THE PRE-TRANSPLANT PREDICTION OF ACUTE CELLULAR REJECTION FOLLOWING LIVER TRANSPLANTATION

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DECLARATION

I declare that this thesis has been composed by me and that the work contained within it was performed by me except where clearly stated otherwise. The work was performed while I held a post at the Centre for Liver and Digestive Disorders and the Scottish Liver Transplant Unit at the Royal Infirmary of Edinburgh. I have not submitted this thesis for any other professional qualification.

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ABSTRACT

The aim of this thesis was to investigate the effect of various parameters in patients with chronic liver disease before liver transplantation and their influence on the occurrence of acute rejection following transplant. This may be useful in tailoring immunosuppression to avoid adverse effects in patients less likely to develop acute rejection.

Firstly a retrospective analysis of patients transplanted between 1992 and 1997 was undertaken. This showed that patients who were younger and had less severe liver disease were more likely to suffer acute rejection. It also showed that acute rejection was less likely to occur in patients with depleted protein stores as measured by mid-arm muscle circumference and those with alcoholic liver disease. Multivariate analysis found that a depleted mid-arm muscle circumference was independently associated with a reduced incidence of acute rejection.

A lymphocytotoxic cross-match between the patients serum and the donor T lymphocytes was also studied. This did not show any influence on a single episode of acute rejection but a postitive test was found in significantly more patients with recurrent acute rejection and in those with early graft failure.

The role of cytokines in acute rejection is not clear but animal and human studies had suggested that tumour necrosis factor alpha (TNF-α) played some role. Polymorphisms in the genes encoding TNFα, interleukin 10 and transforming growth factor beta1 (TGFβ1) which influence *in vitro* production of cytokines were examined

in transplant patients. This showed an increase in the TNFα 2 polymorphism at position –308 in patients with acute rejection but no association with IL-10 or TGFβ1 polymorphisms.

Pre-transplant levels of TNFα and IL-10 were measured following stimulation of peripheral blood mononuclear cells with lipopolysaccharide from patients with chronic liver disease. PBMC were preincubated with different immunosupressants. There was increased production of stimulated TNFα pretransplant in patients who went on to develop acute rejection. No relationship was found between IL-10 production and acute rejection.There were differences in the effects of tacrolimus, cyclosporin and dexamethasone on the production of both cytokines.

The pre-transplant immune status of patients was assessed by contact sensitisation to diphenylcyclopropenone (DPC). This demonstrated that patients unable to mount an immune response to DPC did not require treatment for acute rejection following liver transplantation. It also demonstrated a correlation between the strength of reaction to DPC and the severity of acute rejection.

CHAPTER ONE- INTRODUCTION

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1. HISTORY

The first orthotopic liver transplant in humans was carried out by Starzl in 1963 followed by the first reports of extended survival in 1967 (Starzl, 1968) with the emergence of immunosuppression with antilymphocyte globulin, azathioprine and corticosteroids. The improvement in liver preservation and the introduction of cyclosporin in1979 (Calne et al.,1979) led to significant improvement in survival. The realization that the procedure was surgically feasible with adequate survival led to acceptance by the National Institute of Health in 1983 that orthotopic liver transplantation was no longer experimental. At present around 4000 transplants are performed each year in the United States (UNOS data source) and around 3500 in Europe (European Liver Transplant Registry).

The first series from the United Kingdom was reported in 1973 from the Kings-Cambridge group (Williams et al.,1973). There are now seven centres within the U.K. which perform orthotopic liver transplantation, and in total they perform around 500 transplants per year (United Kingdom Transplant Support Services Authority).

The majority of orthotopic liver transplants are performed in patients with end-stage chronic liver disease, acute liver failure when the native liver is unlikely to recover spontaneously, and intra-hepatic malignancies. The proportion of patients transplanted for each indication reflects the geographical variation in the causes of acute and chronic liver disease with chronic hepatitis C related cirrhosis now being the commonest indication worldwide (Terrault, 2000).

2. REJECTION

The initial experiments in liver transplantation were carried out in dogs, and it became clear that rejection of the transplanted liver was a major problem (Moore, 1960). The use of immunosuppressive therapy in the form of anti-lymphocyte serum and azathioprine and the finding of less severe rejection in pigs did however provide some hope that long-term survival may be possible in humans.

Rejection occurs as a result of genetic disparity between the donor and recipient. In syngeneic animals there is no rejection of organ transplants. Rejection in liver transplantation can be hyperacute, acute or chronic (Adams and Neuberger, 1990).

2.1 INCIDENCE OF REJECTION

Hyperacute rejection in liver tranplantation is rare and results in early graft loss. It is thought to be caused by preformed antibodies resulting in activation of complement and graft destruction.

Acute cellular rejection occurs in between 24-80% of liver transplants (Fisher et al, 1995). The incidence varies depending on the initial immunosuppression regimens. There is no evidence to suggest that acute cellular rejection increases mortality. There are reports suggesting that morbidity is increased in patients who suffer from acute cellular rejection (Fisher et al.,1995) although this is not every centre's experience (Neuberger, 1995). A recent report from the U.S. suggests that there is a significant

cost associated with the treatment of an episode of acute cellular rejection (Martin et al., 1997).

Recent reports of graft outcome following acute rejection suggest that graft outcome following a single episode of rejection is not detrimental (Dousset et al., 1998; Wiesner et al., 1998; Avollo et al., 1998; Neuberger and Adams, 1998). The studies by Avollo and Wiesner suggest in fact that graft outcome may in fact be improved following a single episode of acute rejection. Severe acute rejection does however seem to adversely affect graft outcome, as does acute rejection in patients transplanted for hepatitis C related liver disease (Wiesner et al.,1998).

The incidence of chronic rejection is diminishing and the number of grafts lost now is around 5% (Wiesner et al., 1999). The improvement in immunosuppression is likely to be the principal reason for this.

2.2 HISTOPATHOLOGY

The histological changes of acute cellular rejection typically present as a triad which was first observed by Snover et al. in 1984. The triad consists of portal inflammation, bile duct damage and venous endothelialitis.

(A) Portal inflammation consists of a mixed infiltrate containing lymphocytes, neutrophils, eosinophils and often blast like cells. The inflammatory infiltrate can spill over into the periportal parenchyma.

(B) Bile duct inflammation and damage varies from a minority of ducts being infiltrated by inflammatory cells to most or all ducts showing degenerative changes , such as nuclear pleomorphism or cytoplasmic vacuolisation of the epithelium. Focal luminal disruption and duct loss can also occur.

(C) Venous endothelialitis involving the portal and or hepatic venules ranges from lymphoid attachment to the luminal surface of the endothelium, to subendothelial lymphocytic infiltration affecting most venules with extension into the perivenular parenchyma.

Figure 1 shows allograft biopsies showing no acute rejection and the typical features of acute cellular rejection.

The occurrence of other histological findings such as arteritis and perivenular necrosis without inflammation, hepatocyte ballooning and interstitial haemorrhage do occur in acute rejection but are poorly reproducible.

2.3 GRADING SYSTEMS

At least two of the above triad are required for a histopathological diagnosis of acute rejection. The need for a simple, reproducible and clinically relevant grading system has been acknowledged and an international consensus document was published in 1997 (Demetris et al.,1997). The development of grading systems by individual centres eg. Pittsburgh and Birmingham, made it difficult for comparisons to be made between centres in considering the requirement for additional therapy. The first

attempt at making a grading system using multiple pathologists and patients from more than one centre came from the National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation database in 1995 (Demetris et al.,1995), but it was felt to be too difficult to follow. The consensus document in 1997 proposed a system incorporating an overall grade i.e. indeterminate (portal infiltrate fails to meet criteria for acute rejection), mild - affecting only a minority of portal triads and confined to the triad, moderate- infiltrate expanding most or all triads and severe- as moderate with periportal spillover and moderate to severe perivenular inflammation that extends into the hepatic parenchyma and is associated with perivenular hepatocyte necrosis. In addition to this global assessment the authors proposed a rejection activity index (RAI) which was a semiquantitive score equal to the sum of the severity (0-3) of inflammation/damage occurring within the portal triad, bile duct and venous endothelium. Therefore a RAI of 0 indicated no acute rejection whereas an RAI of 6 indicated moderate rejection. It was felt that the RAI would be most useful in academic centres when it could be used in evaluating new treatment schedules etc.

3. DEFINITION AND CLINICAL FEATURES

3.1 Acute rejection

Acute cellular rejection has been defined by an International Working Party (1995) as inflammation of the allograft elicited by a genetic disparity between the donor and recipient, primarily affecting interlobular bile ducts and vascular endothelia including portal and hepatic veins and occasionally the hepatic artery and its branches.

Acute rejection usually occurs within the first 30 days following transplantation. In mild acute rejection there are often no clinical findings. However in severe acute rejection the patient may complain of malaise and be pyrexial with swelling and tenderness of the graft. Occasionally, ascites develops due to the increase in intrahepatic pressure secondary to liver swelling.

Acute rejection is difficult to diagnose without histology. Liver enzymes are often increased and a rise or cessation in the fall of bilirubin is seen, but these are neither sensitive nor specific. No correlation has been found between liver tests and the histologic severity of acute rejection (Adams and Neuberger, 1998). The eosinophil count may be raised during acute cellular rejection (Dollinger et al., 1996) and various serum markers of immune activation and adhesion molecules have shown associations with rejection but with poor sensitivity and specificity (Adams et al., 1989;Lalli et al., 1992; Fabrega et al., 2000). The diagnosis of rejection therefore usually requires histology. Most centres obtain percutaneous core needle biopsies although some centres use a fine-needle aspiration technique.

3.2 Chronic rejection

Chronic rejection is defined by two main histopathologic features -loss of bile ducts and obliterative vasculopathy. These components usually co-exist but may occur independently. The diagnosis is difficult to make on a single biopsy as the foam cell arteriopathy usually involves larger vessels. The loss of small bile ducts in more than 50% of portal triads is indicative of chronic rejection although it can be seen in other complications e.g. bile duct strictures and cytomegalovirus infection. A recent

consensus document has been published proposing a standardised definition (International panel, 2000).

Chronic rejection usually does not occur before 60 days following transplantation and may develop after an unresolved episode of acute rejection or following multiple episodes of acute rejection. In some cases it may occur indolently over a period of months to years with no clinically apparent acute rejection episodes. In addition to the genetic disparity between the donor and recipient there have been reports of other risk factors such as recipient age, CMV infection (Evans et al., 2000) and low cyclosporin levels in the early postoperative days (Wiesner et al., 1999).

4.1 TREATMENT OF ACUTE REJECTION

The treatment of established acute rejection is with high dose corticosteroids. The threshold for treatment varies between centres and individuals. The regimes used vary and include oral prednisolone (100-200mg/day for 3 days), intravenous hydrocortisone (up to 1g/day for 3 days) or methylprednisolone (500-1000mg/day for 3 days). There is little evidence to support any particular regimen. The use of monoclonal antibodies directed against T lymphocytes e.g. OKT3 have not proved to be of benefit in the initial therapy of acute rejection but may be of benefit in the treatment of steroid-resistant rejection. Recent evidence has suggested that an increase in the dose of tacrolimus alone may be sufficient for the treatment of acute rejection (Boillot, 1998).

The vast majority of episodes of acute rejection respond to corticosteroids. In some cases however there is no improvement in liver function tests, and repeat biopsy reveals ongoing rejection. The rate of steroid resistant rejection varies from 7-18% and can depend on the immunosuppressive agents used. The treatment of steroid resistant rejection is with a switch from cyclosporin to tacrolimus (Klintmalm et al, 1993) or in some cases with OKT3 monoclonal antibody. There is some evidence to suggest that late acute rejection occurring after 30 days may be less steroid responsive with the identified risk factors being concomitant viral infection and low immunosuppressant levels (Cakaloglu et al., 1995; Mor et al., 1992). There is an increased incidence of chronic rejection in patients who suffer late acute rejection (Neuberger and Adams, 1998) or steroid-resistant rejection (Wiesner et al, 1999). The reason for the different outcomes of early and late acute rejection is not clear. It may be that late rejection represents a more aggressive form of rejection reflecting T cell escape from immunosuppression. Alternatively it may be that the more severe episodes of acute rejection are clinically apparent after the initial post-operative period when acute rejection is actively sought (Neuberger and Adams, 1998).

4.2 TREATMENT OF CHRONIC REJECTION

There is no therapy for advanced chronic rejection except re-transplantation. Studies have shown that a switch to tacrolimus from cyclosporin early in the process before the bilirubin is above 170 μmol/l may improve graft function and lead to a reduction in bilirubin (Sher et al., 1997) although no controlled study has been done.

5. MECHANISM OF ACUTE REJECTION

5.1 MAJOR HISTOCOMPATIBILITY COMPLEX

The polymorphic membrane-bound glycoproteins termed the Major Histocompatability Complex (MHC) are involved both in the recognition of self and in the activation of the immune system. MHC class I molecules are found on almost all nucleated cells whereas MHC class II molecules are largely limited to cells of the immune system. The antigen presenting cells (APC) express MHC class II molecules on their surface with antigenic polypeptides which can be recognised by T lymphocytes. Some of the terms used in transplantation immunology are outlined below.

Term	Definition
Syngeneic	Genetically identical, e.g. monozygotic twins, mice of same strain
Allogeneic	Genetically non-identical but of the same species, e.g. cadaveric liver transplant
Alloresponse	Immune response to the antigens present in an allogeneic graft
Alloantigen	Target antigens in an allogeneic graft

In transplantation of solid organs, genetic disparity exists between donor and recipient. The allogeneic MHC molecules are recognised as foreign by host T lymphocytes (Lechler, 1990) and in the case of liver transplantation it is thought that donor APC such as dendritic cells migrate from the liver and encounter host T-

lymphocytes in host lymph nodes. This is the called the direct recognition pathway. The evidence for the involvement of this pathway in acute rejection comes from various approaches. There is an increase in the precursor frequency of T cells recognizing an allogeneic MHC molecule directly some 100 times that of T cells responding to the same MHC indirectly (Liu et al., 1993). Secondly, the reduction of donor APC within grafts leads to a marked reduction in acute rejection (Lechler et al., 1982). In addition pretreatment of donor grafts to increase the number of dendritic cells leads to rejection of grafts which are normally accepted indefinitely (Steptoe et al, 1998). Finally, adoptive transfer of T cell lines specific for direct recognition of rat allogeneic MHC lead to acute rejection in immunocompromised recipients (Braun et al., 1993). A schematic representation of the direct recognition pathway is shown in figure 1.2.

The indirect pathway involves the processing of donor derived MHC-molecules by host APC which then present this peptide in the context of self MHC to host T lymphocytes. The indirect pathway is thought to play less of a role in allorecogniton and rejection although there is evidence to support it in liver transplantation (Molajoni et al., 1997) and it may have a role in chronic rejection (Shirwan, 1999). A schematic representation of the process of indirect recognition is shown in figure 1.3.

