequent reporting of results. New large multicentre trials of immunosuppressant agents where patient and graft survival are principal end-points should address the aetiological categories of transplant failure within a system such as the one proposed.

The categories devised in the present classification serve as a basis for the general areas examined in relation to assessment and modulation of post-transplant complications examined in this thesis. Assessment and management of primary graft dysfunction is examined in chapter 3 and 10 respectively. Rejection, which remains the major cause of late graft injury, is examined with respect to identifying a high risk category in chapter 6, a novel immune marker in chapter 5 and finally the potential value of the new immunosuppressant agent FK506 (tacrolimus) in controlling the process in chapter 8. The importance of pretransplant clinical status is investigated in a high-risk group of emergency recipients transplanted for ALF in chapter 7 and the potential for modulation of HBV-related disease recurrence in chapter 9. A subgroup of technical complications (vascular complications) are assessed in chapter 4. Finally, the potential for alleviating immunosuppression-related transplant failures is examined through the ultimate step of withdrawal of the pharmacological agents in chapter 11.

CHAPTER 3

RELATIONSHIP BETWEEN EARLY LIVER GRAFT VIABILITY AND ENZYME ACTIVITIES IN EFFLUENT PRESERVATION SOLUTION

3.1 BACKGROUND AND INTRODUCTION

Immediate and early graft function following liver transplantation is largely determined by the quality of the donor organ at retrieval and the secondary influences of hypothermic preservation, flush and reperfusion injury. These processes, as outlined in section 1.2, may also play a role in several post-operative complications including frequency of rejection, the development of vascular and biliary complications and overall graft survival. As was demonstrated in the previous chapter, graft failure secondary to deficient organ viability and storage injury is a major cause of retransplantation and mortality.

Attempts to predict graft viability based upon assessments of the donor liver prior to retrieval have the potential advantage of being clinically useful in the process of graft selection (Oellerich et al 1989, Yamaoka et al 1992). However, in clinical practice a reliable and readily performed parameter has not been identified, with donor organ assessment still based upon loosely defined clinical and, in some centres, histological criteria (D'Alessandro et al 1991). Markers of donor liver status prior to retrieval are also of limited and indeed diminishing value due to the current clinical requirements which are enforcing increased utilisation of previously considered inferior quality grafts. This practice reflects the widening gap between organ donation rates and recipient waiting-lists present in many programmes. Hence, markers of graft viability which reflect not only the

donor organ status but also the injuries sustained following procurement may be of more practical value. Accordingly, a measurement of graft injury performed at the end of the ischaemic period should provide, by incorporating most of the post-procurement phases of injury, a more sensitive guide to viability than if performed on the donor. Such a measurement, albeit performed once the transplant is irretrievably underway, could well serve as a useful clinical parameter in the prediction of early graft function and additionally could be employed to examine the effects of experimental strategies in donor management and new retrieval and preservation / flush protocols.

In this investigation, liver enzyme activities present in the organ preservation solution washed out from the graft at the end of ischaemic storage and prior to reperfusion were examined. It was postulated that release of enzymes into this fluid over the ischaemic period may reflect the functional integrity of the graft. Two standard laboratory indices of hepatocellular damage, aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were examined. These enzymes were chosen because of their different localisation in the hepatocyte (LDH - cytoplasmic predominantly, AST - mitochondrial and cytoplasmic). Additionally, because of evidence indicating the importance of microvascular integrity in early graft function and the particular damage to sinusoidal lining cells (SLC) during hypothermic preservation, two recently proposed markers of sinusoidal cell damage, (purine nucleoside phosphorylase (PNP) and an isoenzyme of creatine kinase (CK-BB) were also evaluated (Rao et al 1990, Vaubourdolle et al 1991). PNP is reported to be primarily localised in the cytosol of hepatic endothelial and Kupffer cells and is postulated to represent a good index of oxidative injury to the liver particularly following reperfusion.

3.2 SUBJECTS

3.2.1 Patients and samples

Effluent preservation fluid was collected from 53 randomly selected (34 consecutive) primary elective adult liver recipients following gravity flushing of the graft, with 0.15M NaCl at room temperature, after completion of the suprahepatic inferior vena cava (IVC) anastomosis and immediately before that of the portal vein. Samples were collected at 1 minute intervals from the infrahepatic IVC over a five minute period into plain tubes and centrifuged (2000g for 10 minutes) to remove cell debris and stored at -20°C prior to assay. Donor organ retrieval was performed in the standard manner with UW solution at 4°C used for organ cooling and preservation. Immunosuppression in the study population was standardised to a conventional CyA based triple therapy regimen. All transplants were ABO isocompatible. Recipients in whom the pretransplant clinical status is recognised as significantly influencing early post-operative outcome were excluded (emergency transplantation for acute liver failure, presence of significant preoperative renal failure or retransplantation). Two patients in whom post-operative technical complications were considered to have significantly influenced early graft function were also excluded (HAT 1, hepatic artery stenosis (HAS) 1).

3.2.2 Biochemical measurements

Total AST and LDH activities were assayed using the automated Spotchem SP-4410 analyzer (BioMen Ltd., Croydon, UK). PNP activities were determined as previously described (Hoffee et al 1978). Total CK was determined using the optimised standard method recommended by the Deutsche Gesellschaft fur Klinische Chemie performed on a Cobas Bio analyzer (Roche Products, Welwyn Garden City, UK) at 30°C. CK

isoenzymes were separated by agarose electrophoresis using the Helena REP automated electrophoresis system (Helena Laboratories, Gateshead, UK). The resulting bands were reacted with the substrate creatine phosphate and in a coupled series of reactions a change from NAD to NADH occurs which can be scanned densitometrically under UV light to obtain a percentage for CK-MM, CK-MB and CK-BB. The CK-BB activity was then calculated from the total CK. Enzyme activities in the effluent fluid are presented as area - under-the-curve [AUC] totals derived from the 5 serial one minute samples.

3.2.3 Assessment of early graft viability

Early graft viability was assessed by measurement of standard laboratory indices of liver function for the first 5 days following transplantation (daily serum AST and total bilirubin, prothrombin time: international normalised ratio [INR]) and graft survival at one month post-transplant. Correlation coefficients were calculated to examine what relationships existed between activities of the different effluent enzymes and whether these activities were related to the post-transplant serum liver function tests and / or the presence of impaired graft function. This latter condition was defined as being present in patients in whom the serum AUC AST totals during post-operative days 1-3 exceeded the population mean activity plus one standard deviation. Enzyme activities were also examined in relation to the period of graft cold ischaemia in addition to the circulating platelet count (AUC totals of post-transplant days 1-5) and frequency of rejection in the first month. The combined cold and rewarming (poikilothermic) ischaemic time was defined as the interval from donor aortic cross-clamp until recipient portal vein unclamping.

Results are expressed as mean ± standard error mean (SEM). Differences were calculated for statistical significance by the t-test or non-parametric (Mann Whitney U) test where appropriate. P values less than 0.05 were considered to be statistically significant.

3.3 RESULTS

3.3.1 Effluent preservation fluid enzyme activities

The first sample retrieved during initial graft rinsing contained between 79-94% of the total content for all four enzymes measured. Insignificant amounts of the enzymes were present in the wash-out fluid collected from the later 3 - 5 minute sample time-points. The mean (SE) [range] AUC activities of the 2 markers of hepatocellular damage (AST 1440 (295) [35-10800] IU/L and LDH 4540 (739) [500-31700] IU/L) in the five samples were considerably more elevated than the two enzyme markers of sinusoidal cell damage (PNP 119 (30) [0-521] IU/L and CK-BB 53 (18) [0-473] IU/L). Large variations in enzyme activities between patients were present and are reflected in the standard errors and ranges. The ratio between the AST and LDH activities in washout fluid was similar to the ratio of reference ranges in serum (~ 1:4). The CK-BB assay, as performed in this study, was unable to detect this isoenzyme in a significant proportion of patients (n=9, 18%) with other samples having activities at the lower end of the assay sensitivity. The PNP assay did not detect any significant activities in the wash-out fluid of 5 patients.

Correlations between the effluent enzyme activities were examined. Strong correlates existed between the two hepatocellular enzymes ([$AST_{effluent\ (e)}$] vs [LDH_e] r=0.75, p<0.001). Unexpectedly, similarly strong relationships were also found between these 2 enzymes and PNP activities (where detectable): [AST_e] vs [PNP_e] r=0.906, p<0.001 and [LDH_e] vs [PNP_e] r=0.833, p<0.001. No correlation between AST_e or LDH_e and CK-BB_e was present and unexpectedly, no relationship between the 2 proposed markers of sinusoidal cell injury could be demonstrated ([PNP_e] vs [CK-BB_e] r=0.16, ns).

3.3.2 Relationship between effluent enzyme activities and early graft viability

In those transplants where graft loss occurred within the first post-operative month (n=10), analysis of the effluent fluid enzyme activities demonstrated that the median AST_e concentration was significantly elevated in these recipients compared to those in whom the graft survived (1840 vs 695 IU/l, $p<0.05$) (table 3.1). Median LDH_e and PNP_e activities were also higher in graft non-survivors but did not achieve statistical significance. With respect to patients where there was a significant impairment of early graft function (see definition above) (n=9), none of the enzyme activities in the effluent fluid were significantly elevated compared to those recipients with non-impaired graft function.

A statistical correlation was found between the effluent AST_e activity and the post-operative $AST_{serum\ (s)}$ activity ($r=0.32$, $p<0.05$). The post-operative AST_s activity was significantly more related to the AST_e activity than the other measured effluent enzymes (LDH r=0.26, PNP r=0.18, CK-BB r=0.04).

When the effluent AST_e and LDH_e activities were examined in quartiles, relationships with graft outcome and ischaemia were demonstrated (table 3.2). Those patients in the lowest effluent fluid AST_e quartile had the shortest period of cold and rewarming ischaemia and experienced only one graft loss (92% one month graft survival). Whereas those patients in the highest AST_e quartile group also had the highest AST_s activities and a significantly inferior early graft survival (62% one month graft survival, $p<0.05$). No relationship between effluent AST_e activities and the prothrombin time / INR was present. With respect to effluent fluid LDH_e activities, a similar relationship between increased activities and prolonged ischaemia and high post-operative AST_s activities was noted. Those patients in the higher effluent AST quartile groups had a progressive tendency to increased rejection frequency in the first month post-transplant (group 1, 4 of 13 (31%),

group 2, 4 of 13 (31%). group 3, 7 of 13 (54%) and group 4, 8 of 12 (67%). No relationship between activities of the four enzymes in the effluent fluid and early post - operative platelet count was shown. For example in relation to the AST quartile groups, no significant difference in the median AUC platelet count of the day 1-3 totals between the quartiles was shown (189, 173, 90 and 192 x10^9/L in groups 1-4 respectively).

3.4 DISCUSSION

Determination of changes in the biochemical composition of the solution preserving a donor organ is an obvious but neglected source of information regarding the processes which occur during hypothermic storage. In this study, significant release of several liver derived enzymes into this fluid was demonstrated. Moreover, the activity of AST was found to be a discriminative marker for post-operative graft viability providing further evidence for the importance of donor organ quality and preservation in determining early liver graft function. That an enzyme released from hepatocytes was valuable is notable given the general belief that these cells are spared the effects of preservation injury as compared to non-parenchymal ones (Blankensteijn et al 1991, Clavein et al 1992). Whether other measurements performed in effluent preservation fluid, such as determination of lactate and ammonia levels which have been shown to be useful discriminants of allograft viability in an animal model would be of superior value clinically to the liver-derived enzyme is unknown (Shimada et al 1993).

With respect to the two different hepatocellular enzymes, the activities of effluent AST were more closely related to post-transplant graft function than those of LDH. This result is consistent with a previous study performed in an isolated rodent liver perfusion model which similarly demonstrated the superiority of AST activities in the circulating perfusate -

Table 3.1. Enzyme activities in the effluent preservation fluid collected from recipients with severely impaired graft function within the first 5 days following transplantation and in those who experienced graft loss by 1 month.

	GRAFT FUNCTION			GRAFT SURVIVAL		
	normal (n=42)	impaired (n=9)	p value	survivors (n=41)	non-survivors (n=10)	p value
Effluent*						
hepatocellular						
AST	866	1300	0.13	695	1840	<0.05
LDH	3122	3670	0.2	2730	3150	0.2
sinusoidal						
PNP	69	93	ns	64	89	ns
CK-BB	24	34	ns	35	46	ns
Serum**						
AST	746	2800	<0.05	1200	2300	<0.05
INR	3.8	5.6	0.1	4	3.9	ns

*area-under-curve totals, **area-under-curve totals post-transplant day 1-3

Table 3.2 Effluent fluid enzyme activities presented in quartile groups in relation to graft function and survival.

Effluent Enzyme Activities		Graft Outcome			
		Graft survival (1 month)	AST* (IU/L)	bilirubin** (umol)	INR*
Effluent AST (IU/L) Quartile: mean [range]					
125 [30-175]	(n=13)	12 (92%)	788	538	4.9
440 [188-866]	(n=13)	12 (92%)	624	628	4.1
1267 [867-1564]	(n=13)	11 (85%)	1130	912	3.8
2817 [1654-10820]	(n=12)	7 (58%)	1340	901	3.6
Effluent LDH Quartile					
855 [500-1322]	(n=13)	13 (100%)	695	507	3.9
2271 [1444-3145]	(n=13)	9 (69%)	788	584	4.6
4707 [3309-6331]	(n=13)	11 (85%)	764	938	3.9
10650 [6866-31762]	(n=12)	9 (75%)	2060	853	4.1

* median area-under-the-curve post-transplant day 1-3 totals
** median area-under-the-curve post-transplant day 1-5 totals

- as a discriminative test of liver viability compared to LDH (Iu et al 1987). Whether the cellular localisation of AST, which is located in both the cytoplasmic and mitochondrial compartments, in contrast to LDH which is cytoplasmic only, explains the superior predictive value is speculative. However, this may be an important difference in view of the previous demonstration that the sensitive test of hepatocyte mitochondrial function (arterial ketone body ratios) is of value in discriminating donor organ viability prior to retrieval (Yamaoka et al 1992). Moreover, a close relationship between loss of mitochondrial function and hypothermic preservation was found in a rodent model (Kim et al 1992). Further studies evaluating the mitochondrial fraction of total AST or of other mitochondrial markers in effluent UW fluid may be valuable. The closest relationship between AST activities in this fluid was with serum activities of this enzyme and marginally less with serum bilirubin. The absence of a relationship with prothrombin time probably reflects the variable loss and administration of coagulation factors peri - operatively.

Several previous studies have demonstrated the functional importance and particular susceptibility of sinusoidal lining cells (SLC) to hypothermic preservation and reperfusion injury (for reviews see Blankensteijn et al 1991, Clavein et al 1992, Caldwell-Kenkel et al 1989, Currin et al 1990). It was therefore surprising to find no clear relationship between early post-operative graft function and the markers of SLC damage evaluated. These results do not necessarily contradict the previous findings referred to as technical limitations for those markers exist. The close correlation which was found between PNP activities and the hepatocellular enzymes would suggest that this enzyme may be also released from hepatocytes and not just from sinusoidal and Kupffer cells as had originally been suggested (Rao et al 1990). Indeed, more recent reports have also suggested that PNP is not a specific marker of sinusoidal cell function with variable release from both parenchymal and non-parenchymal cells (Mochida et al 1992, Brass and Mody 1995).

Activities of CK-BB also turned out to be a disappointing method of assessment of SLC damage with activities often being undetectable. Whether levels of hyaluronic acid would be a better index of sinusoidal damage or early graft viability as has been shown in a study of small bowel preservation is unknown (Mueller et al 1993). Since SLC are especially sensitive to reperfusion injury, it is perhaps not surprising that markers of this cell damage were low in the particular samples chosen to study which were collected prior to reperfusion.

Since enzyme activities present in the liver preservation fluid were evaluated at a single time point, it is difficult to draw conclusions as to the contributions to the total measured amounts of enzymes from the various processes which could have caused cellular release following organ procurement. To determine the influences of graft flushing, hypothermic preservation and rinsing, an examination of serially collected preservation fluid samples would be required. Such a study could not be performed clinically except in the context of redundant or rejected donor livers. From the present data, although a relationship between enzyme activities and the period until portal vein unclamping (i.e cold and rewarming ischaemic periods) was demonstrated, it is clear this is not the only variable to account for the significant differences in activities noted between patients. Graft quality at retrieval is probably the most significant variable in determining susceptibility to storage and of subsequent enzyme release into the preservation solution. Another potential source of enzyme release into the preservation fluid is the effect of the initial in-situ flushing of the liver during retrieval and of course the rinsing at the end of preservation the efflux of which was collected in this study. Suggestions that UW fluid used for initial flushing is superior to other solutions has presently not been substantiated and it remains unclear whether variations in this protocol would play a major role in enzyme release over the preservation period (Adam et al 1991, Morgan et al 1990). Similarly, various physical characteristics of the graft rinsing solution may be important in determining early organ

viability through effects on mitochondrial function (Takei et al 1991). Saline rinsing, as performed in this study, has significant limitations with this solution having no oncotic or antioxidant properties. Carolina rinse may have advantages as a flush solution (Currin et al 1990, Sanchez-Urdazpal et al 1993). A significant limitation to the determination of effluent fluid composition is that no information on the impact of isolated cold or in particular warm ischaemia can be assessed, with the latter period increasingly recognised as playing a significant role in early graft function (Dunne et al 1994)

CHAPTER 4

ASSESSMENT OF VASCULAR COMPLICATIONS BY ANGIOGRAPHY AND RELATIONSHIP TO GRAFT DYSFUNCTION AND SURVIVAL

4.1 BACKGROUND AND INTRODUCTION

From the data presented in chapter 2 and other recent reports, one of the major persisting causes of graft loss is an arteriopathic process either involving major vessels or the microvasculature of the liver graft. In addition to the lesions caused by HAT or HAS, vascular injury has been demonstrated in such serious graft complications as EGD and both acute and chronic allograft rejection. Devascularisation of a liver graft in the early post-operative period usually results in catastrophic graft dysfunction progressing rapidly to death unless surgical or thrombolytic recanalisation or preferably retransplantation is performed. Similar but normally less severe graft dysfunction results from segmental HAT or anastomotic stenosis. Vascular insufficiency from a major vascular event beyond the first few months post-transplant may be tolerated following development of collateral circulation, but complications such as non-anastomotic biliary strictures and graft abscesses can develop.

EGD is an umbrella term used in the present investigation to describe serious liver dysfunction occurring within the first seven days after transplantation and includes both PGN and graft function which deteriorates rapidly after an initial period of stability. This latter condition may result from identifiable clinical complications such as severe acute

cellular rejection, HAT or ABO incompatibility but in many instances no clearcut precipitating cause can be identified, although graft ischaemia, systemic sepsis, preservation / reperfusion injury, viral disease recurrence and unrecognised rejection have been suggested as possible aetiological factors (Carithers et al 1988, Fagan et al 1992, Hubscher et al 1989).

The vascular component to liver allograft rejection is underestimated. Histological assessment of a percutaneous biopsy specimen from a rejecting graft, in which few arterioles will be present, often does not recognise the arterial-directed element of the rejection process. Terms derived by histopathologists such as 'vanishing-bile-duct - syndrome' which infer damage to the biliary epithelial cells alone to describe chronic liver rejection reflect this limitation (Vierling and Fennel 1985). In acute cellular rejection, an arteritis is a poor prognostic sign but rarely recognised (Snover et al 1987). Similarly in chronic rejection, the presence of an obliterative arteriopathy, which is the hallmark of other vascularized solid organs undergoing chronic rejection, may go unnoticed in rejecting livers. This histological phenomenon normally indicates an irreversible process in heart, lung and renal allografts.

The diagnosis and significance of the vascular pathologies outlined above have not been systematically evaluated in a single study. In this investigation, a retrospective analysis of the findings using hepatic angiography, which is presently the optimal tool for detecting vascular pathology was performed. This study attempted to define the role of arteriopathic processes and the value of angiography in monitoring and assessing graft dysfunction in a series of 50 consecutive patients undergoing this examination.

4.2 SUBJECTS

4.2.1 Clinical groups

Fifty (50) consecutive patients undergoing hepatic angiography following orthotopic liver transplantation in the Kings College Hospital and Cambridge transplant programmes between July 1987 and March 1991 completed the study, this population representing 9.7% of the total transplant activity of four hundred and sixty four (464) patients over this period. All donor livers during this period were harvested in the UW preservation fluid and only two patients received a graft from an ABO-incompatible donor. The study population had serial liver function tests performed and most underwent assessment of the hepatic arterial tree with duplex doppler ultrasonography prior to angiography.

The patients could on the basis of the clinical syndrome be divided into the following three groups: *Group A* comprised eighteen patients with a clinical diagnosis of EGD — 11 patients with PGN and 7 with delayed graft dysfunction. In each patient the peak serum AST activity exceeded 2000 IU/l and the prothrombin time was greater than three times the control within the first seven days following transplantation. *Group B* were made up of 16 patients with clinically suspected hepatic artery obstruction, either HAT or HAS who presented with either biliary leakage / stricture n=5 (median time 50 days) or relapsing bacteraemia / hepatic abscess(es) n=6 (median time 72 days) or deteriorating graft function with a liver biopsy suggestive of ischaemic damage n=5 (median time 40 days). *Group C* had 16 patients in whom histological features of chronic allograft rejection as evidenced by an absence of interlobular or small septal ducts in 50% or more of portal tracts with or without an associated foam cell arteriopathy in addition to biochemical evidence of deteriorating graft function were present (Portmann et al 1988).

4.2.2 Angiographic investigations

Arterial access for angiography was via the transfemoral route, an abdominal flush aortogram was performed initially in all patients followed by selective injections of the hepatic artery when an abnormality was suspected. Indirect portography by superior mesenteric artery injection was carried out to evaluate the portal vein if this failed to opacify in the flush angiogram. Imaging was performed by digital subtraction angiography (General Electric) and the radiographs were reviewed at a joint clinico - radiological conference (JK - see acknowledgements).

4.3 RESULTS

Group A: Angiograms in this group with EGD were performed at a median time of three days (range 1-7 days). In nine of the 18 patients the angiograms were normal. Of the nine with abnormal angiograms anastomotic HAS and HAT were seen in two patients and one patient respectively. In the remaining six patients with early graft dysfunction in this group angiography demonstrated a characteristic appearance of delayed filling of the donor hepatic artery in association with narrowing of the main recipient and donor arterial vessel and pruning of the peripheral intra-hepatic arterial tree. There were no apparent significant differences in the biochemical profile of those with and without abnormal angiographic appearances — normal angiogram mean AST 2186 IU/L, INR 3.6, arterial obstruction mean AST 2574 IU/L, INR 4.2 and arteriopathic group mean AST 2668 IU/L, INR 3.6. All patients in this group with the exception of the three patients with arterial obstruction were treated empirically for acute cellular rejection with supplemental steroids (hydrocortisone 1gram b.d. for three days, 1 gram o.d. for a further two days and if there was no response following this regimen, methylprednisolone 1gram/day for

three days was added). A satisfactory response to anti-rejection therapy was accepted on the basis of a reduction of the AST level to below 100 IU/l and a prothrombin time not greater than three seconds of the normal control. This response was obtained in six (66.7%) of the nine patients with normal angiograms and two (33.3%) of six patients with diffuse arterial changes. Retransplantation was achieved in only one of the patients with evidence of a diffuse arteriopathy and the longer-term graft and patient survival in the sub-group with angiographically determined changes was 33% as compared to 78% in the nine patients with normal angiograms. Histological evaluation of liver biopsies, obtained either at retransplantation or early postmortem in 12 patients, revealed a common pattern within the group of a severe coagulative necrosis often with a mixed but predominately polymorphonuclear (PMN) cell infiltrate. In those with arteriopathic changes there was evidence in three of the five available biopsies of acute cellular rejection in addition to severe graft infarction. Those patients (n=5) with a dense PMN cell infiltrate had clinical evidence of severe systemic sepsis.

Group B In this group with clinically suspected HAS or HAT the angiograms were performed at a median time of 4 weeks (range 17 days to 14 months) after transplantation. Either thrombosis, n=10, (figure 4.1) or stenosis, n=6, (figure 4.2) was confirmed in all cases at angiography. Screening duplex doppler ultrasonography performed in all patients had suggested the presence of arterial blockage or narrowing in only 10 of the 16 patients. All the stenotic lesions were present at the site of anastomosis of donor and recipient artery. Patients with confirmed thrombosis were listed for retransplantation, 6 of the 10 patients undergoing the procedure, and those with clinically significant stenosis underwent balloon angioplasty. There was a marked disparity in prognosis within this group with graft and patient survival being only 10% and 30% in the HAT group respectively compared to 100% survival for the HAS group.

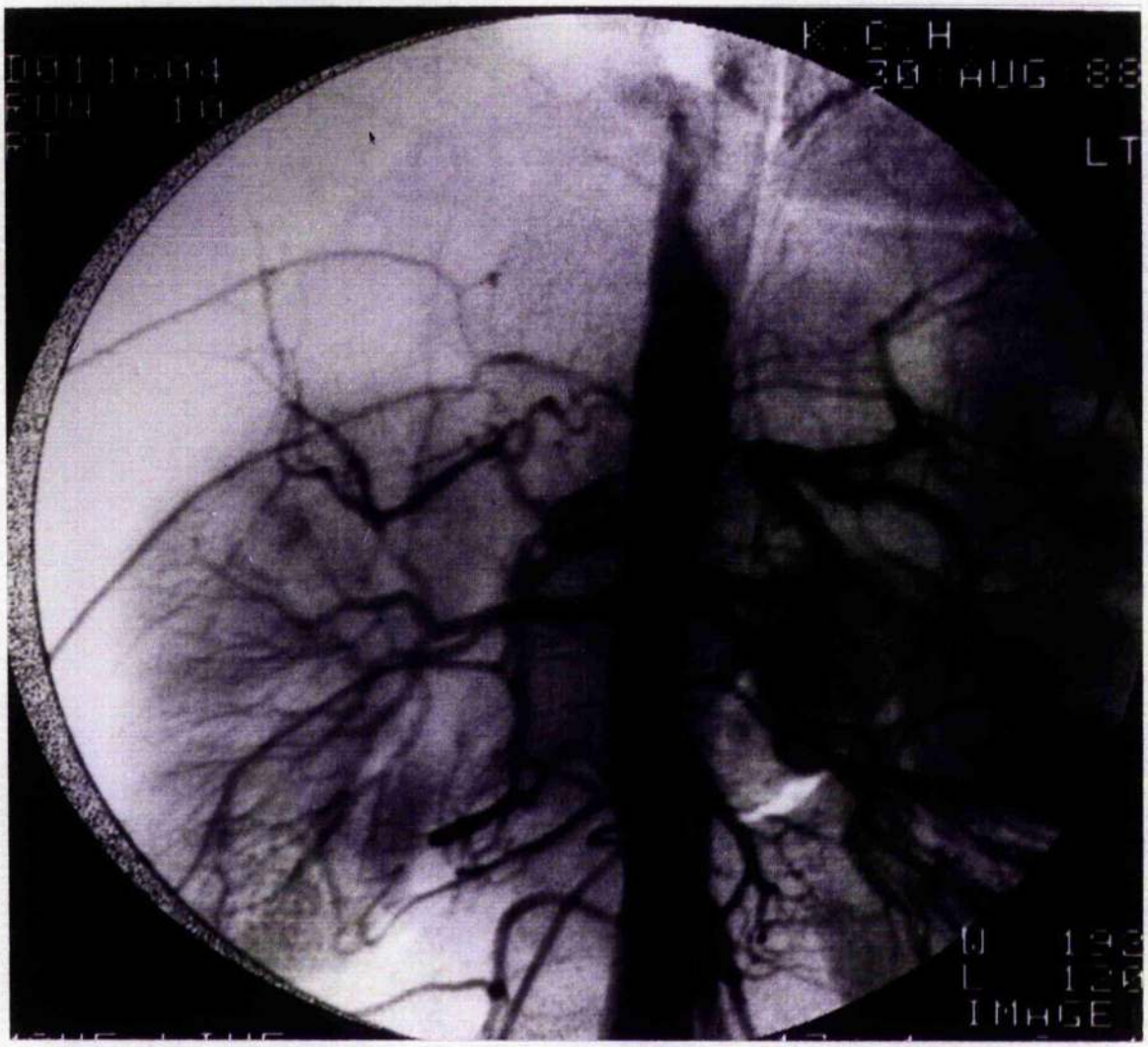


Figure 4.1 Hepatic artery thrombosis. Absent filling of the hepatic artery demonstrated on selective angiography.

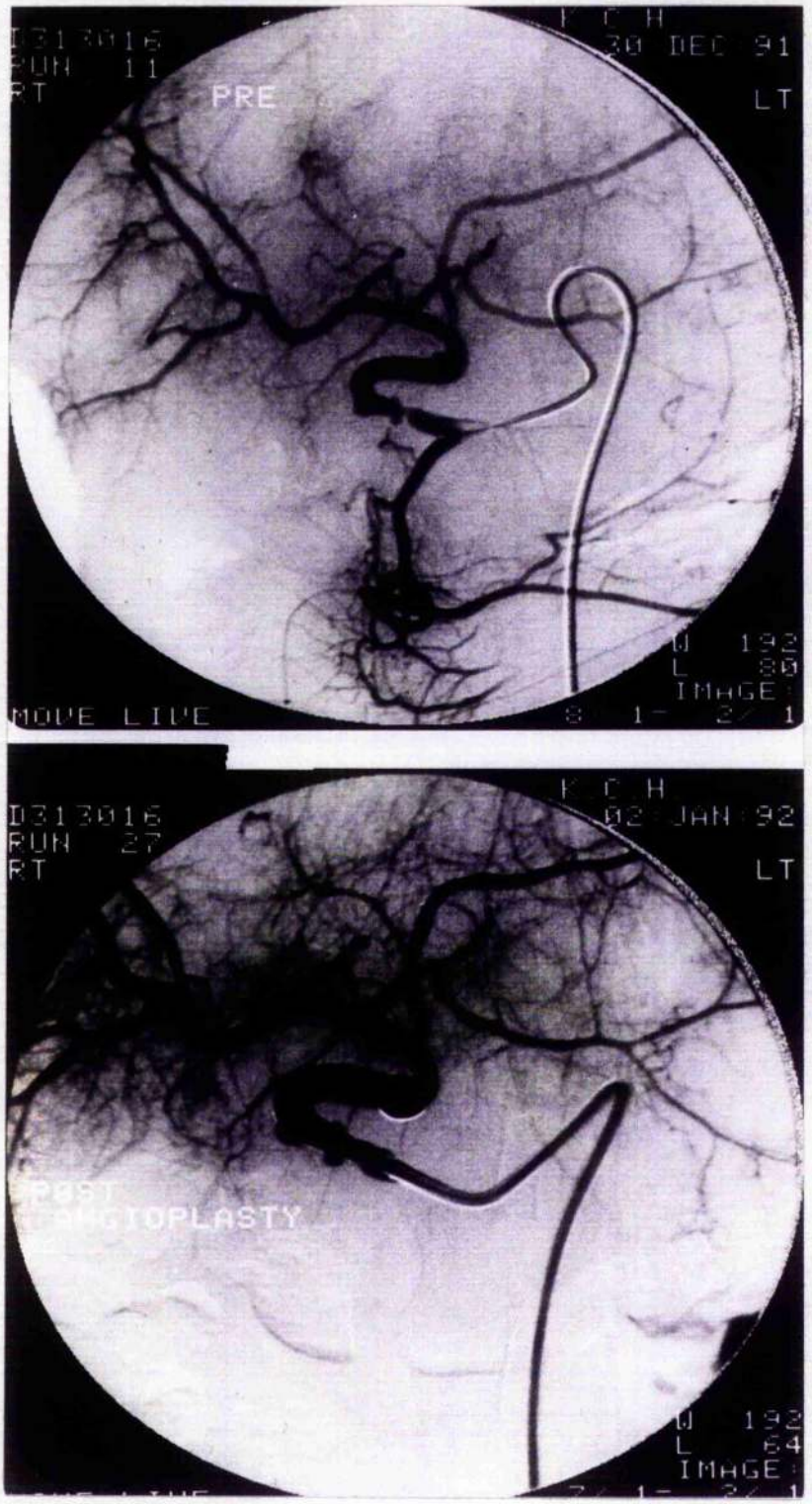


Figure 4.2 ***Hepatic artery stenosis. Significant anastomotic arterial stenosis demonstrated on selective hepatic arteriography (above) and relieved by percutaneous angioplasty (below).***

Group C The sixteen patients with chronic rejection in this group had angiograms performed at a median time of 11 months following transplantation (range three months to three years). Patients in this group were observed for a mean follow-up period of three months following angiography, with regular clinical and histological review to detect any reversibility of the rejection process, prior to being listed for retransplantation. All patients in the group received a conventional maintenance triple therapy immunosuppression regimen of prednisolone, azathioprine and CyA. These latter levels were maintained at 100-120 µg/l (*monoclonal RIA, Incstar)* for the first six months following transplantation. In the presence of histologically confirmed chronic rejection levels were maintained at 120 - 180 µg/l. Two patients later in the series were switched to tacrolimus and low dose prednisolone as salvage therapy following clinical deterioration. A pattern of vascular abnormalities ranging from peripheral pruning of the intra-hepatic vasculature with diminished perfusion (figure 4.3A) to a constrictive sclerosing pattern of the intra and extrahepatic arterial tree with a beading appearance (figure 4.3B) was noted in 10 of the 16 patients. There was evidence of a foam cell arteriopathy on standard histological criteria in only three of the group, all of whom also demonstrated arterial lesions at angiography. Graft survival in patients with radiographic changes was very poor with only one graft in 10 surviving, this latter patient showing a successful 'rescue / salvage' with tacrolimus. Two patients of the six with no evidence of arteriopathy either histologically or at angiographically spontaneously reversed the histological pattern of chronic rejection and now have normal graft function.

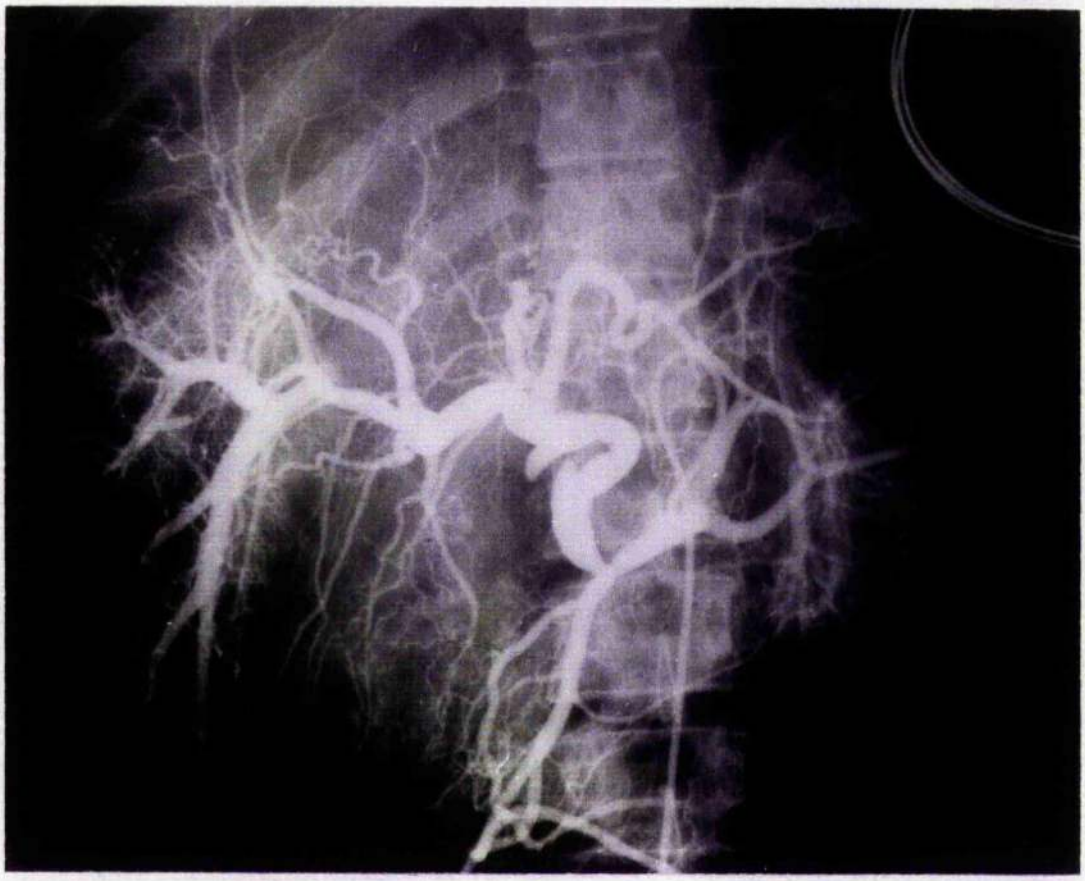


Figure 4.3A Development of angiographic evidence of rejection arteriopathy in a patient over a four week time interval. Normal hepatic angiogram present at this stage.

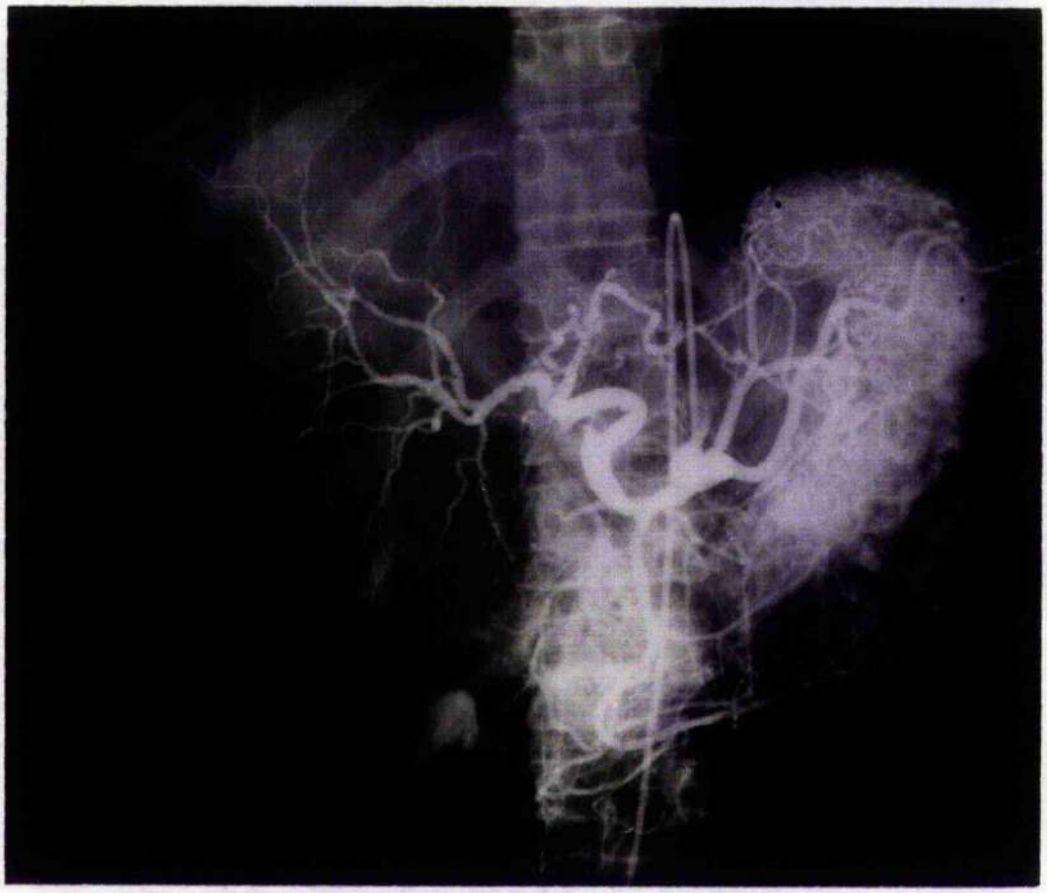


Figure 4.3B Chronic allograft rejection. Severe constrictive and beading appearances in the hepatic vasculature of the same patient who now has established chronic rejection of the allograft which developed over a 4 week interval.

4.4 DISCUSSION

The findings using angiography in the present series show clearly the value of this investigation in the assessment and management of patients after transplantation as well as in the prediction of outcome. In patients suffering from EGD, 33% of the patients had angiographic evidence of an arteriopathic process involving both the recipient and the donor vasculature and this pattern was associated with reduced graft survival compared to those patients with a normal angiogram. The involvement of both the donor and recipient vasculature is fascinating and suggests a non-discriminant vasoactive process rather than one determined by tissue characteristics alone. EGD remains a poorly understood phenomenon. The descriptive pathological term, massive haemorrhagic necrosis, has been used in one centre to describe the histological picture of the allograft present in this condition (Hubscher et al 1989). In this programme, the term non-thrombotic graft infarction to emphasise the clinical and histological features of graft infarction in the absence of macrovascular hepatic arterial thrombosis is preferred. The pathological appearances of severe haemorrhagic necrosis in the absence of major vessel obstruction, in conjunction with our angiographic evidence, leads to the hypothesis that microcirculatory failure is the common pathogenic pathway of different aetiological processes in this devastating complication. Acute cellular rejection has been considered by other groups to be the cause of early graft dysfunction and in our group there was supportive evidence of cellular rejection in 60% of patients as shown by histological examination of the graft at retransplantation or autopsy. In the 40% of patients with no evidence of acute rejection a common factor of severe systemic sepsis appeared to be the precipitating factor of the early graft dysfunction. In practical terms, from this data the hepatic arteriogram is the investigation of choice in this clinical situation and urgent

retransplantation should be considered when an abnormal study is demonstrated.

A reduction in total hepatic arterial flow was first observed in an experimental animal model of unmodified acute cellular rejection and confirmed recently in human transplantation using implanted doppler probes (Groth et al 1968, Payen et al 1989). Furthermore in a previous angiogram study, in which acute cellular rejection was histologically confirmed in the majority of cases, angiographic appearances were demonstrated similar to those above in 50% of patients (White et al 1987). The hepatic endothelial cell, which demonstrates increased expression of the class II major histocompatibility complex (MHC) antigens and may act as an antigen-presenting cell during rejection, is a probable target in the rejection process (Adams et al 1989). Indeed the presence of an arteritis on pathological assessment of liver biopsies, found in 2-5% of patients with other histological criteria of acute rejection, appears to be a marker for particularly severe acute rejection as described previously (Snover et al 1987). Administration of supplemental anti-rejection therapy in the group of EGD with arteriopathic changes, to treat presumptive acute cellular rejection, appeared to confer no benefit in either graft or patient survival. This suggests that the vascular pattern demonstrated on angiography represents either particularly severe, steroid resistant acute cellular rejection which, once established, is no longer reversible by controlling the rejection process or that this pattern is non-specific to rejection.

The need for angiography in the detection of large arterial vessel compromise was shown by the findings in the second group of patients with suspected arterial thrombosis or anastomotic stenosis. Although duplex doppler ultrasonography may be used as a screening investigation it's sensitivity was only 60% as compared to angiography. It may also give false-positive signals in the presence of collaterals and in the paediatric population (Kubota et al 1990). These results confirm the poor graft and patient survival

in established HAT. Early recourse to angiography when this complication is suspected, even in the presence of a normal doppler ultrasound, is essential to obtain a definitive diagnosis and to expedite retransplantation where possible.

The results in patients with chronic allograft rejection demonstrate the superior sensitivity of angiography to routine histopathology in the detection of hepatic arterial lesions and suggest that the presence of arteriopathy may be an important determinant in graft survival. Chronic rejection arteriopathy primarily involves large septal or segmental hepatic artery branches and therefore often cannot be identified on histological assessment of needle liver biopsies which remains the standard method of assessing the severity of rejection processes. A recent paper has reported reversibility of the chronic rejection process in a small proportion of patients, and in this series 3 of sixteen patients demonstrated reversibility either by switching to tacrolimus or increasing conventional immunosuppression (Hubscher et al 1991). Two of these patients had entirely normal angiograms and the one patient with moderate arteriopathic changes is on tacrolimus. The role of rejection arteriopathy in the pathogenesis of interlobular bile duct loss (a characteristic feature of chronic allograft rejection) is controversial, but there is increasing evidence of both ischaemic and immunological factors in this lesion (Oguma et al 1989). In the study by Oguma et al., based on histometric analysis, the severity of bile duct loss was found to be proportional to the degree of obliterative arteriopathy. It remains unproven whether this biliary destruction is dependant on the arteriopathy or merely occurs in parallel. The well-described association of large biliary duct strictures with arterial insufficiency and chronic allograft rejection supports the hypothesis that graft ischaemia may be an important co-factor in intra-hepatic biliary duct destruction (O'Grady et al 1988a). In a recent clinical and histopathological review of chronic rejection it has also been argued that a sub-group of patients have a primarily vascular directed rejection which leads to an ischaemic hepatic injury and has a worse prognosis than biliary directed

rejection. It is also well established in renal and cardiac allograft rejection that the microvascular supply to the graft is an important determinant in graft survival and that the presence of an obliterative arteriopathy leads inevitably to graft loss (Rao et al 1988, Chomette et al 1988). From these observations, it can be recommended that angiograms should be performed routinely in patients with suspected chronic allograft rejection who do not demonstrate unequivocal arteriopathic lesions at histopathology in view of the sensitivity of this investigation in detection of arterial lesions and its predictive value in graft survival.

The clinical limitations of the present recommendations primarily reflect the invasive nature of conventional angiography. However with the insensitivity of duplex sonographic assessment as shown in this study and others and the present clinical application of magnetic resonance angiography poorly characterised, clinicians for the foreseeable future will rely on this method as the gold-standard assessment of vascular graft pathology. Prospective evaluation of the place of angiography in assessing vascular pathology would be valuable, particularly in the area of vascular rejection which has previously been considered irreversible. Tacrolimus, on the basis of the isolated case presented herein, may modify the natural history of this process.

CHAPTER 5

ASSESSMENT OF ACUTE LIVER ALLOGRAFT REJECTION BY NITRIC OXIDE GENERATION

5.1 BACKGROUND AND INTRODUCTION

Acute liver allograft rejection is traditionally diagnosed by histopathological assessment of a percutaneous biopsy specimen, a method which has several limitations. An inevitable risk of biopsy complications exists, a delay in diagnosis occurs and in certain patients the presence of a thrombocytopaenia or a coagulopathy contraindicates the procedure. Several novel techniques have been suggested to overcome these limitations. Fine-needle aspiration biopsies of the liver graft are technically safe and simple, but are also limited by technical failures in sampling the relevant cell population and the laborious sample processing and interpretation. Immunological monitoring has also been proposed as an alternative method of assessing the rejection response. Phenotyping peripheral circulating lymphocytes, cytokine measurements and various other putative markers of immunological damage have been proposed. Difficulties arising from cost, assay complexity and reproducibility have limited the introduction of these methods into clinical practice.

The generation of nitric oxide (NO) as an adjunct in monitoring of rejection is evaluated in this chapter. The synthesis of NO by the L-arginine / NO biosynthetic pathway mediates a wide spectrum of biological functions including roles in cytotoxic host defence mechanisms and in immunoregulation (Moncada et al 1991a). This pathway is induced in

inflammatory cells by various cytokines including interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-2 (IL-2), all of which have been shown to be involved in the pathogenesis of the in-vivo allograft response (Li et al 1991, Denis et al 1991, McCall et al 1991, Hibbs et al 1992, Vierling et al 1992). The induction of the enzyme which mediates this increased production of NO can be inhibited by therapeutic doses of glucocorticoids which may contribute to the anti-inflammatory mechanism of action of these agents (Moncada et al 1991b). In addition, NO appears to have an important modulatory role in lymphocyte proliferation (Efron et al 1991, Fu et al 1992). Increased production of NO in the allo-antigen response has recently been shown in a sponge allograft model and in an animal model of transplantation (Langrehr et al 1991a, Langrehr et al 1991b).

In this investigation, the levels of the stable end-products of NO generation and their relationship with the allograft cellular response in liver graft recipients were studied. These levels have been correlated with circulating TNF-α, an important mediator in the allograft response and, interleukin 2 receptor (IL2R) positive lymphocytes as a marker of peripheral lymphocyte activation and proliferation. In addition, a comparison of NO production between primary transplant patients receiving a CyA-based immunosuppression regimen to a tacrolimus one was performed.

5.2 SUBJECTS

5.2.1 Patients and controls

Samples were collected from 50 consecutive primary liver recipients prospectively entered into this study at the time of transplantation and a cohort population of 10 consecutive

patients with established and histologically confirmed chronic allograft rejection. The primary recipients received either a triple therapy immunosuppression regimen of CyA (120-150 ng/ml whole blood, Incstar RIA), azathioprine (1 mg/kg/day) and prednisolone (0.4-0.8 mg/kg/day) (n=24), a dual therapy regimen of tacrolimus (0.10-0.15 mg/kg/day) and low-dose prednisolone (0.1-0.3 mg/kg/day) (n=22) or, in the presence of toxicity to either CyA or tacrolimus, a regimen of azathioprine and prednisolone (n=4). Treatment allocation was prospectively randomised as part of the European multicentre trial of primary tacrolimus versus CyA based immunosuppression which was being conducted during the period of this investigation. NO generation was compared in those with histologically confirmed rejection, whether or not clinically significant, to those with other post-transplant complications which included cytomegalovirus (CMV) infection (n=3), vascular stenosis or thrombosis (n=3), significant renal impairment (n=3) and systemic or hepatobiliary related sepsis (n=4). Patients clinically well (absence of major graft or systemic complications) and with good graft function (AST and bilirubin levels less $\leq$ x2 upper limit of normal range) were considered as having a 'stable' post-operative course.

5.3 METHODS

5.3.1 Samples

Venous blood was collected serially, on a daily basis for the first two weeks and then twice weekly until day 28 after transplantation and whenever a liver biopsy was performed. Blood was collected on ice into endotoxin-free tubes (vacutainer) and plasma separated within 20 minutes by centrifugation at 200 g for 10 minutes. Aliquots were stored at –20° C until assay. Separate aliquots of whole blood were collected in EDTA for study of peripheral compartment lymphocytes.

5.3.2 Determination of nitric oxide end-products

The stable end-products of NO generation were assayed by chemiluminescence, either directly or after reduction of nitrate (NO_3-) to nitrite (NO_2^-) with nitrate reductase as described previously (figure 5.1) (Palmer et al 1987, Schulz et al 1992). Nitrate reductase (20 mu), FAD (120 mM), NADPH (14.4 mM) were added with phosphate buffer solution to the plasma samples and incubated for 1 hour at 37^0C to derive total NO_2^-. Volumes ranging from 5-100 μl were then added to a reaction vessel refluxing 6% aqueous sodium iodide and acetic acid under which conditions NO_2^- is reduced to NO which is then removed into a gaseous phase by a constant stream of nitrogen which also removes any contaminated oxygen (which reacts with the NO). The NO is then mixed with generated ozone to form a chemiluminescent product which is measured by a detector coupled to an electronic integrator. The area under the peak signal is proportional to the NO_2^- in the sample. The assay was calibrated using standard solutions of $NaNO_2$. The direct assay measures NO derived from acid labile products (NO_x), which include NO_2^- and possibly some nitroso-compounds (co-efficient of variation 9-12%). After reduction this assay additionally measures NO_3- which predominates by approximately 20 fold (co - efficient of variation 6-15%).

5.3.3 Measurement of TNF-α and IL2R-positive lymphocytes

TNF-α was assayed using a commercial ELISA kit (British Biotechnology Ltd. UK) with a threshold sensitivity 5 pg/ml. The proportion of IL-2R lymphocytes was determined using CD25 monoclonal antibodies (Dako Ltd., UK). Analysis was performed using FACS analyzer (Becton and Dickinson).

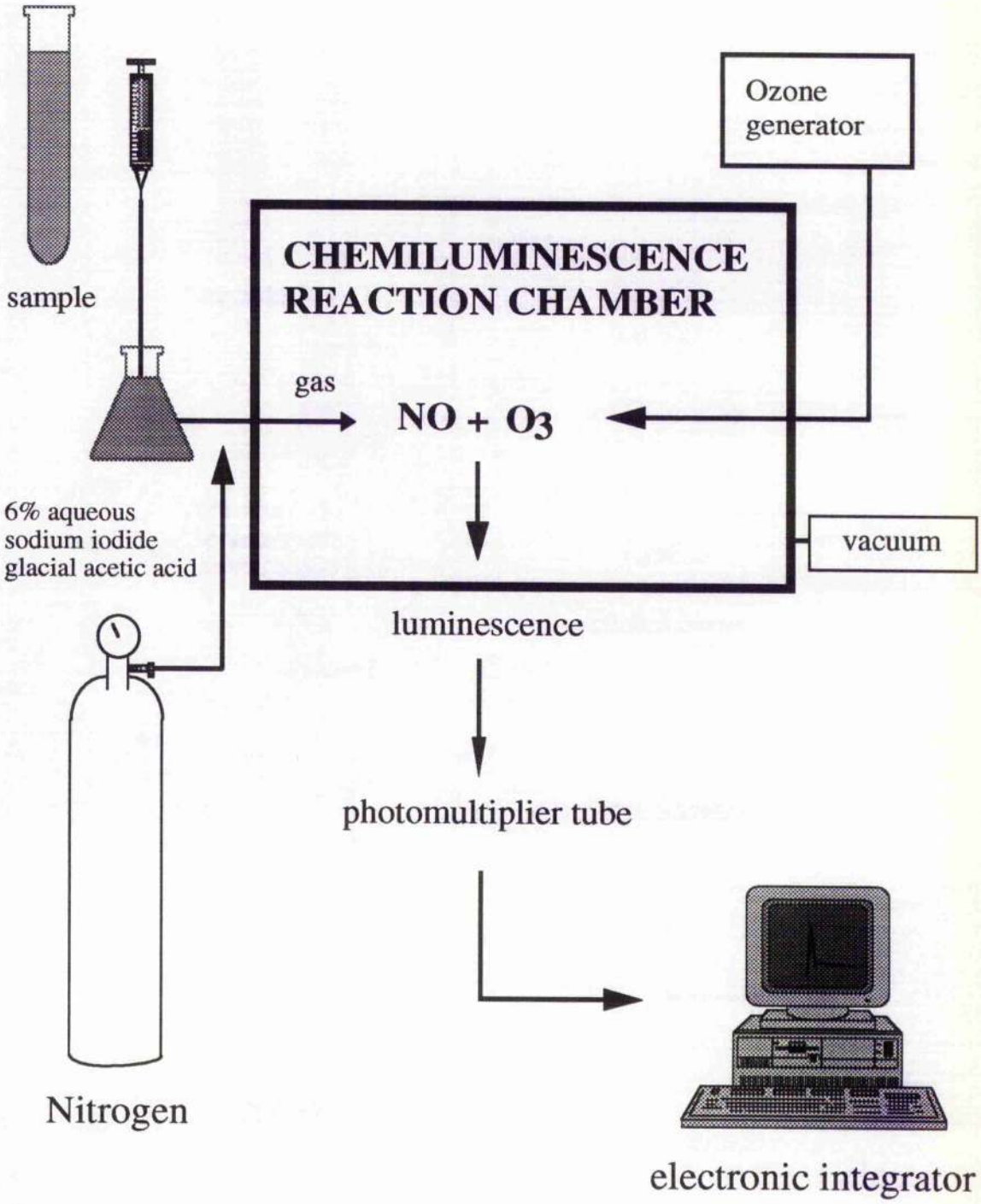


Figure 5.1. Schematic representation of the nitric oxide end-product analysis

5.3.4 Incidence and grading of acute allograft rejection

Percutaneous liver biopsies were performed at day 7 following transplantation and whenever an episode of rejection was clinically suspected (n=67). Three patients were excluded from analysis because a liver biopsy was contra-indicated during an episode of suspected rejection. Allograft rejection was graded by histological assessment into four categories on the basis of previously published criteria — none/grade 0, mild/grade 1, moderate/grade 2 and severe/grade 3 by an independent pathologist (Snover et al 1987). Clinically significant rejection requiring a course of high dose supplemental glucocorticoids (hydrocortisone 1 g b.d. for three days and 1 g o.d. for two days) was defined as the presence of clinical and biochemical evidence of graft dysfunction accompanied by either grade 2 or 3 histological evidence of rejection. Recipients who experienced an episode of grade 1 rejection were not treated with anti-rejection therapy.

Results are expressed as mean (SEM) and compared by the students paired or unpaired t - test where appropriate. Regression analysis was performed to determine the relationship between variables and a logistic regression performed to determine the most discriminating variable in the prediction of an episode of acute clinical rejection.

5.4 RESULTS

5.4.1 Nitric oxide generation and allograft rejection

Twenty-eight recipients had 33 episodes of histologically confirmed acute rejection (grade 1 18, grade 2 11, grade 3 4). All but four of these episodes occurred during the first 5-10 days of the post-transplant period. Plasma levels of NO_x were higher in patients during an episode of acute clinical rejection (n=15) than patients with either stable graft function,

other complications or chronic allograft rejection (figure 5.2). In contrast there was no significant correlation between plasma NO_3^- levels and the occurrence of rejection (table 5.1). There was a strong correlation between the histological grade of allograft rejection and the plasma NO_x level. The mean concentrations for rejection grade 0 and 1 (0.58 µmol, 0.61 µmol respectively) were significantly less than those for grade 2 or 3 rejection (mean level 2.28 µmol, p 0.001) (figure 5.3). In the patients developing clinically significant rejection, serial median concentrations of plasma NO_x rose to a peak during the rejection episode and decreased with resolution. The mean increase in NO_x concentration from pre-rejection (-3 days) 0.96 µmol to rejection was 1.44 µmol ($p<0.01$). Following administration of supplemental glucocorticoids, plasma NO_x concentrations were reduced to below the pre-rejection concentrations, with a mean reduction of 1.88 µmol by seven days post-treatment ($p<0.01$) (figure 5.4). Levels began to decline by day 2 in all patients following supplemental steroids. Concentrations of NO_3^- were not significantly reduced by the supplemental glucocorticoids (p 0.07).

5.4.2 Relationship between nitric oxide end-products and other circulating markers of rejection

In the first 30 consecutive patients in the study (which included 10 patients with significant clinical rejection), the relationship between plasma NO_x levels and circulating TNF-α and peripheral IL2R positive lymphocytes was studied. There was a correlation with TNF-α ($r=0.451$, $p<0.001$) with this parameter showing marked elevations during complications other than rejection. There was also a correlation between plasma NO_x and IL2R positive lymphocytes ($r=0.787$, $p<0.001$). These three variables in addition to plasma NO_3^- were compared as predictors of rejection in a logistic regression analysis.

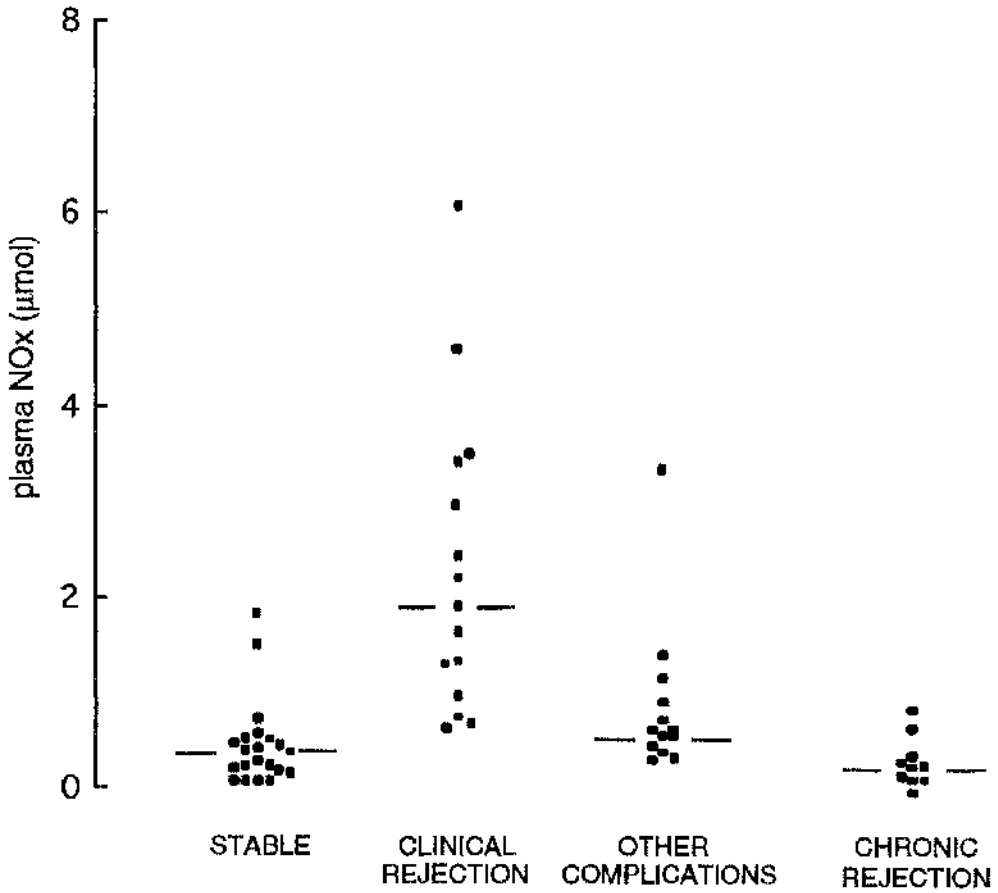


Figure 5.2 ***Plasma NO_x levels during episodes of clinically significant rejection were significantly elevated compared to recipients with 'stable' graft function ($p<0.0001$), non-rejection complications ($p<0.01$) or chronic rejection ($p<0.05$).***

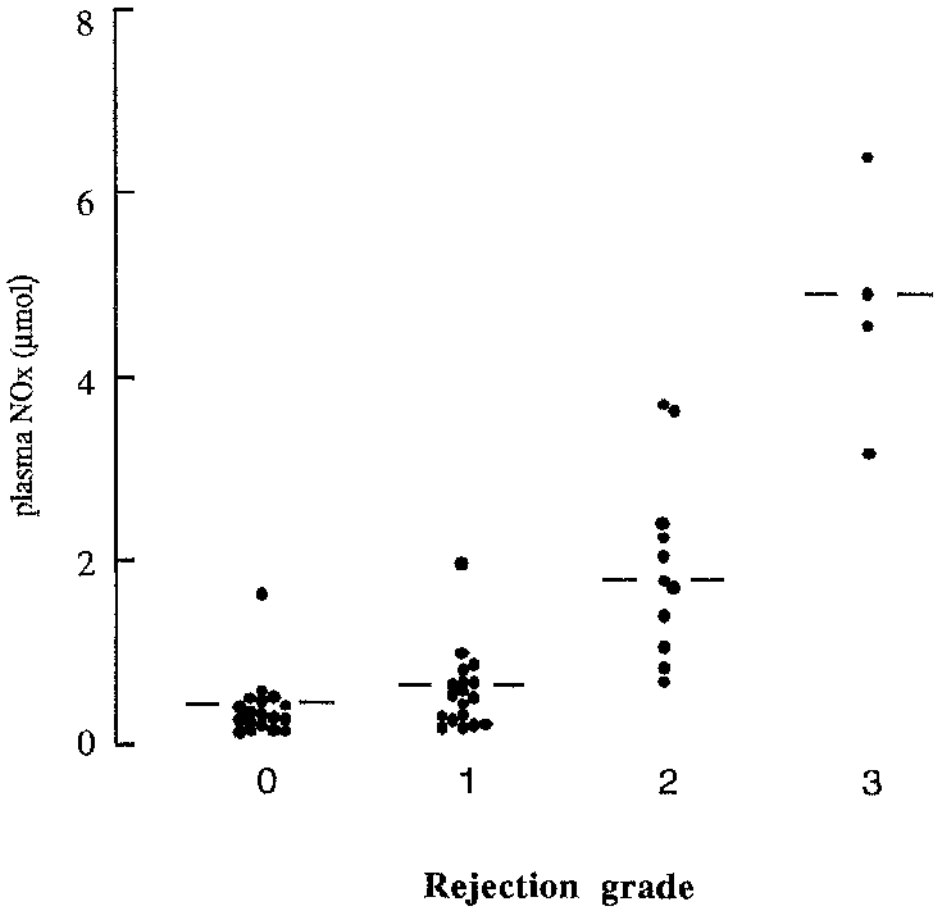


Figure 5.3 Peak plasma NO_x levels related to histological grades of rejection. These levels were significantly higher in grades 2 and 3 than either group 0 or 1 ($p<0.01$ and p 0.001 respectively).

Plasma NO_x was found to be the most discriminating variable in both univariate and multivariate analyses (table 5.1). An analysis of the predictive values of isolated measurements of these variables during an episode of rejection based on the median+SEM values of the four parameters (NO_x, NO_3-, TNF-α and IL2R positive lymphocytes) on the day of histological diagnosis was performed. All parameters were reliable negative predictors, with plasma NO_x and IL2R lymphocytes additionally demonstrating high positive predictive values for an episode of rejection. Representative examples of the measured parameters during graft rejection (case 1) and stable function (case 2) are presented in figure 5.5.

5.4.3 Relationship between nitric oxide generation and immunosuppression

Plasma concentrations of NO_x and the proportion of IL-2R lymphocytes were significantly lower in patients receiving tacrolimus-based immunosuppression than in those on a CyA regimen in the early post-operative period (table 5.2). This difference in plasma NO_x was consistent with the trend towards reduced frequency of an episode of acute rejection in the tacrolimus versus CyA treated patients of both clinical (4/21) 19% versus (8/21) 38% respectively (p 0.18) and histological rejection (11/21) 52% and (16/21) 76% respectively (p 0.10). Those recipients (n=4) not receiving either CyA or tacrolimus had the highest levels of NO_x in the study period, in particular during the first 10 days post-transplant (mean levels 1.84 μmol, p 0.05) with 3 of the 4 patients experiencing clinical rejection.