The expression of MHC class I molecules in normal liver is only weak or negative on hepatocytes (Fleming, 1981;Steinhoff, 1988), whereas biliary epithelium and vascular endothelium express these molecules strongly (Daar, 1984). MHC class II molecules are largely on the Kupffer cells in normal liver with both hepatocytes and biliary epithelium having no expression (Steinhoff, 1988;Daar, 1984). During acute allograft

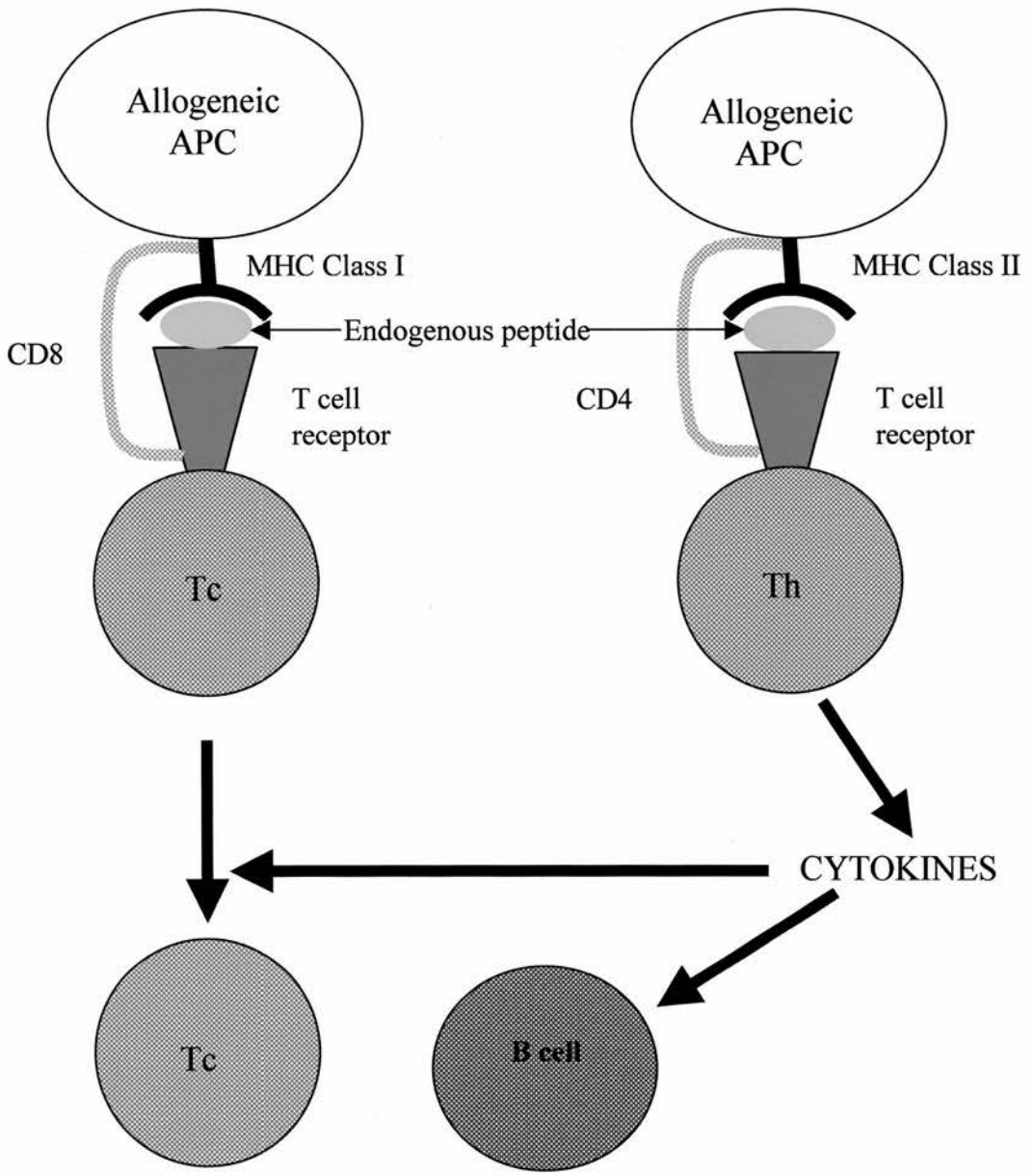


Fig 1.2. Proposed mechanism of direct recognition pathway. (APC = antigen presenting cell, MHC =major histocompatibility complex,CD = cluster of differentiation, Tc = cytotoxic T cell, Th = helper T cell)

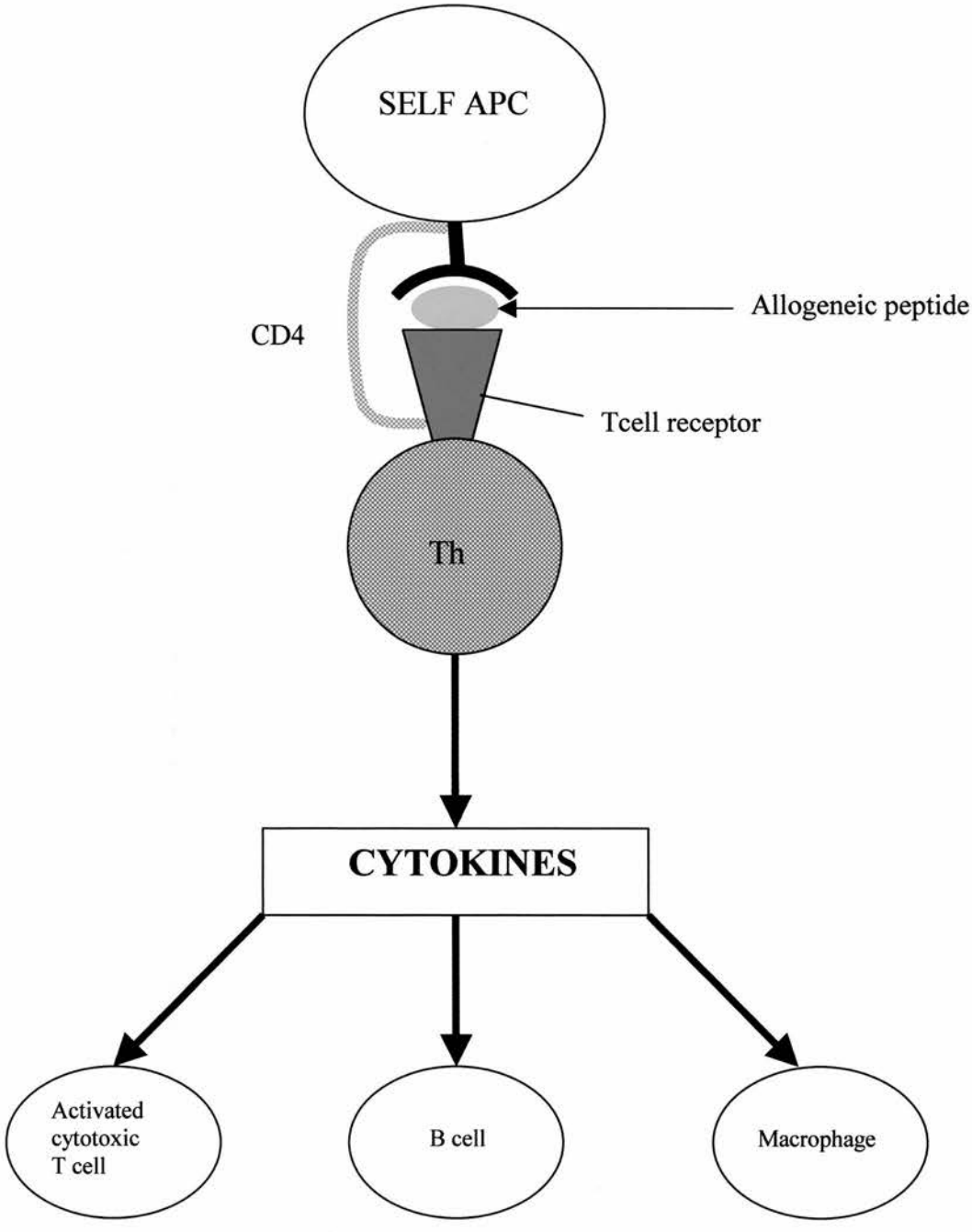


Figure 1.3. Indirect pathway of recognition of alloantigen by self antigen presenting cell (APC) resulting in activation and proliferation of cytotoxic T cells, B cells and macrophages.

rejection there is altered expression of MHC molecules with an induction of MHC class I on hepatocytes and MHC class II on biliary epithelium and vascular endothelium (Steinhoff, 1988;So, 1987). The induction of MHC molecules leads to different liver components becoming targets for the immune response in graft rejection. There is evidence to suggest that MHC expression correlates with rejection (So, 1987) although this was not substantiated by Rouger et al. in 1990.

5.2 INFILTRATING CELLS

The inflammatory infiltrate in acute cellular rejection is composed principally of activated T cells, B cells, activated lympoblastoid cells. Studies investigating the phenotype of the T cells have been conflicting. Ibrahim et al.(1993) found the portal infiltrate to be composed principally of CD8+ T cells whereas Wong et al.(1998) found little difference between the number of CD4+ and CD8+ T cells. A study by Dollinger et al.(1998) however, found an increase in CD4+ T cells in rejecting liver tissue. This study also showed these cells to express markers of proliferation. These CD4+ cells are thought to be the principal source of cytokines which are responsible for the further activation and expansion of the immune response. The cytotoxic T cells (CTL) are thought to be the principal source of damage to the graft. There is evidence that the mechanism of cell death is by apoptosis rather than necrosis. The two major pathways utilised by the CTL are the Fas/Fas ligand pathway and the perforin-dependent granule-exocytosis pathway by the release of granzyme B. Studies involving tissue from liver, heart and kidney allografts have shown an increase in granzyme B during or preceding acute rejection rejection (Krams et al., 1995;Hayashi et al., 1995;Legros-Maida et al., 1994;Lipman et al., 1994). It is likely that the

Fas/FasL pathway is also used as a pathway of allograft damage (Krams and Martinez, 1998).

5.3 CO-STIMULATION

In addition to the antigen specific recognition reaction involving MHC and T cell receptors there is an additional, non antigen-specific, co-stimulation signal which is required for an effective immune response. The best characterized receptor on T cells is the CD28 molecule although there are other molecules on T cells which can serve as co-stimulatory receptors. The ligands for CD28 are B7-1 (CD80) and B7-2 (CD86) which are present on activated antigen presenting cells. CTLA4 is also expressed on T cells and binds B7-1 and B7-2 with an inhibitory signal.

In the absence of co-stimulatory signals, the T cell which encounters antigen does not divide and does not produce appreciable levels of cytokine. The outcome for the T cell which does not receive a co-stimulatory signal is either anergy (Gimmi, 1993) or apoptosis (Noel, 1996).

Another pathway recently characterised and thought to play a role in T cell stimulation is the CD40:CD40 ligand pathway. CD 40 is expressed on Bcells, dendritic cells and macrophages. The CD40 ligand (CD154) is expressed on activated CD4 T cells. Stimulation of the CD40 molecule on B cells results in signals for antibody production and B7 expression (Hancock et al, 1996;Ranheim et al., 1993). CD 40 stimulation on APC leads to the production of cytokines and adhesion molecules involved in T cell activation (Guo et al., 1996).

The role of these molecules for potential targets for therapy in transplantation is significant. The delivery of CTLA4-Ig to the donor liver prior to transplantation prevented rejection in a rat model (Olthoff et al., 1997). The blockade of both co-stimulatory pathways has also led to prolonged rejection-free survival of renal transplants in primates (Kirk et al., 1999).

5.4 TOLERANCE

The definition of tolerance is not straightforward. The initial use of the term by Starzl implied a reduced amount of immunosuppression to maintain graft function. Others have used the term clinical tolerance when immunosuppression has been withdrawn with no apparent rejection of the allograft. The finding that donor cells were found in distant host lymphoid sites led to the hypothesis that microchaemerism may lead to tolerance. However a study of immunosuppression withdrawal did not find any difference in rejection rates between recipients with microchaemerism and those without (Devlin et al., 1998).

Immunological tolerance however is defined as an unresponsiveness to donor antigens. The mechanism of tolerance in animal models where no immunosuppression is required is not clearly understood, but T cell anergy has been proposed as a possibility. This has been proposed to occur by a mechanism whereby T cell receptors encounter donor antigen but do not receive a co-stimulatory signal and therefore do not elicit an immune response when this antigen is encountered again. There are studies which support this theory (Turka et al.,1992; Pearson et al.,1994) although this

is unlikely to be the sole mechanism. Studies investigating the systemic hypo-responsiveness to donor antigen induced by liver transplantation have found that cells are in fact still responsive *in vitro* leading to the term split tolerance (Dahmen et al, 1994). This suggests that there is in fact an active regulation or suppression of these donor reactive cells *in vivo.*

5.5 CYTOKINES

As indicated previously the augmentation of the immune response following allorecognition involves the release and action of the soluble mediators termed cytokines. Cytokines such as tumour necrosis factor alpha (TNF-α) can induce damage via cell receptor pathways. The principal source of TNF-α is thought to be infiltrating monocytes from the recipient (Teramoto et al., 1998). The other principal cytokines are produced by $CD4^+$ T cells. These cytokines are responsible for the upregulation of MHC expression, T cell proliferation, cytotoxic T cell differentiation and alloantibody production. There are thought to be distinct subsets of CD4 cells which produce distinct cytokine patterns, Th 1 cells produce IL-2, IFNγ, and TNFβ promoting cellular responses while Th2 cells produce IL-4, IL-5, IL-6 and IL-13 and support antibody production.

The immunoregulatory role of the Th2 cytokines led to a proposal that these cells may be beneficial in achieving long term graft survival. Experimental models of transplantation have indeed shown a predominance of Th2 cytokines in long term graft survival. However other studies involving abrogation of the co-stimulatory pathways by CTLA4-Ig or anti-CD40 ligand have shown a reduction in both Th1 and Th2 cytokines (Krams and Martinez, 1998)

In the case of human liver transplant rejection, several investigators have studied intra-graft cytokine levels in grafts with and without acute rejection. These have shown differing results. Some investigators have found an increase in the TH1 type cytokines IL-2 and IFNγ (Bishop et al,1990 and Gorcynczki et al.,1994) while others have found little or no increase in IL-2 (Martinez et al.,1992; Conti et al.,1999). The TH2 type cytokines IL-4 and IL-5 were found to be increased during rejection by Martinez (1992) and Conti (1999). Interleukin-10 which has more of an immunoregulatory role has not been found to be increased in serum or in the graft at times of rejection (Conti et al,1999) . The pro-inflammatory TNF-α has been found to be increased during rejection in the graft with infiltrating monocytes being the major source(Hoffmann et al., 1993) and other workers have found this cytokine to be increased in the serum at times of rejection (Imagawa et al.,1991;Kita et al.,1996). These findings are consistent with the development of multiple, concomitant cellular pathways of immune-mediated injury to the liver.

6. IMMUNOSUPPRESSION

The efficacy of immunosuppressive agents has largely been responsible for the improvement in outcome of orthotopic liver transplantation. There are many different regimens combining agents used throughout the world. Some units use antibody induction therapy with a delayed introduction of calcineurin inhibitor i.e. cyclosporin or tacrolimus. Almost all regimens would include one of the calcineurin inhibitors. The other immunosuppressants commonly used are azathioprine and a glucocorticoid,

in particular prednisolone. Newer agents include mycophenylate mofetil and rapamycin.

6.1 INDUCTION THERAPY

The use of polyclonal antilymphocyte serum began in 1963 and since that time several antibody preparations have been developed and used. Antithymocyte globulin (ATG) and anti-CD3 antibody (OKT3) have been the principal therapies used but have failed to show superior outcome compared with triple therapies with cyclosporin, prednisolone and azathioprine. One of the reasons for this was an increase in infectious complications and post transplant lymphoproliferative disorder (PTLD) (McDiarmid et al,1991). In recent years there has been an interest in more selective monoclonal antibodies which are directed against the IL-2 receptor and do not appear to increase the incidence of PTLD (Jonas et al.,1997). Studies using these newer agents have shown a low incidence of acute rejection but no difference in graft survival (Langrehr et al., 1997; Nashan, 1996).