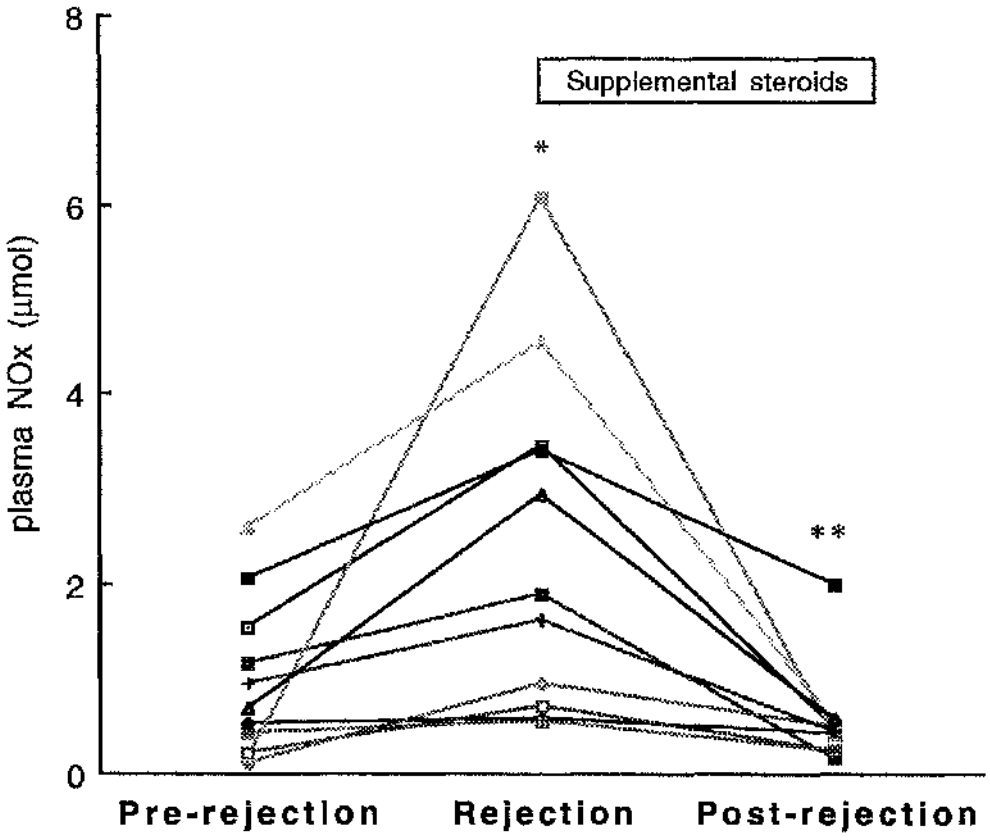


*Figure 5.4 Serial plasma NO_x levels in 12 recipients under either cyclosporin A or tacrolimus immunosuppression during an episode of rejection. Levels rose significantly from prerejection (-3 days) to rejection (*p <0.010) and decreased following administration of supplemental anti-rejection therapy (**p <0.01).*

Table 5.1 ***Comparison of the predictive powers of the 4 parameters (NO_x, NO_3-, TNF-α, IL2R lymphocytes [based on mean +SEM on day of histological diagnosis]) in detecting an episode of clinical rejection.***

	positive predictive value %	negative predictive value %	univariate analysis p value	multivariate analysis p value
NOx	73	95	<0.001	0.06
NO3-	33	87	0.09	0.27
TNF	25	92	0.26	0.40
IL-2R	66	89	0.075	0.43

Table 5.2 ***Plasma NOx (mean) levels in the early post-operative period in recipients receiving either a tacrolimus (FK506) or CyA-based primary immunosuppression regimen.***

	Cyclosporin A	Tacrolimus
DAY 3	0.606	0.681
DAY 5	0.924	0.534
DAY 7	1.260*+	0.496
DAY 12	0.524	0.419

+*levels risen from early post-operative period (day 3) ($p<0.001$)
levels higher in patients receiving cyclosporin A compared to tacrolimus ($p<0.05$)

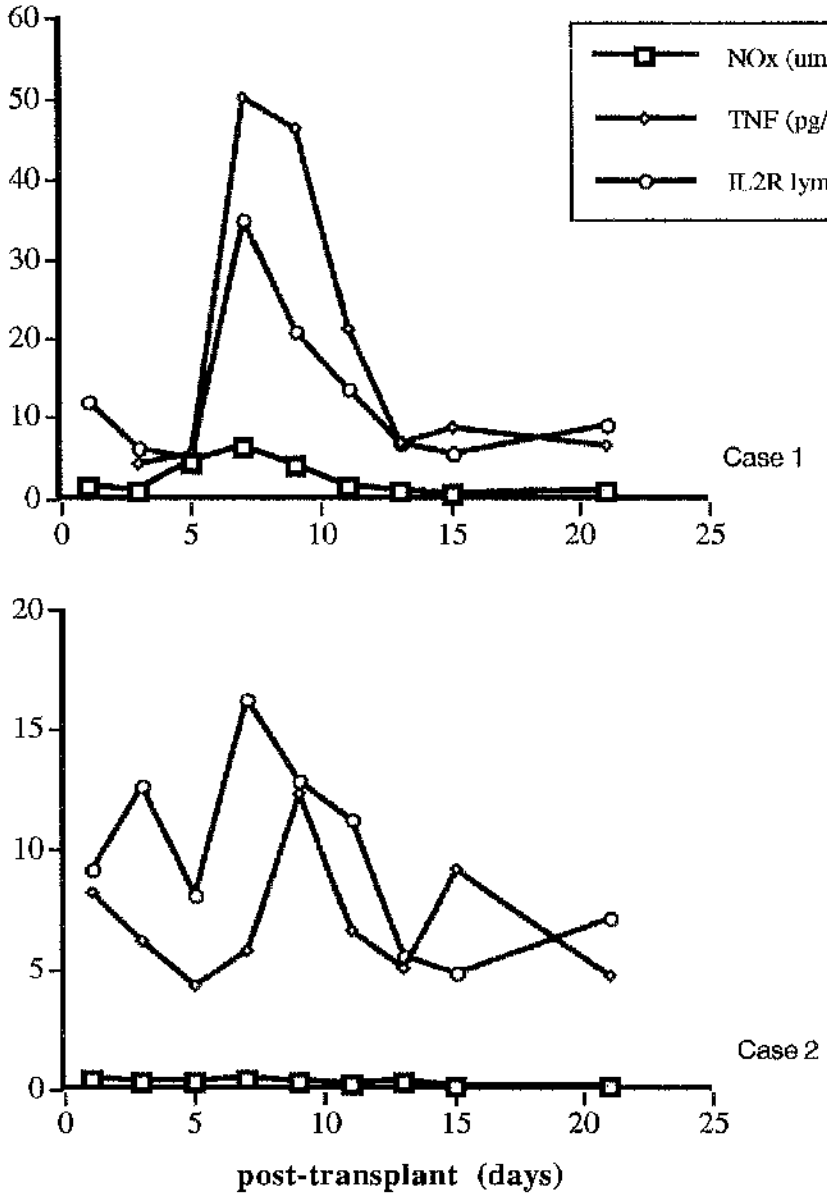


Figure 5.5 Representative examples of serial data during rejection [day 7] (case 1) and stable graft function (case 2) (note different scale). An increase in NOx levels by post-operative day 5 in the rejection episode was detected (i.e. levels were increased 2 days prior to histological confirmation). In case 2, NOx levels were consistently low. Although the levels of the 2 other parameters studied were typically lower than during rejection unexpected fluctuations which were difficult to attribute to clinical events occurred.

5.4 Discussion

The present study shows the potential value of immunological monitoring by measurement of NO generation in human liver allograft rejection. A close relationship between NO end-products and rejection was demonstrated. NO itself is very unstable, reacting with a variety of molecules to form NO_2^-, NO_3^- and other nitroso-compounds, either directly or after decay of intermediates. In this study, a particular correlation between elevations in NO_x (acid labile nitroso-compounds), rather than NO_3^-, and rejection was shown. The reasons for this discrepancy are presently not clear, however it is likely that NO_x is formed directly from a reaction with NO. In contrast NO_3^-, in addition to being a product of NO, is derived predominately from the diet as previously reported (Knight et al 1987). Further work is required to identify the stable product that correlates with rejection and which may be either NO_2^- and / or stable nitroso - compound(s).

NO may contribute to the pathogenesis of rejection following activation of the immune system as a cytotoxic molecule. The absence of systemic hypotension in allograft rejection suggests that the systemic vasculature is not a source for this increased production of NO as it is in endotoxic shock. The source of this increased production is likely to be the graft - infiltrating cells and / or the hepatic vasculature and parenchymal cells, following induction of NO synthase by products of the infiltrating cells. The elevated local concentrations of NO in the allograft are likely to be directly injurious to target cells in rejection, mediating damage in liver rejection to vascular endothelium and inhibiting hepatocyte protein synthesis (Palmer et al 1992, Curran et al 1991).

Administration of exogenous IL-2 increases NO production in a clinical population (Hibbs et al 1992). The correlation between NO_x and IL-2R lymphocytes in this study suggests

that at least one of the factors involved in the production of NO is activation of inflammatory cells by IL-2. Hence the suppression of NO production with tacrolimus and CyA immunosuppression may be mediated through their inhibition of IL-2 production. The markedly reduced NO production in the tacrolimus group matched the lower frequency of rejection in these patients and is consistent with this agent's superior immunosuppression (Starzl et al 1989). The rapid reduction in NO_x production following supplemental steroids during allograft rejection, is consistent with previous studies showing that induction of nitric oxide synthase in inflammatory cells can be inhibited by pharmacological doses of glucocorticoids, which may be a contributing mechanism of action in their ability to reverse cellular rejection (Moncada et al 1991b). The finding that NO_3- concentrations were not significantly reduced after supplemental glucocorticoids supports the superior validity of NO_x as a measure of NO production. A recent study of urinary NO_3- excretion in an experimental rodent model of acute cardiac allograft rejection has found virtually identical results to this clinical investigation (Winlaw et al 1994). In that study, an 8-fold rise in urinary NO_3- excretion (measured by the Greiss reaction) was detected during rejection in untreated animals whereas treatment with steroid and CyA immunosuppression abrogated these peaks. Whether differences in the assay and the compartment which was measured explain the differences between the studies in relation to the particular value of NO_3- measurements is speculative. Urinary levels were not measured in the study from this unit and the Greiss reaction is too insensitive to be used in clinical investigations (Archer 1993).

In addition to having a role in the pathogenesis of allograft rejection, NO production may also be important in regulation of the immune response. This molecule has been shown to be required for optimal DNA synthesis in human lymphocytes, although at higher concentrations it appears to have a species-dependent ability to inhibit proliferation following mitogenic or allo-antigenic presentation (Fu et al 1992, Langrehr et al 1991b).

Despite the potential value of immunological monitoring of allograft recipients, this is rarely employed clinically because of the expense and complexity of assay systems involving lymphocyte and cytokine preparations. Measurement of plasma NO_x, a stable end-product of NO production, is a cheap measurement which is potentially available within minutes of sampling and which appears to give superior predictive values in detecting acute allograft rejection.

The limitations which confound all attempts at immunological monitoring in allograft rejection is the non-specific activation of the immune system in conditions such as sepsis. This variable is not surmounted by measurement of NO, although this parameter did appear the best of the variables evaluated. Although there was a close correlation with activated lymphocytes, there is no definite evidence at present that these cells were the source of NO. Several other cells including Kupffer cells, hepatocytes and endothelial cells, which are known to produce NO, could contribute to the circulating levels. Immunohistochemical localisation of the inducible nitric oxide synthase in graft tissue during rejection would be one approach to determine the source of the circulating NO.

CHAPTER 6

IDENTIFICATION OF RECIPIENT ETHNIC ORIGIN AS A RISK FACTOR IN CHRONIC LIVER ALLOGRAFT REJECTION

6.1 INTRODUCTION

Demographic characteristics have previously not been widely examined in relation to liver allograft survival. The age of the recipient, as discussed in section 1.6, is of some importance with, not unexpectedly, extremes of age being associated with inferior results. No effect of recipient gender is reported. Data on the influence of ethnic recipient background has been inadequately examined in liver transplant patients. In contrast in renal transplantation, it has been established that the ethnic origin of recipients is an important factor in determining allograft survival. Although there are variations between centres, data from the large renal-transplant registries both in the United States and Europe have demonstrated an inferior long-term allograft survival in recipients of black / Afro - caribbean origin (Opelz et al 1989, Takemato et al 1989). Moreover, this ethnic background in separate multivariate analyses has been shown to be an independent risk factor for renal graft survival (Sanfilippo et al 1986). There is limited data regarding the effect of racial origin of either donor or recipient on outcome in other transplanted organs, with no published reports on pancreatic, heart or lung transplantation. In the one large study to date on liver recipients from the University of Pittsburgh, the 120 adult black recipients had a reduced two-year patient survival as compared to non-black recipients in their programme, which they later attributed to a difference in pre-transplant clinical status (Teperman et al 1989).

In this investigation, an analysis of patient and graft survival, incidence and contribution of chronic allograft rejection to graft loss and rate of retransplantation was performed.

6.1 SUBJECTS

6.1.1 Patients and methods

The patient groups comprised all paediatric and adult patients undergoing primary orthotopic liver transplantation in the transplant programmes of Kings College hospital and Addenbrokes hospital, Cambridge between January 1, 1984 and August 1, 1991 was performed. No exclusions were made except for multi-organ transplants and unavailability of ethnic origin data (n=8). All donors and recipients were ABO compatible except in exceptional urgent circumstances (0.7%). Patient selection for transplantation was standardised on clinical criteria and organ allocation was decided on the basis of ABO - compatibility and anatomical suitability with no consideration to the ethnic origin of the donor or recipient or HLA matching. Diagnosis of chronic allograft rejection was made by a single pathologist based on previously published and established criteria (Portmann et al 1988).

6.1.2 Ethnic and racial groups

The patients were divided into four groups based on ethnic origin — *group 1* included those of north European origin (n=441), *group 2* those of south European / mediterranean origin (n=121), *group 3* those of middle east / central Asian origin (caucasoid n=83) and *group 4* patients of Afro-caribbean / eastern Asian origin (non-caucasoid n=28).

6.1.3 Statistical analysis

Actuarial graft and patient survivals were calculated using life table analysis on BMDP-PC software and statistical significance estimated with the Mantel-Cox or Breslow test. The

statistical significance of differences in the demographic data, the incidence of retransplantation and chronic rejection were estimated with the chi-test (x2) or students t - test where appropriate. A multivariate Cox regression model to determine independent risk factors in graft survival within the four groups was performed with the primary liver disorders HCC, HBV and NANB viral hepatitis and chronic allograft rejection. A P value <0.05 was considered to be significant.

6.2 RESULTS

6.2.1 Demographic variations

Comparison of the demographic data showed significant differences between the groups (table 6.1). The average age of the patients making up groups 2, 3 and 4 (south European, mediterranean / central Asian and Afro-caribbean / eastern Asian patients respectively) was significantly less than group 1 (north European patients) ($p<0.001$). Groups 2 and 3 were male-dominated with group 1 having a majority of female recipients ($p<0.001$ vs groups 2, 3 and 4). The aetiology of the liver diseases varied between the groups with groups 2, 3 and 4 having significantly less patients with PBC ($p<0.001$ vs group 1) and an increased proportion of recipients undergoing transplantation for post-hepatitic cirrhosis ($p<0.001$ vs group 1), this category of liver disease accounting for approximately 40% of transplants in these groups. In group 4, an increased proportion of patients were transplanted for primary liver malignancy compared to the other groups ($p<0.01$ vs total). The proportion of recipients undergoing transplantation for FHF was approximately equal between the groups with the exception of group 2 who had a reduced proportion in this category.

Table 6.1 ***Comparison of the demographic data of the liver transplant recipients according to the different groups (group 1 - north European, group 2 - south European, group 3 - mediterranean / central Asian and group 4 - Afro-caribbean / eastern Asian).***

	GROUPS			
	1	2	3	4
n (%)	441 (65.5)	121 (17.9)	83 (12.3)	28 (4.2)
Age				
mean	35.3	29.6	23.8	27.4
Sex				
male	160	74	56	19
female	281	47	27	9
Original Liver Disease				
primary biliary cirrhosis	101 (23)	17 (14)	6 (7)	1 (4)
post-hepatitic cirrhosis	34 (10)	51 (42)	35 (42)	8 (29)
chronic active hepatitis	30 (6.7)	10 (8)	6 (7)	2 (7)
alcoholic liver disease	21 (5)	12 (10)	2 (2)	1 (4)
biliary atresia	53 (12)	2 (2)	4 (5)	2 (7)
α1-antitrypsin deficiency	20 (5)	2 (2)	2 (2)	0 (0)
Wilson's disease	15 (3)	1 (1)	3 (4)	0 (0)
primary liver malignancy	28 (6)	7 (6)	5 (6)	6 (21)
Budd-Chiari syndrome	13 (3)	4 (3)	0 (0)	1 (4)
primary sclerosing cholangitis	32 (7)	3 (2)	7 (8)	1 (4)
fulminant hepatic failure	62 (14)	6 (5)	11 (13)	4 (14)
other	32 (7)	6 (5)	2 (2)	2 (7)

6.2.2 Patient and graft survival

In the early post-operative period — defined as mean period of hospitalisation prior to discharge following transplantation for all the patients in the study (8 weeks) — there was no significant differences in either patient or graft survival between the groups (figure 6.1). At 1, 3 and 3.5 years following transplantation, group 4 had a significantly inferior patient survival than all the other groups ($p<0.05$) (figure 6.2). There was no significant differences in patient survival rates between the other groups. Graft survival rates were similar for the European groups (groups 1 and 2) although there appeared to be a marginally inferior outcome in the south European group (e.g. 48.5% vs 53.5% at 3 years n.s.). Groups 3 and 4 had inferior graft survival rates at 1, 2 and 3 years post - transplantation with the non-caucasoid group having the worst outcome ($p<0.01$ and $p<0.001$ respectively versus group 1 at 1, 2 and 3 years) (figure 6.3).

6.2.3 Chronic allograft rejection and retransplantation

European recipients, either of northern or southern European origin, had similar incidence of chronic allograft rejection at just under 6%. In the non-European groups 3 and 4 this complication was recorded in a significantly greater number — 12.6% of the population (p 0.002 vs groups 1 and 2) with a separate rate of 12.0% for group 3 ($p<0.01$ vs groups 1 and 2) and 14.3% — the highest rate — in group 4 ($p<0.05$ versus groups 1 and 2) (table 6.2). In a multivariate analysis of independent risk factors in graft survival (analysis of chronic rejection, HCC and HBV-related cirrhosis), chronic rejection and HCC were found to contribute independently to graft loss within all the groups (p value 0.02 and p value 0.05 respectively). Chronic allograft rejection was the single largest indication for retransplantation in the groups, accounting for between 38 - 100% (table 6.2).

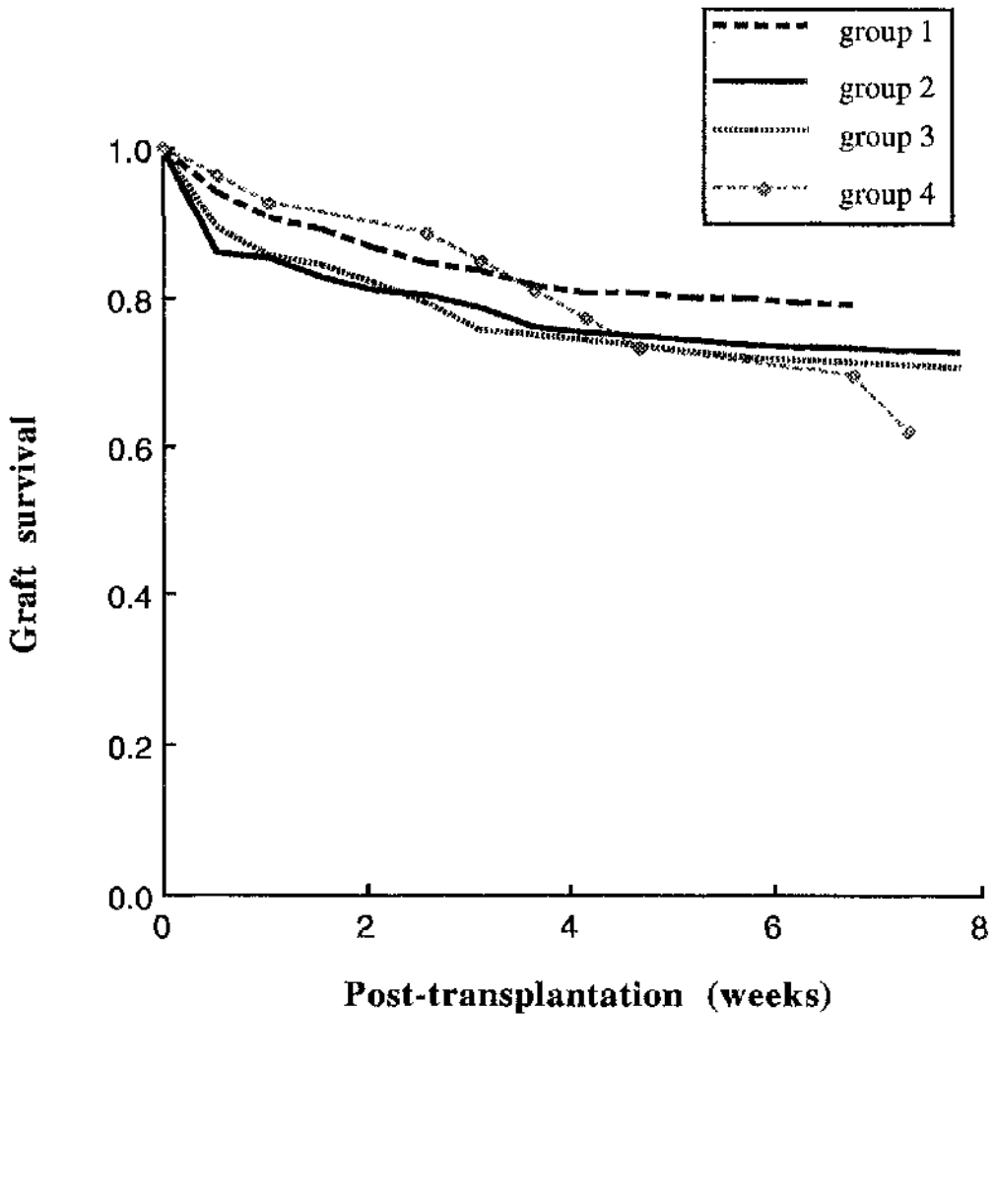


Figure 6.1. Early graft survival up to the mean period of hospitalisation following transplantation (8 weeks) in patients from the different ethnic groups.

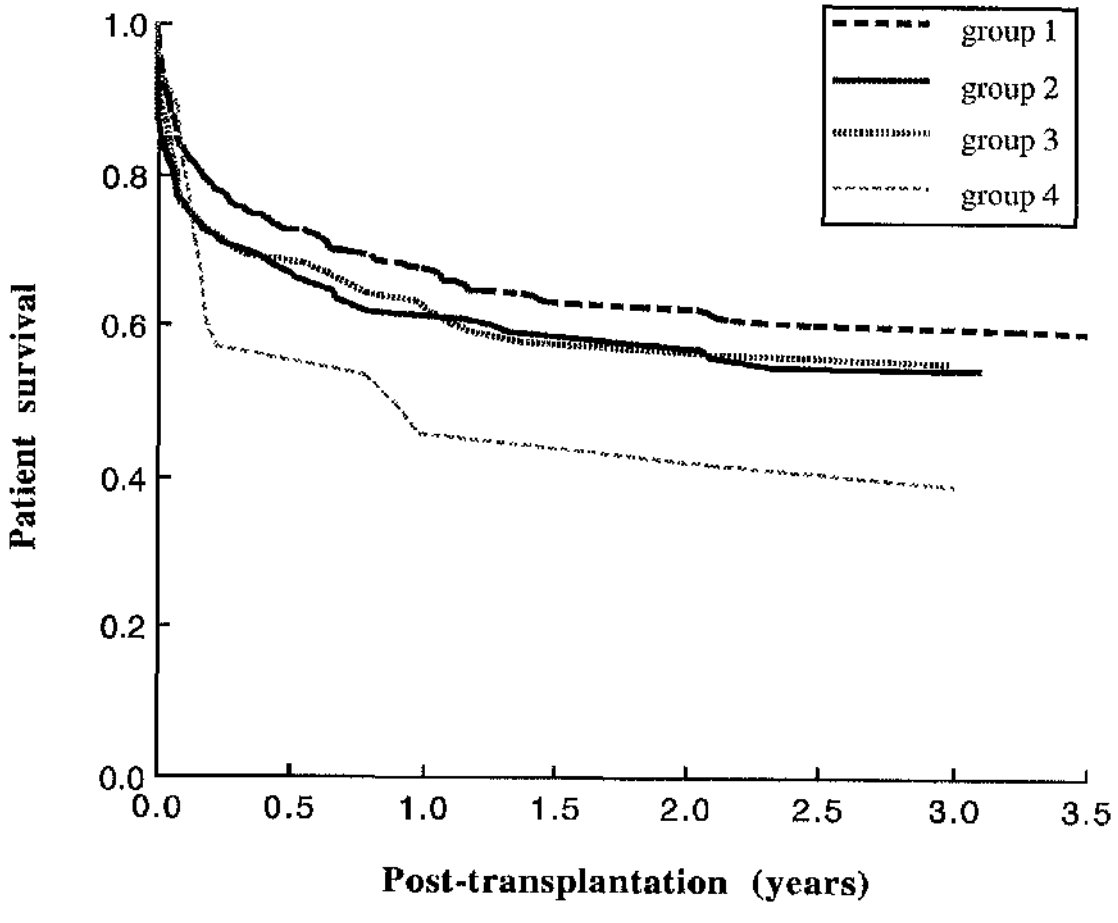


Figure 6.2 Actuarial patient survival in the different ethnic groups up to three years following transplantation.

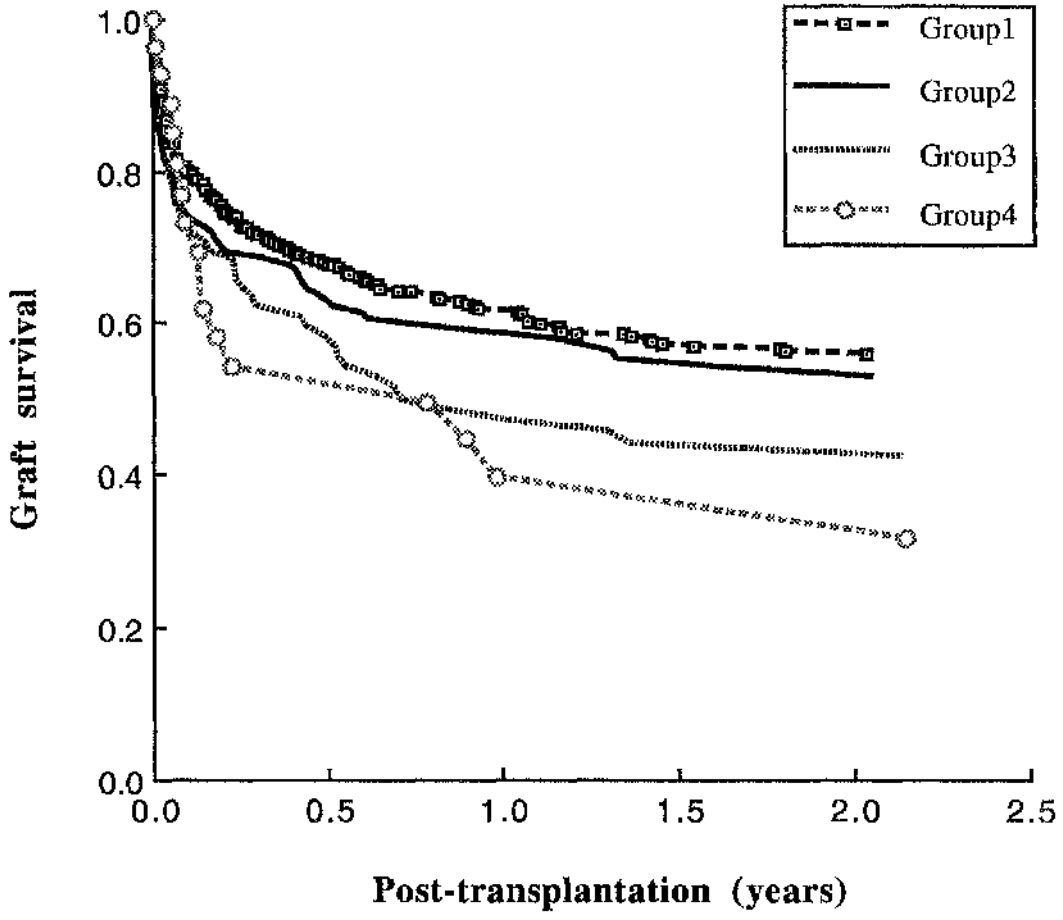


Figure 6.3 Actuarial graft survival divided by ethnic groups up to two years post-transplant. The non-European groups (groups 3 and 4) have lower success rates.

In the period between 1984 - 1987, the maintenance immunosuppression regimens comprised either CyA monotherapy or CyA in combination with low dose prednisolone (CyA levels maintained between 120-150 µg/l, monoclonal RIA). Subsequently from 1987 to the present day, the patients are maintained on a triple therapy regimen (prednisolone 0.1 mg/kg/day, azathioprine 1 mg/kg/day and CyA levels maintained between 100-120 µg/l, monoclonal RIA) with the exclusion of twenty-six patients in the study population who were on a combined tacrolimus ± steroid regimen. There were significant differences in distribution of the groups between the two principal immunosuppression regimens with larger proportions of groups 2, 3 and 4 being distributed in the later triple therapy regimen compared to group 1 (p value 0.005) (figure 6.4).

6.4 DISCUSSION

The findings in this large study in liver transplantation supports the evidence from renal transplant programmes that the ethnic origin of the recipients is an important risk factor in graft loss mediated to some extent through variable rates of chronic allograft rejection. The recipients in the present study from a non-European and in particular a non-caucasoid ethnic background have inferior graft survival rates compared to an indigenous European population in a programme utilising donor organs retrieved from a predominately caucasian population. The United Kingdom Transplant Support Service Special Authority (UKTSSA) estimates that greater than 95% of the multi-organ donor population pool in the UK are white, caucasian and from either the UK or Ireland; unfortunately no data is available on individual donors preventing an analysis of specific donor-recipient pairings (personal communication UKTSSA) but the possibility of a spontaneous racial match between non-indigenous recipients to UK donors would be virtually negligible.

Table 6.2 ***Frequencies of chronic rejection and the contribution to elective retransplantation and graft loss between the different ethnic groups***

	GROUPS			
	1	**2**	**3**	**4**
n,(%)				
Chronic rejection	26 (5.9)	7 (5.8)	10 (12.0)	4 (14.3)
Graft loss due to chronic rejection	23 (5.2)	7 (5.8)	10 (12.0)	4 (14.3)
Retransplantation on account of chronic rejection (%)	4.8	5.8	12.0	14.3

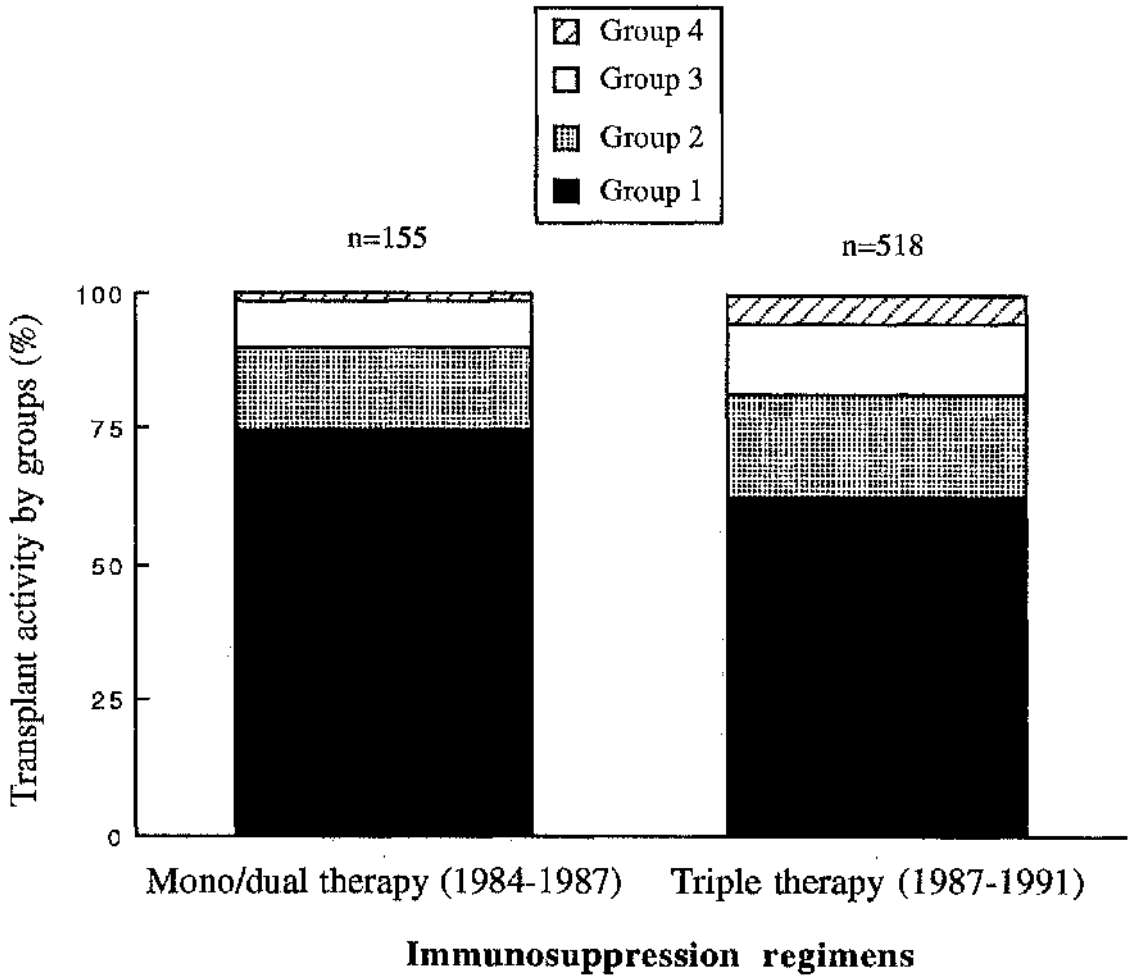


Figure 6.4 Distribution (%) of all the recipients, divided by ethnic group, as managed with the two immunosuppression regimens over the study period.

The influence of ethnic background on outcome is demonstrated clearly in groups 2 (south European / mediterranean origin), and 3 (middle east / central Asian origin), which although similarly matched demographically for age, gender and recipient disease categories had considerably different outcomes after transplantation. The variable incidence of chronic allograft rejection (a complication which alongside HCC is an independent risk factor in graft survival) contributes to and largely explains these ethnic variations in outcome. The comparative data above suggests that the variable rates of chronic rejection between ethnic groups cannot be explained by simple demographic features but is due to some inherent population differences as yet undetermined. To date the only risk factors for chronic rejection have comprised reactivation / reinfection of CMV, HLA match / mismatching and positive lymphocytotoxic crossmatching (O'Grady et al 1988b, Batts et al 1988). However the present results are consistent with the data emerging from the large American and European renal registries which, with the aid of large population groups, have demonstrated that the ethnic origin of transplant recipients is an important factor in graft survival, with a pattern of reduced graft survival secondary to chronic rejection in black recipients (Dunn et al 1989). There have however been no previous studies of this inferior outcome extending into other non-caucasoid populations or into recipients of a middle east / central Asian origin as has been demonstrated in the present study.

Several explanations have been proposed for the inferior outcome in black renal recipients which include non-compliance with immunosuppression therapy and higher rates of hypertension but the present consensus is that an immune-mediated mechanism leading to chronic rejection is more likely to be involved (Sanfilippo et al 1986). Although multivariate analyses have confirmed that ethnic origin is an independent risk factor for an inferior outcome following renal transplantation, there is no convincing evidence from these studies that HLA mismatching (a prime candidate for the increased risk of chronic

rejection) contributes to this phenomenon (Sanfilippo et al 1986, Kondo et al 1987). This apparent lack of importance of HLA mismatching in renal allografts may be misleading in the light of the recently described disparity between clinical serological HLA typing and functional HLA status and the heterogeneity of HLA antigens, as assessed by the newer techniques of polymerase chain reaction and oligohybridisation, in minority ethnic groups (Fernandez et al 1991). What were previously considered to be beneficial matches in ethnic minorities will have to be reconsidered with this functional disparity and the heterogeneity of these groups compared to the more homogenous white donor populations. The role of HLA matching / mismatching in liver transplantation remains controversial, as no attempt is made to beneficially match HLA antigens prior to organ allocation and in this units series spontaneous beneficial matching of either three / four HLA A / B antigens has only occurred in three patients in a series of four hundred patients (Donaldson et al 1991a). Other possible explanations for this increased rate of chronic rejection, independent of HLA status, is the higher immune responsiveness of black compared to white renal recipients which has been demonstrated in these patient populations (Kerman et al 1991). This observation may also explain why black recipients of both cadaveric grafts from black donors and live-related grafts also have an inferior outcome (Milgrom et al 1989). The possible effect of socio-economic status among non - caucasian recipients and consequent non-compliance is not an appropriate explanation among our patients. The great majority of non-caucasian recipients within the Kings programme are privately funded individuals from above average socio-economic status referred from around the world and in whom non-compliance is not recognised as a problem.