6.2 CYCLOSPORIN

The introduction of this naturally occurring lipophilic endecapeptide derived from the fungus *Tolypocladium inflatum Gams* revolutionised solid organ transplantation. Cyclosporin binds to cyclophilin within the cytoplasm of cells and this complex binds to calcineurin preventing the dephosphorylation of nuclear factor of activated T cells (NFAT). This nuclear factor is responsible for stimulating the transcription of many genes. Its inhibition therefore reduces the transcription of certain cytokines, in

particular interleukin 2, which leads to a reduction in the activation and expansion of T cells (Andus and Lafferty, 1982).

Adverse effects are common and in the large multicentre studies in the United States and Europe as many as 40% of patients experienced headache and insomnia. A similar percentage developed hypertension and gastrointestinal symptoms. Renal impairment occurred in 20-40% of patients in the first 12 months (Henry, 1999). End-stage renal failure does occur as a result of cyclosporin therapy (Fisher et al., 1997). New onset renal impairment occurs in only 1-2% after 2 years of treatment (Roberts et al., 1998). In 5 years of follow up malignancies occurred in 7.2% of patients on cyclosporin (Wiesner et al., 1998).

The predisposition to infection is difficult to quantify exactly but around 20 % developed urinary tract infections in the first year. The other common adverse effects seen with cyclosporin are gingival hypertrophy and hirsutism.

6.3 TACROLIMUS

This macrolide derived from the fungus *Tsudakalemide* was introduced to clinical practice in 1987. It is similar in its action to cyclosporin although 10-100 times more potent.

The adverse effects are similar but not identical to cyclosporin. Tremor and alopecia occur more frequently with tacrolimus, as does new onset diabetes mellitus (19%) . Diabetes was reversible in half of the patients with either a reduction in dose or

discontinuation of the drug. Malignancies occurred in 6.4% of patients in a 5 year study period.

Comparisons between the two calcineurin inhibitors have been made in two large multicentre trials which compared the Sandimmune preparation of cyclosporin and tacrolimus (The U.S. multicenter FK506 liver study group,1994; European FK506 multicentre liver study group, 1994). These studies revealed acute rejection, steroid-resistant rejection and chronic rejection to be reduced in the tacrolimus group. There was, however, no difference in the graft and patient survival. The microemulsion preparation (Neoral) of cyclosporin has replaced Sandimmune, and the doses of tacrolimus used now have reduced. Therefore the above trials cannot be applied directly to current practice and the results of the TMC trial comparing Neoral and a reduced dose of tacrolimus are awaited.

6.4 AZATHIOPRINE

This drug is an imidazole derivative of 6-mercaptopurine and was first used in 1961 in transplantation. Its mode of action is as an anti-metabolite interfering with cell division and therefore inhibiting proliferation and differentiation of T cells in response to antigenic stimulation.

The major adverse effect is bone marrow suppression with leucopenia being more common and often necessitating a reduction in dosage. The other adverse effects are gastrointestinal upset, pancreatitis, rash and hepatotoxicity.

6.5 CORTICOSTEROIDS

These drugs have multiple effects on the immune system and until recently have been a mainstay of long term immunosuppressive therapy. The principal adverse effects of corticosteroids are obesity, diabetes mellitus, osteoporosis, hypertension, depression, cataract and rarely avascular necrosis of the femoral head.

Recently many centres have withdrawn steroid therapy within the first six months.

6.6 MYCOPHENOLATE MOFETIL

This drug inhibits inosine monophosphate dehydrogenase and therefore inhibits the *de novo* synthesis of purines (Allison et al, 1994). It has less nephrotoxic effects and may have a role in sparing calcineurin inhibitor induced adverse effects although no randomized data is available at present.

A schematic representation of the proposed sites of action of the immunosuppressants commonly used and the newer agents is shown in Figure 1.4.

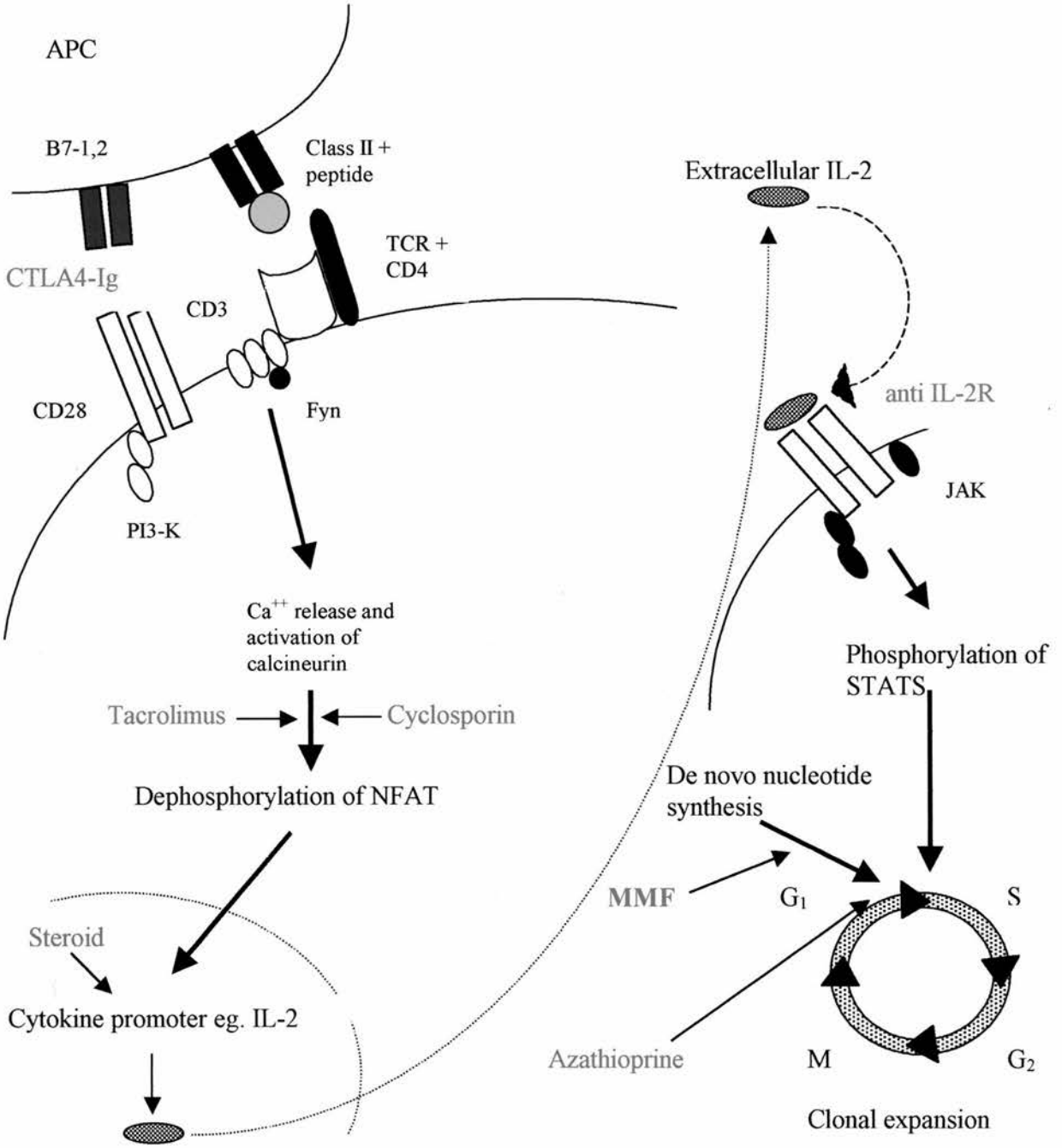


Figure 1.4. T cell activation and cytokine release leading to clonal expansion and the principal action of immunosuppressants. (APC = antigen presenting cell, TCR = T cell receptor, STAT = signals transducers and activators of transcription, Il-2R = interleukin-2 receptor, NFAT = nuclear factor of activated T cells, MMF = mycophenolate mofetil)

7. CONCLUSION AND AIMS

The occurrence of acute cellular rejection is a common event following orthotopic liver transplantation but this does not appear to adversely affect graft outcome in the majority of patients. However, severe acute cellular rejection and acute rejection in patients with hepatitis C infection does adversely affect graft outcome. The benefit of identifying patients of increased risk of severe acute rejection would therefore be to monitor their immunosuppression carefully to ensure it is adequate.

The adverse effects of immunosupppressants are multiple both in the early post-operative period and later on. The major early complication of transplantation related to immunosuppression is infection, which has significant morbidity and mortality. Recent evidence also suggests that early immunosuppressant levels influence chronic renal impairment (Fisher et al., 1998). In patients with a low risk of acute rejection there may be scope to reduce initial immunosuppression to prevent infection and possibly prevent problems with renal function in the longer-term. One would also expect a reduction in *de novo* malignancies in the longer term if there was a reduction in immunosuppression.

The purpose of this thesis was to investigate predictors of acute rejection pre-transplant in the hope that this information could be used in the future to vary immunosuppresion on an individual basis depending on the individual's predicted risk of acute rejection. The influence of clinical and nutritional parameters, cytokine polymorphisms, stimulated cytokine production and contact sensitisation to a neo-

antigen was investigated in the recipient. The effect of recipient pre-formed antibodies to donor cells was also investigated.

CHAPTER TWO

THE INFLUENCE OF RECIPIENT CHARACTERISTICS ON ACUTE REJECTION

1. INTRODUCTION

Acute cellular rejection is common following orthotopic liver transplantation, usually occurring in the first three weeks following transplantation. There is an increase in morbidity associated with acute cellular rejection (Fisher et al, 1995), although more recent reports suggest that a single episode of acute rejection may well improve graft outcome (Wiesner et al, 1998; Avollo et al, 1998). Most, if not all centres, administer a protocol immunosuppression regimen to all recipients with adjustments made only for weight and with the same target levels for calcineurin inhibitors for all recipients. The adverse effects of immunosuppression are multiple both in the short and longer term. It may be appropriate to tailor immunosuppression on an individual basis as all patients are not at the same risk of developing acute rejection.

Retrospective studies have shown original disease to be important in the occurrence of acute rejection (Farges et al.,1996;Berlakovich et al, 1996;Wiesner et al, 1997; Adams et al.,1993). More recent reports have suggested other risk factors such as race, age of the recipient, serum creatinine, presence of oedema and HLA-DR mismatch (Wiesner et al, 1998). Late acute rejection has been associated with viral infection and with sub-therapeutic levels of immunosuppression. The aim of this study was to investigate markers of immune status pre-transplantation in patients with chronic liver disease to identify their value in predicting the risk of developing acute cellular rejection.

2. METHOD

2.1 PATIENTS

The case notes and dietetic records of 121 consecutive patients transplanted between November 1992 and April 1997 receiving their first hepatic allograft were examined for the following information- sex, aetiology of liver disease, age at liver transplantation, Child's group, triceps skin fold thickness and mid-arm muscle circumference, preoperative albumin, creatinine and lymphocyte levels. Nutritional parameters were compared with normal values for age and sex and classified as depleted if they were below the 5th percentile and marginal if between the 5th and 15th percentile (Bishop et al, 1981). Other factors investigated were donor age, cold ischaemia time and number of HLA DR mismatches between donor and recipient and T cell lymphocytotoxic crossmatch. HLA typing was carried out at the Scottish Blood Transfusion service tissue typing laboratory using standard techniques (only 81 recipient/donor types were available). The lymphocytotoxic test was performed in the same laboratory using standard NIH techniques. Mean follow up was 36 (1-60) months.

Acute cellular rejection was defined as rejection requiring treatment with high dose steroids. Most patients (80%) had a protocol biopsy around seven days, although this was dropped from the protocol for one year. Indications for liver biopsy at other times were abnormal liver enzymes or slowly resolving liver function tests following rejection therapy. All patients with acute rejection except one had a biopsy before treatment was commenced. Acute rejection was graded as mild, moderate or severe

according to the rejection activity index (Demetris et al., 1997). Late acute rejection was defined as occurring after the first 30 days.

Immunosuppression was with triple therapy consisting of cyclosporine (10mg/kg/day) or tacrolimus (0.1mg/kg/day), azathioprine (2mg/kg/day) and prednisolone(20mg/day). The target trough levels were 175-200 mmol/l for the first 6 months, 125-150 mmol/l for 6-12 months and then 100-125 mmol/ml. Cyclosporin was initially given intravenously, beginning within the first 6 hours post-transplant for the first 48 hours, until the microemulsion preparation (1995) became available when it was then given via a nasogastric tube. Tacrolimus was used in 5 patients (randomly assigned as part of a clinical trial) with target levels of 10-15ng/l for the first 3 months and then 5-10ng/l.

2.2 STATISTICAL ANALYSIS

The numerical variables were assessed using student's t-test for the difference between two means. The existence of an association between acute rejection and specific categorical variables was verified by use of Chi-square tests. Those categorical or numerical variables significantly associated with acute rejection were tested on multivariate analysis using a stepwise approach. All statistical tests were performed by means of the SPSS statistical software package, release 9.0.0 (1998).

3.RESULTS

Acute cellular rejection required treatment in 64 (53%) of 121 patients. The severity of these episodes is shown in table 2.1. Nineteen patients (15%) had more than one episode of acute rejection treated. Nine of these patients required a second course of methylprednisolone within two weeks of the initial early rejection episode. Two of these patients went on to receive OKT3. One patient with two episodes of acute rejection developed chronic rejection. Five patients had 3 or more episodes of acute rejection requiring treatment, 4 of whom went on to lose their graft from chronic rejection. Eighteen (95%) of these patients were female (p=0.007) and 4 of the five patients with a positive crossmatch had recurrent early rejection. No other variable was significantly different in the group with recurrent rejection as shown in table 2.2.

Eleven patients had an episode of late acute rejection - 5 of which were associated with immunosuppresive levels below target levels, three at the time of CMV infection and 3 with no known attributable factor. The histological severity of these lesions was mild in 4 patients and moderate in 7 patients. One of these patients has developed chronic rejection resulting from poor compliance.

Eight (7%) patients developed chronic rejection. All patients were female (p=0.04). The mean age of patients with chronic rejection was 47.5 +/- 5.2 (S.E.) compared with 47.6 +/- 1.2 (p=0.34) of patients without chronic rejection. There was no difference between the two groups in the variables measured including aetiology, CMV infection, HLA DR mismatch or initial severity of acute rejection (table 2.3). Patients who had recurrent (5 out of 19) or late rejection (2 out of 11) were more

likely (p=0.03) to develop chronic rejection than those who had a single episode of early acute rejection (1 out of 34).

The occurrence of acute rejection related to original disease is shown in table 2.4. Patients with alcoholic liver disease had less acute rejection than patients with non-alcoholic liver disease (p=0.01). Patients with chronic viral disease (3 HCV,1 HBV) were grouped together as there has been no difference in acute rejection rates between patients transplanted for chronic HBV or HCV infection in our experience.

Table 2.5 shows recipient age, preoperative albumin levels, lymphocyte counts, and the cold ischaemia time . The patients with acute cellular rejection were younger than those without (p=0.007). The occurrence of acute rejection with respect to recipient sex, donor age, serum creatinine pre-transplant and the number of HLA DR mismatches is shown in table 2.6. Patients in Childs group A had more acute rejection than those in groups B and C (p=0.04). Median ages were used for grouping.

Nutritional parameters of patients with chronic liver disease as measured by anthropometry (n=75), shown in table 2.7, did predict less acute rejection in those patients who had depleted mid-arm muscle circumference (p=0.01).

Stepwise logistic regression analysis of those variables found to be significant on univariate analysis indicated that mid-arm muscle circumference was the only factor independently associated with acute rejection (p=0.01). Patients with a depleted mid-arm muscle circumference suffered less rejection.

TABLE 2.1. Histologic severity of acute rejection on day 7 biopsy (n=93, as not all patients had day 7 biopsy) in recipients with no treatment, early and recurrent acute rejection.