This present European study appears to reach different conclusions from the only previously published reports on this subject from two liver transplant programmes in the USA. The University of Pittsburgh which restricted analysis to outcome in black and

hispanic americans demonstrated a reduced patient survival at 2 years in black compared to white recipients (less than 50% vs 70%, p 0.015). They concluded however that allowing for pretransplant clinical status as assessed by UNOS there was no significant difference in outcome although this scoring system was only applied to a relatively small number of the study group (Teperman et al 1989). A smaller study of 23 black recipients did not show any significant difference in outcome although there was a trend towards lower survival in this ethnic group (Gonwa et al 1991). In this study, pre-clinical status did not appear to explain the differences with similar frequencies of patients between the groups undergoing transplantation for fulminant hepatic failure and no differences in early peri-operative mortality. It is also noteworthy that higher proportions of the ethnic minorities groups were transplanted in the later part of this study when there had been additional immunosuppression in addition to improved patient selection and surgical technique.

The results of this study indicate that further prospective analysis of the role of HLA matching / mismatching and immunological responsiveness in stratified ethnic groups undergoing liver transplantation is required. A further study of donor organs retrieved from non-indigenous populations and transplanted into a uniform indigenous population may also yield important information on the effects of organ donation across ethnic groups.

CHAPTER 7

IMPORTANCE OF PRETRANSPLANT CLINICAL STATUS IN OUTCOME OF EMERGENCY TRANSPLANTATION FOR ACUTE LIVER FAILURE

7.1 BACKGROUND AND INTRODUCTION

Pretransplant clinical status is an important determinant of outcome in all liver recipients and was proposed in chapter 2 as meriting a specific category of clinico-pathological determinants causing graft failure. The inferior survival of elective recipients with poor nutrition, renal failure and advanced cirrhosis has already been established (Cuervas - Mons et al 1986). In contrast to this data, no information is available on the influence of pre-transplant clinical status on the outcome of emergency transplantation for ALF where the recipients are often critically ill. Transplantation is now established as an important component of the overall care of patients with acute hepatic failure, although selection of the cases that need to be transplanted is difficult due to variability in both the prognosis of different aetiological / clinical categories and the accompanying effects of multisystem critical illness (O'Grady et al 1988c). One year patient survival rates following transplantation are around 50-60% and many recipients have a slow convalescence requiring prolonged periods of intensive care (Bismuth et al 1987, Schafer et al 1989, Emond et al 1989). Indeed it has been argued that liver grafts would be better used for elective cases where survival rates of 80-90% can be obtained (Chapman et al 1990). In this centre, the decision to consider a transplant is based on certain clinical and laboratory indicators of prognosis found in a multivariate analysis of patients with fulminant hepatic failure (FHF) which identified a survival without transplantation of less than 10% (O'Grady et al 1989). Other groups use different criteria based around either factor V

levels or serial assessment of liver size with CT scanning and liver biopsy (Bernuau et al 1991, Van Thiel et al 1993). Following initial selection and listing for transplant, some patients deteriorate before a donor organ becomes available with the development of absolute contraindications which include severe haemodynamic disturbance, presence of uncontrolled cerebral dysfunction or systemic sepsis. As yet no detailed analysis of other parameters of pretransplant clinical status which could have a significant influence on the post-operative course has been reported.

In this present study, the outcome of the first 100 consecutive adult patients who underwent emergency transplantation for ALF in relation to aetiology, severity of the liver failure and associated systemic critical illness was analysed. Additionally, an attempt to elucidate which clinical parameters or scoring systems of pretransplant status could be used in the prediction of early hospital outcome was performed.

7.2 SUBJECTS

7.2.1 Patients and clinical categories

Data from the first 100 adult patients who underwent emergency liver transplantation (98 orthotopic and 2 auxiliary heterotopic transplants) for either FHF (n=79) or late-onset - hepatic failure (LOHF) (n=21) [standard classifications (Trey and Davidson et al 1970, Gimson et al 1986)] in this unit's series between January 1984 and September 1992 were analysed. These 100 patients represented 13% of consecutive cases admitted with ALF (n=759) during the study period. Median age of those transplanted was 32 years [range:11-63] with a predominant female sex ratio [F:M, 70%:30%]. Determination of aetiological category was based upon standard clinical, virological and laboratory indices with histological assessment of the explanted liver available in all cases. The number and proportion of all cases admitted to this unit who underwent

transplantation were as follows: hepatitis A virus (HAV) 4/36 (11%), NANB / indeterminate 51/125 (41%), paracetamol hepatotoxicity 21/446 (5%), fulminant Wilsons disease (FWD) 9/14 (64%), idiosyncratic drug hepatotoxicity 7/26 (26%) and miscellaneous causes (which comprised HBV 1, halothane toxicity 2, fulminant presentation of autoimmune hepatitis (AIH) 2 and Budd-Chiari syndrome 1, 6/83 (7%) (table 7.1). To allow examination of potential pre-transplant risk factors in relation to outcome, the study population was additionally divided into 2 patient groups: non-paracetamol induced liver failure (n=79 [FHF n=58, LOHF n=21], group 1) and those with paracetamol hepatotoxicity (n=21, group 2).

7.2.2 Patient selection and immunosuppression

Consideration for transplantation in FHF patients, since 1988, was based on the clinical criteria of O'Grady as already referred to (n=58; group 1 n=37, group 2 n=21). All patients with LOHF were considered for transplantation in view of the poor prognosis of this group (Gimson et al 1986). The presence of irreversible brainstem dysfunction, increasing inotrope dependence and culture-positive systemic sepsis resistant to 48 hours antimicrobial therapy were absolute medical contraindications to proceeding with transplantation. Additionally in patients who self-administered a deliberate paracetamol overdose, a history of a severe chronic psychiatric disorder or repeated suicide attempts were also contraindications. Immunosuppression schedules were based upon either a dual therapy prednisolone / CyA regimen [1984-1987] (n=23), CyA based triple therapy regimen [1987-1992] (n=69) or a tacrolimus / low dose corticosteroid schedule [1990-1991] (n=8). Modifications to induction immunosuppression regimens, in particular reduction or withdrawal of CyA, were often required due to the high incidence of postoperative renal and neurological complications. No anti-lymphocyte preparations were administered as induction immunosuppression. For donor organ preservation

Eurocollins solution (n=11) was used prior to 1987 and UW solution (n=89) thereafter.

7.2.3 Parameters examined in relation to post-transplant outcome

Pretransplant parameters examined as risk factors were the static variables: age, gender, transplant year, aetiology, 'time from admission to transplantation' and in the case of paracetamol-overdose cases 'time from overdose to transplantation / hepatectomy'. The latter variable was included as 3 patients underwent total hepatectomy as a 'holding procedure' prior to the eventual transplantation. On admission to the Liver Failure Unit and again immediately prior to transplantation dynamic variables examined comprised encephalopathy grade, presence of cerebral oedema, indices of systemic illness (organ system failure and apache 111 score) and standard serum biochemical and haematological parameters (bilirubin, AST, albumin and creatinine levels, INR; mean control 15s, total white cell and platelet count). In the paracetamol hepatotoxicity cases only, arterial pH was also evaluated. Multiple organ failure was quantitated using standard criteria with the addition of an episode of cerebral oedema or grade IV hepatic coma constituting neurologic failure during a 24-hour period (Knaus et al 1985). Apache 111 score was calculated as previously described with the additional adoption of a standardised scoring system for the assessment of hepatic encephalopathy dependent upon coma grade (grade 1 - 3 points, grade 2 - 8 points, grade 3 - 13 points, grade 4 (without cerebral oedema) - 24 points and grade 4 (with cerebral oedema) - 33 points (Knaus et al 1991). In patients who required mechanical ventilation (all paralysed and sedated according to established clinical practice) encephalopathy grade was later assumed to be that present at the time ventilation was commenced except when cerebral oedema developed which was equated to grade IV encephalopathy. Actuarial patient survival was calculated by life-table analysis and statistical differences estimated by the log-rank test.

Figure 7.1 ***Summary clinical data of transplanted liver failure patients divided into those experiencing non-paracetamol liver failure (group 1) and severe paracetamol hepatotoxicity (group 2). Significant variations in the clinical parameters, related to liver failure or systemic illness characteristics, are present between the aetiological categories.***

	NON-PARACETAMOL (1)						PARACETAMOL (2)	TOTAL
characteristic	NANB / indeterminate FHF (n=30)	LOHF (n=21)	HAV (n=6)	Wilsons disease (n=9)	drug (n=7)	other (n=6)	paracetamol (n=21)	(n=100)
sex								
male	6	7	3	4	1	4	5	30
female	24	14	3	5	6	2	16	70
age								
mean	33	37	30	19	34	38	30	32
(range)	(11-54)	(11-54)	(12-63)	(14-26)	(19-50)	(13-48)	(17-50)	(11-63)
apache 111	50 (18)	50 (25)	57 (26)	46 (29)	48 (25)	41 (13)	74 (21)	55 (24)
INR	6.9 (4.3)	3.8 (1.7)	7.0 (3.5)	4.6 (3.5)	9.5 (6.3)	6.0 (4.9)	8.2 (4.0)	6.5 (4.2)
AST (IU/L)	704 (1208)	245 (264)	316 (171)	173 (212)	744 (666)	517 (438)	2034 (2058)	787 (1317)
bilirubin (μmol)	410 (140)	538 (174)	492 (93)	714 (204)	427 (230)	369 (233)	146 (81)	413 (223)
encephalopathy grade * (%)								
I	3 (10)	9 (43)	0 (0)	6 (68)	1 (14)	1 (17)	0 (0)	20 (20)
II	6 (20)	4 (19)	1 (17)	1 (11)	2 (29)	2 (33)	0 (0)	16 (16)
III	9 (30)	3 (15)	2 (33)	2 (22)	1 (14)	2 (33)	8 (38)	27 (27)
IV	12 (40)	5 (24)	3 (50)	0 (0)	3 (43)	1 (17)	13 (62)	37 (37)
cerebral oedema (%)	10 (33.3)	2 (10)	2 (33)	0 (0)	2 (29)	1 (17)	10 (48)	27 (27)

apache 111, INR, AST, bilirubin expressed as mean (standard deviation)
encephalopathy grade and cerebral oedema are expressed as totals (%)

Other differences between the groups were estimated by the chi-square test. Those parameters significantly different by univariate analysis (Mann-Whitney U test) between survivor and non-survivors at two months were entered into a stepwise logistic regression model stratified for the two groups in the prediction of early [two-month] survival. Reasons for graft and patient loss were determined following combined clinical and pathological assessment with post-mortem reports available for all deaths. Results are expressed as median or mean (standard deviation) [SD] where appropriate.

7.3 RESULTS

7.3.1 Pretransplant Characteristics

A significant deterioration in the majority of parameters was noted during the interval from admission to the time of transplantation (table 7.2). At transplantation, the median prothrombin time was greater than 100 seconds [INR 6.5] [SD 4.2]) and serum bilirubin 411 [223] umol (group 1: prothrombin time 62 s [INR 4.15], bilirubin 474 umol; group 2: prothrombin time 122s [INR 8.2], bilirubin 109 umol). All patients had experienced hepatic encephalopathy with approximately two thirds of patients (64%) in either grade III or IV hepatic coma and a clinical episode of cerebral oedema occurring in 27%; those patients with paracetamol hepatotoxicity had significantly worse grades of encephalopathy and rates of cerebral oedema (table 7.1). At transplantation, the mean apache 111 score was 55 [24] with 32 patients requiring renal replacement therapy. In group 1, the mean periods [hours (range)] from admission and listing to transplantation were 132 (9-264) and 65 (7-216) hours respectively. In group 2, the median period from overdose and liver failure unit admission to transplantation / hepatectomy was 4 days (range 3-8 days) and 40 hours (range 14-144 hours) respectively. Over the 8 year study period, an increase in the median apache 111 scores at the time of transplantation was detected (figure 7.1).

7.3.2 Patient and graft outcome

Two month actuarial patient survival rates in the whole series was 67% (66% in group 1 [non-paracetamol] and 71% in group 2 [paracetamol]). Within those patients experiencing FHF who were selected for transplantation on the basis of the Kings College Hospital criteria for a poor prognosis since 1988, 38 of 58 (66%) were alive at two months. In patients with non-paracetamol FHF, a significant variation in patient survival in relation to aetiology was noted (figure 7.2). All patients transplanted for FWD were alive at two months in contrast to those with idiosyncratic drug reactions in whom only 1 from 7 survived. For the other aetiological categories, no significant variations in outcome were seen. In patients with NANB / indeterminate FHF or LOHF, the two-month patient survival rate was 69% with no significant difference in outcome between fulminant or late - onset presentations (67% and 71% respectively). Retransplantation was required in 9 patients within the first 2 months, with only 2 of these patients surviving long-term. Reasons for graft and patient loss were determined following combined clinicopathological assessment with explant postmortem reports available for all cases. The indications for retransplantation (confirmed by explant pathological examination) were massive haemorrhagic necrosis [2], arterial thrombosis / graft size disparity [3], fungal graft sepsis [2], and intractable acute cellular rejection [2]. The largest proportion of deaths were due to systemic sepsis developing as a primary complication (47%). This complication resulted in death at a median of 13 days post-transplant. Additionally, a number of other patients with other complications died with infection as a secondary terminal event. This complication usually precluded retransplantation with the exception of 2 patients who had isolated fungal graft sepsis (see above).

Table 7.2 ***Clinical parameters on admission and at transplantation in both groups: (non-paracetamol liver failure [group 1] and severe paracetamol hepatotoxicity [group 2]). Deterioration in a range of important indices (particularly encephalopathy and multisystem illness) was apparent during this variable interval.***

	Group 1 (n=79)		Group 2 (n=21)	
variable	admission mean (SD)	transplantation mean (SD)	admission mean (SD)	transplantation mean (SD)
bilirubin (μmol)	443 (190)	485 (193)**	104 (13.9)	146 (19)*
(mg/dl)	26.1 (11.2)	28.5 (11.4)	6.1 (0.8)	8.6 (1.1)
AST (IU/L)	518 (582)	470 (809)	3214 (1892)	2034 (2057)
prothrombin time (INR)	4.6 (3.4)	6.0 (4.1)***	8.1 (3.3)	8.2 (4.0)
albumin (mg/dl)	3.0 (0.6)	3.3 (0.7)**	3.8 (0.8)	4.0 (0.7)
encephalopathy grade^	1	3***	1	4***
cerebral oedema (%)	4.2	22**	9.5	43***
creatinine (μmol)	131 (88)	153 (122)	272 (148)	349 (176)
mg/dl	1.75 (1.17)	2.04 (1.63)	3.62 (1.97)	4.65 (2.35)
WCC (x109)	12.4 (7.0)	12.4 (6.2)	17.2 (6.6)	10.1 (5.5)***
platelets (x 109)	201 (132)	160 (162)***	154 (98)	68 (52)***
organ system failures^	0	1***	0	2**
apache 111	38 (18)	50 (22)***	49 (23)	74 (21)***
arterial pH	–	–	7.28 (0.14)	7.35 (0.10)

^ encephalopathy grade and OSF expressed as median

* p <0.05, ** p<0.01, *** p<0.001

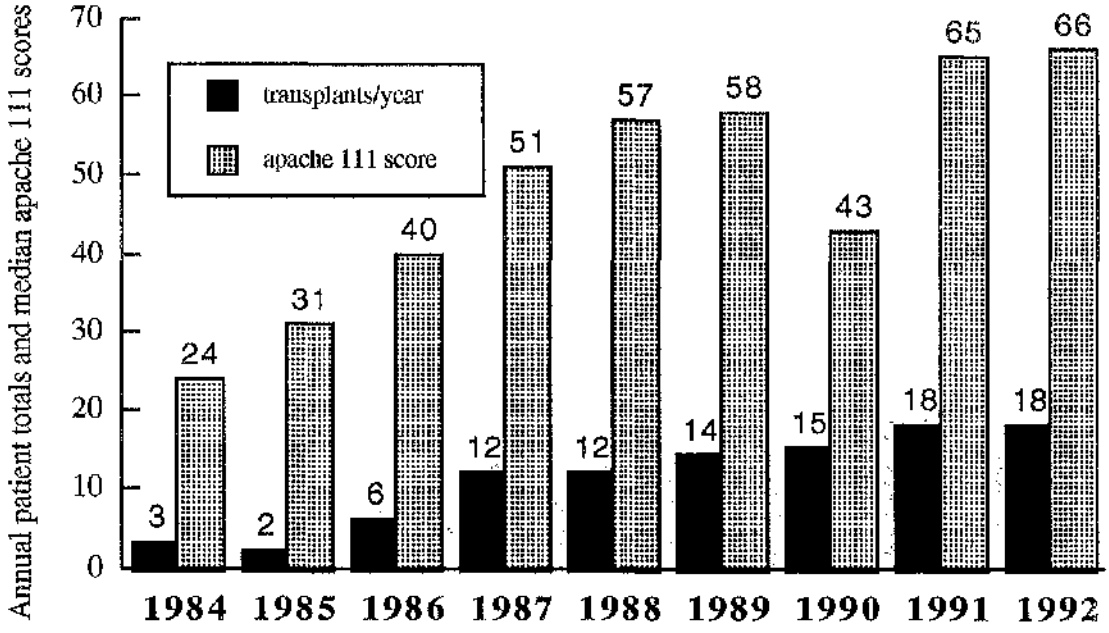


Figure 7.1 Distribution of the study population of the 100 recipients transplanted for acute liver failure with regard to year of transplantation (March 1984 to September 1992). The median apache 111 score at the time of transplantation is also shown. It is apparent that these are tending to rise annually probably reflecting the increased use of emergency transplantation in severe paracetamol hepatotoxicity and lengthening delays in organ allocation.

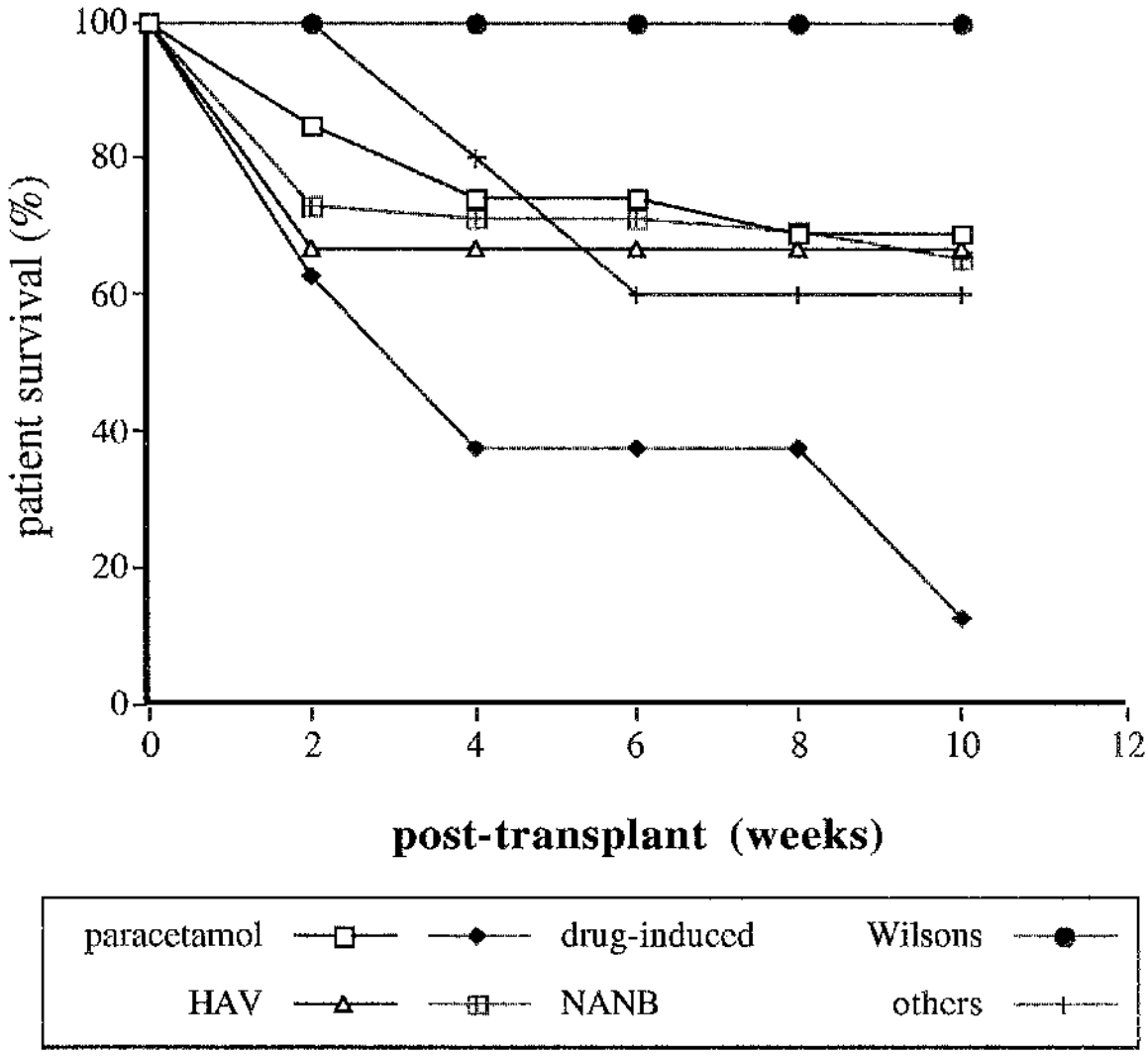
Fungal infection was detected in 8 patients and was associated with death in 7. Graft loss from rejection was found in 12 patients. Possible disease recurrence from viral graft reinfection (Fagan et al 1992) in 8 cases of NANB hepatitis was noted and usually led to death within the first 2 weeks post-transplant. Miscellaneous events leading to early 2 - month mortality were neurological complications in three patients (cerebral oedema/infarction [2], intracerebral haematoma [1]), GVHD [2], PGN [1], aortic dissection [1], myocardial infarction [1] and a gastrointestinal bleed [1].

7.3.3 Pre-transplant risk factors in early hospital mortality

In non-paracetamol-induced liver failure, no *static* variables other than aetiology (FWD) and idiosyncratic drug reactions as described above were significantly related to 2-month survival. Of the *dynamic* variables examined *on admission* to the Unit, the serum creatinine (2.1 [1.6] mg/dl non-survivors vs 1.4 [0.6] mg/dl survivors, p 0.02) was the only parameter significantly different in a univariate analysis. In contrast, by the time of *transplantation*, in addition to serum creatinine which remained significantly elevated in non-survivors, the two intensive care indices of systemic critical illness (organ system failure and apache III score) also discriminated survivors from non-survivors (table 7.3). When these significant parameters were entered into a stepwise logistic regression model to predict 2-month mortality, serum creatinine at the time of transplantation was the only independent variable (r=0.33). A serum creatinine >2.7 mg/dl (200 μmol) at transplantation had a 42% sensitivity and a 92% specificity for 2-month mortality.

In paracetamol-induced liver failure, the 'time from ingestion to transplantation / hepatectomy' was the only *static* variable significantly different between survivors 4 [1] day vs non-survivors 6 [1] days, p<0.01). No patient transplanted at day 7 or greater from the time of drug ingestion survived to two months.

Figure 7.2 ***Early actuarial patient survival following transplantation in the various aetiological liver failure indications. With the exception of idiosyncratic drug reactions and fulminant Wilson's disease (filled symbols), the outcome was independent of aetiology.***



The 'time from admission to transplantation / hepatectomy' also approached statistical significance with survivors waiting for a graft on average less than non-survivors, 41 [14] hours vs 67 [52] hours (p 0.09). With respect to *dynamic* variables on admission, no parameter examined could significantly distinguish survivors from non-survivors. However by the time of transplantation, total serum bilirubin and apache 111 score were significantly different in survivors vs non-survivors (table 7.3). Significant correlations between serum bilirubin and 'time from overdose to transplantation / hepatectomy' existed (r=0.56) with a lesser association between the bilirubin level and apache 111 score (r=0.29). In a stepwise logistic regression of 2 month survival with the significant variables above entered, the apache 111 score at the time of transplantation was the only independent determinant (r=0.51). If one patient who died from a technical complication (severe post-operative bleeding) on day 3 was excluded from the analysis, arterial pH would have been the strongest parameter by a significant margin. In a univariate analysis, the mean (median) pH at transplantation in non-survivors was 7.21 (7.23) vs 7.38 (7.37) in survivors ($p<0.001$). A pH below 7.30 at transplantation had a sensitivity of 80% and a specificity of 94% for 2-month hospital mortality.

With respect to hepatic encephalopathy at the time of transplantation (whole population), a trend towards a reduction in patient survival was seen with increasing coma grade. The 2 - month survival rates were 80% in patients transplanted in grade 1, 81% in grade 2, 63% in grade 3 and 59% in grade 4. The prognosis for patients transplanted in grade IV coma is significantly worse than those in grade I in a univariate analysis ($p<0.05$). In those patients who experienced a clinical episode of cerebral oedema (usually with grade 4 coma), the 2-month patient survival rate was 68%.

Table 7.3 Summary of clinical and biochemical data in group 1 and 2 for survivors and non-survivors at 2 months post-transplant calculated at the time of transplantation.

variable	Group 1		Group 2	
	survivors (n=52) mean (SD)	nonsurvivors (n=27) mean (SD)	survivors (n=16) mean (SD)	nonsurvivors (n=5) mean (SD)
bilirubin (µmol)	504 (192)	443 (181)	121 (47)	233 (126)**
(mg/dl)	29.6 (11.3)	26.1 (10.6)	7.1 (2.8)	13.7 (7.4)
AST (IU/L)	418 (354)	654 (1315)	1782 (1593)	2791 (3292)
prothrombin time (INR)	5.8 (3.8)	6.7 (4.8)	8.9 (4.6)	7.9 (2.5)
albumin (mg/dl)	3.2 (0.7)	3.4 (0.7)	4.5 (0.9)	3.8 (0.6)
encephalopathy grade^	2	3*	4	3
cerebral oedema	23%	26%	44%	40%
creatinine (µmol)	117 (81)	190 (147)*	333 (167)	402 (214)
(mg/dl)	1.56 (1.08)	2.53 (1.96)	4.44 (2.22)	5.36 (2.85)
WCC (x109)	11.6 (5.5)	12.0 (5.8)	9.1 (5.3)	13.1 (5.7)
platelets (x 109)	155 (116)	153 (117)	67 (36)	74 (94)
OSF	0.5	1*	2	2
apache 111	48 (20)	58 (23)*	68 (19)	92 (16)*
arterial pH	–	–	7.36 (0.09)	7.26 (0.13)

^ encephalopathy grade and OSF expressed as median

* p <0.05, ** p<0.01

7.4 DISCUSSION

Pretransplant clinical status has a major impact on the outcome of transplantation in acute liver failure. Given that the selection of recipients, at least in this centre, is based on identification of the most severely ill (often greater than 90% expected mortality) it is not surprising that markedly deranged systemic and liver parameters influence the outcome. Several important observations emerge from this large study explaining the inferior survival figures. Graft loss and death in this series of transplanted ALF patients were predominately due to the development of systemic sepsis. This susceptibility to infection, present in patients with ALF from an early stage, is continued and possibly initially aggravated in the early post-transplant period with the introduction of immunosuppression regimens which include high dose corticosteroids (Kusne et al 1988, Canalese et al 1982, Rolando et al 1990). In addition, conventional strict criteria applied to graft assessment may through necessity be set aside in the emergency transplant situation when there is a desperate need for an organ. Early graft dysfunction following use of inferior organs is likely to further predispose to sepsis. The high incidence of fungal infection post - transplant associated with death is in keeping with previous findings from this unit of the frequency of this complication in patients with ALF managed conservatively (Rolando et al 1991). Antifungal prophylaxis regimens, although never tested at clinical trial, have been incorporated clinically in the later series at this unit.

Patients with ALF are usually so critically ill that they are unable to undergo the normal rigorous pretransplant assessment of cardiorespiratory status which may explain why normally avoidable postoperative complications ensued with one patient dying from myocardial infarction, in the presence of coronary artery disease, and another from aortic dissection (present at the time of transplantation). Possible disease recurrence may also

have been a significant cause of early graft failure in patients transplanted with fulminant NANB hepatitis (Fagan et al 1992). Graft dysfunction and sepsis presenting at the end of the first week with a rapid deterioration to graft failure and characterised by 'massive haemorrhagic necrosis' on histological examination may represent recurrence (Hubscher et al 1989). Viral particles were demonstrable in the explanted graft in a proportion of cases in the study by Fagan. The low mortality rate related to cerebral complications including cerebral oedema was encouraging and may reflect both the patient selection policy and peri-operative monitoring with intra-cranial pressure transducers in high-risk patients adopted at this centre (Potter et al 1989). The results of retransplantation were poor when carried out within the first two months post-transplant, probably reflecting the patients very poor clinical condition at that time.

With respect to identification of pretransplant clinical parameters which may predict post-transplant outcome, surprisingly, clinical characteristics of the primary liver disease were poor discriminators of survival. The various aetiological categories had similar outcomes with the exception of idiosyncratic drug reactions (poor outcome) and FWD (100% survival). Liver failure from idiosyncratic drug reactions managed conservatively is similarly associated with a high mortality, a phenomenon which is presently not explained (O'Grady et al 1988c). The excellent results achievable in FWD recipients confirms the suitability of these patients for emergency transplantation and is probably a reflection of their less rapid downhill clinical course. Additionally, these patients may benefit from usually being fit and young and a decision to list can be taken as soon as the diagnosis is made reflecting the dismal outcome of this condition if managed conservatively (Sternleib et al 1984). In relation to the two largest aetiological groups, no significant difference in patient survival between NANB / indeterminate liver failure and paracetamol hepatotoxicity was detected despite this latter group having the worst indices of both liver

damage and systemic critical illness (apache 111 scores). The similar outcomes may reflect the use of a prognostic scoring system which identifies patients in these groups with a similarly poor prognosis - approximately 10% survival - if managed conservatively (although the criteria are different) and whose prognosis might be expected to improve equally following transplantation. The 2 month patient survival rate of approximately 68% in patients with FHF who were selected for transplantation on the basis of these criteria represents a considerable improvement on that achievable with medical management.

No relationship between outcome and the year the transplant was carried out (8 year study period) was noted. This initially surprising result is in line with the recent plateauing of outcome, as yet not adequately explained, reported for all recipients by the large transplant registries as previously referred to in section 1.1 (Belle and Detre 1993, ELTR et al 1992). The apparent lack of improvement in overall results in these patients is probably best explained by the significant increases in apache 111 scores over time i.e. sicker patients, as indicated by multisystem illness, are being transplanted. An increasing use of sub-optimal organs is also likely to prevent further improvements in outcome in this particular clinical population where the rapid restoration of adequate hepatic function is so crucial.

In the non-paracetamol liver failure group, the critical illness scores at the time of transplantation and the presence of renal dysfunction were the most significant parameters correlating with outcome. Both renal impairment and development of systemic sepsis (an association recognised in both acute and chronic liver disease), are known to be associated with an inferior outcome in patients experiencing FHF managed conservatively (O'Grady et al 1988c, Bismuth et al 1987). It would appear that transplantation does not entirely remove this deleterious effect with these results supporting previous reports which have

found that renal dysfunction is an important pretransplant clinical variable in determining outcome after transplant for cirrhosis (Cuervas-Mons et al 1986a, Arroyo et al 1993). In terms of clinical decision-making, the serum creatinine / renal impairment identifies a sub - group of liver failure patients whose prognosis would appear to be only moderately improved by transplantation.

In patients with paracetamol hepatotoxicity, the effects of the multi-system illness (as reflected in the highest apache 111 scores in the study) was a more important factor in outcome than the severity of the liver failure which indeed is often improving at the time of transplantation. The prognostic significance of high apache 111 score reflects the widespread effects of severe liver failure. Determination of the apache 111 score, as part of the clinical review at the time of proposed transplantation, would appear to allow a subjective appraisal of post-transplant outcome. Although critical illness scoring systems are not widely applied in liver failure / transplant programmes, adoption of these scores in general intensive care units has considerably improved the accuracy of clinical judgements on likely patient hospital mortality. Serum bilirubin levels, which rise with time after paracetamol overdose in the severe cases considered for transplantation, were also associated with a poor outcome and predictably strongly correlated with the period from overdose to hepatectomy / transplantation. Prolongation of this interval leading to a greater opportunity to develop serious complications not surprisingly led to an inferior outcome and reinforces the superurgent requirement for early graft allocation. The indicative value of arterial pH in this group is not surprising as the onset of acidosis in multisystem failure (not explained by other causes) reflects critically low tissue oxygen extraction / tissue hypoxia, a marker of severe often preterminal illness.

This study has focused selectively on the role of pretransplant parameters which are

probably the most important variables influencing outcome where the patients are so critically ill at the time of transplantation. However, other variables such as donor organ quality, ischaemia times, intra-operative events and control of rejection all contribute to eventual outcome. Excellent early graft function can be expected to reduce the influence of the pretransplant parameters described here.

It is apparent from this data that the final decision to proceed to transplantation, when a donor liver becomes available, needs to be a separate decision from the initial selection of potential candidates, a policy previously expressed by the French group who recently evaluated the Kings criteria for FHF (Pauwels et al 1993). With greater delays in organ availability likely, definition of risk factors at the time of transplantation may permit a more informed basis for deciding which patients are too ill to have a successful outcome. In the initial report on liver transplantation for paracetamol hepatotoxicity from this unit, the authors described the 'window period' of opportunity for a successful outcome which this study emphasises and shows can be applied to other acute liver failure aetiologies (O'Grady et al 1991).