	No rejection	Mild	Moderate	Severe
No treatment	5	31	6	0
Single epsiode of acute rejection		2	22	8
Recurrent episodes of acute rejection		0	13	6

Table 2.2. Characteristics of recipients developing recurrent rejection.

	Single episode	Recurrent rejection
Gender – male	14	1
female*	20	18
Aetiology – PBC	11	11
non-PBC	23	8
Severity of rejection		
Mild	2	0
Moderate	23	13
Severe	8	6
Child-Pugh class – A	6	2
B	6	7
C	16	6
MAMC – normal	15	8
marginal	5	1
deplete	3	2
TSF normal	9	3
marginal	2	3
deplete	12	5
HLA DR mismatch- 0	1	0
1	13	5
2	11	4
Lymphocytotoxic crossmatch		
Positive	0	4
Negative	32	11

* $p = 0.007$, χ^2 test

Table 2.3. The occurrence of chronic rejection.

	No chronic rejection	Chronic rejection
Gender – male	39	0
female*	74	8
Aetiology- PBC	44	5
non-PBC	69	3
Severity of early rejection		
Nil	6	0
Mild	39	0
Moderate	49	3
severe	11	3
Rejection episodes		
none	57	0
single- early	33	1
recurrent or late*	23	7
HLA DR mismatch		
0	7	0
1	36	4
2	33	1

* $p< 0.05$, χ^2 test

TABLE 2.4. Acute cellular rejection in different aetiologies.

Aetiology	No rejection No. of patients	Acute rejection No. of patients
Primary biliary cirrhosis	24	25
Alcoholic liver disease	14	4
Primary sclerosing cholangitis	6	8
Chronic viral disease	2	2
Autoimmune hepatitis	1	5
Cryptogenic cirrhosis	2	3
Paracetamol induced acute liver failure	5	7
Non A-E acute liver failure	3	4
Others	0	6

TABLE 2.5.The occurrence of acute rejection related to age at transplantation and pre-operative levels of albumin and lymphocytes, and cold ischaemia time.

Variable (mean +/- se)	No rejection	Acute rejection	Significance
Age (yrs)	51.3 (1.44)	44.3 (1.66)	p = 0.007
Albumin (g/l)	30.2 (0.77)	31.8 (0.76)	p = 0.59
Lymphocyte count ($x10^9$)	1.26 (0.10)	1.41 (0.12)	p = 0.22
Cold ischaemia time (hrs)	10.6 (0.4)	10.6 (0.4)	p = 0.30

TABLE 2.6.Sex, donor age, HLA DR mismatches, Child's group and pre-transplant creatinine in acute rejection.

	No Rejection	Rejection	Significance
Sex			
female	37	45	p=0.53
male	20	19	
Donor age			
< 41	26	32	p=0.75
> 41	28	31	
HLA DR mismatches			
0	2	5	p=0.48
1	20	20	
2	19	15	
Lymphocytotoxic crossmatch			
Positive	1	4	p=0.39
Negative	39	43	
Severity of liver disease			
ALF	8	12	p=0.04
Child-Pugh A	1	9	
Child-Pugh B	17	15	
Child-Pugh C	31	28	
Pre-transplant creatinine(μmol/l)			
<160	41	58	p=0.13
>160	13	9	

Note: Cut-off value for donor age =median value. Creatinine cut-off to allow comparison with other published data (Wiesner et al., 1998)

TABLE 2.7. Acute rejection in different nutritional groups.

	No rejection	Acute rejection	Significance
Triceps skinfold thickness			
normal	15	14	
marginal depletion	5	5	p=0.97
deplete	21	21	
Midarm muscle cicumference			
normal	17	25	
marginal depletion	5	8	p=0.01
deplete	18	6	

Significance by χ^2 test.

4.DISCUSSION

The occurrence of acute cellular rejection in 53% of patients is in keeping with other reported series. Recent evidence suggests that a single episode of acute cellular rejection does not adversely affect graft outcome (Wiesner et al, 1998; Dousset et al., 1998). However the use of immunosuppressive agents is not without its problems, with infection being the main early complication, and recent evidence suggesting that early levels of cyclosporin are predictive of late renal failure (Fisher et al, 1998). The value of predicting acute cellular rejection may therefore be twofold. On the one hand those more likely to suffer from acute rejection could have their levels of immunosuppressants kept at the present accepted levels but those predicted to be less likely to suffer from acute rejection could receive reduced immunosuppression.

Acute rejection is principally a T lymphocyte mediated response and therefore the parameters investigated were principally those known to affect cell mediated immunity, as well as a few others which have been suggested to influence the incidence of acute rejection. The finding that the cold ischaemia time was not different between the rejection and non-rejection groups is contrary to Wiesner et al. (1998) although the longest cold ischaemia time in our group was 15 hours. In the above study the incidence of acute rejection was increased in grafts with a cold ischaemia time longer than 15 hours. Other centres have also found no increase in rejection associated with cold ischaemia time (Shackleton et al.,1995).

The role of HLA mismatching in acute cellular rejection is not clear. Many centres, like ours, have found no association with DR mismatches (Donaldson et al, 1993; Chen et al., 1994) but this is not every centre's experience (Wiesner et al., 1998) . The role of a positive lymphocytotoxic crossmatch is discussed in a later chapter with a larger number of patients.

The finding that acute cellular rejection is more likely in younger patients is not surprising, as cell-mediated immunity is known to decrease with age (Goodwin et al., 1982; Dworsky et al., 1983). Similarly the finding that severity of liver disease is related to the incidence of rejection is not unexpected, as cell mediated reactivity has been shown to be related to severity of liver disease (Zipprich et al., 1982; Konigstedt et al., 1986).

The influence of original disease has been known for some time and other authors have noted a reduction in acute cellular rejection in alcoholic liver disease compared with primary biliary cirrhosis or with non-alcoholic liver disease (Farges et al, 1996; Berlakovich et al., 1996; Wiesner et al, 1997). A study from Birmingham also found a reduction in acute rejection in patients transplanted for hepatitis B infection (Adams et al., 1991). A proposed reason for this may be that patients with alcoholic cirrhosis are often severely compromised nutritionally, although recent evidence suggests that the degree of nutritional disturbance is related to severity of disease rather than to aetiology (Caregaro et al., 1996). The reduced acute rejection reported in hepatitis B infection may reflect the immune status of the recipient as this infection is thought to exist in a chronic state in those with impaired cell mediated immunity (Adams and Neuberger, 1998).

The effect of renal impairment on cell-mediated immunity in chronic liver disease has not been investigated. Renal impairment *per se* does affect cell-mediated immunity as measured by skin testing (Giacchino et al., 1982) and may have clinical implications (Kelly, 1996). One previous report (Wiesner et al., 1998) has suggested that liver patients with higher creatinine pre-transplant are less susceptible to acute rejection following transplantation. This data although showing the same trend do not show statistical significance. Serum creatinine is not a reliable indicator of creatinine clearance and it may be that this is a more potent predictor of impaired cell-mediated immunity.

An involvement of nutritional status in cell-mediated immunity has been proposed for some time (Neumann et al, 1976; McMurray et al., 1981). Mid-arm muscle circumference pre-transplant in this study was the only independent predictor of acute cellular rejection. This may reflect a relative inability to mount a cell-mediated response to foreign antigen in patients with protein energy malnutrition. Infection following transplantation has been found by some to cause a significant difference in survival in those with a poor nutritional state (Selberg et al., 1997). Fat stores as measured by triceps skinfold thickness do not appear to reflect immune capabilities to the same extent.

Chronic rejection is now uncommon and in our small experience the only risk factors identified were female sex and recurrent acute cellular rejection. The latter risk factor has been found in other centres (Candinas et al, 1995). Cytomegalovirus infection has been found to be a risk factor in some centres (O'Grady et al., 1988; Arnold et al,

1992; Candinas et al., 1995; Evans et al.,2000). It was the policy of our unit to transplant CMV negative recipients with CMV negative donor livers until 1997 which may account for this not being a risk factor in our group, as infection was rare.

5.CONCLUSIONS

Acute cellular rejection requiring treatment is significantly associated with recipient age, severity of liver disease and mid-arm muscle circumference. Acute rejection occurs less frequently in recipients transplanted for alcoholic liver disease.

Recurrent acute cellular rejection occurs more frequently in females and is significantly associated with the development of chronic rejection.

CHAPTER THREE

THE INFLUENCE OF A POSITIVE LYMPOHCYTOTOXIC CROSSMATCH ON HEPATIC ALLOGRAFT OUT COME

1. INTRODUCTION

2. METHODS

    2.1 Patients

    2.2 Crossmatch

    2.3 Organ Preservation

    2.4 Follow up and Statistics

3. RESULTS

4. DISCUSSION

5. CONCLUSIONS

1.INTRODUCTION

In the early days of solid organ transplantation it became apparent that a positive lymphocytotoxic crossmatch was associated with hyperacute rejection of renal allografts (Kissmeyer-Nielsen et al., 1966; Patel and Teraski, 1969). The mechanism of hyperacute rejection is dependent on complement damage following the combination of donor antibody and recipient cells. Hepatic allografts appeared to be resistant to these preformed antibodies and the early reports suggested no adverse effects on graft rejection or outcome (Iwatsuki et al., 1984; Krom et al, 1984; Gordon et al., 1986; Moore et al, 1987).

However, reports then began to emerge of cases of hyperacute hepatic allograft rejection (Hanto et al., 1978; Bird et al., 1989; Starzl et al., 1989) and subsequent to this a series of reports indicating poorer graft outcome in those grafts transplanted against a positive lymphocytotoxic crossmatch (Takaya et al, 1992; Katz et al, 1994; Nikaein et al., 1994; Charco et al.,1996). This, however, was not every centre's experience (Lobo et al, 1995; Donaldson et al., 1995; Goggins et al., 1996; Fujita et al., 1997).

The liver transplant unit in Scotland was initiated in 1992 and like many units performed liver transplants without reference to cytotoxic donor-specific antibody status. The role of these antibodies in allograft rejection and graft survival was investigated.

2.METHODS

2.1 PATIENTS

During the period from December 1992 to June 1997 145 adult patients received their first orthotopic liver transplantation. Thirty-three patients were not tested for lymphocytotoxic antibody against a specific donor for technical reasons. No ABO blood group incompatible transplant was carried out. Immunosuppression was commenced within the first 6 hours with intravenous hydrocortisone (100mg twice daily) and azathioprine (2mg/kg/daily). Cyclosporin (5mg/kg/12 hourly) or tacrolimus (0.05mg/kg/12 hourly) was commenced within the first twenty four hours.

2.2 CROSSMATCH TEST

The recipients' sera obtained immediately before liver transplantation were tested for cytotoxic antibody against donor lymphocytes using the standard NIH technique. Donor T-lymphocytes were obtained from lymph nodes and one microlitre of patient's serum was added for 30 minutes at room temperature. Five microlitres of rabbit complement were added for an additional 1 hour at room temperature and ethidium bromide and acridine orange was added to stain cells. The crossmatch test was interpreted as positive when more than 20% of donor T-lymphocytes were killed by recipient serum and negative when less than 20 % of cells were killed.

2.3 ORGAN PRESERVATION

All of the liver allografts were preserved with University of Wisconsin solution.

2.4 FOLLOW UP AND STATISTICS

Acute cellular rejection was defined as clinical, biochemical and histological evidence of rejection requiring treatment with high dose steroids. Hepatic allografts were considered to be lost if the recipient died or when the graft was replaced because of poor or non-function.

Mean follow up was 28 months (12-60 months).

Survival rates were calculated by the method of Kaplan-Meier with Breslow log rank tests for significance. Statistical comparisons were made by Student's t- test for the difference between means, chi-square analysis and Fisher's exact test.

3.RESULTS

Twelve (10.7%) of the 112 recipients receiving their first hepatic allograft had a positive antidonor lymphocytotoxic antibody test. One hundred (89.3%) had a negative crossmatch. The characteristics of these patients are detailed in table 3.1 and show no statistical differences between the two groups. There was however a preponderance of females within the positive crossmatch group (82% vs. 59%). In addition 67% of the positive crossmatch group had autoimmune liver disease (primary biliary cirrhosis, primary sclerosing cholangitis or auto-immune chronic active hepatitis) compared with 38% in the negative crossmatch group. The donor factors, which may be important in graft function, were not different between the positive and negative crossmatch groups i.e. mean donor age (42.1 vs.43.1), use of pressors/inotropes (67% vs. 63%), peak aminotransferases (36.0 U/l vs. 40.7 U/l) and hypernatraemia (150 mmol/l vs. 147 mmol/l).

Two patients in the positive crossmatch group had not either been pregnant or received a blood transfusion. Six patients in this group had been pregnant and received a transfusion.

Figure 3.1 shows the graft survival for those with a positive and a negative crossmatch. The twelve month graft survival was 58% in the positive crossmatch group and 81% in the negative group ($p= 0.03$). The patient 12 month survival was 83% and 90% ($p= 0.41$) for those with a positive and negative crossmatch.(Figure 3.2)

Three of the 5 grafts lost in the positive crossmatch group were retransplanted in 1-4 days. The other 2 patients died within 5 days. The first had fibrin thrombi within the lungs and dilatation of the right heart. The second died from sepsis. Within the negative crossmatch group 4 patients were re-transplanted within 5 days - 3 for primary non-function and the other for anastomotic hepatic artery thrombosis. The other 8 early losses were from sepsis (4), cerebral complications (2) or cardiac arrest at operation (2). The reasons for the other 7 graft losses were death from sepsis (1), re-transplantation for chronic rejection (4), hepatic artery thrombosis (1) and disease recurrence (1).

Table 3.2 shows the episodes of acute cellular rejection in both groups in the grafts that survived greater than 7 days, demonstrating a significant increase of rejection requiring treatment beyond one dose of high dose steroids in the positive crossmatch group within the first 30 days.

Table 3.3 shows the outcome of grafts transplanted against a positive crossmatch. This shows that 3 of the 7 patients with a strongly positive crossmatch lost their graft, while the remaining patients had a mean of 2.25 rejection episodes compared with 2 graft losses and a mean of 1 rejection episode in the 5 patients with either weakly positive or positive crossmatches.

Table 3.1. Details of patients and immunosuppression.

Crossmatch	Positive	Negative	P
No. of grafts	12	100	
Age	54.1 (10.2)	47.4 (12.9)	0.08
Male/female	2/10	41/59	0.07
Cold ischaemia time (hrs)	10.5 (3.2)	10.4 (2.5)	N.S.
Immunosuppression- cyc,pred,aza/tacr,pred,aza	9/3	91/9	N.S.
Indication for transplant-			
PBC	7	33	N.S.
Alcoholic liver disease	1	20	N.S.
P.S.C.	1	9	N.S.
Viral disease	0	4	N.S.
Fulminant hepatic failure	0	18	N.S.
Tumour	1	0	N.S.
Autoimmune hepatitis	1	5	N.S.
Cryptogenic cirrrhosis	1	7	N.S.
Others	0	4	N.S.

cyc= cyclosporin, pred = prednisolone, aza= azathioprine, tacr = tacrolimus

Significance tests- Student's t-test, χ^2 test

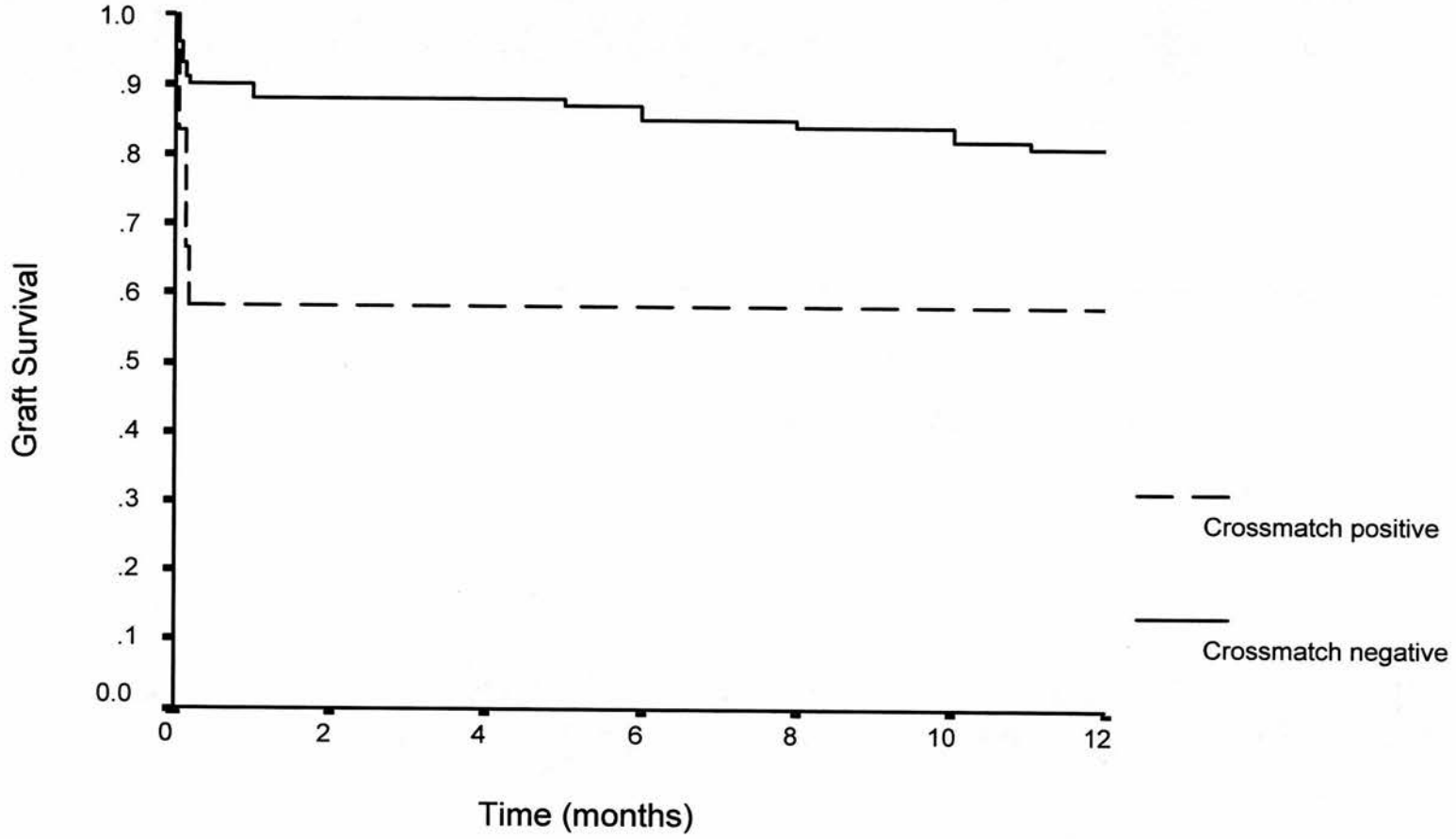


Figure 3.1. Graft survival curves according to lymphocytotoxicity crossmatch (p=0.03, log rank test).

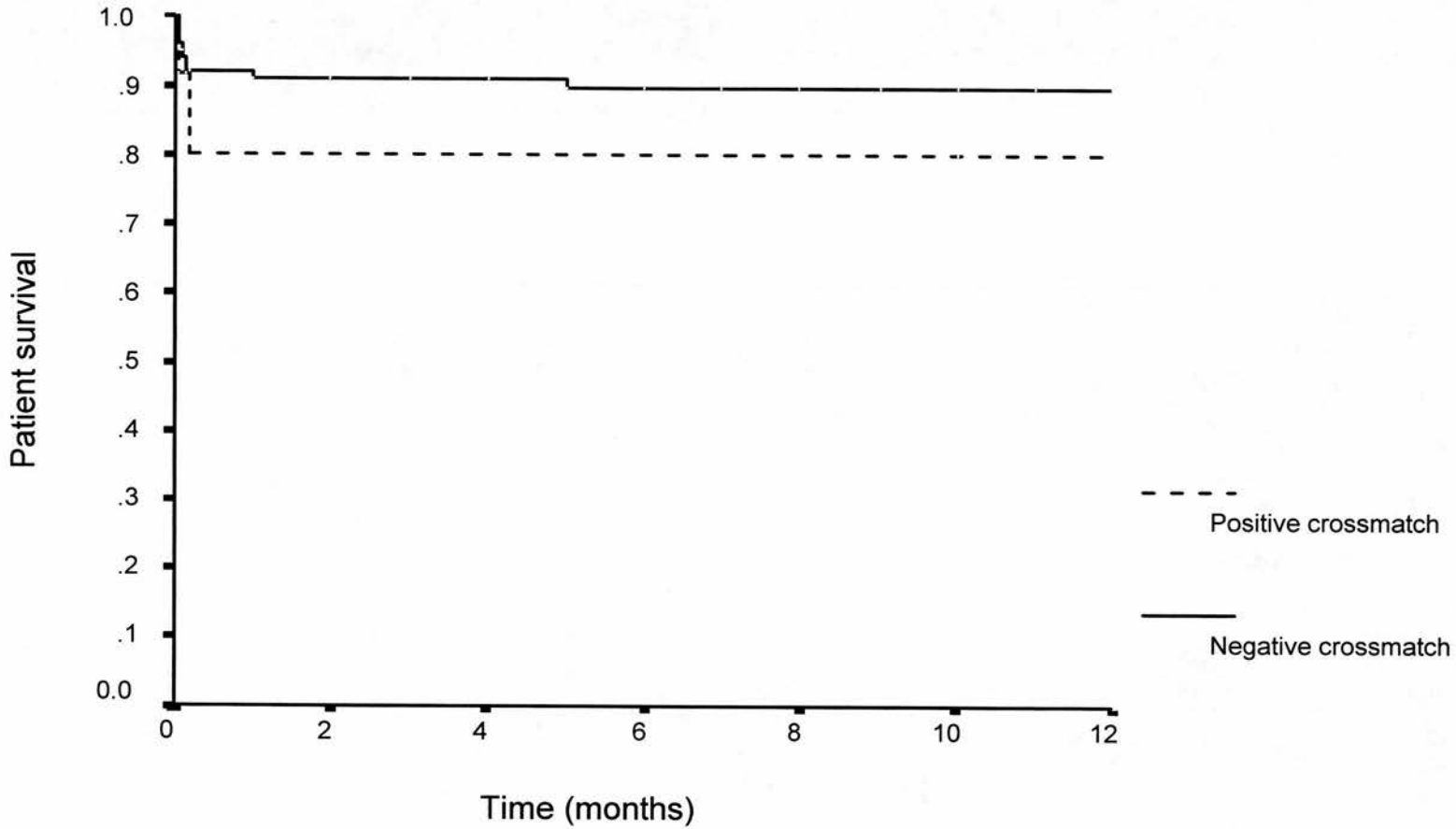


Figure 3.2. Patient survival according to lymphocytotoxicity crossmatch.

Table 3.2. Acute cellular rejection in positive and negative crossmatches.

Crossmatch	Positive (n=7)	Negative (n=88)	P
One episode of acute cellular rejection	6 (86%)	46(52%)	0.09
More than 1 episode of acute rejection within 30 days	4 (57%)	4 (5%)	0.0006

Significancec test, χ^2 test.

Table 3.3. Outcome of grafts with a positive crossmatch

Patient	Cell death	Immuno-suppression	Graft status	Episodes of ACR requiring therapy
Number 1	41-80%	cyc,aza,pre	died 48 months	0
Number 2	41-80%	cyc,aza,hyd	died day 5	
Number 3	81-100%	nil	died day 1	
Number 4	21- 40 %	cyc,aza,hyd	failed day 1	
Number 5	81-100%	cyc,aza,pre	functioning 27 months	2
Number 6	81-100%	cyc,aza,pre	functioning 22 months	4
Number 7	81-100%	cyc,aza,hyd	failed day1	
Number 8	81-100%	cyc,aza,hyd	functioning 12 months	2
Number 9	21-40%	tac,aza,pre	functioning 12 months	2
Number 10	81-100%	tac,aza,pre	functioning 12 months	1
Number 11	81-100%	tac,aza,hyd	failed day 2	
Number 12	21-40%	tac,pre	failed 7 months	1

4. DISCUSSION

The results from this study of 112 patients receiving their first hepatic allograft for either acute or chronic disease revealed an overall graft and patient survival of 78% and 89%. Initial graft loss was significantly worse where preformed cytotoxic anti-donor antibodies existed.

In recent years the standard NIH crossmatch has been joined by more sensitive methodologies in detecting immune reactivity i.e. long incubation NIH assay, antihuman globulin (AHG) procedure and indirect immunofluorescent flow cytometry (Kerman, 1994).

The clinical relevance of these procedures in liver transplantation is questionable as they may be oversensitive, and recent studies using flow cytometry revealed no impact on graft survival (Talbot et al., 1995; Goggins et al., 1996). It may well be that the liver is able to neutralize antibody in low titre but in some grafts the protective mechanisms are over whelmed and the antibody persists. The poor outcome of these patients was reported by Manez et al. (1993).

The data in our study do not distinguish between immunoglobulin M or G antidonor antibody. There is evidence in primary recipients of renal allografts that IgM reactivity does not confer an adverse effect on allograft outcome (Iwaki et al.,1988; Kerman et al, 1991)). However, in liver transplantation IgM reactivity has been shown to adversely affect graft outcome albeit less so than IgG reactivity (Katz et al., 1994).

The rate of positive crossmatches is similar to other centres, although the studies reported to date have used different criteria for a positive crossmatch, varying from greater than 10 % cell death to 50 % cell death.

The poor early outcome of grafts transplanted across a positive crossmatch found in other centres was confirmed in our population. Of particular interest was the loss of grafts with so called primary non-function. The pre-perfusion biopsies of these grafts did not show any architectural abnormalities or steatosis and the donor risk factors for graft loss were not different between the two groups. The histological findings of these failed grafts were not dissimilar to those described by Demetris et al. (1992). The strict criteria which have been suggested by the same group for hyperacute rejection were not met but it is possible that immunoligical damage contributed to graft loss.

The findings of acute cellular rejection requiring additional treatment to a single course of methylprednisolone is also interesting. This has been reported by other groups (Takaya et al., 1991) using standard crossmatching techniques and more recently by McCarthy et al. (1999) using flow cytometry crossmatching. The reason for this finding is not entirely clear. It is noteworthy that there were more episodes of rejection in the strongly positive crossmatch group compared with the less positive crossmatches and it may well be that the liver cell injury resulting from anti-donor antibodies results in a clinicopathological syndrome similar to acute cellular rejection. In support of this is the finding in this study that there is no increase in the incidence of chronic rejection in our positive crossmatch group as steroid resistant and relapsing acute cellular rejection have been shown to be more likely to progress to

chronic rejection (Wiesner, 1999). An early study from the Mayo clinic which used AHG lymphocytotoxicity testing has suggested an increase in the vanishing bile duct syndrome (Batts et al., 1988) in patients with a positive crossmatch and this was supported by findings in other centres (Takaya et al.,1992; Katz et al., 1994). Recent studies from other centres however failed to find this association (Nikaein et al, 1994; Charco et al., 1996; Lobo et al., 1995; Donaldson et al., 1995; Goggins et al., 1996).

5. CONCLUSIONS

The outcome of grafts in patients with a positive crossmatch is worse than those with a negative crossmatch but the patient survival is not affected. There appears to be an increase in episodes of acute rejection requiring treatment particularly in patients with strongly positive crossmatches.

CHAPTER FOUR

THE INFLUENCE OF RECIPIENT GENETIC POLYMORPHISMS IN TUMOUR NECROSIS FACTOR ALPHA, INTERLEUKIN 10 AND TRANSFORMING GROWTH FACTOR BETA ONE GENES

1.INTRODUCTION

Cytokines play a major role in the inflammation and immune responses which take place in allorecognition and rejection. There are a number of cytokines that have been implicated in these responses. The proinflammatory cytokines tumour necrosis factor alpha (TNF–α) and interleukin 6 (IL-6) can cause endothelial cell activation, up-regulate cell adhesion molecules and MHC expression as well as recruiting other immune cells to the site of inflammation (Rink and Kirchner, 1996). Other cytokines may have a modulatory function such as interleukin 4 (IL-4) and interleukin10 (IL-10).

IL-10 inhibits the proliferation of T cells by inhibiting IL-2 production and reducing the synthesis of proinflammatory cytokines such as TNF-α, IL-6 and IL-10. It also downregulates surface expression of MHC class II molecules on antigen presenting cells (Bhol and Ahmed, 1997).

There is evidence for increased production of TNF-α in patients undergoing acute cellular rejection following orthotopic liver transplantation in both serial measurement of serum levels and intra-graft messenger RNA (Imagawa et al., 1990; Martinez et al., 1992). Other studies have revealed increased intra-graft expression of other pro-inflammatory cytokines such as IL-6 (Gorczynki et al, 1996). The measurement of IL-10 in serum and graft have shown no changes during liver allograft rejection (Bishop et al., 1993; Conti et al.,1998) although one study suggested an increase prior to episodes of acute rejection (Platz et al., 1996).

Transforming growth factor beta one (TGF-β1) is a multifunctional cytokine with effects on the immune response and tissue healing. It is chemotactic for inflammatory cells such as monocytes and neutrophils and can inhibit the production of TNFα from T and B lymphocytes (Wayne et al., 1994). It is a potent inducer of both the synthesis and deposition of extra-cellular matrix. In the context of transplantation there has been interest in the role of TGF-β1 in the development of chronic allograft rejection, which results in fibrosis of the transplanted organ.

The propensity of an individual to develop acute rejection following liver transplantation is difficult to predict. One possible reason for individual variation may be differences in cytokine production related to polymorphisms in cytokine genes.

The aim of this study was to investigate polymorphisms known to affect the production of cytokines TNF-α (Louis et al., 1998), IL-10 (Turner et al., 1997) and transforming growth factor $beta_1$ (TGF-β_1) (Awad et al., 1998) *in vitro* in a liver transplant population and the occurrence of allograft rejection. Many other polymorphisms exist in other cytokine genes but as yet have not been shown to have an effect on the function of the gene. The polymorphisms investigated are not linked.

2.MATERIALS AND METHODS

2.1 PATIENTS

Patients were transplanted at the Scottish Liver Transplant Unit between November 1992 and November 1998. All patients were adults receiving primary transplants. There were no ABO incompatible transplants in this group. Immunosuppression was with triple therapy consisting of cyclosporin or tacrolimus, azathioprine (1-2mg/kg) and prednisolone (20mg tapering to 5 mg at three months post transplant).

Acute rejection was defined as rejection treated with high dose corticosteroids based upon clinical, biochemical and histological evidence- using the accepted Banff criteria. Chronic rejection was diagnosed histologically - foamy cell arteriopathy and/or 50% of portal tracts without a bile duct.

2.2 CONTROLS

Cytokine genotypes were also established for healthy Caucasian controls. The number varies as the original studies of function were carried out at different time points and more controls had been recruited for the IL-10 functional study.

2.3 CYTOKINE GENOTYPING

DNA extraction

Genomic DNA was obtained from whole blood by phenol extraction and ethanol precipitation following proteinase K (Boehringer Mannheim) digestion.