CHAPTER 8

TACROLIMUS (FK506) IMMUNOSUPPRESSION MODIFIES REJECTION AND SYSTEMIC COMPLICATIONS FOLLOWING TRANSPLANTATION FOR FULMINANT HEPATIC FAILURE

8.1 BACKGROUND AND INTRODUCTION

The outcome of liver transplantation when carried out for FHF remains inferior to elective recipients as described in the previous chapter and previously reported (Belle et al 1991, see previous references). Selection of patients with liver failure for transplantation is on the basis of biochemical or clinical prognostic criteria which indicate an extremely low probability of survival if treated medically (Bernuau et al 1991, O'Grady et al 1989). As shown in chapter 7, these critically ill patients are at high risk of bacterial and fungal infection despite prophylactic antimicrobial therapy and close infection surveillance (Rolando et al 1990, 1991). Patient loss in these high-risk patients in the early postoperative period is often related to the development of severe systemic sepsis and multiorgan failure (Quiroga et al 1991). The risk of these complications in all liver recipients, but particularly in those patients transplanted for FHF, is probably modified by the immunosuppression regimens used (Ascher et al 1988, Kusne et al 1988). Conventional regimens based upon CyA and azathioprine include administration of high-dose corticosteroid dosage schedules in the early postoperative period. Patients experiencing liver failure should be considered to be immunodeficient. Accordingly, they are at particular risk from the non-specific immunological effects mediated by high dose corticosteroid schedules which constitute a major component of conventional induction immunosuppression regimens in the event of a rejection episode (Cupps et al 1982).

Tacrolimus, a potent immunosuppressant agent recently the subject of controlled clinical trials, has been proposed to allow withdrawal of azathioprine and considerably reduce maintenance and supplemental corticosteroid schedules through improved control of rejection. These modifications in immunosuppression may represent major advances in post-transplant care with the early uncontrolled clinical trials of tacrolimus based regimens demonstrating both reductions in sepsis and improved patient and graft survival (Starzl et al 1989). The advantages of such a regimen may be most readily appreciated in the high risk patients transplanted for liver failure.

In this investigation, the outcome of the patients transplanted for FHF who were entered into the controlled, European multicentre trial of tacrolimus versus a conventional CyA - based immunosuppression regimen (CBIR) from this centre are analysed in relation to the clinical course of the early post-transplant period. To put these patients in context, the efficacy data of all 50 recipients entered from the participating centres is initially reported (unpublished data used with permission of Fujisawa GMBH).

8.2 SUBJECTS

8.2.1 Patient population

50 liver recipients transplanted for FHF (as defined by Trey and Davidson) and who were entered into a multicentre, open, prospectively randomised, parallel-group study comparing primary tacrolimus immunosuppression (n=22) to a CBIR (n=28) in liver transplantation were studied. Randomisation was performed separately in this sub-group before surgery using a 1:1 randomisation schedule. Data is presented on a minimum follow-up for six months. 17 patients (34%) from this total number were entered in this trial from this unit and have been examined in considerably more detail with respect to efficacy and safety.

8.2.2 Immunosuppression protocols

In the first part of the trial, the initial intravenous (IV) dose of tacrolimus of 0.075 mg/kg (four hour infusion) was administered within six hours after closure of the abdominal wall. This dose was repeated every 12 hours for three days and followed by oral tacrolimus therapy at a dose of 0.30 mg/kg day in two divided doses. Subsequently, this regimen was modified to 0.03-0.05 mg/kg (12 hour infusion) repeated every 12 hours for three days with the same oral dosing schedule. Dose modifications on the basis of graft function, efficacy, drug monitoring and drug-related toxicity were performed. Plasma levels in the first phase of the trial were maintained initially between 1-2 ng/ml with later trial protocol dose reductions allowing levels below this range based on clinical parameters (assay method: Sep-pak, room temperature separation, ELISA). Corticosteroid administration in this treatment group was standardised with initial doses of prednisolone (or equivalent) commenced at 20 mg/day with reductions as clinically indicated. Azathioprine was not routinely administered in this group except if desired during tacrolimus interruption or withdrawal.

The CyA based regimens were centre-specific, with each programme maintaining their preferred optimal immunosuppression regimen at the time of protocol design (1989) throughout the study period. All induction maintenance regimens included CyA (2-6 mg/kg day), azathioprine (1-3 mg/kg day) and corticosteroids (0.2-10 mg/kg day) with anti-lymphocyte globulin administered in induction schedules additionally in three centres. In this centre, this regimen in the early post-operative period comprised a triple therapy schedule of CyA (target whole blood levels 120-150 ng/ml, monoclonal RIA, *Incstar*), azathioprine (dosage:1 mg/kg/day) and prednisolone 60 mg/day. Maintenance immunosuppression was similarly based, in the absence of drug-related complications, on a triple therapy regimen with CyA levels maintained between 100-150 ng/ml and prednisolone dosage at 0.08-0.10 mg/kg/day.

Supplemental anti-rejection protocols were similarly centre-specific with this regimen in this centre comprising an initial course of hydrocortisone 1 g twice daily IV for two days followed by 1 g once daily for three days. Rejection episodes resistant to this regimen were treated with an additional course of methylprednisolone 1 g once daily IV for three days.

8.2.3 Efficacy and safety parameters

Patient and graft survival was analysed using Kaplan-Meier methods and the survival times compared between the treatment groups using the generalised Wilcoxon test. As a large proportion of clinical events were in the early post-transplant period this test was employed which is more sensitive for early events in life-table analysis. Patients who died or underwent retransplantation were defined as having graft failure. The same conventions were employed for the time to first rejection / intractable rejection with distribution of events between the study groups compared with the Cochran-Mantel-Haenszel procedure. Protocol liver biopsies were performed on day 7 and whenever a rejection episode was clinically or biochemically suspected. In the detailed analysis of patients from this unit, outcome of the study population was determined by measurement of routine clinical and biochemical parameters, in addition to prognostic scoring systems (apache 111 and therapeutic intervention scoring system [TISS]) used in the assessment of critically ill patients (Knaus et al 1989, Knaus et al 1991). These results are expressed as median [mean] and compared using chi-squared methods or Fishers exact test where appropriate.

8.3 RESULTS

8.3.1 Patient and graft survival

From the complete series of fifty (50) patients, survival at six months in the tacrolimus recipients was 72.7% compared to 60.7% in the CBIR treated patients (ns). A consistent trend towards improved patient survival was detected in favour of tacrolimus treated patients with this pattern apparent from week 1 (95.5% versus 82.1%) (figure 8.1). A similar benefit was also noted for primary graft survival in the tacrolimus group with a survival at six months of 72.7 % compared to 53.6% in the CBIR group (ns).

8.3.2. Rejection and graft function

A significant reduction in the cumulative rate of a first acute rejection episode was demonstrated in the tacrolimus group compared to the CBIR patients at one week (19.3% versus 59.1%) with considerably lower rejection frequency in the early post-operative period (figure 8.2). Similarly, there was a marked reduction in the intractable rejection rate between the groups. The intractable rejection rate of 22.6% in the CBIR group by six months with a rate of 6.2% in the tacrolimus treated patients (table 8.1). Notably, the rates of rejection and in particular intractable episodes were considerably higher in fulminant patients than elective recipients irrespective of immunosuppression, although these rates were always lower in the tacrolimus treated patients.

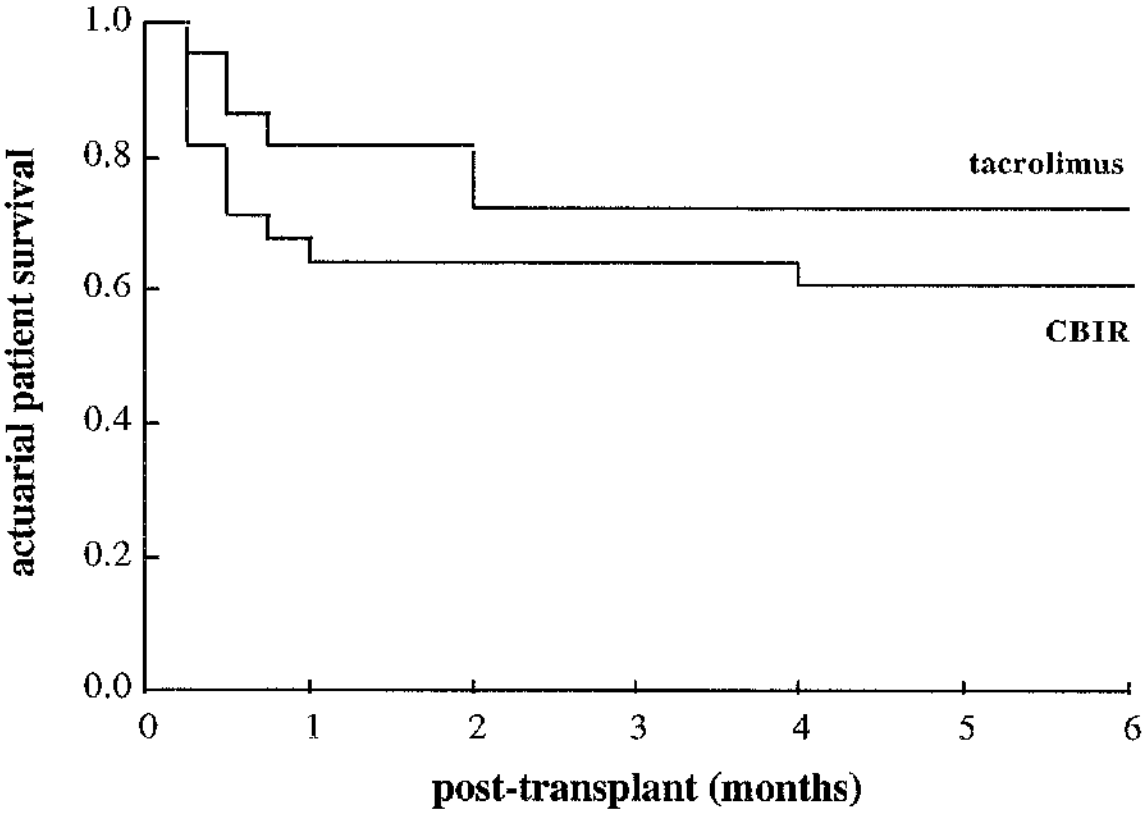


Figure 8.1 Cumulative actuarial patient survival to 6 months following transplantation in the patients transplanted for fulminant hepatic failure who were administered either a tacrolimus (n=22) or CyA (n=28) based immunosuppression regimen (CBIR).

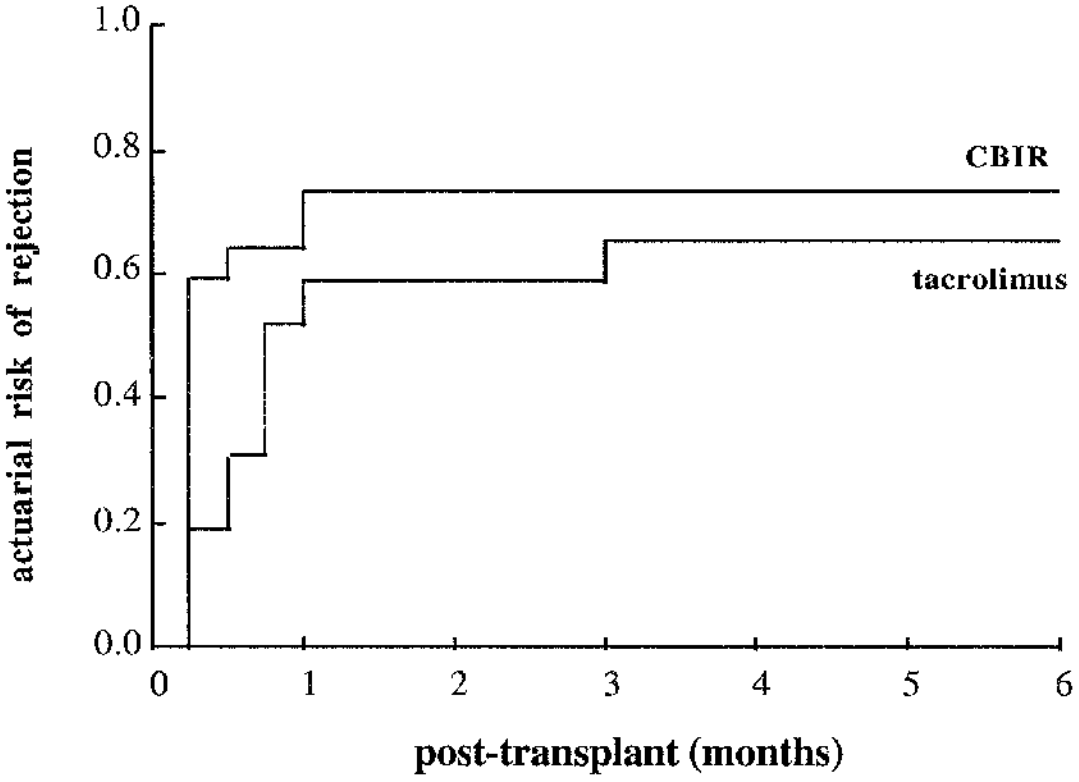


Figure 8.2 Lower cumulative risk of acute allograft rejection in those patients administered tacrolimus compared to conventional CyA based regimens (CBIR) following transplantation for fulminant hepatic failure.

Table 8.1 ***Cumulative incidence of acute (including intractable) rejection rates at 6 months in both emergency and elective patient groups in the 2 immunosuppression groups***

		Tacrolimus (%)	CBIR (%)
recipients	*rejection*		
FHF (n=50)	acute	65.5	73.2
	intractable	6.2	22.6
Elective (n=490)	acute	40.5	53.7
	intractable	3.1	9.4
All (n=540)	acute	42.0	54.7
	intractable	2.9	10.1

8.3.3. Kings College Hospital Experience

The clinical characteristics of the two treatment limbs of the study population were not significantly different in the treatment groups with a similar pretransplant status and donor organ characteristics (table 8.2). All donors and recipients were ABO compatible. The median grade of encephalopathy in the tacrolimus (2.5) and the CyA recipients (3) was similar, with evidence of cerebral oedema present in 2 of 8 and 3 of 9 patients respectively. Renal replacement therapy was required in 3 of 8 of the tacrolimus recipients and 4 of 9 CyA recipients prior to transplantation. The actuarial one-year patient and graft survival at six months post-transplant or at retransplantation was 87% (7 of 8) in the tacrolimus group and 53% (5 of 9) in our CyA treated patients (ns). Current follow-up of the population reveals an actuarial patient survival of 75% (6 of 8) in the tacrolimus and 44% (4 of 9) in the CyA group (ns). The causes of the two deaths in the tacrolimus recipients was segmental hepatic infarction leading to retransplantation and subsequent sepsis / multi-system organ failure (MOF) and primary sepsis / MOF. In the CyA treated patients, patient loss (n=5) was due to graft versus host disease / MOF, chronic rejection requiring retransplantation and subsequent development of haemorrhagic pancreatitis / MOF and primary sepsis / MOF (3 cases).

Apache 111 and TISS scores were lower in the tacrolimus recipients particularly in the first post-transplant week and 5 of 8 (63%) tacrolimus-treated patients were discharged from the ICU by the end of the first week compared to 3 of 9 (33%) of the CyA-treated recipients (table 8.3). The median period to self ventilation in survivors was 3 days in the tacrolimus group and 5 days in the CyA recipients (ns). Early graft function was superior in the tacrolimus limb as assessed by routine clinical measurements of serum levels of aspartate aminotransferase (AST) and bilirubin (table 8.4).

Table 8.2 ***Clinical and demographic data of the 17 patients entered into the European tacrolimus primary liver transplant study from this unit prior to transplantation with donor organ ischaemia times and intra-operative blood loss.***

	Tacrolimus (n=8)	**Cyclosporin A** (n=9)
age (yr)		
median	34	35
range	18-53	17-64
sex		
M|F	2\6	3\6
aetiology		
paracetamol	2	2
nonA-nonB	3	2
hepatitis A/B		4
wilsons	2	
drug	1	1
apache 111		
median	53	62
range	19-91	19-78
infection		
suspected	3\8	4\9
confirmed	3\8	2\9
donor organ		
total ischaemia time (hrs)	12.6	9.5
surgery		
intra-operative blood loss (mls)	2617	3250

Table 8.3 Critically ill prognostic scores (Apache 111 and TISS) in the early post-transplant period in the Kings College Hospital patient series.

	APACHE 111			TISS		
post-Tx day	cyclosporin A	tacrolimus	p value	cyclosporin A	tacrolimus	p value
1	71	58	ns	37	35.5	ns
3	67	55	0.05	37	28	0.02
7	53	45	0.08	14	7.5	ns
14	46	40	ns	8.5	4	N/A

In the tacrolimus treated patients, levels of AST were significantly reduced in the first week following transplantation in comparison to those on CyA with the largest difference between the regimens noted on the seventh postoperative day (78 vs 326 IU/l, $p<0.05$). Similarly, the serum bilirubin did not rise significantly in the tacrolimus patients in comparison to the CyA ones with levels lower in tacrolimus recipients throughout the early post-transplant period (e.g. post-operative day 7: 188 vs 352 μmol, $p<0.05$). Reflecting the clinical parameters above, the median intensive care unit admission (either discharge or death) was lower in the tacrolimus group compared to the CBIR group (median period: 6 vs 11 days).

Thirteen episodes of acute rejection proven histologically occurred in the CyA recipients with all patients experiencing at least one episode (1.38 episodes per patient). Control of rejection was achieved following one cycle of supplemental corticosteroids in nine episodes with a further four episodes requiring an additional cycle of steroids. Two patients developed intractable rejection and were switched to rescue therapy with tacrolimus. In one, rejection was successfully reversed with the other patient requiring retransplantation. In the tacrolimus limb, only four episodes of rejection were documented all of which were reversed by one cycle of supplemental corticosteroids in four patients (0.5 episodes per patient) (p 0.02). The total steroid requirements in the first thirty days following transplantation (prednisolone equivalent dose/day graft survival) was 150 mg/day in the CyA treated patients as compared with 69 mg/day in those administered tacrolimus (p 0.01).

A higher incidence of infection episodes in the CyA limb (2 [2.11 per patient]) compared to the tacrolimus limb (1 [0.88 per patient]) ($p <0.01$) was noted with sepsis a major contributing factor to all deaths in the study population. The majority of these infection episodes were either of confirmed or suspected bacterial sepsis with the number of viral infections insufficient for analysis.

Table 8.4 Early graft function as assessed by serial liver function tests (serum bilirubin and amino aspartate transferase [AST]) in the first month following transplantation.

	AST (IU/L)			Bilirubin (μmol)		
day	CyA	Tacrolimus	p	CyA	Tacrolimus	p
3	303 (106)	152 (40)	<0.05	193 (31)	184 (64)	ns
7	326 (100)	78 (23)	<0.05	352 (64)	188 (48)	<0.05
14	105 (17)	70 (31)	ns	357 (60)	182 (77)	<0.05
28	119 (43)	74 (20)	ns	70 (10)	35 (7)	<0.05

Two patients in the CyA limb additionally developed systemic / cerebral nocardiosis at 6 and 8 months post-transplant. There were no protozoal infections in the tacrolimus-treated group.

Major neurological complications were infrequent in this study population despite the severity of pre-transplant hepatic encephalopathy. There were no episodes of seizures in either group with one patient in the tacrolimus group experiencing a possible episode of central nervous system toxicity (aphasia persisting until the second post-transplant week). No significant psychiatric or confusional episodes were seen. Renal impairment was common in these patients with dialysis required in 3 of 8 tacrolimus recipients and 4 of 9 CyA recipients (the same patients who required pretransplant dialysis) with calculated creatinine clearance rates similar between the groups. The clearance rates were 53 ml/min at seven days, 70 ml/min at three months and 82 ml/min at 12 months in the tacrolimus group with the rates of 58 ml/min, 81 ml/min and 85 ml/min in the CyA group respectively (ns).

8.4 DISCUSSION

This present study demonstrates the potential benefits available with a tacrolimus-based immunosuppression regimen in those high-risk recipients transplanted for FHF compared to conventional schedules. In tacrolimus recipients, an improvement in a range of standard clinical and biochemical parameters in relation to both graft function and systemic illness was demonstrated. Easier control of allograft rejection and a reduction in the prevalence of severe systemic sepsis are the main explanations for the superior graft function and outcome in the tacrolimus recipients. The potent immunosuppression available with this agent permitted a substantial reduction in total steroid requirements which will have been a

major contributing factor to the reduction in systemic sepsis. The reduction in steroid exposure is mainly derived from a decrease in the frequency of rejection and hence high - dose supplemental corticosteroid schedules (which in this centre comprise methylprednisolone 1g daily for three days).

Liver function can be severely affected by systemic sepsis with the development of a prolonged cholestatic syndrome, and it is therefore interesting to note the lower level of bilirubinaemia in the tacrolimus-treated compared to the CyA-treated patients (Carima et al 1982). Other possible explanations for the differences in graft function may be the hepatotoxic and hepatotropic profiles of these major immunosuppressants. CyA has well - documented cholestasis effects, a phenomenon not reported under tacrolimus (Kahan et al 1987). This latter agent also appears to have significant hepatotropic properties with an early report suggesting that this may be considerably superior to CyA (Francavilla et al 1991).

Excellent long-term graft function in those recipients immunosuppressed with tacrolimus monotherapy supports the withdrawal of corticosteroids and azathioprine from maintenance regimens and thereby removing the serious long-term complications of these agents. There would not appear to be a major hazard to this undertaking in patients transplanted for FHF in whom there is no evidence that primary disease recurrence would be promoted by removal of these agents. One notable exception to this principle would be the small number of patients transplanated as an emergency for a fulminant presentation of AIH. Disease recurrence in this group may be reduced by continued immunosuppression with steroids and azathioprine (see section 11.5.1). The attendant hazards from long-term azathioprine administration, particularly in relation to hepatotoxicity, which are avoided with a tacrolimus monotherapy schedule were discussed in chapter 2. It is not clear whether tacrolimus has any advantage over a CyA-regimen when it comes to a steroid - sparing effect. Experience from the programme in Birmingham, UK, would appear to indicate that early steroid withdrawal may be possible in both treatment schedules without

a deterioration in graft function (Dmitrewski et al 1994).

The absence of any major neurological complications or long-term renal impairment in these patients was reassuring. These patients who were all encephalopathic and had a high prevalence of renal dysfunction were at particular risk from immunosuppressant related neurotoxicity and / or nephrotoxicity. In this present study, no significant difference in the toxicity profile of the major immunosuppressants was detected.

As a separate observation, it is interesting that the rates of rejection (including intractable) were higher in the patients in this population of FHF transplanted patients compared to elective recipients emphasising the requirement for potent immunosuppression in this group. The mechanisms underlying this phenomenon require to be determined.

The importance of immunosuppression in affecting outcome in the early post-operative period is probably best examined in high-risk patients where the benefits and pitfalls of regimens are amplified and readily detectable. This may explain why the benefits of tacrolimus immunosuppression are more obvious in these patients with ALF than in the elective recipients where the benefit in survival is less than 10% at one year. An improved control of rejection should deliver not only reduced immunological graft failures (which is dramatic with tacrolimus) but also reduced deaths from sepsis which this separate analysis would tend to support. The particular trial from which the study population was entered suffered from a number of major design deficiencies which included major positive and negative biases to the trial agent. Centres had the option of converting patients experiencing intractable acute and chronic allograft rejection from CyA to tacrolimus which inevitiably artifically supported the survival figures in the control limb. Additionally, the influence of the major dose reduction in the tacrolimus schedule may significantly alter the efficacy and toxicity profile.

CHAPTER 9

IMMUNOPROPHYLAXIS AND IMPROVED PATIENT SELECTION REDUCES DISEASE RECURRENCE FOLLOWING ELECTIVE TRANSPLANTATION FOR HEPATITIS B SURFACE-ANTIGEN POSITIVE PATIENTS

9.1 BACKGROUND AND INTRODUCTION

Disease recurrence following transplantation was identified in chapter 2 as a major cause of graft damage and loss. In particular, the recurrence of hepatic malignancies and of primary viral transplant indications were most problematic. Improvements in patient selection for malignancy with exclusion of cholangiocarcinomas and large multifocal HCC has reduced the recurrence rate in this indication. Alongside these improvements, increased awareness of the predisposing features for recurrence of HBV infection following transplantation and the clinical availability of hepatitis B immunoglobulin (HBIG) should allow improvement in the outcome of HBsAg-positive recipients. End - stage liver disease from HBV infection remains the largest potential population of liver recipients worldwide. The natural history of this infection after liver transplantation has previously been described from this unit in a cohort of HBsAg positive patients in whom selection for transplantation was not determined by pre-transplant viral serology and who did not receive long-term passive immunoprophylaxis (O'Grady et al 1992). Following serological recurrence, a significant proportion of patients go on to develop either cirrhosis or the distinct syndrome of *fibrosing cholestatic hepatitis* a condition which is characterised by a unique histological pattern in the presence of a rapidly progressive deterioration in graft function (Davies et al 1991). The outcome of this clinical group

serves as important control data in the assessment of possible improvements in patient selection and the use of immunoprophylaxis in modifying disease recurrence and the nature and severity of associated graft injury. Considerable uncontrolled data suggests that HBV DNA-negative recipients have a lower rate of serological recurrence and that long-term passive immunoprophylaxis regimens further reduces the recurrence rate (Samuel et al 1991). Co-existent hepatitis delta virus (HDV) infection has also been described as delaying serological recurrence of HBV and diminishing graft injury (Samuel et al 1992).

In this study, this units experience in a group of HBsAg-positive recipients transplanted for related chronic liver disease and who have received systematic and prolonged immunoprophylaxis alongside a stricter patient selection policy (HBV DNA-negative or HDV-positive candidates preferred) was compared with the previous experience in historical untreated controls as reported above. Additionally, in this present series of patients a rapid steroid withdrawal schedule was initiated as a modification to our triple therapy immunosuppression regimen to offset the potential role of corticosteroids in promoting HBV replication (Tur-Kaspa et al 1986). The consequences of co-existent HDV infection in relation to both serological and disease recurrence and overall patient survival have also been examined.

9.2 SUBJECTS

9.2.1 Patients and methods

The study series comprises 56 HBsAg-positive elective liver transplants who survived beyond the early postoperative period (21 days) and who were divided into two groups based upon the introduction of immunoprophylaxis and a rapid steroid withdrawal regimen in April 1989. The earlier cohort (group 1) comprised 29 HBsAg-patients who

underwent transplantation between December 1975 and April 1989 (24 male, 5 female, median [range] age 37 [23-63] years). With the exception of the three earliest patients, immunosuppression was based upon a CyA triple therapy regimen with no modification made because of the presence of HBV infection. One patient received a short-term course of polyclonal anti-HBsAb in the early post-transplant phase. The median (minimum) follow-up in this group was 5.9 (4) years. The second cohort (group 2) was made up of 27 HBsAg-patients who were transplanted between May 1989 and May 1992 (23 male, 4 female, median [range] age 43 [29-60] years). All patients in this group, were immunosuppressed in the early phase with this units conventional CyA triple therapy regimen and underwent a rapid corticosteroid withdrawal regimen (prednisolone 40 mg at week one, 30 mg at week two, 20 mg at week three and 10 mg at week four with discontinuation where possible by three months). The median (minimum) follow-up of group 2 was 2.2 years and 10 months. No patients with HBV-related fulminant liver disease were included in this study.

9.2.2 Immunoprophylaxis with hepatitis B immunoglobulin

HBIg (polyclonal anti-HBs immunoglobin, United Kingdom Blood Transfusion Service, British Products Laboratory, Ltd.) was administered to the group 2 patients in a standard regimen based upon a modification of the schedule recommended by the Paris group. In the anhepatic phase of the transplant, 5000 IU of HBIg was administered intravenously and in the early post-operative phase 2000-5000 IU administered intramuscularly on consecutive days until clearance of HBsAg was demonstrated. Further administration was determined by circulating anti-HBs titres with the object of maintaining levels above 100 IU/l indefinitely (2000-5000 IU per dose) [Paris group administered 10000 IU daily for the first week with additional 10000 IU when anti-HBs titres approached 100 IU/l]. If HBsAg persisted or recurred in serum further immunoprophylaxis was withheld.

9.2.3 Pre-transplant hepatitis B, C and D virus status

HBV status prior to transplantation was determined by standard commercially available kits. Radioimmunoassay or microparticle capture enzyme immunoassay was used to measure HBsAg, HBeAg, anti-HBs and anti-HBe (Ausria II, Abbott HBe (rDNA), Ausab, Abbott, North Chicago, IL). Total anti-delta antibody was tested by radioimmunoassay or enzyme immunoassay (Sorin Biomedica, Italy). HBV DNA was detected in group 1 by a quantitative dot-blot technique as previously described and in group 2 detected and quantified by the Hepatitis B viral DNA assay (Abbott, North Chicago, IL) (Fagan et al 1985). Hepatitis C antibody (HCVAb) was determined retrospectively by second generation enzyme immunoassay (United Biomedical. Inc., Lake Success, N.Y., U.S.A). Based upon the pre-transplant serological profile, the patients in the two groups were divided into three sub-sections: (a) non-replicative HBV infection (HBV DNA seronegative), (b) replicative HBV infection (HBV DNA seropositive or HBeAg positive where HBV DNA unavailable) and (c) HBV infection (irrespective of replication status) with co-existent HDV infection (tables 9.1 and 9.2).

Actuarial rates of HBsAg recurrence and patient survival were calculated by life-table analysis and statistical significance compared with the log-rank test. Circulating HBV DNA titres were compared with the Mann-Whitney U test with other differences in frequencies between groups estimated with the chi-square test (with continuity correction where appropriate).

Table 9.1 ***Pretransplant hepatitis B serology in the historical control patients who were selected for transplantation on clinical need and did not receive immunoprophylaxis (group 1).***

Case	HbsAb	HbeAg	HBeAb	HBVDNA	Anti-HDV
(a) HBV alone without viral replication					
1		-	N.D.	N.D.	N.D.
2	-	-	-	-	-
3	-	-	+	-	-
4	-	-	+	-	-
5	+	-	+	-	-
6	-	-	+	-	-
7	-	-	+	-	-
8	-	-	-	-	-
9	-	-	+	-	-
(b) HBV alone with viral replication					
10	-	-	+	11	-
11	-	-	+	10	-
12	-	-	+	10	-
13	-	-	+	76	-
14	-	+	-	N.D.	-
15	-	+	-	21	-
16	-	-	+	98	-
17	-	+	-	131	-
18	-	+	-	N.D.	-
19	N.D.	+	N.D.	N.D.	-
20	-	-	+	11	-
(c) HBV and delta					
21	-	-	-	-	+
22	-	-	+	-	+
23	-	-	-	-	+
24	-	-	+	-	+
25	-	-	+	12	+
26	-	-	+	15	+
27	-	-	+	9	+
28	-	-	+	-	+
29	-	-	+	-	+

Table 9.2 ***Pretransplant hepatitis B serology in the latest patients who underwent a more rigorous selection procedure and who were administered long-term passive immunoprophylaxis (group 2).***

Case	HBeAg	HBeAb	HBVDNA	Anti-HDV	HCVAb	
(a) HBV alone without viral replication						
1	-	-	-	-	-	
2	-	+	-	-	-	
3	-	+	-	-	ND	
4	-	+	-	-	+	
5	-	weak +	-	-	-	
6	-	+	-	-	-	
7	-	+	-	-	-	
8	n/a	n/a	-	-	-	
9	n/a	n/a	-	-	-	
(b) HBV alone with viral replication						
10	+	-	+	-	-	
11	-	+	+	-	-	
12	+	-	+	-	-	
13	+	-	+	-	-	
14	+	-	+	-	-	
15	+	-	+	-	-	
(c) HBV and delta						
16	-	+	-	+	-	
17	-	+	-	+	-	
18	ND	ND	-	+	ND	*
19	-	+	-	+	-	
20	-	+	+	+	+	*
21	-	+	-	+	-	
22	-	+	-	+	+	
23	-	+	-	+	+	
24	-	+	-	+	+	
25	-	+	+	+	-	
26	-	+	-	+	-	
27	-	+	-	+	-	

* precore mutant

9.3 RESULTS

9.3.1 Serological recurrence

In the earlier group of patients, 9 (31%), 11 (38%) and 9 (31%) patients were in the non - replicative, replicative and HDV-positive sections respectively prior to transplantation and 9 (33%), 6 (22%) and 12 (44%) patients in group 2. The six HBV DNA-positive recipients in group 2 had been recruited early during the second series as they were otherwise good candidates for an elective transplant. Comparison of rates of serological recurrence in the two groups shows a significant reduction since 1989, with one-year actuarial recurrence rates of 90% in group 1 and 48% in group 2 ($p<0.05$) (figure 9.1). In addition, the median time to recurrence has also been significantly extended (group 1 one week [0-34 weeks] and in group 2 10 weeks [0-32 weeks]) ($p<0.05$). When considered in relation to the state of pretransplant replication, virtually all patients as assessed by the presence of HBV DNA experienced serological recurrence of HBsAg irrespective of whether or not they were administered HBIg (11/11 in group 1 and 5/6 in group 2). Immunoprophylaxis appeared to have more beneficial effects in the subsections of non - replicating and HDV-positive patients. Within the HBV DNA-negative recipients, recurrence was significantly reduced at one-year from 7 of 8 patients (78%) in group 1 to 3 of 9 (33%) in group 2 ($p<0.05$). Similarly in the HDV-positive recipients, a beneficial effect from immunoprophylaxis was apparent with the one-year recurrence rate in group 1 89% as compared to 33% in group 2 ($p<0.05$) (table 9.3).

9.3.2 Hepatitis B serological findings and HBV DNA levels

Following initial detection of serum HBsAg, levels of serum HBV DNA rose steadily in all patients, becoming detectable at a median of 2 weeks.