Polymerase chain reaction (PCR)

DNA was amplified using PCR performed on PTC-100 thermal cycler (Genetic Research Instrumentation Ltd). Each 30 µl reaction mixture contained 2 µl test DNA (50-200 ng), 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 200 µM each dATP, dCTP, dGTP and dTTP (Gibco BRL), 2.5mM $MgCl_2$ (except for TGF-β where 1.5 mM $MgCl_2$ was used) 0.4 M betaine monohydrate (Sigma), 0.5 µM each primer and 1U *Taq* polymerase (Gibco BRL). After an initial melting time of 5 min, samples were subjected to 30 rounds of 95°C for 30 s. and 72°C for 60 s., with a final extension time of 5 min. at 72°C. Primers used in each reaction and their annealing temperatures are shown in table 4.1. The amplified products were monitored by electropheresis on a 2% agarose gel with ethidium bromide (10 mg/l).

Polymorphism analysis (promoter region for TNF-α and IL-10, leader sequence for TGFβ1)

After specific PCR reactions were performed, two 5' biotinylated oligonucleotide probes (Genosys Biotechnologies Ltd., Pampisford, UK) were used to positively identify each polymorphism in cytokine gene promoters by a dot blot technique. Two µl of PCR product was blotted onto Hybond N+ nylon transfer membrane (Amersham). The double stranded DNA was seperated by treating membranes with denaturing solution (0.5M NaOH and 1.5M NaCl) for 5 minutes and then neutralising solution (1.5M NaCl and 0.5M Tris, pH 7.5) for 60s. The membranes were baked in an oven at 80°C for 10 min and the DNA was immobilised on to membrane by cross-

linking in a UV Stratalinker (Stratgen) at 120 mJoules. The membranes were incubated in 50 ml tubes (Falcon, Becton Dickinson) with 10 ml of 5x SSC hybridisation buffer (where 5x SSC is 0.75M NaCl and 0.075M NaCitrate) with 0.5% blocking agent (milk powder), 0.1% N-lauryl sarcosine and 0.02% sodium dodecyl sulphate (SDS) for 30 min at 42.5^{0}C. Following this 400 ng of specific probe, shown in table 4.2, was added to each tube and allowed to hybridise for 90 min at 42.5^{0}C. The membranes were washed twice in 5x SSC containing 0.1% SDS for 5 minutes at room temperature and then stringency washed in 1x SSC with 0.1% SDS for 30 minutes at the temperature appropriate for each cytokine. The membranes were washed for 60 sec. in 0.15 M NaCl and 0.1M Tris buffer, pH7.5 incubated for 30 minutes at room temperature in the same buffer containing 0.5% milk powder as blocking agent to reduce non-specific binding and then incubated with streptavidin/horse radish peroxidase conjugate (Amersham) for 30 minutes at room temperature before detection by chemiluminescence using the ECL system (Amersham). X-ray films were developed and binding of allele specific probes was used to determine genotypes.

2.4 STATISTICAL ANALYSIS

Statistical analysis was carried out using Chi squared tests with a value of $p< 0.05$ deemed as significant. The Bonferroni correction was applied in multiple comparisons.

Table 4.1.Primers and annealing temperatures for cytokine gene polymorphism.

Primer pairs	**Sequence**	**T (^{0}C)**
TGF-β_1 sense	5'-CTTCACCAGCTCCATGTCGACAG-3'	60^{0}C
TGF-β_1 antisense	5'-ACTGCGCCCTTCTCCCTG-3'	60^{0}C
TNF-α -308 sense	5'-ACTCAACACAGCTTTTCCCTCCA-3'	66^{0}C
TNF-α –308 antisense	5'-TCCTCCCTGCTCCGATTCCG-3'	66^{0}C
IL-10 sense	5'-ATCCAAGACAACACTACTAA-3'	55.5^{0}C
IL-10 antisense	5'-CGAGCTTTAAAAGATAGTTCC-3'	55.5^{0}C

Regions amplified : TGF-β_1 +691 to +965

TNF-α -331 to -224

IL-10 -1115 to -528

Table 4.2. Sequences of oligonucleotide probes.

Probe	Sequence	T°C
TGF-β_1 codon 10*C Coding strand	5'-GCTGCTGCCGCTGCTGC-3'	58°C
TGF-β_1 codon 10*T Coding strand	5'-GCTGCTGCTGCTGCTGC-3'	
TGF-β_1 codon 25*G Coding strand	5'-GCCTGGCCGGCCGGCCG-3'	62°C
TGF-β_1 codon 25*C Coding strand	5'-GCCTGGCCCGCCGGCCG-3'	
TNF-α -308 * G Coding strand	5'-GAGGGGCATGGGGACGG-3'	56°C
TNF-α -308 * A Non-coding strand	5'-CCCGTCCTCATGCCCCTC-3'	59°C
IL-10 –1082* G Coding strand	5'-TTCTTTGGGAGGGGGAAG-3'	47°C
IL-10 –1082* A Non-coding strand	5'-ACTTCCCCTTCCCAAAGAA-3'	
IL-10 –819*C Coding strand	5'-CAGGTGATGTAACATCTCTCGTGC-3'	61°C
IL-10 –819*T Non-coding strand	5'-GCACCAGAGATATTACATCACCTGT-3'	
IL-10 –592*C Coding strand	5'-CCGCCTGTCCTGTAGGAA-3'	48.5°C
IL-10 –592*A Non-coding strand	5'-TTCCTACAGTACAGGCGGG-3'	

3.RESULTS

3.1 AETIOLOGY

Analysis of genotypes was carried out in 144 patients who received a transplant and survived more than 30 days. The distribution of the cytokine genotypes in the different aetiologies and normal controls are shown in tables 4.3-4.6. In primary sclerosing cholangitis and autoimmune hepatitis there was an increase in the TNF – 308 A (TNF2) allele compared with controls. In primary biliary cirrhosis however there was an increase in the TNF-308 G allele (TNF1) compared with controls.

No statistical difference was seen in any aetiology with respect to polymorphisms in the IL-10 gene or in the TGF-β1 gene.

Table 4.3. Distribution of TNF-α -308 genotypes in different aetiologies and controls.

Aetiology	TNF-308*G/G	TNF-308*G/A	TNF-308*A/A	Significance
Normal controls (n=106)	65(61)	24(23)	17(16)	
PBC (n=61)	34(56)	23(38)	4(7)	p=0.02
ALD (n=25)	17(68)	6(24)	2(8)	n.s.
PSC (n=16)	5(31)	5(31)	6(38)	p=0.02
Viral (n=7)	4(57)	3(43)	0	n.s.
Auto-immune (n=8)	0	4(50)	4(50)	p=0.001
Acute -				
Paracetamol (n=12)	6(50)	6(50)	0	n.s.
Other (n=8)	3(38)	4(50)	1(12)	n.s.
Others	1	4	2	

Significance test, χ^2 test.

Table 4.4. Distribution of IL-10 polymorphisms at position -1082 in different aetiologies and controls.

Aetiology	IL10 -1082 A/A(%)	IL10-1082 A/G(%)	IL10-1082 G/G(%)
Normal controls(n=330)	93(28)	138(42)	99(30)
PBC (n=61)	15(25)	28(46)	18(30)
ALD (n=25)	9(36)	12(48)	4(16)
PSC (n=16)	3(19)	9(56)	4(25)
Viral (n=7)	2(29)	3(42)	2(29)
Auto-immune (n=8)	2(25)	4(50)	2(25)
Acute paracetamol (n=12) other (n=8)	 2(17) 2(25)	 6(50) 5(62)	 4(33) 1(12)
Others (n=9)	2	1	3

Table 4.5. Distribution of TGF-β1 polymorphisms at position +869 in patients and controls.

Aetiology	TGF+869 C/C (%)	TGF +869 C/G (%)	TGF+869 G/G (%)
Normal controls	12(11)	46(48)	41(43)
PBC (n=61)	7(12)	34(56)	20(33)
ALD (n=25)	3(11)	7(28)	15(60)
PSC (n=16)	4(25)	6(37)	6(37)
Viral (n=7)	1(14)	5(72)	1(14)
Auto-immune (n=8)	0(0)	3(38)	5(62)
Acute - paracetamol (n=12) other (n=8)	1(8) 1(12)	7(58) 6(75)	4(33) 1(12)
Others	1	3	3

Table 4.6. Distribution of TGF-β1 polymorphisms at position +915 in patients and controls.

Aetiology	TGF+915 T/T	TGF+915 C/T	TGF+915 C/C
Normal controls	1(1)	19(18)	86(81)
PBC (n=61)	2(3)	7(11)	52(85)
ALD (n=25)	0(0)	4(16)	21(84)
PSC (n=16)	0(0)	3(19)	13(81)
Viral (n=7)	1(14)	1(14)	5(72)
Auto-immune (n=8)	0(0)	2(25)	6(75)
Acute - paracetamol (n=12) other (n=8)	0(0) 0(0)	1(8) 2(25)	11(92) 6(75)
Others	1	0	6

3.1 ACUTE REJECTION

The overall incidence of acute cellular rejection in this group was 48%. The incidence of acute rejection in the different aetiologies is shown in table 4.7.

The occurrence of acute rejection in patients with the different TNF-α genotypes is shown in table 4.8.The results for IL-10 and TGF–β_1 genotypes are shown in tables 4.9 and 4.10. There was a significant difference between the rejection and non-rejection groups with respect to TNF–α –308 genotype. The increased incidence of acute rejection was in those patients who were homozygous A/A ($p< 0.02$, Bonferroni correction). When acute rejection was further classified as a single episode or recurrent, there was no significant difference between genotypes with recurrent acute rejection occurring in 6,8 and 2 patients for the genotypes G/G, G/A and A/A, respectively. Combining TNF–α genotype with IL-10 genotype did not show significant differences as shown in table 4.11.

Table 4.7. The occurrence of acute rejection according to aetiology of liver disease.

AETIOLOGY	NO REJECTION	ACUTE REJECTION
Primary biliary cirrhosis (n=61)	30	31
Alcoholic liver disease (n=25)	17	6
Primary sclerosing cholangitis (n=16)	6	10
Autoimmune hepatitis (n=8)	4	4
Chronic viral hepatitis (n=7)	3	4
Acute liver failure (n=20)	12	8
Others (n=7)	3	4

Table 4.8. The incidence of acute rejection with respect to TNF-α -308 genotype

TNF–α -308 genotype	G/G	A/G	A/A
No acute rejection	42	30	4
Acute rejection	28	25	15*

* $p<0.01$, χ^2 test. ($p<0.02$ following Bonferroni correction)

Table 4.9. Acute rejection with respect to IL-10 -1082 genotype (not significant, χ^2 test).

IL-10 –1082 genotype	A/A	A/G	G/G
No acute rejection	21	38	16
Acute rejection	16	30	22

Table 4.10. Acute rejection with respect to TGF-β1 +869 and +915 genotype (not significant, χ^2 test).

	$TGF\beta_1$ +869 genotype			$TGF\beta_1$ +915 genotype		
	C/C	C/T	T/T	C/C	C/G	G/G
No rejection	8	37	32	1	11	65
Acute rejection	10	34	23	3	9	55

Table 4.11.The combination of different IL-10 and TNFα genotypes and the occurrence of acute rejection

IL10 -1082	TNF-α-308 A/A			TNF-α-308 G/A			TNF–α–308 G/G		
	A/A	G/A	G/G	A/A	G/A	G/G	A/A	G/A	G/G
Acute rejection	2	10	3	6	11	8	8	9	11
No acute rejection	1	1	2	7	14	7	11	24	7

4.DISCUSSION

4.1 AETIOLOGY

The role of cytokines in the aetiology and progression of liver disease is not completely understood. The data presented in this chapter suggests that there may be an association between polymorphisms associated with an increase in TNF-α production and primary sclerosing cholangitis and autoimmune hepatitis. The data presented here also suggests an association between primary biliary cirrrhosis and the polymorphism associated with a low production of TNF–α.

There has been a recent interest in polymorphisms in cytokine genes as some of these polymorphisms have functional significance. There has been some published work in primary biliary cirrhosis already suggesting an increase in the polymorphism TNF1 (G at position -308) in those developing endstage disease (Jones et al., 1999),although this has not been supported by another study from the UK (Gordon et al, 1999). In support of this there is *in vitro* data showing that production of TNFα from patients with PBC is less than in controls and patients with PSC (Broome et al., 1992). The data regarding acute liver failure secondary to paracetamol poisoning and TNF–α polymorphisms suggests no role for progression of disease (Bernal et al., 1998)

There is work suggesting an increase in TNFα in patients with autoimmune hepatitis (Hussain et al., 1994; Maggiore et al., 1995) and although the number of patients with autoimmune hepatitis was small in this study they all possessed at least one TNF2

allele which is highly significant compared with normal controls and other aetiologies. This study, however, does not indicate if this is an aetiological predisposition or an increased propensity to progress to endstage liver disease requiring assessment for liver transplantation. A recent publication from Cookson et al. (1999) reported an increase in the TNF2 gene in patients with type 1 autoimmune hepatitis, although there was no data on progression of disease (Czaja et al.,1999).

The findings relating to primary sclerosing cholangitis are also interesting as the aetiology of this liver disease is unknown although presumed immunological. The findings of 65% of patients with the TNF2 allele compared with 38% of controls is similar to the recent report from the King's college group who found 58% of patients to possess the TNF2 allele compared with 29% of controls (Bernal et al., 1999). This group found no correlation between genotype and disease progression or outcome. This allele may therefore determine susceptibility to PSC as in other autoimmune diseases. There is also *in vitro* evidence to suggest that TNFα production from PBMC is increased in patients with PSC compared with controls (Broome et al, 1992). Primary sclerosing cholangitis occurs often in patients with inflammatory bowel disease particularly ulcerative colitis. TNF–α polymorphisms have been investigated in both Crohn's disease and ulcerative colitis with a modest reduction of the TNF2 allele in Crohn's disease but no difference from controls in ulcerative colitis (Louis et al,1996;Bouma et al,1996). It therefore appears that the TNF2 allele confers susceptibility to PSC and not to inflammatory bowel disease.

The functional significance of the polymorphisms in the interleukin-10 promoter and the transforming growth factor–β1 in disease has been supported by studies in

humans suggesting a relationship between genotype and cytokine levels in inflammatory bowel disease for the former (Tagore et al., 1998) and fibrotic lung disease (Awad et al, 1998) for the latter. IL-10 plays an important role in modulating T cell responses and theoretically the impaired production of IL-10 may allow the potentially autoreactive T cells to proliferate with a breakdown in peripheral tolerance. There is data reporting a decrease in IL-10 mRNA in the livers of patients with PBC and PSC (Mitchell et al.,1997) and also some *in vitro* data to suggest that T cells from patients with PBC produce less IL-10 in response to antigen when compared with controls (Jones et al.,1997. However our finding that there is no association between PBC and PSC and IL-10 genotype is in support of the published data (Przemioslo et al., 1999).

TGFβ1 mRNA has been shown to be increased in cirrhosis of various aetiologies suggesting a role in fibrogenesis (Bedossa, 1995; Baer et al.,1998). Studies investigating TGFβ1 in autoimmune hepatitis have shown an increase at the time of inflammation but levels have reduced to baseline and were similar to controls following treatment (Bayer et al, 1998). This study did not identify any difference in genotype between patients with cirrhosis and normal controls.

4.2 ACUTE REJECTION

Acute rejection following liver transplantation is an unpredictable event which often requires treatment with an increase in immunosuppression, usually in the form of high dose corticosteroids.

The role of polymorphisms in cytokine genes that influence their production is largely unknown with respect to acute cellular rejection in liver transplantation. This may have some bearing on future immunosuppressive strategies if there is a relation between genotype and rejection.

The pro-inflammatory cytokine TNF-α has many effects in the inflammatory process and there is evidence to suggest that the polymorphism at position -308 in the promoter region of the gene influences disease. Individuals possessing the so called TNF2 genotype (-308 A) have been shown to have worse outcome in cerebral malaria and non-Hodgkins lymphoma (McGuire et al., 1994; Warzocha et al., 1998). The influence of this polymorphism in rejection of solid organ allografts has been reported in both kidney and heart transplantation (Sankaran et al., 1999; Turner et al.,1997), the latter only showing a statistical difference when combined with IL-10 genotype. The Nco1 polymorphism in the TNFα gene, which may influence TNF-α production in vitro, has recently been reported to have no influence on the occurrence of acute rejection (Freeman et al, 1999). This polymorphism is not linked to the polymorphism at position –308. The large increase in relative risk associated with cerebral malaria (MacGuire et al., 1994) outcome was associated with the homozygous TNF2 genotype, and it may be that this genotype has more of an effect on TNF-α production than the Nco1 polymorphism.

The finding that 79% of patients who were homozygous for TNF2 required treatment for acute rejection compared with 40% of patients who were homozygous for TNF1 suggests that it may predispose to the occurrence of acute cellular rejection in liver transplantation. The finding that there was no difference in genotypes in patients with

recurrent acute rejection may indicate that the initial recipient recognition of donor antigen is the only response influenced to a sufficient level by TNF–α genotype.

The role of IL-10 in acute cellular rejection is not straightforward. It has many anti-inflammatory properties and one may therefore expect that a polymorphism leading to high production both *in vitro* (Turner et al.,1997) and *in vivo* (Tagore et al., 1998) may protect against acute rejection. However the studies investigating IL-10 in acute rejection in liver transplantation both in the graft and serum have failed to show differences between grafts with or without rejection. Studies investigating acute renal allograft rejection have shown increased IL-10 intragraft expression (Xu et al, 1995; Sutharanthiran and Strom, 1998) and the recent study reporting cytokine genotypes in renal allografts showed an increase in rejection in the polymorphism associated with high IL-10 production (Sankaran et al., 1999). Cardiac allografts in animal studies have shown an exacerbation of rejection with the addition of IL-10 and the measurement of intragraft levels of IL-10 in human grafts do not predict acute rejection (Lagpp et al., 1996). As already indicated the single study of cytokine genotyping in heart transplantation has shown that a combination of a genotype corresponding to high TNFα production and low IL-10 production was associated with high levels of rejection in the early post transplant period (Turner et al., 1997). There was however no reduced rejection in those with the high IL-10 producer genotype. The effect that IL-10 has on B cells, causing them to proliferate and increase the humoral response, may be important in renal transplantation and this may be one of the reasons for the apparent difference in the effect of IL-10 in the rejection of different organs.

In this study there is no evidence that IL-10 polymorphisms either alone or in combination with specific TNF–α polymorphisms influence the occurrence of acute cellular rejection. There is a widespread belief that acute cellular rejection in hepatic allografts is different to that in other solid organ allografts as evidenced by the lack of requirement for HLA matching and the improved outcome of grafts suffering a single episode of acute rejection (Wiesner et al.,1998; Dousset et al.,1998). Therefore it may not be too surprising that the influence of polymorphisms differs between organs.

Transforming growth factor β is a cytokine with immunosuppressive and profibrotic actions. The data presented suggest no influence of TGFβ1 genotype on acute rejection. There is evidence to suggest that in the case of lung allografts there is a significant effect on graft outcome with respect to lung fibrosis, which is influenced by TGF–β genotype (Awad et al., 1998). Chronic rejection of hepatic allografts is not common and it occurred in less than 5% of this study population. Larger numbers of patients will be required to determine if TGF–β1 genotype influences hepatic allografts in a similar fashion.

5.CONCLUSIONS

There is an increase in the TNF2 allele in patients who develop endstage liver disease from primary sclerosing cholangitis and type 1autoimmune hepatitis compared with controls. Patients with endstage PBC however have an increase in the TNF1 allele.

There is an increase in acute cellular rejection in patients who are homozygous for the TNF2 allele. There is no association between acute cellular rejection and polymorphisms of the interleukin 10 and transforming growth factor β1 genes.

CHAPTER FIVE

THE EFFECT OF PRE-TRANSPLANT CYTOKINE PRODUCTION IN VITRO AND ACUTE REJECTION

1.INTRODUCTION

The treatment of choice for end-stage liver disease is orthotopic liver transplantation. The prevention of acute cellular rejection requires powerful immunosuppression with the mainstay of treatment provided by the calcineurin inhibitors cyclosporin and tacrolimus. The principal mechanism of their immunosuppressive action is by the inhibition of interleukin 2 transcription by preventing the activation of nuclear factor of activated T cells (NFAT) (Rao et al., 1997). The effect of these drugs on the production of other cytokines is largely inhibitory(Ruhlmann and Nordheim, 1997), although others such as interleukin-6 are enhanced (Murayami et al., 1994). Moreover tacrolimus and cyclosporin have differential effects on the production of some cytokines (Han et al., 1995; Hutchinson et al., 1998).

Acute rejection is not an entirely predictable event and the effects of powerful immunosuppressive agents have multiple adverse effects including acute renal impairment and increased susceptibility to infection, and in the longer term chronic renal damage and malignancy. Liver allograft loss is very infrequently a consequence of rejection, and some patients may well be over-immunosuppressed. The effects of pre-transplant parameters on acute rejection have recently been reported on (Wiesner et al., 1998). Age, severity of liver disease, renal failure and nutritional status have been shown to influence the occurrence of acute rejection. If pre-transplant parameters can reliably identify a propensity for rejection it may well allow tailoring of immunosuppression.

The role of cytokines in acute cellular rejection following orthotopic liver transplantation is unclear. There is an increase in pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and IL-6 in both graft and serum at the time of acute cellular rejection (Imagawa et al., 1990; Gorczynski et al., 1996; Tilg et al.,1990). Interleukin-5 has also been shown to be increased during acute rejection in the graft (Martinez et al., 1992). By contrast interleukin-10 appears to be unchanged at the time of acute rejection.

The pro-inflammatory cytokines TNF-α and interleukin 6 (IL-6) can cause endothelial cell activation, up-regulate cell adhesion molecules and MHC expression, as well as recruiting other immune cells to the site of inflammation. Other cytokines may have a modulatory function such as interleukin 4 (IL-4) and interleukin10 (IL-10).

IL-10 inhibits the proliferation of T cells by inhibiting IL-2 production and reducing the synthesis of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-10. It also downregulates surface expression of MHC class II molecules on antigen presenting cells.

Cytokine production in endstage liver disease varies with aetiology (Simpson et al., 1997;) and the pre-transplant levels have not been studied with respect to acute rejection post-transplant. The aim of this study was to investigate the production of TNF-α and interleukin-10 by patients with end-stage liver disease awaiting transplantation and explore any relationship with acute cellular rejection. The effect of different immunosuppressants was also investigated.

2 MATERIALS AND METHOD

2.1 PATIENTS

All patients were studied while free from sepsis and in a stable condition while on the waiting list for orthotopic transplantation.

Thirty ml. of heparinised blood was obtained from patients and healthy controls. The blood was separated on a density gradient (Histopaque, Sigma Chemical Co.,St Louis, MO) and 0.5 x 10^6 peripheral blood mononuclear cells cultured in RPMI 1640 () supplemented with 10% fetal calf serum (Gibco, Paisley,U.K) with L-glutamine and streptomycin added to 12 well plates (Iwaki microplate, supplied by Bibby Sterilin, Stone, UK).

Stock solutions of tacrolimus (gift from Fujisawa) and cyclosporin (Sigma Chemical Co.,St Louis, MO) were prepared after first dissolving the drug in ethanol according to the manufacturers instructions.

Dexamethasone (Sigma Chemical Co.,St Louis, MO) was dissolved in sterile water and stock solutions prepared.

Immunosuppressants were stored at -20° C and thawed just prior to use and added to10ng and100ng per ml. final concentrations in duplicate and preincubated for 30 minutes before stimulating with 200ng of *Escherichia coli* O 111:B4

lipopolysaccharide (Sigma Chemical Co., St Louis, MO). Controls did not have any LPS added. Culture was performed in a 5% CO_2 incubator at 37° C.

Supernatants were removed after 20 hours, centrifuged at 2000g for 10 minutes and stored at -20° C until enzyme-linked immunosorbent assay (ELISA) testing.

2.2 MEASUREMENT OF TUMOUR NECROSIS FACTOR–α

TNF–α levels were measured using an ELISA according to the manufacturers (Quantikine, R and D systems Europe, Abingdon, UK).

Ninety-six well plates coated with a murine monoclonal antibody which binds TNF–α in the assay standard or in the supernatants. The bound TNF–α is next revealed by the use of polyclonal antibody against the TNF-α which is conjugated to horseradish peroxidase . The bound enzymatic activity is demonstrated by its oxidative action on the substrate tetramethylbenzidine in the presence of hydrogen peroxide. After the reaction has been stopped by the addition of sulphuric acid the colouration obtained is measured at 450nm. The observed optical density is proportional to the concentration of TNF-α.

Assay protocol

The reconstituted TNF-α standard containing 1 000 pg/ml used to prepare assay calibrators by performing serial dilution (1:2) with dilution buffer.

All supernatants to be tested were diluted 1 in 2 with dilution buffer.

Assay procedure:

Antigen fixation: 200µl of test sample (standard or sample) was added with 50µl of assay diluent to each well. After an incubation of 2 hours at room temperature, 3 successive washes with wash buffer was performed.

Addition of conjugate: After the last washing cycle , 200µl of TNFα conjugate was added to each well. After incubation for 1 hour at room temperature another 3 successive washes were performed.

Addition of substrate: Following the last wash, 200µl of substrate solution (tetramethylbenzidine and hydrogen peroxide) was added. After an incubation of 20 minutes at room temperature 50µl of 2M sulphuric acid was added to each well.

Plate reading: The optical density of each well was determined within 30 minutes using a microtitre plate reader set to a wavelength of 450 nm.

Calculation of results: The standard curve was obtained using log-log paper. The average for each standard, control and test sample was obtained for each duplicate reading. The concentration of TNFα calibrators (pg/ml) was plotted on the x-axis, and the absorbance values of the samples was used to determine TNFα values. The value obtained was multiplied by 2 to get the concentration of the test sample.

2.3 MEASUREMENT OF INTERLEUKIN-10

The measurement of IL-10 levels was carried out using an ELISA (R and D systems), the principle of which has been outlined above.

Assay protocol

The reconstituted IL-10 standard containing 500pg/ml was used to prepare assay calibrators by performing serial dilution (1:2) with dilution buffer.

All supernatant samples to be tested were diluted 1 in 5 with dilution buffer.

Assay procedure

Antigen fixation :200µl of standard or ample were added to each well which was coated with monoclonal antibody against IL-10. After incubation for 2 hours at room temperature , 4 successive washes were performed with wash buffer.

Addition of conjugate: Following washing 200 µl of polyclonal antibody with conjugated horseradish peroxidase was added. After incubation for 1 hour at room temperature washing was performed four times.

Addition of substrate: Following the last wash, 200µl of substrate solution (tetramethylbenzidine and hydrogen peroxide) was added. After an incubation of 20 minutes at room temperature 50µl of 2M sulphuric acid was added to each well.

Plate reading: The optical density of each well was determined within 30 minutes using a microtitre plate reader set to a wavelength of 450 nm.

Calculation of results: The standard curve was obtained using log-log paper. The average for each standard, control and test sample was obtained for each duplicate reading. The concentration of Il-10 calibrators (pg/ml) was plotted on the x-axis, and the absorbance values of the samples were used to determine IL-10 values. The values obtained were multiplied by 5 to get the concentrations of the test samples.

Time course experiments showed maximum production of cytokine after 12 hours, and concentration curves showed stimulation to be maximal over 20ng of LPS.

Samples from two patients and from a healthy control at different time points showed a variation in measured levels of TNF–α of 1-14% (mean 6%) and 3-12% of IL-10 (mean 8%).

2.4 CYTOKINE POLYMORPHISMS

A number of patients had polymorphisms for TNF-α and IL-10 performed at the University of Manchester as outlined in chapter 4.

2.5 ACUTE REJECTION

Acute rejection was defined as rejection requiring treatment with high dose corticosteroids. The diagnosis was made on clinical and histological grounds.

2.6 STATISTICAL ANALYSIS

The sign test was used to compare paired samples of cytokine concentrations. Mann-Whitney U test was used to compare differences between patients and controls, patients with and without acute rejection. Analysis of variance (ANOVA) was used to investigate a difference between aetiologies.

3.RESULTS

TNF-α was measured in 21 patients (9 primary biliary cirrhosis, 6 alcoholic liver disease, 4 chronic viral disease, 2 cryptogenic cirrhosis) and in 8 healthy controls.

Interleukin-10 was measured in 18 of the above patients (6 primary biliary cirrhosis,6 alcoholic liver disease, 4 chronic viral disease, 2 cryptogenic cirrhosis) and 6 healthy controls.

3.1 TUMOUR NECROSIS FACTOR ALPHA

The production of tumour necrosis factor alpha (TNF-α) following LPS stimulation of PBMC in patients (1220 pg/ml ± 130, mean ± SEM) and controls (820 pg/ml ± 190) following LPS stimulation is shown in table 5.1, and there was no significant difference. The production of TNF-α pretransplantation was significantly increased in patients (1575 ± 190) who went on to develop acute cellular rejection requiring treatment following transplantation compared with patients who did not have rejection (950 ± 130) as shown in figure 5.1. There were significant differences between rejectors and non-rejectors in TNFα production with pre-incubation with the calcineurin inhibitors but not with 100ng of dexamethasone.

A ROC curve for TNFα production and rejection was used to determine cut off values (area under the curve 0.792, p = 0.02). A contingency table using a cut off value of TNF production of 1260 pg/ml is shown in table 5.2.The sensitivity was 78%,

specificity 75%, positive predictive value of 0.70 and negative predictive value of 0.81.

The effect of pre-incubation with the different immunosuppressants in the inhibition/augmentation of TNF-α production is also shown in table 1 and more fully in figures 5.2-5.5. TNF–α production in patients was significantly inhibited by dexamethasone but not cyclosporin or tacrolimus (all p values < 0.0001 with respect to either dose of dexamethasone compared with LPS, both doses of tacrolimus and both doses of cyclosporin). Dexamethasone 100ng significantly inhibited TNF-α production more than 10ng of dexamethasone ($p<0.0001$). In normal controls the production of TNF-α was also significantly inhibited by dexamethasone in a similar dose dependent manner. However TNF-α production was also inhibited by tacrolimus 10ng (0.008) and 100ng ($p=0.04$) but not cyclosporin 10ng ($p=0.18$) or 100ng ($p=1.00$). The differential effect of immunosuppressants led to significant differences in TNFα production between patients and controls not seen with LPS alone.