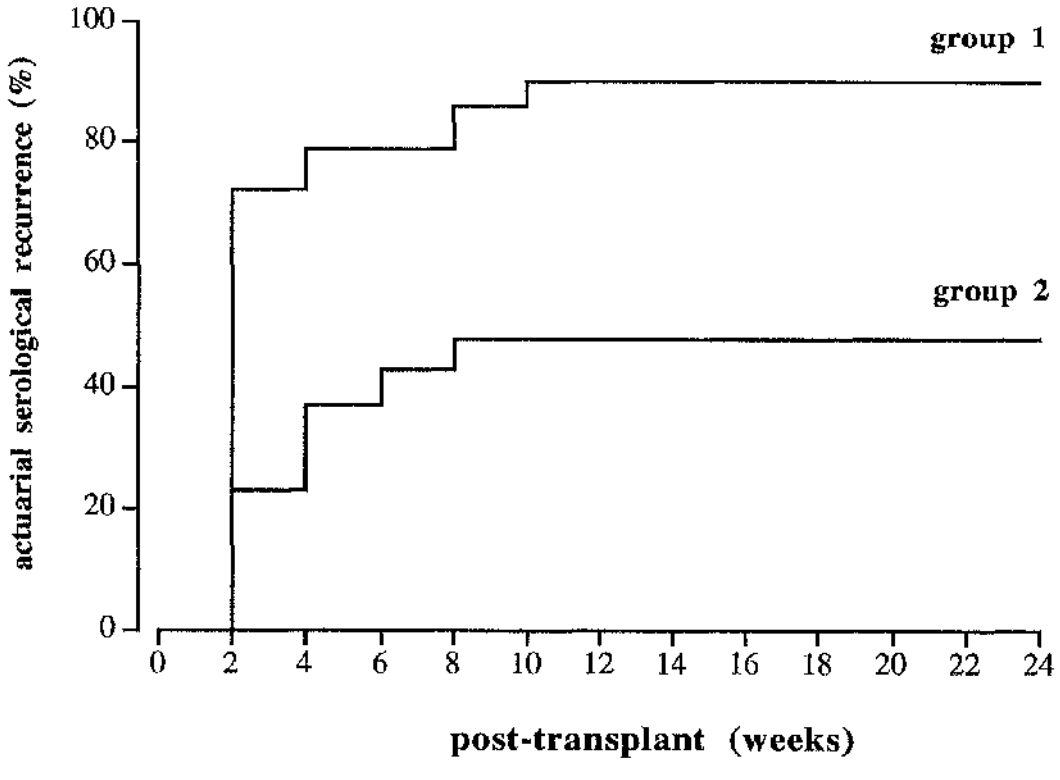


Figure 9.1 Reduced HBsAg recurrence in those patients selected more rigorously for transplant and who received long-term immunoprophylaxis

Table 9.3 HBsAg serological recurrence in relation to pretransplant HBV and HDV recipient status

HBV status	**Actuarial HBsAg recurrence (%)**		
	6 months	12 months	18 months
Group 1			
HBV DNA positive (n=11)	91	91	91
HBV DNA negative (n=9)	57	78	78
HDV positive (n=9)	89	89	89
Group 2			
HBV DNA positive (n=6)	84	84	84
HBV DNA negative (n=9)	22	33	33
HDV positive (n=12)	25	33	33

The median titre of HBV DNA rose from 58 pg/40µl prior to transplantation to a maximum level of greater than 800 pg/40µl (dot-blot assay) following recurrence in the replicating recipients (11/11) in group 1 and from a median pretransplant level of 45 pg/ml to 843 pg/ml (768-1282 pg/ml) (Hepatitis B viral DNA assay) in this category of patients in group 2 (5/6).A difference in the levels of maximum HBV DNA titres attained in relation to pretransplant HBV status was noted with the median titres amongst group 1 patients [insufficient recurrence in group 2] being 800 µg/40µl in non HDV-positive patients compared to 181 µg/40µl in the HDV-positive recipients ($p<0.05$).

Following recurrence, those patients who were HBeAg-negative prior to transplantation invariably developed HBcAg positivity with loss of anti-HBe if this had been present previously. Of the 6 patients who have become HBV DNA-negative following HBsAg recurrence (4 patients in group 1, 2 in group 2), 5 had coexistent HDV infection. In 3 of these patients, clearance of HBV DNA has been associated with seroconversion of HBeAg positivity with the development of anti-HBeAb. Of a total of 6 patients who have cleared HBsAg, 4 patients were HDV-positive with the remaining two being HBV DNA - negative prior to transplantation. No HBV DNA-positive patient in the series has seroconverted HBeAg or cleared circulating HBV DNA.

9.3.3 Patient survival

Actuarial patient survival was significantly improved in the most recent patients compared to the earlier group 1 cases with one and two years survival of 62% and 48% [group 1] and 86% and 80% [group 2] ($p<0.05$) (figure 9.2). A comparison of actuarial patient survival in the recipients classified by their pre-transplant status demonstrated that since (1989) there has been an improvement in survival of the pre-transplant HBV DNA - positive patients at current follow-up (1/11 [9%] group 1 vs 4/6 [67%] group 2, p 0.05)

(table 9.4). In the non-replicating recipients, there is no significant difference in patient survival. Taking all the non HDV-positive patients together in group 2 irrespective of pre - transplant replicative status, current survival is 11/15 (73%) versus 5/20 (25%) in group 1 ($p<0.05$). A comparison of those patients who experienced recurrence in group 1 (25/29) and in group 2 (12/27) reveals that HBV directly led to death of the patients in 12/29 [41%] and 4/27 [15%] respectively ($p<0.05$). The median period to patient loss in group 1 was 9.5 months and 7 months in group 2. The better patient survival in HDV-positive recipients in group 1 (89% one-year survival) has been maintained in the group 2 series (92%). Taking the two groups together, HDV-positive patients have a one-year patient survival of 90% (19/21) versus 45% (16/35) in the HDV-negative patients ($p<0.05$). The current one-year survival in all HDV-positive recipients and patients in group 2 is similar to a consecutive cohort of 100 cirrhotic HBsAg-negative patients who underwent elective transplantation from (1989)-(1991) (86% versus 89%, ns) and who survived beyond 3 weeks.

9.3.4 Graft injury

In the recent series there appears to have been a reduction in the graft injury associated with serological recurrence. Amongst the 12 patients in group 2 who have experienced recurrence, fibrosing cholestatic hepatitis (FCH) has occurred in 3 (11%) patients (3, 9 and 12 months respectively) with subsequent patient loss despite retransplantation in two. This is to be compared with 7 (24%) patients with FCH in group 1 (median onset 6.5 months) leading similarly to rapid death (a clinical suspicion of FCH was also present in a further 3 cases but no tissue was available for histological confirmation).Thus the development of confirmed FCH has accounted for 10/16 [63%] of the total HBV recurrence-related deaths. In the remaining 9 patients in group 2 experiencing HBV recurrence, one patient died 4 months post-transplant with massive hepatic necrosis present in the peri-mortem biopsy, chronic active hepatitis (CAH) is currently present in five (fibrosis is present in three), and minimal or mild chronic hepatitis in four.

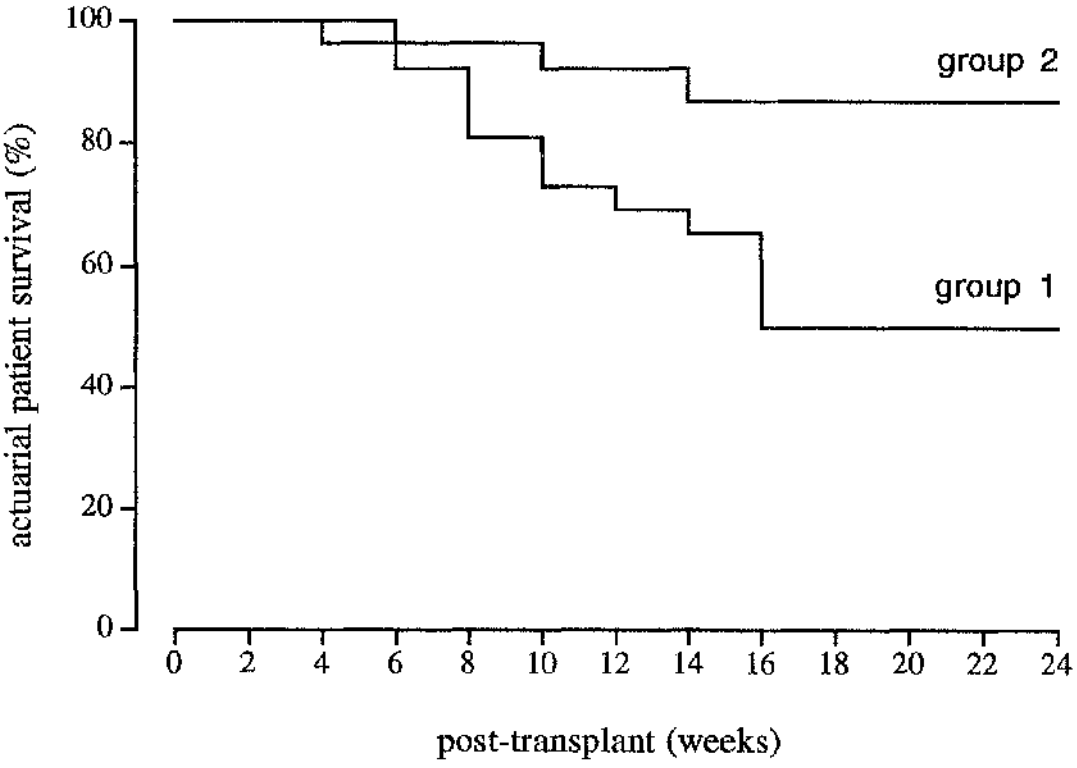


Figure 9.2 Improved patient survival in recipients (group 2) who underwent the new selection and treatment policy.

Table 9.4 Patient survival in relation to pre-transplant HBV and HDV recipient status

HBV status	**Actuarial patient survival (%)**		
	6 months	12 months	18 months
Group 1			
HBV DNA positive (n=11)	87	45	9
HBV DNA negative (n=9)	89	56	56
HDV positive (n=9)	89	89	89
Group 2			
HBV DNA positive (n=6)	80	80	67
HBV DNA negative (n=9)	89	78	78
HDV positive (n=12)	92	92	92

There have been no deaths in the recent patients from hepatic decompensation consequent on development of chronic active hepatitis or cirrhosis. All together there were seven patients [5 in group 1, 2 in group 2] who underwent retransplantation for severe graft injury following HBsAg recurrence at a median post-operative period of 24 weeks (8-56 weeks). Circulating serum HBV DNA in those patients immediately prior to retransplantation was greater than 1000pg/ml in all cases measured. Six of these patients died either from rapid HBsAg recurrence or early multi-organ failure with four of this group developing a post-transplant course characterised by rapid HBV serological recurrence and early graft failure despite administration of large prophylactic doses of HBIg in three patients. Four patients died within one week, and one patient each in the fourth and twenty-second week after retransplantation.

9.4 DISCUSSION

Recurrence of HBV following liver transplantation is the main reason for the restriction of this management option in HBV-related end-stage liver disease. Recurrence is presently one of the commonest causes for transplant failures beyond the early post - operative period and has resulted in several centres declining to transplant all HBsAg - positive patients. However, long-term passive immunoprophylaxis appears to significantly modify HBV recurrence and associated graft injury. The benefits are clearest in those HDV-negative recipients in whom, with a moderate reduction in the frequency of recurrence, there is a significant improvement in patient survival. Within this sub-group, immunoprophylaxis appears to reduce serological recurrence especially in the non - replicating recipients, whereas in the replicating recipients although recurrence is not prevented there is also an improvement in patient survival. This data on the benefits of immunoprophylaxis, in relation to HBV DNA status, is similar to those results obtained from the other large published series by Samuel et al (1991). In their HBV DNA-

negative recipients with chronic liver disease, 2 year actuarial HBV recurrence rate was reduced to 29% whereas in the replicating recipients the rate remained high at 96% (33% and 83% in our comparative groups). Similarly, there was no significant difference in 2 year patient survival between replicating and non-replicating recipients (52% vs 60% respectively, ns [67% and 78% respectively in our patients]). These data do not justify the exclusion of HBV DNA-positive patients from consideration for elective transplantation.

In addition to decreasing the frequency of HBsAg serological recurrence, HBIg may modify graft injury in those recipients who do experience recurrence. Amongst the possible explanations for this effect of HBIg, the timing of HBV recurrence (delayed with immunoprophylaxis) may be crucial if there is a graft susceptibility factor or if the level of concurrent immunosuppression is playing a substantial role in the pathogenesis of graft injury. In the early post-transplant phase, the graft which is often already compromised by the common post-transplant complications (which include other viral infections) may be at particular risk of further insult in the form of HBV recurrence. An analogy exists with the increased graft susceptibility to vascular complications in the early post-operative period. A major arterial thrombosis is often tolerated at least in the medium-term as a late-onset complication but has disastrous sequelae if occurring in the early post-operative period as discussed previously. Delaying recurrence beyond the early post-operative period also avoids the potential additive risk of a high level of immunosuppression in enhancing viral replication and antigen load in liver cells due to the glucocorticoid-responsive element of HBV. Steroid requirements as part of both maintenance and supplemental regimens are maximal in this period due to the level of immunosuppression required to control the rejection process. The precise effects of other immunosuppressant agents, which inhibit T-cell activation, on HBV replication require to be determined. Severe rejection problems were not experienced with the rapid steroid withdrawal regimen. Indeed there is some evidence that HBsAg-positive patients

may be at lower risk of allograft rejection than other recipients which could permit further reductions in maintenance immunosuppression (Adams et al 1991).

The value of pretransplant anti-viral therapy with α-interferon in all HBsAg-positive patients and in particular with the replicating candidates requires to be defined. Attempts to elicit seroconversion and / or inhibition of HBV replication prior to transplantation with administration of α-interferon is often accompanied by significant morbidity in these patients who have end-stage chronic liver disease and at present there is no evidence that this treatment reduces the HBV reinfection rate following transplantation (Marcellin et al 1992). However a range of new anti-viral agents are becoming available (such as Lamuvidine and gancyclovir) which will require clinical assessment as will the use of concurrent anti-viral chemotherapy with immunoprophylaxis regimens.

These results demonstrate a reduced frequency of HBsAg recurrence in HDV-positive recipients receiving immunoprophylaxis. The presence of HDV also appears to modify the severity of graft injury with patient survival in HDV-positive recipients comparable to HBsAg-negative recipients. Successful use of HBIg in post-HBV / HDV hepatitis recipients appears to prevent graft injury although HDV RNA can still be detected by reverse transcriptase-PCR in the first post-transplant year (Samuel et al 1991). Although there is no difference in current patient survival in HDV-positive recipients whether they received HBIg or not, the rate of serological recurrence is lower in the treated group and this may translate into a difference in patient survival with longer follow-up. The beneficial effect of co-existent HDV infection is likely to be a reflection of this agents ability to inhibit HBV replication and our data which indicates lower peak HBV DNA levels after viral recurrence in these patients and a high proportion of patients who have subsequently either cleared HBV DNA or HBsAg also being HDV-positive supports this hypothesis (Genesca et al 1987).

There appears to be no current clinical role for retransplantation following the graft

damage consequent on HBsAg recurrence. We have had no survivors in this situation irrespective of further immunoprophylaxis and a recent series has reported only one survivor from a study population of twenty patients (Crippin et al 1992). As shown by the findings in this present study the poor outcome may reflect the very high HBV DNA levels present in the recipients prior to retransplantation.

The anti-HBs Ig regimen which was followed is based upon the schedule recommended by the Paris group with HBIg being given in dosages sufficient to maintain anti-HBs titres greater than 100 IU/l without discontinuation in the absence of recurrence. The requirement for indefinite immunoprophylaxis is based upon the identification of HBV DNA by PCR in extra-hepatic sites which serves as a basis for graft reinfection (Feray et al 1990). With late graft reinfection reported one year beyond transplantation and the absence of data based upon PCR in relation to the persistence or clearance of extra - hepatic foci of HBsAg following prolonged HBIg, we currently follow an indefinite immunoprophylaxis regimen. Although our total recurrence rate in HBIg-treated patients is similar to non-fulminant HBV patients from the Paris group, our median period to recurrence was significantly shorter which may reflect a lower dosing schedule in the early post-operative period. Furthermore, three-year serological recurrence rates of less than 10% have recently been reported by Gugenheim et al (1992). in non-replicating recipients where polyclonal HBIg was administered to maintain anti-HBs titres above 500 IU/l. If, as appears likely, a dose-response effect to immunoprophylaxis does exist, there are considerable implications with respect to the long-term costs of high dose regimens.

CHAPTER 10

N-ACETYLCYSTEINE IMPROVES INDOCYANINE GREEN EXTRACTION AND OXYGEN TRANSPORT DURING HEPATIC DYSFUNCTION INCLUDING THAT FOLLOWING LIVER TRANSPLANTATION

10.1 BACKGROUND AND INTRODUCTION

Morbidity and mortality in the immediate post-transplant phase is commonly related to primary graft non-function or ischaemia as discussed in section 2.3. These graft complications which are associated with reduced hepatic blood flow often result in systemic critical illness requiring intensive care. Although urgent retransplantation is often the preferred management option in this situation, maximal medical treatment is required to prevent the development and reduce the severity of multi-organ failure which if established can preclude regrafting and ultimately lead to death. Pharmacological haemodynamic intervention in this period of critical illness is directed at improving both systemic and splanchnic circulations.

It has previously been established that inadequate systemic oxygen delivery (DO_2) and consumption (VO2) with accompanying tissue hypoxia is associated with the development of multi-organ failure and a reduced patient survival following major surgery and during critical illnesses (Bland et al 1985). This phenomenon may in part reflect impairment of regional circulation and particularly hepatic-splanchnic O_2 and nutrient delivery which, as assessed by low gastric intramucosal pH and indocyanine green (ICG) extraction, are associated with a poor clinical outcome (Gutierrez et al 1992, Gottlieb et al 1984, Kholoussy et al 1984). Although the relationship between systemic oxygen transport

parameters and regional indices of tissue oxygenation are poor, strategies achieving supranormal oxygen transport to both these circulations can improve survival (Shoemaker et al 1988, Boyd et al 1994).

In liver disease, the only systematic studies of systemic haemodynamics and oxygen transport have been performed in FHF, a condition which is also haemodynamically characterised by a 'supply dependent' oxygen consumption, multi-organ failure and tissue hypoxia (Bihari et al 1987). In contrast, the functional importance of impairment in the hepatic-splanchnic circulation has been well described in a range of liver disorders including those following transplantation (Yamanaka et al 1992, Frenette et al 1994).

Pharmacological modulation of haemodynamics in severe illness would ideally employ an agent which would improve both systemic and regional DO_2 and not stimulate cellular aerobic metabolism. Concern is mounting that those agents presently administered to modulate systemic oxygen transport may paradoxically promote hepatic-splanchnic ischaemia or increase oxygen requirement through stimulation of tissue metabolism (Ruokonen et al 1993, Giraud et al 1984, Reinhart et al 1994). In the particular experience of liver intensive care, potent α_1-agonists are associated with reductions in VO_2 despite their undoubted inotropic effects, a phenomenon which most probably reflects the increasingly intense regional vasoconstriction present at increased dosages (Wendon et al 1990). Novel pharmacological agents which improve systemic and regional circulations, without a penalty in relation to direct stimulation of tissue metabolism or a deterioration in gas exchange, require to be identified.

Previous studies from this unit have previously shown that infusion of N-acetylcysteine improves systemic haemodynamics and oxygen supply and consumption in FHF, a property which may account for this agents beneficial effect on survival in this disorder

(Harrison et al 1990, Harrison et al 1991). It is presently unknown what effect this agent has on either systemic haemodynamics or the deranged hepatic circulation found in most forms of acute or decompensated chronic liver disorders including those present following transplantation. This present study was designed to evaluate and compare the haemodynamic effects of N-acetylcysteine and prostacyclin infusions on systemic and hepatic circulatory disturbances present in both liver recipients with normal graft function and those experiencing complications following liver transplantation in the early post - transplant period. Since this studied was also extended to non-transplant disorders and the same effects were observed, the combined results are presented for the interest of the reader.

10.2 SUBJECTS

10.2.1 Patients

15 patients (9 M, 6 F, median age 36 years, range;16-62) with liver dysfunction and accompanying critical illness, who were haemodynamically stable, entered this study. The population comprised eight liver transplant recipients who were either studied on the first post-operative day (n=5) or later (post-operative day 2-8) at the time of a critical complication (n=3). Graft function was within the normal post-operative range in 3 of this group, primary graft dysfunction was present in 2, severe acute rejection in 2 and septic shock in 1. The remaining 7 patients were either experiencing sepsis syndrome as a late complication in the convalescent phase of FHF (n=3) or had critical decompensation of a chronic liver disorder (n=4; alcoholic hepatitis 2, AIH 1, PSC 1). Four patients were receiving inotropic agents during the course of the study. All subjects required mechanical ventilation (Siemens Servo 900C; volume control mode with a PEEP of 4 cm H_2O). No alteration in ventilator settings, paralysis or sedation infusions was permitted during the

study period. A stable inspired oxygen requirement. and normalisation of the pulmonary capillary wedge pressure were present at least 2 hours prior to study commencement. Crystalloid and vasoactive drug infusion rates were unchanged and no colloid administration was permitted during the investigation.

10.2.2 Measurement of haemodynamics and gas exchange

Placement of a systemic arterial cannula and a conventional thermal dilution pulmonary artery flotation catheter (Edward Laboratories 7 French, Irvine, California) had been performed as part of this units routine clinical practice. These catheters were connected to electronic pressure transducers which in turn were connected to a physiological monitor (Marquette Series 7005; Marquette Electronics Inc, Milwaukee, WI, USA) from which heart rate, central venous, pulmonary capillary wedge, mean systemic and pulmonary artery pressures were determined. A 5F fibreoptic thermistor catheter (PV-2025 Pulsiocath 5FTL, Pulsion Medizintechnik, Munich, Germany) was inserted into a femoral artery and connected to an integrated fibreoptic physiological monitoring system (COLD Z - 02 system, Pulsion Medizintechnik). The pulmonary artery catheter was connected to the standard physiological monitor for conventional determination of cardiac output by thermodilution and thereafter to the COLD Z-02 system for the measurements available from thermal dye double indicator methodology (Pfeiffer et al 1991). Conventional haemodynamic measurements and calculations were performed to standard protocols (Varon and Civetta 1990, Harrison et al 1992). Radial arterial and distal pulmonary artery samples were analysed for oxygen tension (AVL 995; AVL Medical Instruments UK Ltd, Stone, UK) and for haemoglobin content and saturation (AVL 912). Following determination of these measurements, ICG (0.5 mg per kg) dissolved in 10 mls of ice cold (4-10ºC) 5% dextrose was injected into the proximal port of the pulmonary artery catheter and derived measurements recorded from the COLD-Z-02 system.

10.2.3 Administration of N-acetylcysteine and prostacyclin

Following determination of baseline values, prostacyclin (Flolan; Burroughs Wellcome, Crewe, England) was administered at a continuous infusion rate of 5 ng/kg/min for 60 minutes at which time measurements were repeated. A 60 minute interval was then allowed to wash-out the effects of this agent before repeating a second baseline measurement. Thereafter, N-acetylcysteine was administered (Parvolex; Duncan Flockhart & Co Ltd. Greenford, England) at 150 mg/kg in 250 ml 5% dextrose over 15 minutes and then 50 mg/kg in 250 ml 5% dextrose for 45 minutes at a rate of 62.5 ml per hour. Control infusions (without N-acetylcysteine) at the volumes and rates described above were administered in 8 patients. Of these latter patients, 4 were studied as an addition to the protocol between the 2 treatment infusions, 3 were new patients and 1 patient was studied 28 hours before the treatment schedules. Administration of prostacyclin was not permitted 1 hour or N-acetylcysteine 12 hours before the study infusions were commenced. Two patients had previously received prostacyclin and six N-acetylcysteine. In those patients in whom benefit in systemic haemodynamics, oxygen transport or ICG extraction was found, the appropriate infusion was maintained.

Results are expressed as mean ± SD with differences between baseline and treatment values compared by a two-tailed paired Students t-test if normally distributed or by the Wilcoxon signed rank if non-parametric. Percentage changes are also expressed as mean with 95% confidence limits.

10.3 **RESULTS**

10.3.1 Haemodynamics and oxygen transport

N-acetylcysteine and prostacyclin infusions both increased the mean cardiac index (table 10.1). This effect of the N-acetylcysteine infusion was observed in 14 of the 15 patients, and could be attributed to both a reduction in systemic vascular resistance (mean change -12% [95% CI, -4, -19]) alongside a moderate inotropic action as seen by an increase in left ventricular stroke work index [LVSWI] (mean change +11% [95% CI, 0, +21]). Mean arterial pressure was not significantly altered by N-acetylcysteine. In contrast, the increase in the cardiac index with prostacyclin was secondary to its significant vasodilatatory action (systemic vascular resistance index, mean change -22% [-12, -31]), an effect which was accompanied by a tachycardia and reduction in mean systemic arterial pressure. There was no change in LVSWI. Similarly, mean pulmonary arterial pressure and the pulmonary vascular resistance index, which were unaffected by N-acetylcysteine, were reduced during the prostacyclin infusion.

Although similar improvements in oxygen delivery were seen with both agents, only the infusion of N-acetylcysteine increased mean global VO2 (150[30] to 169 [25] ml/min/m^2) (p 0.002). This effect was found in 14 (93%) patients with a mean increase of 15% [95% CI,+3, +27]. The oxygen extraction ratio, which was unaffected by N-acetylcysteine, showed a trend towards a reduction during the prostacyclin infusion. Neither agent affected the pulmonary capillary wedge or central venous pressures. Control volume infusions did not elicit any significant change in haemodynamic parameters.

Table 10.1 Haemodynamic, oxygen transport and indocyanine green clearance profiles before and during the infusions of prostacyclin and N-acetylcysteine.

variable	Baseline 1	PgI2	change %	Baseline 2	NAC	change%
Haemodynamics						
heart rate	89 (26)	94 (25)	+6 (+3,+9)	88 (26)	94 (26)	+7 (+2, +11)
mean arterial pressure	75 (18)	68 (22)*	-8 (-20, +3)	74 (17)	75 (16)	0 (-8, +7)
pulmonary capillary wedge pressure	12 (3)	12 (3)	+6 (-10, +21)	11 (3)	11 (4)	-1 (-13, +12)
mean pulmonary arterial pressure	22 (5)	20 (6)	-5 (-18, +7)	22 (6)	23 (5)	+8 (-3, +19)
cardiac index	5.4 (2.0)	6.1 (1.6)**	+18 (+3, +34)	5.6 (1.8)	6.5 (2.2)**	+16 (+9, +22)
stroke volume index	61 (20)	64 (17)	+7 (-3, +18)	63 (18)	69 (20)**	+9 (+4, +15)
left ventricular stroke work index	48 (14)	45 (11)	-1 (-29, +27)	53 (16)	58 (16)^	+11 (0, +21)
pulmonary vascular resistance	165 (88)	131 (67)*	-15 (-39, +8)	173 (65)	160 (71)^^	-3 (-18, +13)
systemic vascular resistance	1055 (484)	802 (370)**	-22 (-31, -12)	1012 (351)	898 (350)*	-12 (-19, -4)
Oxygen transport						
oxygen delivery	670 (214)	753 (198)*	+16 (-2, +35)	667 (154)	751 (166)**	+13 (+7, +20)
oxygen consumption	153 (27)	158 (33)	+3 (-6, +12)	150 (30)	169 (25)**^	+15 (+3, +27)
oxygen-extraction ratio	24 (6)	22 (6)	-8 (-19, +3)	23 (6)	24 (5)	+5 (-8, +17)
Indocyanine green clearance						
plasma disappearance rate	7.6 (4.2)	8.4 (4.8)	+26 (-12, +191)	7.3 (4.2)	11.8 (4.0)**^^	+100 (+9, +191)

values are expressed as either mean (SD) or as (95% confidence limits) with respect to the mean changes
* p<0.05, **p<0.01 comparison with baseline,
^p<0.05, ^^p<0.01 comparison of N-acetylcysteine with prostacyclin

10.3.2 Indocyanine green clearance

N-acetylcysteine infusion resulted in an improvement of ICG clearance in 12 (80%) of the 15 patients with the mean clearance rising from a mean of 7.3% (4.2) to 11.8% (7.1) (p 0.002) (figure 10.1). This increased clearance was often marked with a mean percentage increment of 100% [+9, +191] in the 12 patients; an increase significantly out of proportion to the modest improvements in cardiac index described above. Despite the consistent increase in cardiac index during the prostacyclin infusion, this agent had a variable effect on ICG clearance with approximately similar patient numbers showing an increase or decrease (overall change from a mean of 7.6% [4.2] to 8.4% [4.8]).

10.3.3 Gas exchange

The infusion of N-acetylcysteine had no effect on gas exchange, whereas administration of prostacyclin resulted in a marked deterioration of the pulmonary shunt and alveolar - arterial oxygen gradient (table 10.2). Extravascular lung water also rose significantly during the prostacyclin infusion.

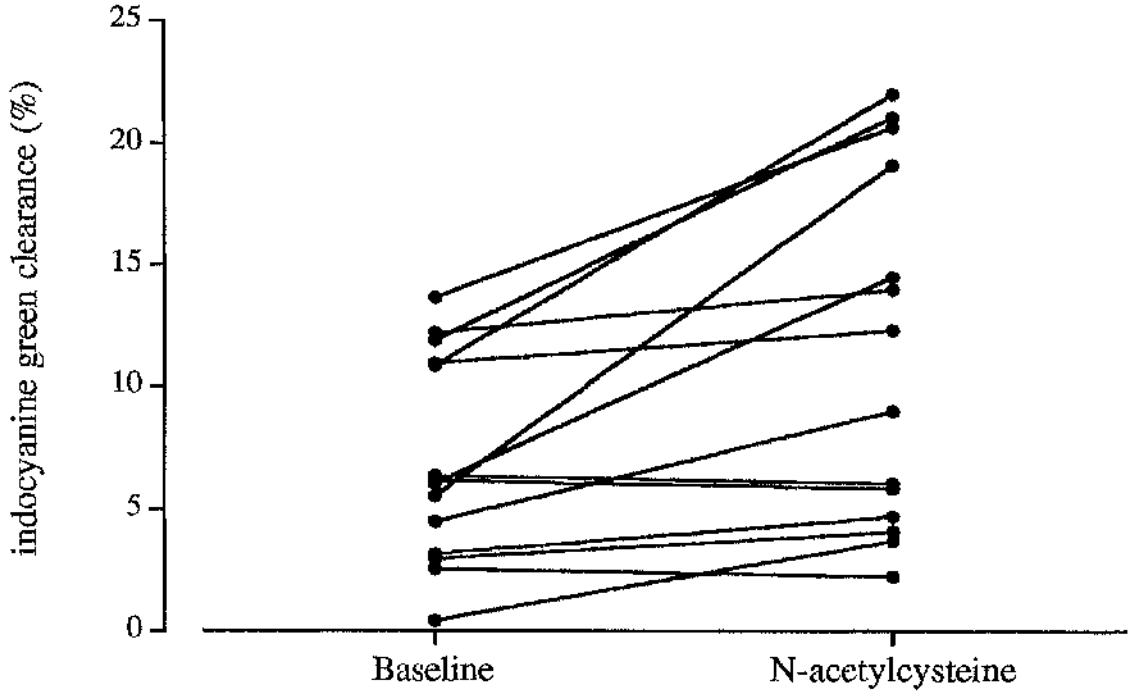


Figure 10.1 Effect of N-acetylcysteine infusion on indocyanine green clearance. Increments were reduced in those patients with low baseline levels.

Table 10.2 Respiratory variables in patients before and during administration of N-acetylcysteine (NAC) and prostacyclin infusions.

variable	Baseline 1	**PgI2**	change%	Baseline 2	**NAC**	change%
respiratory index	0.8 [0.7]	1.4 [1.0]**	122 (+20,+225)	1.1 [0.9]	1.1 [0.8]^	19 (-6, +43)
(Qs/Qt)	16 [7]	24 [8]**	59 (+23, +95)	17 [8]	17 [8]^^	-2 (-15,+10)
(A-a) O_2 gradient	235 [118]	316 [113]**	52 (+11, +94)	263 [133]	278 [116]^^	13 (-2, +28)
EVLWI ($ml/kg/m^2$)	7 [4]	9 [6]*	23 (+4, +42)	8 [6]	9 [6]	104 (-109, +318)

change% = mean percentage change
n [SD], (95 percent confidence intervals)
*p<0.05 ** p<0.01 for comparison between infusion and treatment
^p<0.05, ^^p<0.01 for comparison between N-acetylcysteine and prostacyclin

10.4 DISCUSSION

The results of this study extend the previous observations from this unit on the clinical usefulness of N-acetylcysteine in FHF, with the present evidence indicating both beneficial systemic and regional haemodynamic properties in a variety of liver disorders including those present following transplantation. That improvements in systemic VO2 were possible following administration of this agent infers the presence of tissue hypoxia and an accompanying oxygen debt. The finding of a pathological 'supply-dependency' in liver dysfunction (regardless of the clinical setting) and in particular following transplantation is not well recognised even in liver centres and clinicians should be made much more aware of this phenomenon and the potential of treatment modalities such as N - acetylcysteine in preventing further organ dysfunction and failure.

Consistent improvements in cardiac index and DO_2 were accompanied by increments in systemic VO_2 in the majority of patients following administration of N-acetylcysteine. In contrast infusion of prostacyclin, despite resulting in similar improvements in DO_2 did not affect VO_2. This data provides evidence contrary to the hypothesis that increments in systemic VO2 following increases in DO_2 are a result of mathematical coupling of the cardiac index which is incorporated into both formula (where the reverse Fick equation is employed). The marginal effects of prostacyclin on the VO_2 of patients in this study is consistent with results from a recent study in septic shock but in contradistinction to earlier results in FHF (De Backer et al 1993, Bihari et al 1987). Such variations may be explained by the different severity and characteristics of the haemodynamic disturbance and shunting present in different disease states. Further evidence of different responses in critical illness to haemodynamic manipulation can be seen if comparison is performed of the results in the present cases to those in patients with FHF who were examined in the original report from this unit. In the present series, improvement in mean arterial

pressure with administration of N-acetylcysteine as demonstrated in the patients experiencing paracetamol-induced FHF was not found. The less pronounced inotropic action of N-acetylcysteine in this study group versus FHF patients can be seen from comparison of the mean percentage change in LVSWI; +11 versus +21 for patients in FHF. From this data it can be recommended that clinicians should accordingly assess the effects of any agent they intend to utilise with respect to the haemodynamic changes and underlying disease in any individual patient.

Circumstantial evidence previously existed that N-acetylcysteine infusions may improve regional circulations. Administration of this agent in FHF was accompanied by a reduced frequency of renal failure and cerebral oedema. The improvements in cerebral blood flow with this agent may explain the decreased frequency of cerebral oedema (Wendon et al 1994). Pharmacological nitric oxide donors (which includes N-acetylcysteine), improve cerebral blood flow in experimental systems (Harrison et al 1992, Zhang et al 1994). However, any beneficial effects on the hepatic-splanchnic circulation, as examined in this study, would have wider application given the postulated central role of derangements of these circulations in the pathogenesis of the sepsis syndrome and multi-organ failure (Ritz et al 1973, Dantzker et al 1993). The findings presented here on the improvement in the critical hepatic-splanchnic circulation following N-acetylcysteine are potentially of considerable importance. The large percentage increments in ICG clearance, in many patients out of proportion to the changes in systemic haemodynamics, suggests a significant local effect. These improvements, which were seen over short time intervals, can reliably be interpreted as reflecting blood flow changes rather than dynamic improvements in metabolism / extraction capacity. The improvement in clearance infers an increased local delivery of oxygen, nutrient and other regenerative factors which may promote cellular recovery and function.

Provisional evidence exists indicating that prostacyclin can improve hepatic-splanchnic blood flow in man (Radermacher et al 1993, Hassan et al 1983). However, the effect of infusing this agent on ICG clearance was disappointing overall and, often at the penalty of a deleterious effect on gas exchange and occasionally severe vasodilatation resulting in a reduction in mean arterial pressure. Although individual patients did show significant increases in clearance, no discriminating characteristics of this sub-group could be identified. Whether prostacyclin could be promoting trans-hepatic shunting which would offset any beneficial effect on hepatic blood flow is speculative. The deterioration in gas exchange, which is a recognised complication of this agent, is particularly unwelcome in long-standing cirrhotic patients where pulmonary shunting is already a common feature and may limit this agents clinical usefulness in treatment (Radermacher et al 1990, Rodman et al 1960).

N-acetylcysteine therefore has considerable advantages to prostacyclin in optimising systemic and hepatic-splanchnic haemodynamics and with this the potential for considerable clinical application. Patients who may gain particular benefit are those with impairment of the hepatic or splanchnic microcirculations especially in the context of critical illness, a common set of circumstances in general intensive care. In the context of liver transplantation, those recipients who are experiencing primary graft dysfunction, and who characteristically have pruned intrahepatic vasculature (see section 4.3) and impaired ICG clearance alongside systemic haemodynamic disturbance, could show significant improvement (Jalan et al 1994). The value of N-acetylcysteine in achieving further benefits in post-operative survival achieved through optimising systemic and regional haemodynamics prior to transplantation requires evaluation (Boyd et al 1994). A randomised controlled trial of N-acetylcysteine in improving post-operative survival and reducing complications following transplantation would appear to be justified from the present data.

Some limitations to the haemodynamic measurements used in this study exist. The cardiac output measurements were derived from the now clinically standardised method of thermodilution via a pulmonary artery thermistor catheter. Technical events can reduce the accuracy of this technique - although a minimum of 3 measurements were performed. The method of ICG extraction is novel and is presently the only clinically available method which gives a bedside measurement. This latter feature is a major advantage in liver intensive care when assessment of hepatic blood flow would be useful following some clinical intervention such as ventilation or introduction of an inotropic agent. This technique which relies on reflection densitometry has not previously been validated against current gold-standard methods of ICG clearance. However, the measurement of ICG would appear to be extremely accurate since the COLD machine employing the thermodilution technique is now acknowledged to be the most sensitive method for measurement of extravascular lung water (Pearce and Beazell 1966, Lewis et al 1982).

CHAPTER 11

CLINICAL TOLERANCE IN LONG-TERM LIVER RECIPIENTS AND RELATIONSHIP TO SYSTEMIC DONOR-TYPE MICROCHIMERISM

11.1 BACKGROUND AND INTRODUCTION

Continued requirement for immunosuppression is perhaps the greatest hazard to the long - term welfare of liver recipients given the morbidity and mortality associated with the present anti-rejection agents. In chapter 2, a spectrum of the most serious complications resulting in graft loss and often death which were either initiated or aggravated by immunosuppressants was reported. In long-term patients, the main risk from the global inhibition of immunological surveillance is an increased risk of de novo malignancy and infection (Penn et al 1969a and 1983). Corticosteroids (Baserga and Shubik 1954), antilymphocyte preparations (Allison et al 1967), and azathioprine (Casey 1968) have all been shown to promote the development of malignancies. However, this inhibition of immunity also promotes recurrence in the graft of primary malignant and viral transplant indications which can affect greater proportions of patients. In addition to these effects, each of the drugs which comprise standard maintenance regimens have a significant toxicity profile. Chronic steroid usage is associated with well known systemic effects. Short and medium-term effects of CyA are not well-recognised given the use of this agent for only just over a decade (Kahan et al 1989). The additional clinical hazard of azathioprine with respect to hepatotoxicity - which was previously seen in the early experimental and clinical studies (Haxhe et al 1967, Penn et al 1969b) - is also becoming more recognised (Gane et al 1994, Read et al 1986, Sterneck et al 1991).

Successful withdrawal of maintenance immunosuppressive agents would therefore

represent a major advance in clinical transplantation with benefits derived from a return in natural immunity and a reduction in drug-related toxicity. The potential hazards of such an undertaking would be lessened if clinicians were able to base this on the identification of either a clinical or immunological marker of operational tolerance. The technical limitations surrounding the *in vitro* assessment of donor-recipient immune responsiveness are such that as yet no parameter reliably predictive of tolerance has found clinical application (Wood et al 1993). Considerable interest and debate presently surrounds the thesis recently proposed by Starzl that the establishment and augmentation of systemic microchimerism, following multilineage cell migration from a graft or via additional donor cell infusion, is an important factor in immunomodulation of the allograft response with promotion of donor-specific unresponsiveness and hence long-term graft acceptance (figure 11.1) (Starzl et al 1992a, 1992b, 1993a, 1993b, Fontes et al 1994). This phenomenon of chimerism and its potential biological significance was recognised by Owen in 1945 (Owen et al 1945). These authors demonstrated that in-utero transfer of haemopoeitic and lymphoid cells as well as gonadal cells phenotypically altered dizygotic twin calves. The most obvious manifestation of this transfer forming a chimeric animal was the dual gender expression rendering a naturally female cow a sterile animal with both sex organs, a so-called freemartin (figure 11.2). More interestingly, if skin grafts were performed between the twins they were spontaneously accepted. These historical observations were given further significance in the early liver recipients when it was noted that phenotypic changes in immunoglobulin production occurred in some recipients (Kano and Milgrom 1968). That non-graft cells are transferred at transplantation and can be functional for considerable periods is supported by reports of transfer of passive immunity and even donor-derived immunological conditions such as lymphomas, autoimmune disorders as well as the occurrence of graft-versus-host responses and correction of metabolic defects (Burdick et al 1988, Kano et al 1968, Ramsey et al 1984, Starzl et al 1993c). It has been established that a large pool of passenger cells from the

lymphodendritic lineage are present in liver grafts either in the adjacent hilar lymph nodes or trapped in the sinusoids (Schlitt et al 1993).

The demonstration of clinical tolerance in association with chimerism in long-term liver recipients at the University of Pittsburgh was the stimulus to the clinical trial reported here in which immunosuppression drug withdrawal was attempted in a group of long-term liver recipients who were experiencing a number of side-effects or disorders related to continued immunosuppression. In addition to describing the clinical, biochemical and histological outcome of this patient cohort, it represents the first correlative data on the relationship between operational tolerance and the presence of chimerism in a group of solid organ recipients.

11.1 SUBJECTS

11.1.1 Patients

18 orthotopic liver recipients with a functioning primary allograft for greater than 5 years (median 7 range; 5-11 years) were entered into a programme of immunosuppressive drug withdrawal. All patients had a minimum of one side effect or disorder either initiated or aggravated by maintenance immunosuppression: namely recurrence of HCC [n=1] and HCV related liver disease [n=2], de novo systemic [cervical and breast carcinoma n=2] and skin [n=2] malignancies, post-transplant lymphoproliferative disorder [PTLD] n=1, renal impairment [n=4], diabetes mellitus [n=3], hypertension [n=7], osteoporosis [n=3], severe weight gain [n=1], lipid metabolism abnormalities [n=7], severe hirsutism [n=1], neurotoxicity [n=4], azathioprine related bone marrow toxicity [n=1] and various biochemical derangements (hyperkalaemia, hypomagnesaemia, hyperuricaemia) [n=7]). The aetiologies of the primary disease comprised PBC [n=4], PSC [n=3], Budd-Chiari

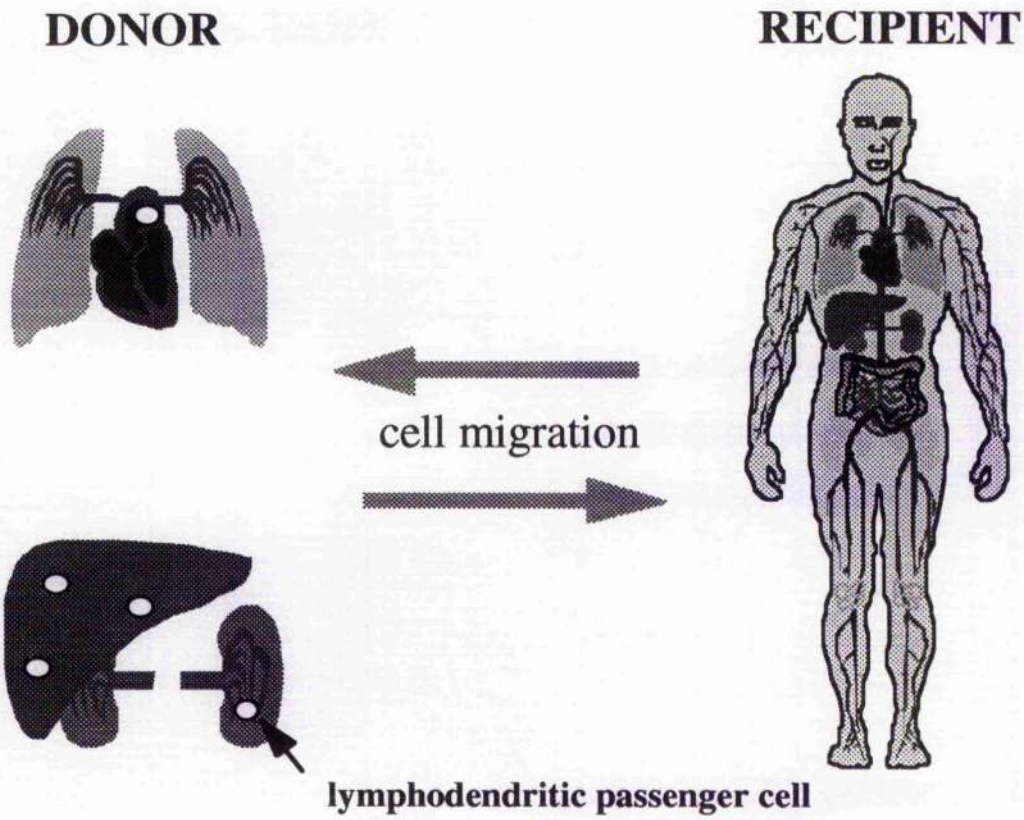
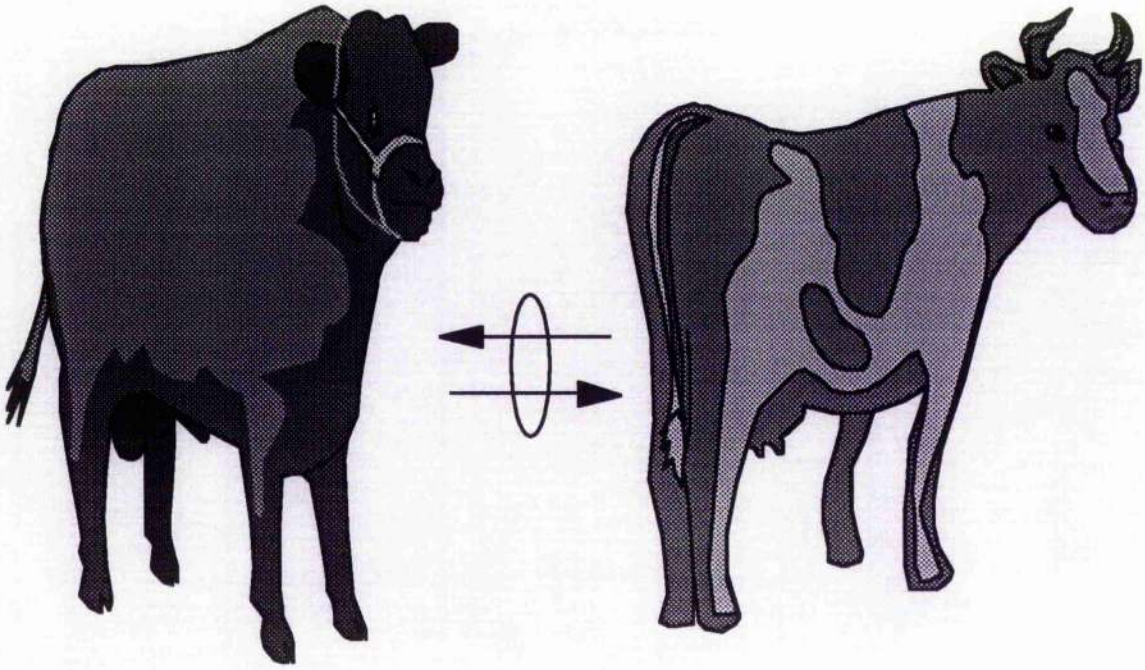


Figure 11.1. ***Following transplantation, so-called passenger cells, migrate from the donor organs and pass into the recipient circulation. It has been postulated that these cells are functionally active and may exert an immunomodulatory role. In liver grafts, recipient Kupffer cell migration establishes this cell population in the donor organ in the early post-operative period.***



Male animal *Original female cow masculinised (Freemartin)*

Figure 11.2. ***In-utero exchange of gonadal cells from the male to the female in these unlike-sexed dizygotic animals renders the female a sterile hermaphrodite (so-called freemartin) and both animals chimeras. Additionally, an exchange of cells from the lymphoid lineage populations creates a degree of tolerance between these animals. Skin grafts performed in later life between them are spontaneously accepted.***

- syndrome [n=2], AIH [n=2], cystic fibrosis [n=1], ALF [n=3], HCV and alcohol - related cirrhosis [n=1], HCC and HCV cirrhosis [n=1], hepatic metastases from primary teratoma [n=1].

11.2.2 Immunosuppression withdrawal protocol

Following baseline determination of standard liver biochemical tests and a liver biopsy to give a complete assessment of graft status as well as exclusion of allograft rejection, systematic immunosuppression withdrawal was performed to a standard protocol. The baseline maintenance regimens varied considerably; one patient was receiving azathioprine and corticosteroids only, one CyA only, three CyA and azathioprine and the remainder were administered all three agents. The median daily dosage schedules were prednisone 5 mg (0.08 mg/kg), azathioprine 75 mg (1.1 mg/kg) and CyA 200 mg (2.7 mg/kg). In those patients still administered corticosteroids, prednisone dosage was gradually withdrawn over a period of 4-6 weeks. The remaining immunosuppressive agents were then discontinued and patient clinical status and biochemical graft function monitored initially in hospital (2 week period) and thereafter in the clinic at 1-4 weekly intervals. All patients experiencing an episode of significant biochemical graft dysfunction (level of any routine biochemical liver parameter x 2-3 upper limit of normal) underwent percutaneous biopsy of the graft and histopathological assessment with liver allograft rejection defined by standard criteria (Snover et al 1987). Immunosuppression was recommenced when episodes of liver biochemical abnormalities were accompanied by any significant deterioration in histological appearance when compared to baseline. Following stabilisation, a further slower weaning schedule of immunosuppressive drug withdrawal was then attempted.

11.2.3 Determination of microchimerism

The presence of donor-derived microchimerism was examined by molecular analysis for class II MHC donor-type genotype sequences in recipient peripheral tissue. Whole blood, bone marrow and skin samples were collected from the patients prior to immunosuppression withdrawal and genomic DNA extracted from which the analyses were performed. Three separate methodologies were then evaluated for the determination of donor HLA-DRB1 alleles following polymerase chain reaction (PCR) amplification of the DNA. These techniques were sequence-specific oligonucleotide analysis (PCR-SSO dot blot) (Doherty and Donaldson et al 1991) (British Society for Histocompatibility and Immunogenetics, Molecular Special Interest Group, c/o University of Bristol, UK), one - step PCR with sequence-specific primers (PCR-SSP) (Olerup et al 1991) (Dynal Inc, Wirral, UK) and two-step nested sequence-specific PCR (PCR-nested SSP) (Bein et al 1991) (British Society for Histocompatibility and Immunogenetics, see above). All recipients had previously been genotyped and assigned HLA-DRB1 specificities by molecular methods (restriction fragment length polymorphism [RFLP] and / or PCR - SSO). Further confirmation of recipient HLA-DRB1 genotype was gained by PCR-SSP examination of genomic DNA retrieved from a fresh protocol liver biopsy. In appropriate male donor / female recipient pairings, the presence of Y gene-specific nucleotide sequences in recipient DNA was also determined by 2 separate primer pairs (Kogan et al 1987, Nakagome et al 1991). See appendix for detailed protocol in relation to the preferred method for determining donor-specific microchimerism (i.e. nested PCR-SSP amplification for DRB1 alleles).

11.3 **RESULTS**

11.3.1 Clinical and histological outcome

Of the 18 patients, 5 experienced no deterioration in graft function continuing without significant abnormalities in biochemical tests and remaining clinically well when off immunosuppressive agents. Three of these patients had developed minor fluctuating elevations of serum AST and biliary enzymes activities to just above the upper limit of normal at some point following immunosuppression withdrawal which finally settled. All 5 patients were transplanted for conditions with a non-immune pathogenesis (Budd-Chiari syndrome 2, alcohol-related liver disease 1, acute liver failure 1, cystic fibrosis-related liver damage 1). Biopsies of the liver graft, performed in 3 at a minimum of 8 months following withdrawal, showed no major changes from findings present at baseline and in particular no evidence of rejection changes.

The remaining 13 patients experienced derangement in biochemical tests such that immunosuppression was recommenced in 12 at a median of 3 weeks (range: 2-24 weeks) after initial withdrawal of drugs. In these patients, several patterns of abnormalities in graft dysfunction were observed with variable and often initially fluctuating elevations in AST activities (median peak value 288 IU/L [range:73-830 IU/L] occurring. These were first noted at a significant level (100 IU/L i.e.≥ x2 upper limit of normal) at a median of 25 days (range:10-176 days) post-withdrawal. Parallel rises in the biliary enzymes (alkaline phosphatase, γ-glutamyltransferase) accompanied these changes in AST. A rise in serum total bilirubin occurred in 7 patients (median peak value 53 μmol; range 28 - 474 μmol) at a median of 32 days following withdrawal. The one patient of these 13 with a deterioration in graft dysfunction in whom immunosuppression was not deliberately recommenced was suffering from a post-transplant lymphoproliferative disorder as well

as severe intra and extrahepatic biliary stricturing consequent on primary sclerosing cholangitis recurrence for which retransplantation was finally carried out at 4 months.

Histopathological assessment of the graft in these 13 cases, during the episodes of biochemical dysfunction, revealed characteristic histological features of acute allograft rejection in 4 patients. One specimen was graded as severe rejection and the others mild to moderate. A more common pattern of a mixed inflammatory portal tract infiltrate composed of predominately lymphocytes with fewer plasma cells and occasional eosinophils was seen in 7 patients (figure 11.3). Two of this group also had severe neutrophilic infiltration of the portal tract. In 3 patients of this group, minimal or mild limiting plate spillover and a lobular hepatitis in the form of occasional spotty necrosis and mixed inflammatory cells present within sinusoids was found. In 2 patients, these features were accompanied by minimal focal endotheliitis. Infiltration within bile ducts was rare. This overall picture of a hepatitis-like disorder appeared to represent exacerbation of similar but less severe baseline findings in 5 of the patients and a de novo appearance in 2 cases. In two patients this histological picture was observed in the context of a co-existing viral illness (Coxsackie in 1, unidentified in other). One further case showed features of acute hepatic necrosis with evidence of previous parenchymal necrosis along with a moderate mixed inflammatory cell infiltrate in post-necrotic areas and portal tracts. Extensive hepatocyte ballooning and regeneration and severe cholestasis were also found. This patient had a preceding history of a viral-like illness and non-steroidal anti - inflammatory agent usage. In the final patient in this group, no significant abnormalities were detected in the liver biopsy. Overall, in these 13 patients no correlation between biochemical abnormalities and accompanying histological features was detected. In particular, no relationship between hepatitis / and or rejection and the height of the serum transaminase activity was found.

The reintroduction of immunosuppression and the schedule followed was determined predominantly on the basis of serum AST activities rather than pathological features. The 4 patients with modest elevations of AST (approximately 150-300 IU/L) were managed with short courses of additional oral prednisolone (30 mg / day 2 weeks, 20 mg one week then gradually reduced to either 5 mg / day or withdrawn) in addition to reintroduction of approximately 50-75% of their maintenance baseline immunosuppression. In 8 patients, with higher AST activities, a conventional high-dose corticosteroid anti-rejection regimen (methylprednisolone 1g for 3 days) was administered in addition to a reintroduction of the complete baseline immunosuppression. Following such changes, AST activities returned to the normal range rapidly in 9 of 12 patients. The remaining 3 patients in whom graft function did not normalise quickly received an additional course of high-dose corticosteroids and, 2 patients underwent temporary conversion to tacrolimus. Elevations in the serum bilirubin level returned to within the normal range considerably more slowly (median 3 weeks following commencement of treatment) than the rises in liver enzymes.

In 2 patients in whom the serum bilirubin was slow to fall and in whom histological features of only mild hepatitis or rejection had been present on biopsy, investigations of biliary drainage were performed. In both patients, percutaneous cholangiograms revealed dilated biliary tracts and stasis within the gallbladder conduit which had been used in the original anastomosis. Biliary reconstruction was subsequently performed with resolution of the biochemical changes.

In these 12 patients in whom immunosuppression was reintroduced and following an interval allowing stabilisation of graft function, a further attempt at drug withdrawal was initiated except in the single case who had experienced a severe episode of acute cellular rejection. On occasion, a weaning regimen was employed which comprised a step-wise progressive reduction of immunosuppressive drugs.

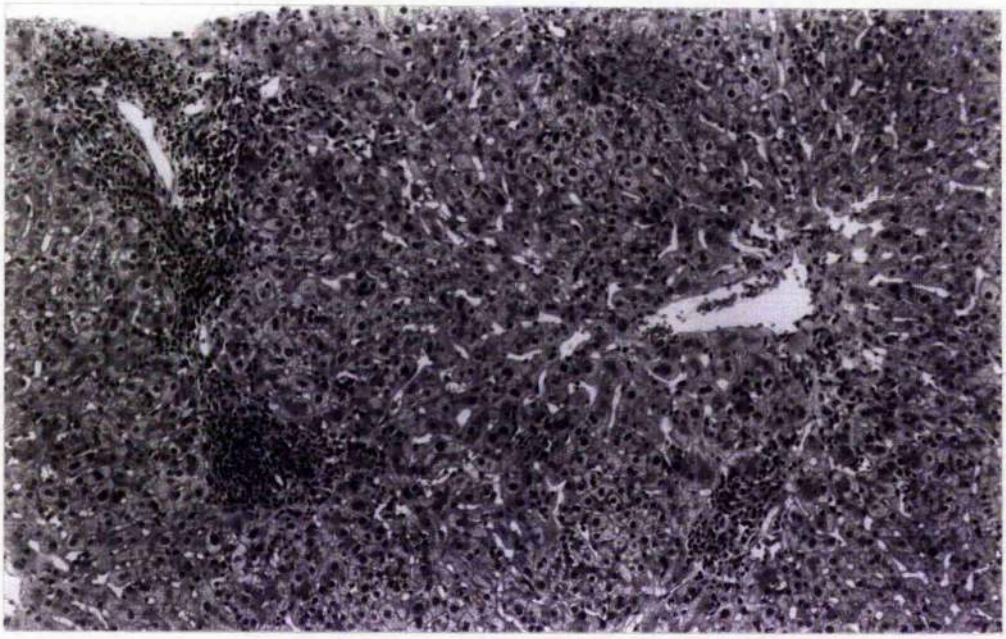


Figure 11.3 Liver histology 8 weeks after partial immunosuppression withdrawal. There is moderate portal inflammation with periportal cell spillover and a mild lobular hepatitis. H&E x80

This schedule is on-going with results presented at a minimum of 8 months following initial commencement. In 9 of the 12 patients, further elevations of liver enzymes have occurred. Despite this common pattern of enzyme elevation, 9 patients were successfully weaned to a significant degree and are presently receiving lower dosages than at baseline, with normal graft function at a minimum of 6 months following first discontinuation. Seven patients are presently receiving less than 50% the baseline corticosteroid and azathioprine dosage accompanied by either whole blood CyA levels below 60 ng/ml (the lower limit for maintenance immunosuppression) (n=5) or complete withdrawal of this agent (n=2). Prednisolone has been stopped in 4 patients and azathioprine in 6. The median daily dosage schedules and percentage [range] changes for the immunosuppressive agents at latest follow-up are prednisolone (0.06 mg/kg (- 25% [100 - 0%]), azathioprine (0.39 mg/kg, (- 64% [100-0%]) and CyA 1.77 mg/kg (-37% [-18 - 100%]).

Of the 18 original patients, 17 are alive and remain clinically well. The single death in a patient 6 years post-transplant, was due to recurrence of hepatocellular carcinoma at eight months after withdrawal of immunosuppressive drugs. Tumour recurrence had been evident at the time of study entry and had been the reason for attempting withdrawal in this patient. A further patient (described above) was successfully retransplantated following drug withdrawal and the one patient with an episode of acute hepatic necrosis, as already referred, has on-going graft dysfunction despite reintroduction of immunosuppression.

11.3.2 Patterns of chimerism

Two-step nested sequence-specific PCR was the most sensitive methodology for the detection of donor-type genotypic sequences. This method detected HLA DRB1 allele specificities of donor-type in 7 (39%) of the 18 patients. PCR-SSO and single-step PCR - SSP methodologies were less sensitive (22% with PCR-SSO and 11% with single-step PCR-SSP) and were abandoned after 12 and 9 patient samples respectively. Demonstration of donor genomic elements by Y-gene primers concurred entirely with the positive and negative results achieved with all of the above techniques and confirmed chimerism in 3 female recipients. No sample resulted in a false-positive result with respect to presence of Y-gene sequences and not the appropriate donor HLA-DRB1 alleles. Of the tissues examined, the presence of donor genomic sequences was found most often in skin. Employing the nested PCR-SSP methodology, donor HLA-DRB1 was found in 5 patient samples from peripheral blood, 4 from bone marrow and 7 from the skin sample.

11.3.3 Relationship between chimerism and operational tolerance

From the 7 patients with evidence of microchimerism, 2 (29%) have been successfully withdrawn and maintained off immunosuppression at 6 months follow-up. A further 3 (43%) have been weaned significantly following partial reintroduction of immunosuppression. The final 2 (29%) chimeric patients experienced severe graft dysfunction. Both these cases experienced an episode of acute allograft rejection confirmed histologically. Of the 11 patients who had no evidence of microchimerism, 3 (27%) have successfully been removed from immunosuppression and a further 6 (55%) weaned significantly. In the final 2 (18%) non-chimeric patients, a return to the baseline immunosuppression level was required.

11.4 DISCUSSION

This report provides further evidence for the unique immunologically privileged position of liver allografts as compared to other solid organ transplants. From the initial observations of Garnier (see section 1.5) that genetically disparate porcine liver allografts were spontaneously accepted in the absence of immunosuppression, considerable experimental and clinical observations of the reduced immunogenicity and tolerance - inducing properties of a liver graft have been reported (Garnier et al 1965, Calne et al 1967 and 1969, Kamada et al 1981). The clinical tolerance which has been demonstrated adds to the observations of a low incidence of immunological graft failures secondary to a reduced susceptibility to hyperacute rejection, resistance to positive cross-match, less frequent and severe acute and chronic rejection and also an ability to induce donor-specific tolerance (Iwatsuki et al 1981, Duquesnoy et al 1989). In the present series, it was possible to either completely withdraw (5 of 18 patients) or significantly reduce (9 of 18) maintenance immunosuppression, to previously considered sub-therapeutic levels. Such results differ from those seen in other solid organ recipients where the hazards of even partial immunosuppressive drug withdrawal from maintenance regimens are well recognised (Stiller and Opelz 1991).

The results in the present study confirms the earlier report from the University of Pittsburgh based on weaning immunosuppression in 15 patients, although a lower proportion of patients exhibiting complete operational clinical tolerance (5 of 18 [28%] vs 12 of 15 [80%]) in that series was seen (Reyes et al 1993). There are several possible explanations for this difference. In the face of a developing episode of graft dysfunction, there was a low threshold for reintroducing the immunosuppressive agents, whereas it was clear from later experience in which withdrawal was performed in a slower manner, as was the procedure followed in Pittsburgh, many of these episodes resolved

spontaneously or required only minor temporary increases in corticosteroid dosages. In addition to different withdrawal regimes, the characteristics of the study populations in the 2 studies are also quite disparate. In the Pittsburgh series, the majority of patients were from the paediatric age group with a large percentage suffering from a post-transplant lymphoproliferative disorder and already on lower baseline immunosuppression. The potential tolerising influence of lymphoma, which in the post-transplant situation is commonly of donor origin (unpublished observations), is one further possible explanation for the different frequencies of tolerance observed in the two series.

The presence of clinical tolerance in long-term recipients even in varying degree has the potential for improving the natural history of liver transplant patients. Longer follow-up is however required before the effect of immunosuppression withdrawal on drug-related toxicity or occurrence of de novo and recurrent infectious and malignant complications can be determined. Reports regarding the efficacy of partial immunosuppressive drug withdrawal in relation to reversal of drug toxicity complications are mixed, particularly when these are long-standing as for instance with chronic CyA-related nephrotoxicity (Sandborn et al 1994). Nevertheless, data from a recent study in renal patients of steroid withdrawal and some preliminary observations suggest that significant reductions in the prevalence of hypertension and glucose and lipid abnormalities can be anticipated (Fabrega et al 1994).

Several novel patterns of liver blood test abnormalities and histological graft appearances were observed following withdrawal of immunosuppressive drugs. The expected biochemical pattern of acute liver allograft rejection as seen in the early post-transplant period with elevations in biliary and hepatocellular enzymes and an associated rise in bilirubin was uncommon. Biochemically, the variable but occasionally very severe

elevations in serum transaminase activities (up to 40 fold rises) indicates a hepatocellular - directed reaction which is consistent with the predominant damage to this cell which is found in pharmacologically unmodified experimental and clinical recipients undergoing rejection (Starzl et al 1969, Williams et al 1973). As was also noted in the Pittsburgh patients, a biochemical flare of enzymes can resolve spontaneously or with minor reintroductions of immunosuppression. This pattern has also been observed in experimental animals when immunosuppression was discontinued (Hunt et al 1967, Porter et al 1969). However, classical features of liver allograft rejection, as characterised by the triad of endotheliitis, portal tract inflammation and bile duct damage, developed in a minority of patients only (4 of 18). More commonly when there was elevation in liver enzymes, the graft biopsy demonstrated moderate or severe portal tract inflammation occasionally with features of a lobular hepatitis. The significance of this pattern is unknown. Whether these graft-infiltrating cells represent an immune response to a latent serologically undiagnosed virus or a previously unrecognised *forme fruste* of allograft rejection is speculative. This unit has previously reported that this histological pattern is a common feature in liver graft biopsies from immunosuppressed long-term recipients and could not be attributed to known hepatitis viruses (Slapak et al 1995). The apparent influence of systemic viral infections on graft function, as seen in possibly 3 patients is of interest, and is in keeping with previous observations from this unit that such infections can be associated with late acute liver allograft rejection in conventionally immunosuppressed patients (Cakaloglu et al 1995). In non-transplanted patients, withdrawal of both chemotherapy and immunosuppression can result in a biochemical and histological flare of an underlying hepatitis (Bird et al 1989, Galbraith et al 1975).

There has been considerable interest in the observation from Starzl's group that the presence of microchimerism, derived from the donor, was a universal feature of long-term liver and renal recipients and was associated with graft acceptance. This phenomenon was

present in approximately 40% of the long-term liver transplant patients studied in this series. The methodology used in this study does allow donor and recipient differentiation at the genomic level and it can be confirmed that the foreign DNA which was detected has donor, rather than third party, characteristics (such as could have been derived from a blood product transfusion). A variation in the detection rate of chimerism in relation to the recipient tissue, with skin having the highest incidence, was also observed. These results with respect to tissue distribution and also the overall proportion of patients with chimerism are, however, virtually identical to a recent study from Subereille performed in long-term renal recipients where they found chimerism in 33% of studied patients again predominantly in the skin (Subereille et al 1994). The preferred method for detection of donor class II MHC, based on the nested PCR methodology for donor HLA DRB1 alleles, is both readily available in molecular transplant HLA laboratories and sensitive (Knoop et al 1994). However, a limitation of this present study is that this technique does not yield any information on the localisation or viability of the detected donor genotype or the cell type from which it is derived. This is a general limitation to PCR-based methodology. Whether, for example, the donor DNA which was detected is shed from the graft, and could be derived from the soluble class I antigen which is released from liver parenchymal cells, or represents lymphoid cells which have migrated and become established from the substantial passenger cell pool present in human liver grafts cannot be determined (Davies et al 1989, Schlitt et al 1993).

In this study, detection of systemic donor-type microchimerism did not allow identification of tolerance and accordingly cannot, as was hoped, be utilized as a tool in the patient selection for prospective drug withdrawal. A major role for microchimerism as either a mechanism or a marker for graft acceptance was not identified in the present series with similar proportions of chimeric patients experiencing rejection and non-chimeric patients exhibiting tolerance. Furthermore, a case-report of intractable acute allograft

rejection developing in a liver recipient 8 years after transplantation, in whom immunosuppression was reduced, in the presence of chimerism has also recently been reported (Schlitt et al 1994). The present results also do not agree with the observation that the presence of chimerism was associated with donor-specific unresponsiveness, as assessed by mixed lymphocyte reaction (MLR) methodology in long-term renal recipients. Furthermore, the sensitivity of *in vitro* observations of anti-donor reactivity, using MLR which employs recipient peripheral circulating rather than graft-infiltrating cells retrieved under the umbrella of immunosuppression is questionable.

Since the presence of chimerism cannot be employed as a marker of tolerance, further investigations into identification of this state are required, taking into account the several controversial but presently unsubstantiated hypotheses relating to the unique position of liver grafts. Whether clinicians can enhance or promote clinical tolerance remains uncertain. It is notable that all these patients and, indeed most liver transplant recipients, have received third-party blood transfusions which are known to inhibit T-cell responsiveness (van Twuyver et al 1993). These transfusion requirements at levels which are very much higher than required for renal transplantation could have functional significance and contribute to the tolerance.

Close clinical surveillance will be required to monitor other effects which may follow from the return of immunocompetence such as recurrence or deterioration of the primary diseases which have an immunological pathogenesis such as AIH or PBC. The hazards which may ensue in this instance are documented in the next section.

11.5 HAZARD OF IMMUNOSUPPRESSION WITHDRAWAL

Recurrence of autoimmune hepatitis following withdrawal of immunosuppression

11.5.1 Background

As outlined in the previous discussion, the return of immunocompetence may promote recurrence of immune-mediated disorders inadvertently controlled by the anti-rejection drug therapy. Disease recurrence after liver transplantation is increasingly recognised as a major cause of graft damage with accompanying patient morbidity and, in certain viral and malignant disorders, mortality as discussed in chapter 2. Amongst the immune-related transplant indications, recurrence is less well documented and not presently considered a major cause of graft dysfunction.

Patients with end-stage AIH accounted for 2.6% of recipients in the ELTR (ELTR et al 1992). This disorder which is characterised by specific HLA associations and disordered immunoregulation frequently responds to immunosuppression. In particular, corticosteroids and azathioprine can induce clinical remission and remain the mainstay of current treatment protocols with anecdotal reports of response to CyA in certain cases (Cook et al 1971, Murray-Lyon et al 1973, Mistilis et al 1985). The use of these particular agents in conventional 'triple therapy' maintenance immunosuppression regimens following transplantation to control liver allograft rejection may, by secondarily inhibiting the autoimmune process, explain why there are so few reports of recurrence of AIH and other immune-related liver disorders. Recurrence of this disorder following transplantation was first reported from this unit in 1984 with a larger series also reported from the University of Pittsburgh (Neuberger et al 1984, Wright et al 1992).

In this report, severe recurrence of AIH in the primary liver graft of a recipient 10 years after transplantation who had received maintenance immunosuppression consisting of CyA alone is described. This case, as well as providing further evidence for recurrence of this disorder and highlighting the necessity for indefinite close clinical and pathological surveillance post-transplant, additionally raises the potential hazards which may ensue with low or indeed as recently proposed withdrawal of pharmacological immunosuppression.

11.5.2 Case report

Patient (CS) initially presented as an 8 year old girl in 1975 with hepatomegaly and elevated serum transaminase activities. Investigations revealed characteristic histopathological features of chronic active hepatitis with elevated serum globulins and autoantibodies (positive for antinuclear antibody (ANA) 1:20, gastric parietal cell antibody (GPC) 1:80). The patient was negative for anti-smooth muscle antibody and retrospective examination of stored serum subsequently demonstrated a positive liver-kidney-microsomal (LKM) antibody at a titre at 1:160. Following the diagnosis she was initially treated with corticosteroids and azathioprine. Due to haematological complications attributed to azathioprine and histological progression of the disease she was transferred to CyA following its clinical introduction in 1982. By 1984 after an 8 year period of clinical remission the patient experienced her first decompensation with the development of ascites and spontaneous bacterial peritonitis and at the age of 17 proceeded to elective orthotopic liver transplantation.

The patient was HLA A and B phenotyped by standard complement dependent microcytotoxicity assay, and DNA analysis was performed for HLA DRB, DQA and DQB alleles by a combination of RFLP and PCR amplification / oligonucleotide probing (Doherty et al 1994). The patient's HLA type was characterised as A1 A24 B8 B39 Bw6

DRB1*0101 DRB1*0301 DRB3*0101 DQA1*0101 DQA1*0501 DQB1*0201 DQB1*0501. The donor was serologically characterised only (data supplied by United Kingdom Transplant Service) as A11 A32, B14 B22, DR2/DR7.

With the exception of surgical correction of a biliary fistula related to T-tube displacement the patient made an uneventful recovery. Induction immunosuppression comprised a dual therapy regimen of corticosteroids and CyA. Satisfactory rehabilitation was achieved and the patient resumed her educational and social activities. Puberty and sexual development which had been delayed now occurred. Annual clinical review in the second post - transplant year confirmed normal liver biochemistry and immunological profiles. At this time-point the patient, now 19 years old, was non-compliant for corticosteroids due to 'unacceptable' cosmetic effects and was subsequently maintained on CyA only within a therapeutic blood level range of 130-500 ug/l (polyclonal Sandimmun assay). In the third post-transplant year, the patient suffered an episode of severe pelvic inflammatory disease with confirmed chlamydial infection and was found to have developed cervical intra - epithelial neoplasia (CIN - colposcopy grade II). Due to this condition and the presence of normal biochemical graft function, CyA levels were maintained towards the lower limit of the above therapeutic range. Clinical reviews, performed on an annual basis, over the next 6 years were satisfactory except for the presence of a clinically palpable spleen.

At 10 years post-transplant, the patient who had remained clinically asymptomatic throughout this time was considered for entry into the programme of immunosuppressive drug withdrawal. A complete assessment of graft function and systemic immunosuppressant-related complications was performed in April 1994 and showed for the first time that the patient had abnormal liver function tests: raised AST 164 IU/l, [normal range (nr) 10-50 IU/L] and total bilirubin 28 umol/L [nr 3-20 umol/L]) levels were noted alongside markedly elevated globulin levels (IgG 49g/l, nr 7-18.6 g/l) and

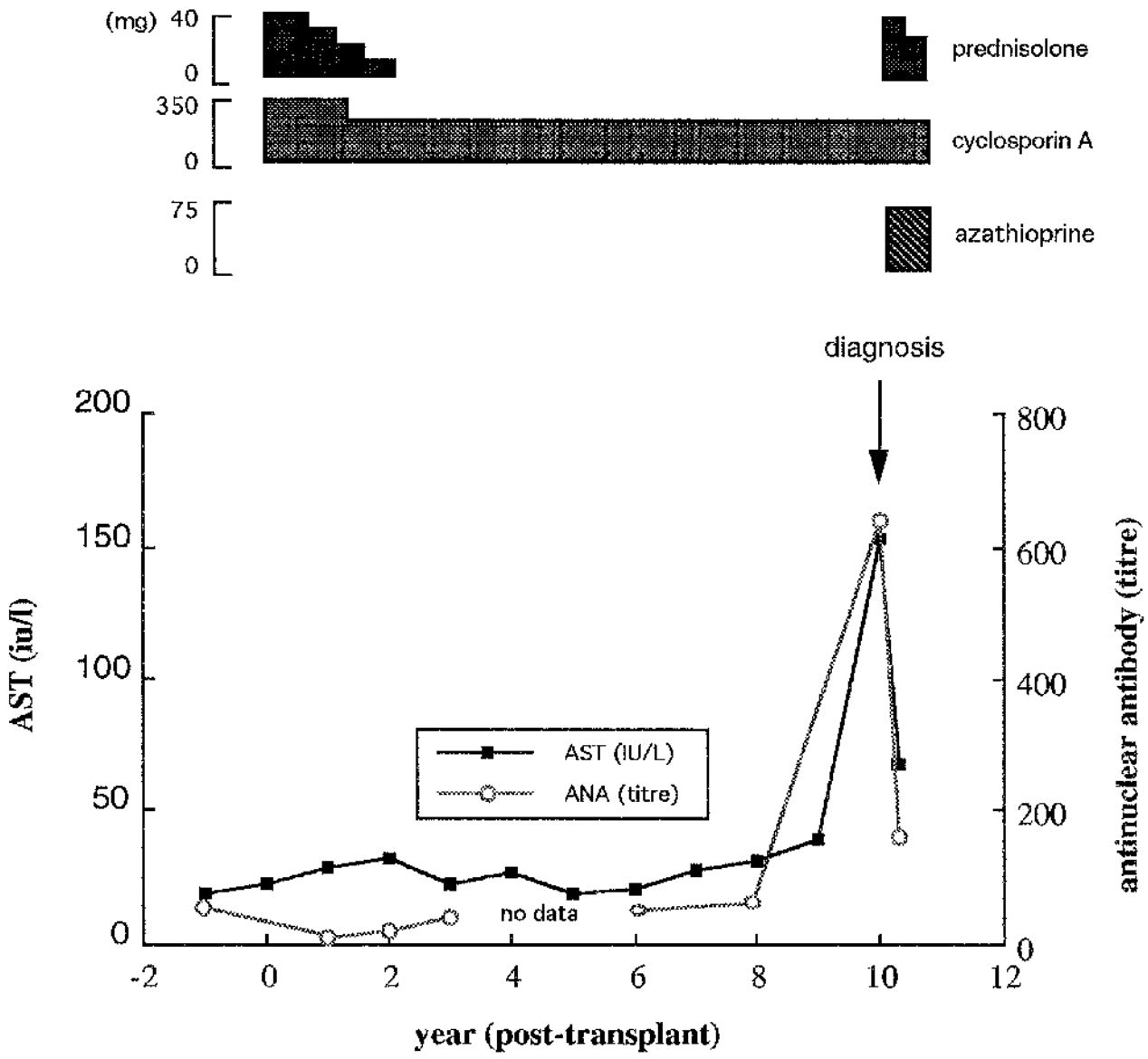


Figure 11.4 ***Serial measurements of antinuclear antibody (ANA) and amino aspartate transferase (AST) levels over the 10 years following transplantation. The titre of ANA fluctuated between 1:20 to 1:80 throughout the clinical course until the time of diagnosis when they rose to an unprecedented titre of 1:640. AST levels were reassuringly maintained in the normal biochemical range up till the point of diagnosis. Concurrent immunosuppression protocols administered over the above period are also shown.***

- autoantibodies (particularly ANF at titre of 1:640 and GPC 1:80) (see figure 11.4). Total serum alkaline phosphatase level was normal at 68 IU/L [nr 30-85 IU/L]. Ultrasound examination of the graft demonstrated a heterogeneous liver parenchymal texture with no evidence of biliary obstruction. A percutaneous biopsy of the graft with histopathological assessment revealed a severe combined lymphocytic and plasma cell infiltrate within the portal tracts accompanied by extensive periportal and bridging collapse. The interlobular bile ducts were preserved. Portal fibrosis with bridging septa formation and parenchymal hyperplasia were also present (figure 11.5). No hepatitis viruses were detectable by conventional serological methods (IgM HAV, HBsAg, IgM HBc antibody [Abbot, North Chicago, Illinois] and HCVAb [United Biomedical Inc. Lake Success, N.Y.] were all undetectable). HCV RNA was not detected in serum (Amplicor, Roche, Basel, Switzerland]. Additionally, there was no serological or immunohistological evidence of cytomegalovirus or Epstein-Barr viral infection. Following the diagnosis of recurrent AIH, corticosteroids (prednisolone 40 mg per day in a reducing regimen) and azathioprine (100 mg per day) were added to CyA and induced biochemical remission by 6 weeks. No follow-up biopsy of the graft is presently available.

11.5.3 Discussion

The disordered autoreactivity which is the hallmark immunological abnormality present in AIH is unlikely to be corrected by liver replacement. Hence, it is perhaps surprising that there are only sporadic reports of disease recurrence following transplantation. In the patient described in this report, serological and histological evidence of disease recurrence was convincingly diagnosed 10 years after transplantation (presently the longest recorded post-transplant interval to recurrence in the literature). Employing the international AIH diagnostic scoring system in relation to the required parameters, the patient had an aggregate score of 15 points consistent with 'definitive' disease (Johnson et al 1993).

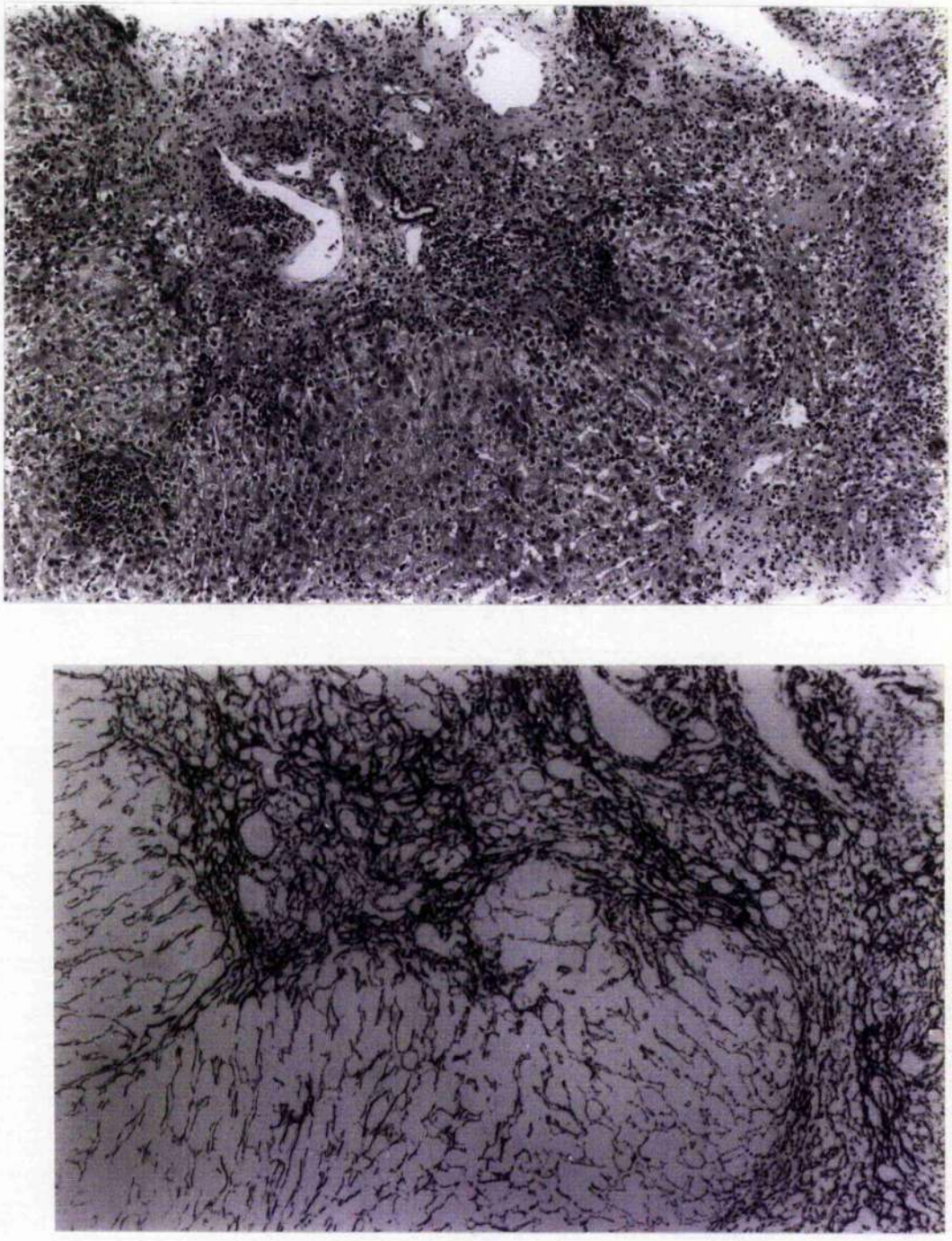


Figure 11.5 ***Liver histology 10 years post-transplant. (a) Prominent fibro-inflammatory portal expansion and bridging parenchymal collapse with active inflammation at the interface between fibrous areas and parenchyma. H & E x 80. (b) same field as (a) stain for reticulin x 80***

The value of this particular scoring system, which was developed in non-transplant patients, in liver recipients is presently not known. Notably, recurrence developed following many years of immunosuppression consisting of CyA alone. This particular regimen which was driven by an unusual combination of patient and medical pressures may, with the benefit of hindsight, be considered a sub-optimal protocol omitting as it does both corticosteroids and azathioprine which are the present mainstays of inducing and maintaining remission in AIH. Withdrawal of these agents from non-transplanted AIH patients during clinical remission is associated with a high incidence of relapse (Hegarty et al 1983). This case report should therefore reinforce clinical caution before considering withdrawal of immunosuppression in this group of long-term liver recipients. Early observations from the University of Pittsburgh programme suggest that withdrawal is accompanied by a high incidence of biochemical graft dysfunction in patients transplanted for autoimmune liver disease (Reyes et al 1993). Although the pathological features of the patients in that report appear inconclusive and no data on autoantibody profiles are given, the presence of elevated liver enzymes in such a population demands close clinical surveillance. In contrast to the experience from the University of Pittsburgh, recurrence of autoimmune hepatitis is a rarely diagnosed complication in this unit's series with only 2 well-documented cases from forty-nine patients transplanted for this indication and who survived at least one year.

The natural history of long-term liver recipients is becoming clearer with clinical and histological follow-up of the first generation of patients. A recent review of long-term survivors in this unit revealed an alarming and unexpectedly high frequency of significant graft pathology, which included late development of primary disease recurrence in asymptomatic patients (Slapak et al 1995). This individual report of AIH recurrence after such a long period of excellent biochemical graft function is further evidence for the requirement of close and indefinite clinical follow-up in liver recipients. Protocol graft

biopsies, performed at least every two years, could be considered part of this surveillance. In this instance, the persistent reluctance of this patient to undergo liver biopsy may have delayed the diagnosis although conventional liver function tests and autoantibody levels were normal up to the eventual time of histological assessment of the graft and diagnosis. However, the severity and characteristics of the pathological damage to the graft demonstrated at the time of initial biochemical abnormality suggest that the disease process had been on-going for a considerable period. Monitoring hepatocellular damage in AIH through standard laboratory indices (levels of AST and total bilirubin) is a hazardous undertaking with normal values being unreliable predictors of concurrent morphological activity (Czaja et al 1981). In this report, the striking discrepancy between the severity of the histological changes and the modest elevations in AST reinforces the comparative insensitivity of routine biochemical liver function tests. This case suggests that in the post - transplant setting there is justification in employing more sensitive markers of autoimmune reactivity and subsequent disease reactivation such as antibodies against the liver-specific membrane lipoprotein preparation (anti-LSP) or against the hepatic asialoglycoprotein receptor (anti-ASGPR) which can be predictive of relapse in the non-transplant setting (McFarlane et al 1984, Treichel et 1993).

A consistent finding of the two previous published reports on post-transplant recurrence of AIH, which is also present in this case, is a mismatch of the donor and recipient for HLA DR3 (present in the recipient, absent in the donor [D-, R+] (Neuberger et al 1984, Wright et al 1992). Similar but less strong associations with recurrence were noted for the B8 locus in the Pittsburgh study. These observations may be a chance finding reflecting the high prevalence of the A1-B8-DR3 haplotype in AIH liver recipients (79% in this units patients) and its comparative rarity in the randomly selected unmatched general donor pool (11% in the local population) (Donaldson et al 1991). This recipient haplotype, which is associated with a range of humoral and cellular immunological abnormalities, appears to

be sufficient to generate AIH even in the presence of a completely unmatched liver. These observations have important implications for the proposed mechanistic role of HLA encoded disease susceptibility (Donaldson et al 1994). T-cell clones mediating the autoimmune process must remain immunoreactive despite liver and hence hepatocyte antigen replacement, indicating the major importance of the T-cell receptor repertoire compared to the apparently less crucial target cell MHC antigens once the autoimmune process is established. The hypothesis of Sheehy which proposes that HLA disease susceptibility alleles inefficiently present disease-associated antigen peptides and thereby prevent clonal deletion of autoreactive T cells would appear to best explain the development of recurrent disease (Sheehy et al 1992).

If a relationship between A1-B8-DR3 present in recipients and subsequent disease recurrence was shown this would be consistent with previous reports that this haplotype is a marker not only of susceptibility, but also of disease aggressiveness and indeed progression to transplantation (Doherty et al 1994, Donaldson et al 1991, Sanchez-Urdazpal et al 1991). Given that no effort to select recipients is based on donor HLA phenotype, it would be difficult to determine whether a match at the above loci confers either further susceptibility to, or protection from, recurrence, as the number of transplants required to generate a sufficient study population is considerable given the rare occurrence of spontaneous random matches.

CHAPTER 12

GENERAL DISCUSSION

Achieving a successful outcome after clinical liver transplantation is an inexact science. Less than 30 years from the date of the first long-term survivor, clinical practice is still evolving rapidly. That a standardised clinico-pathological classification of transplant failures is not available reflects the immaturity of this emerging speciality. The classification proposed in chapter 2 which is based on the clinical experience of this unit, should stimulate discussion on this neglected topic. As with any classification, simplicity is sought at the possible expense of detail with the 8-category scheme a compromise to these principals. Providing the structure of a classification scheme does not however assist in the equally problematic assignment of individual cases to the appropriate categories. A forum may be required amongst potential users of this classification to either refine the proposal or lay guidelines for achieving a consistency in allocating transplant failures.

One of the functions of data which is generated by a classification of pathological events should be to direct research to these major contributing categories. In this thesis, individual investigations in relation to the 6 largest categories from the proposed 8-point classification of transplant failure are examined. Rejection (classified under immunological transplant failure) accounted for the largest proportion of these failures and was separately examined in 3 chapters. Investigations examined a novel demographic risk factor, a new parameter proposed as an adjunct to immunological monitoring and finally the therapeutic efficacy of a newly introduced immunosuppressant in a high-risk recipient population. Identifying those liver recipients at increased risk of rejection could allow closer clinical

surveillance, earlier therapeutic intervention as well as providing insight into the underlying mechanisms of this process. The importance of the ethnic origin of liver recipients was examined in chapter 6. Solid organ donors in the UK, for several cultural and religious reasons, are overwhelmingly of caucasian ethnic origin i.e. are procured from a single and immunologically comparatively homogeneous population. The liver unit at Kings College Hospital is in a unique position to examine the influence of transplanting such donor organs into an ethnically heterogeneous recipient population, given the international referral base from which the transplant population is derived. A penalty of increased rejection and subsequent graft loss in those recipients from non-caucasian origins was demonstrated. Prospective confirmation and examination of the influence of both major and minor HLA disparities (perfomed by molecular analysis) will determine the significance of this phenomenon and the relationship to HLA determinants. It is possible that non-HLA donor or recipient factors may modify an increased susceptibility to rejection. For example, the increased prevalence of end-stage liver disorders secondary to viral hepatitis, which is present in non-caucasian liver recipients, may promote the rejection process. The logistics of graft procurement and allocation would not allow matching recipients and donors for ethnic origin.

Immunological monitoring of rejection remains an attractive, but as yet elusive, standard clinical practice. Conventional diagnosis of rejection in all solid organ allografts relies upon histopathological confirmation as the gold-standard method. Reliance on this method prevents serial and regular assessment of the rejection process and is also accompanied by an appreciable morbidity and occasional mortality. Measurement of nitric oxide generation is a potential clinically useful parameter of the in-vivo allograft response. The assay is robust, stable and cheap and appears to correlate with rejection and its severity. At present, however it is cumbersome to set-up and requires considerable laboratory space and facilities. Real-time analysis of prospective samples comparing measurement of NO

end-products to the presently employed liver function tests should confirm the realistic proposal of employing this agent in post-transplant monitoring. The source of the detected circulating nitric oxide end-products which have has now been found in two different organ allograft investigations of rejection (liver and cardiac) remains speculative. These results provide indirect evidence that the generation is derived from processes present in both disorders. Hence, the immunological cascade present in rejection or possibly endothelial cell damage which is targeted in both organ rejection processes are potential sources for the observed production. Elucidating the precise localisation of this generation has presently confounded immunohistochemical and in-situ hybridsation probing, for the inducible nitric oxide synthase (iNOS) protein and mRNA respectively, in clinical samples (unpublished observations). The precise pathogenic role of NO in inflammatory conditions is not yet adequately defined and was not addressed in this investigation. Proposals for either supplementing or inhibiting NO production during a rejection episode are premature.

The present immunosuppressant agents which are employed clinically have unfavourable therapeutic indices. Efficacy is often only reliably achieved at the expense of elevated dosage regimens with significant accompanying multi-system toxicity. Tacrolimus (FK506) is a novel agent recently introduced to the transplant physicians immunosuppression armamentarium. Early clinical experience with this agent was centred around liver transplantation with provisional evidence indicating favourable properties. The data presented in chapter 7 provides further evidence that this agent is a viable alternative to CyA with potential advantages in relation to superior control of rejection and reduced infection rates. The randomised trial from which the presented data was derived did however have serious flaw designs with, in particular, a negative bias against tacrolimus. In particular, those recipients who were randomised to the conventional CyA based regimens could, in the event of intractable rejection, be administered tacrolimus for

salvage or rescue treatment. A significant proportion of these grafts were successfully rescued and translate into favourable survival data for the conventional regimen. The convincing increase in efficacy which was observed with tacrolimus is not matched by a decline in toxicity. Overall, the safety profile of tacrolimus is broadly similar to CyA with respect to neurotoxicity, nephrotoxicity and diabetogenicity. Whether the benefits of monotherapy with tacrolimus, which appears to provide adequate maintenance immunosuppression, will confer benefits in relation to toxicity over longer follow-up will require study. The pharmaco-economic benefits of a tacrolimus-based immunosuppression regimen compared to a conventional one now require to be evaluated. Whether the reduced intensive care admissions seen in this study were accompanied by shorter hospital stays and reduced admissions over the study period in the large general population of recipients would be of major interest.

From the proposed classification of transplant failures, technical complications then primary graft dysfunction were the next largest groups after the category of immunological transplant failure. The commonest technical complication was hepatic artery thrombosis, which alongside other vascular pathologies, was examined by hepatic angiography in chapter 4. This invasive investigational technique presently represents the optimal method in clinical assessment of graft vascular damage. Future studies will need to address this traditional technique against new and less invasive angiographic radiographic methods such as magnetic resonance imaging. The present study does however confirm the sensitivity of conventional angiography in the diagnosis of the major vascular complications of hepatic artery thrombosis and stenosis and provides additional comparative data on their prognostic significance. The relationship between chronic rejection and vascular damage in liver transplantation, which is poorly characterised, was also examined. The data from this study demonstrates the very unfavourable prognosis of chronic rejection when there is an associated rejection vasculopathy, a phenomenon which

is well recognised in other solid organs allografts. Future studies should address whether tacrolimus, which appears on occasion to be able to reverse the chronic rejection biopsy changes of ductopenia, can also reverse the associated arteriopathy. Finally, the angiographic changes in early graft dysfunction were examined revealing a non-specific pruning of the intrahepatic vasculature consistent with a high-resistance flow pattern. These features are mirrored by the reduced ICG extraction present in both this complication and, indeed to a lesser extent, all grafts in the early post-operative period which was examined in chapter 10. In that investigation, modulation of this parameter pharmacologically was demonstrated with infusion of N-acetylcysteine. This agent appears to have both beneficial systemic and regional haemodynamic properties which may improve graft function. A randomised trial of this agent in the early peri-transplant period is now justified in an attempt to determine whether it will affect clinical parameters of morbidity or mortality before there can be recommendation of routine administration of this agent.

In chapter 3, a biochemical analysis of the effluent preservation fluid which bathed the liver graft during storage was performed to assess whether changes in its biochemical composition could predict those grafts at risk of early dysfunction. As discussed in the chapter, this form of graft assessment although reasonably accurate in this regard cannot assist in organ selection. This pragmatic approach accepts that a significant proportion of utilised donor organs will be of limited viability, reflecting the increasing disinclination of programmes to turn down this scarce resource against a background of appreciable waiting-list mortality rates. The parameters measured in the effluent fluid in this study were comparatively crude indices of endothelial and parenchymal cell function and yielded no information on the potentially important Kupffer cell viability. Further examination of new and more sensitive markers of these cell populations and comparison with more functional indices of viability are required.

Pretransplant clinical status of a liver recipient is undoubtedly a major risk factor for the subsequent post-transplant outcome. This is perhaps most visibly present in the population of critically ill patients transplanted for acute liver failure who were examined in chapter 7. Definition and characterisation of this generally perceived observation into some qualitative or quantative index, which could be employed clinically, is however problematic. The deliberate use of 'hard' data sets such as laboratory parameters and critical illness scoring systems was an attempt to define unfavourable characteristics which could serve as a guide to identifying those patients where the hazards of proceeding with transplantation are unacceptable. Limitations to the present investigation exist, not least of which was the prolonged period over which the study population was derived. Several advances in organ preservation, immunosuppression and general surgical and medical management occurred during this time which may influence outcome. Additionally, the study was retrospective and suffered from small numbers in certain groups. However, this study does provide the first data in this increasingly important area and identifies those indices which should be assessed prospectively.

Recurrence of a disorder in the liver graft which was the primary transplant indication was identified in chapter 2 as accounting for approximately 10% of transplant failures. Previously, such a pathological event resulted predominantly from recurrence of hepatic malignancies. Subsequent retrospective analyses have now identified the major unfavourable clinical characteristics which are asssociated with recurrence in these conditions and accordingly, selection criteria for transplantation are now more refined and stringent. In contrast, the position with regard to transplant selection of HBsAg-positive patients is considerably less well characterised and more contentious. Some centres in the US (where intramuscular immunoprophylaxis is unavailable) now consider these patients to be an absolute contraindication to transplantation. In the single-centre experience described in chapter 9, the impact of long-term passive immunoprophylaxis and a more

rigorous selection procedure were investigated. This data confirms the efficacy of HBIG but stresses the requirement for indefinite administration if recurrence is to be prevented in a significant proportion of patients. Even with this prophylaxis those recipients who are HBVDNA-negative in the peripheral circulation can experience recurrent disease. Further studies which employ more sensitive assessments of HBV replication are required. The protective effect of coexistent HDV infection on recurrence of HBV is remarkable. The logical but possibly unethical conclusion from such powerful data is whether clinicians should be considering deliberate infection of HDV to those HDV-negative recipients who are actively replicating HBV. A further important clinical conclusion from this study is the ability to reduce maintenance immunosuppression in this particular group of recipients. Such a practice may decrease the liklihood of uncontrolled viral replication. Disease recurrence is less well recognised and characterised in those immunological liver disorders which are common liver transplant indications such as PBC and AIH. A case report presented in chapter 11 highlights the potential for recurrence of AIH with sufficient severity to compromise graft viability. In that report the comparatively low maintenance immunosuppression, alongside the well documented recipient HLA specificities, may have contributed to the recurrence. Management protocols cannot be recommended on the basis of one patient but this clinical case may represent a salutatory lesson on the hazards of immunosuppression reduction in such transplant indications, an issue which was examined in considerable detail inthe early part of chapter 11.

Clinical tolerance would greatly promote the health of liver transplant recipients. Immunosuppressant agents are associated with an appreciable morbidity and mortality and yet rejection remains the leading cause of graft failure and death as reported in chapter 2. From the early data presented in chapter 11, it is clear that tolerance allowing withdrawal of pharmacological immunosuppression (so-called operational tolerance) exists to a variable degree in a proportion of long-term liver recipients. However more questions

than answers result from these observations. Why does the phenomenon of chimerism exist in a significant proportion of patients? If microchimerism cannot predict the tolerant population, then what clinical or laboratory parameter can? Certainly, clinical data such as rejection history from the early post-transplant period will have to be perused as will the underlying transplant indication and degree of HLA matching. If this type of clinical data is unhelpful then reliance on *in vitro* assessments of donor-recipient immune responsiveness, with all the attendant pitfalls of this approach, may be required. The population of tolerant patients needs urgently to be characterised immunologically allowing identification of those characteristics which require to be promoted. The intragraft balance of Th1 and Th2 cytokine gene expression or phenotyping the graft - infiltrating cells may yield important differences between the tolerant and non-tolerant populations. Programmes are unlikely to be in a position of being able to identify tolerant recipients within the next few years. Hence, what will be important at a practical level is some form of immune monitoring during immunosuppression withdrawal which will allow prediction of a rejection episode. The limitations of this approach were discussed in chapter 5, but nevertheless, assessment of nitric oxide generation or conventional phenotyping of circulating lymphocyte sub-populations in this distinct and unique clinical circumstance would be of potential value.

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Appendix

Details of donor-type microchimerism determination by the most sensitive nested PCR - SSP methodolgy

Genomic DNA was prepared from recipient whole blood, bone marrow and tissues by phenol/chioroform extraction. 0.5 ml blood or bone marrow was aliquoted and 1 ml red blood cell lysis buffer added and mixed for 2 minutes at room temperature. Cells were pelleted by centrifugation and resuspended, with this process repeated until the supernatant was clear. The following steps were then identical for these cells and the liver and skin tissue specimens.

The samples were resuspended in 300 µl digestion buffer (Tris-NaCl-EDTA solution) and 50 µl of proteinase K added with an overnight incubation at 37°C. In the case of the tissue samples, further proteinase K was normally added (25 µl) for a further 24-48 hours incubation. Following digestion, phenol / chloroform extraction of undigested protein, overnight ethanol precipitation of the DNA at -70°C was performed. The ethanol was removed by speed vacuum evaporation. The DNA was pelleted at 10000 g 5 min and resuspended in 200 µl of distilled water and stored at 4°C prior to analysis.

Following determination of the concentration of the DNA by optical density (measurement of absorbance at 260 nm wavelength using a LKB spectrophotometer), a standard concentration of 0.05 µg/µl DNA was prepared for analysis. The protocol for subsequent genotyping of the DRB1 alleles in the DNA was according to the method described in the literature accompanying the BSHI kit except for in-house modifications as described below.

The Perkin-Elmer 4800 thermal cycler was used for approximately 50% of samples as described in the recommended protocol. However, in the later samples the Perkin - Elmer 9600 thermal cycler was used which required a reduction in the reaction volume from 50µl to 21µl. Accordingly in the first PCR amplification, a 8 µl primer mix (2 µl respectively of DRB7, DRB87, HGH I, HGH II), 2 µl x10 buffer, 2 µl dNTPs (2mM each), 3 µl H_2O, 3 µl glycerol / cresol red was added to 2 µl of denatured DNA. The HGH I and ii primers were used as positive controls for genomic DNA amplification. The DNA was denatured at 94°C for 5min then put immediately on ice.

0.8 µl of *Taq* was added to the stock. Glycerol and cresol red were used to promote heat dissipation and act as a tracker dye when running the gel respectively. A small reduction in cycle numbers in the first round from 23 to 20 was employed to reduce non-specific products and because of the use of glycerol the annealing temperature was decreased from 64°C to 62°C. Since, the faster PCR cycler was now being used, the denaturing and extension times were reduced by 50%. The cycling was commenced with a hot-start.

Similarly in the second round PCR amplification cycles, small modifications to the standard protocol were required. Again a final reaction mix of 20 µl was used which was made up of 8 µl of the 18 primers, 2 µl x10 buffer, 2 µl dNTP, 0.2 µl cresol red, 1 µl glycerol, 1 µl *Taq*, 1.8 µl sterile H_2O and 4 µl denatured and diluted first round product (5 µl first-round product diluted in 495 µl sterile H_2O). In this instance the number of cycles was not altered from the recommended 30, but the denaturing and extension times were reduced by 50% and the annealing temperature increased from 62°C to 64°C with a reduction in the time from 45 to 30 secs.

The final products were best viewed on a 2% rather than a 1% agarose gel (as recommended) with 20 µl of 10 mg.ml ethidium bromide added.