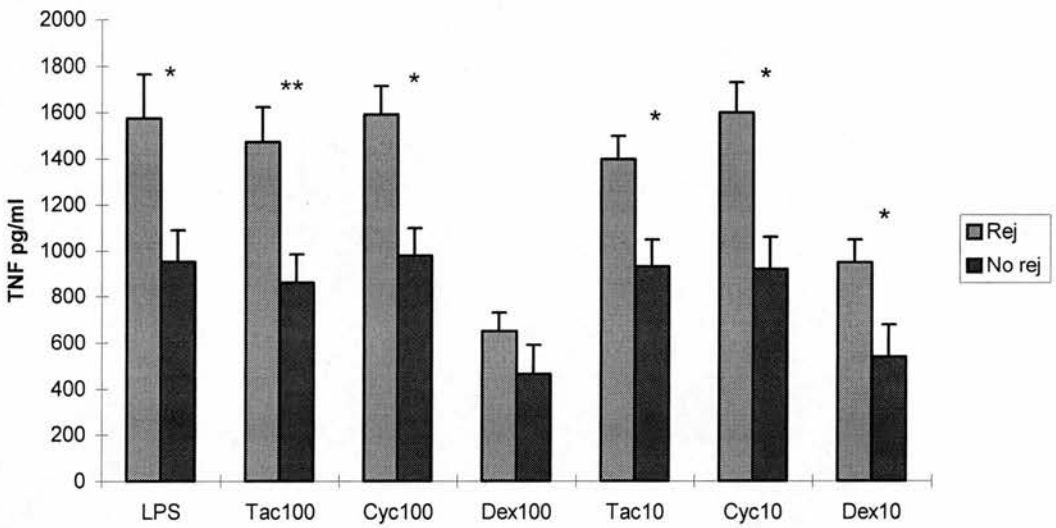
There was no statistical difference in the production of TNF–α, following LPS stimulation, in different aetiologies of liver disease (figure 5.6). The mean values were 1089 ± 200 pg/ml in PBC (n=9), 1315 ± 300 pg/ml in alcoholic liver disease (n=6), 1530 ± 140 pg/ml in chronic viral disease (n=4) and 890 ± 270 pg/ml in cryptogenic cirrhosis (n=2). No statistical difference was observed between aetiologies in the effects of pre-incubation with different immnunosuppresants.

Table 5.1. Production of TNF-α (pg/ml) in patients with liver disease and normal controls. Values expressed as mean (SEM).

	Patients (n=21)	Healthy controls (n=8)
LPS alone	1220 (130)	820 (190)
LPS + tac 100ng	1125 (117)	605 (100)*
LPS + tac 10ng	1130 (106)	590 (125)**
LPS + cyc 100ng	1240 (120)	740 (171)**
LPS + cyc 10ng	1210 (130)	733 (184)*
LPS + dex 100ng	540 (78)	216 (50)*
LPS + dex 10ng	715 (100)	370 (90)
Control	6(3)	20 (6)

* $p< 0.05$, **$p< 0.01$, Mann-Whitney U. Comparison of patients and controls.

Figure 5.1: Production of TNFα pre-transplant in patients with and without acute cellular rejection.(rej = rejection (n=9), no rej = no rejection (n=12)



* $p < 0.05$, ** $p < 0.01$, Mann-Whitney U .

Figure 5.2. ROC curve for TNF-α production and acute rejection.

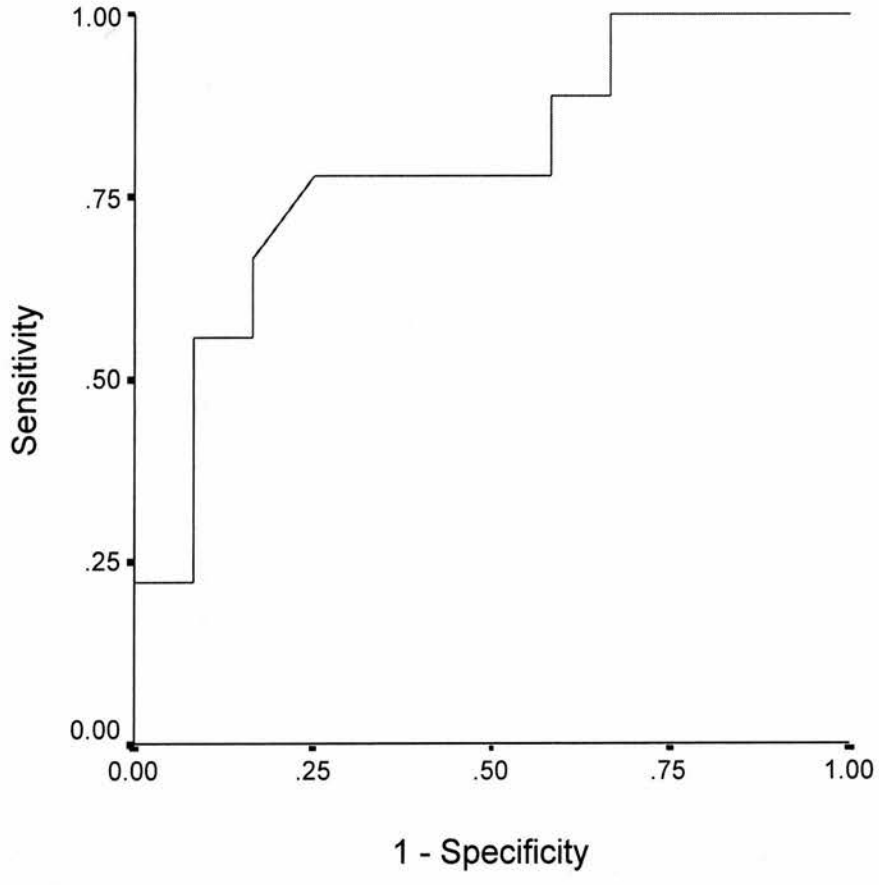
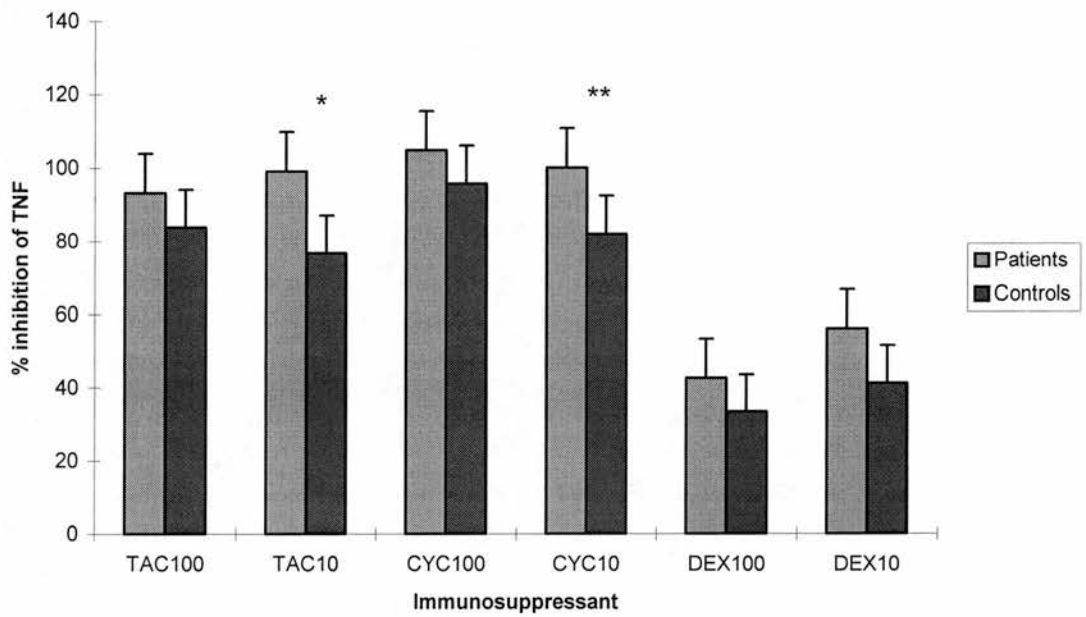


Table 2. Contingency table of TNFα production pretransplant and acute rejection (p =0.03, Fisher's exact test)

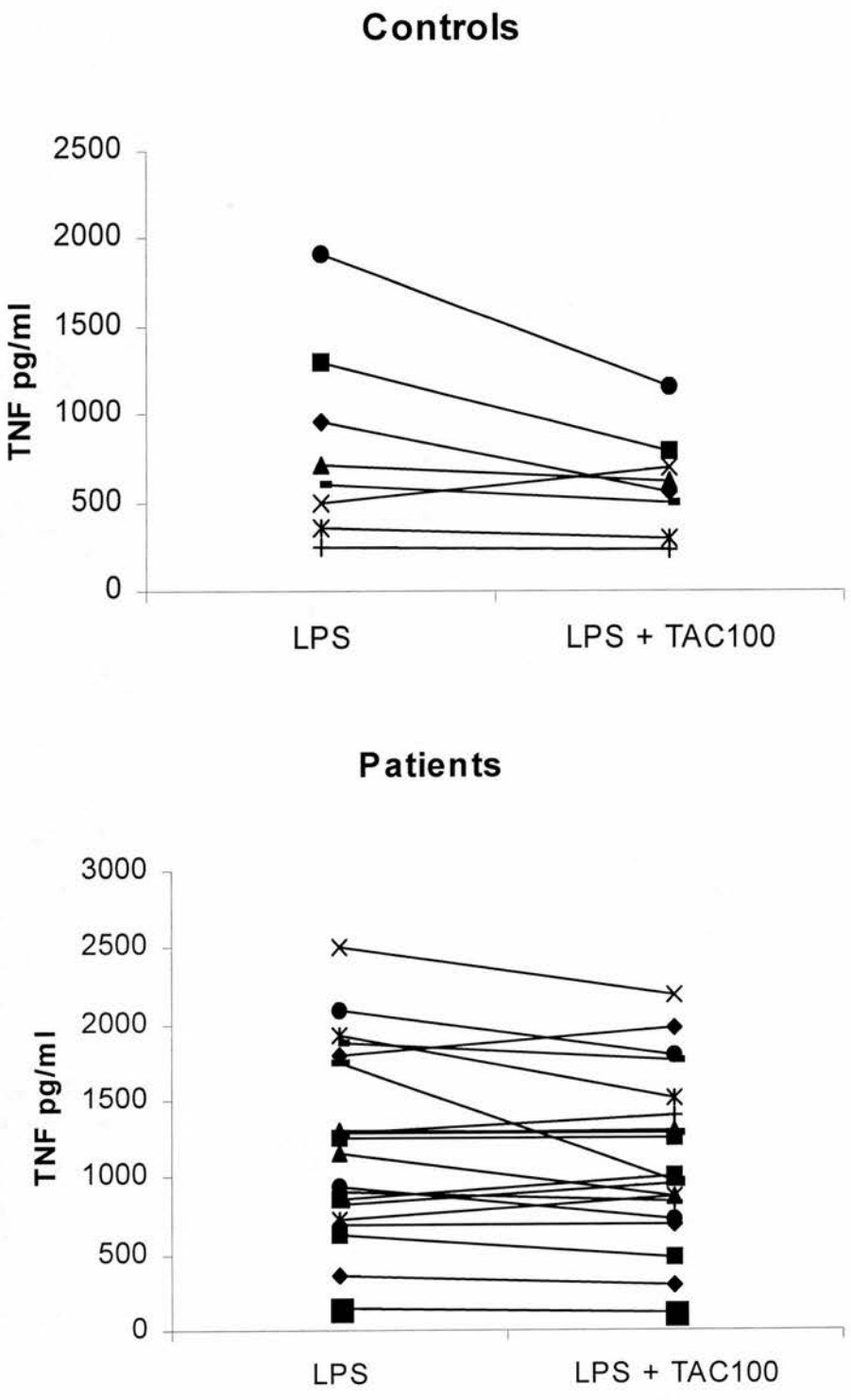
	No rejection	Acute rejection
TNF-α <1260 pg/ml	9	2
TNF-α >1260 pg/ml	3	7

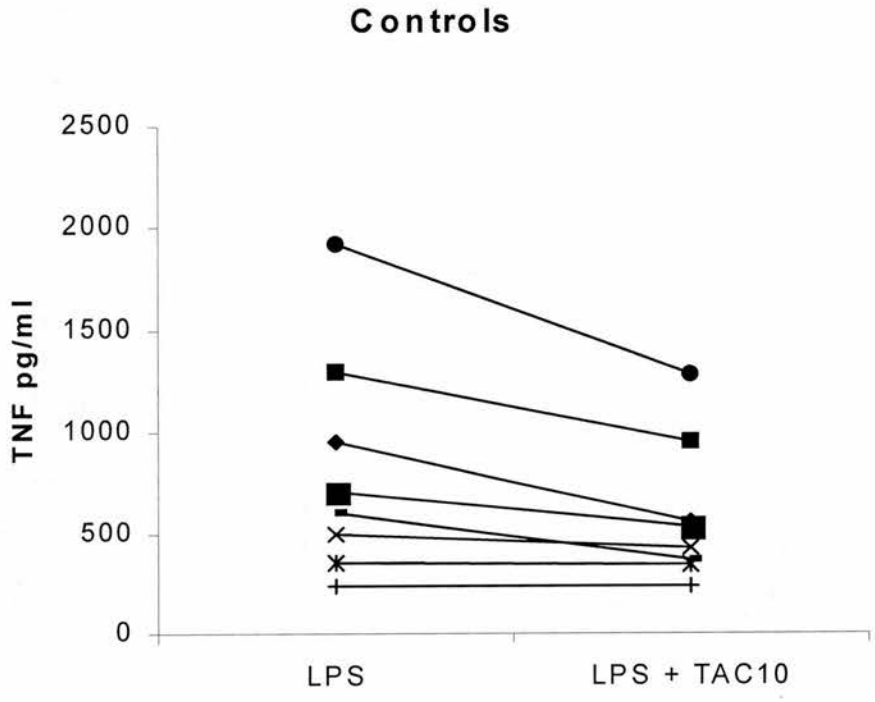
Figure 5.3. Percentage inhibition of TNFα production vs LPS alone by different immunosuppressants in patients (n=21) and controls (n=8). Mean + SEM.



* p=0.02, ** p=0.01

Figure 5.4. Production of TNF-α following stimulation with LPS by patients and controls with and without pre-incubation with tacrolimus 100ng and 10ng.




Controls
2500
2000
1500
1000
500
0
TNF pg/ml
LPS
LPS + TAC10

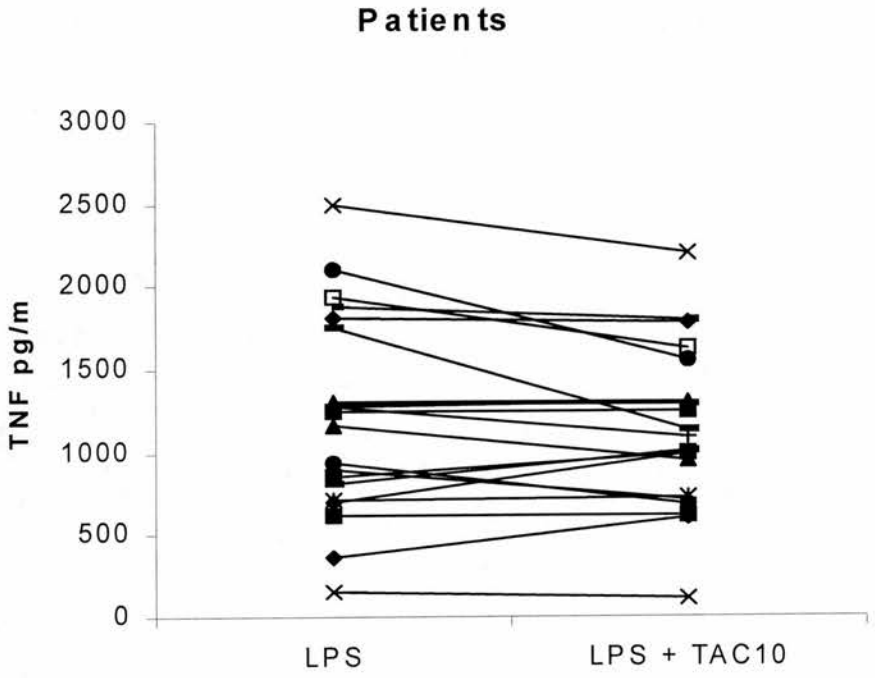
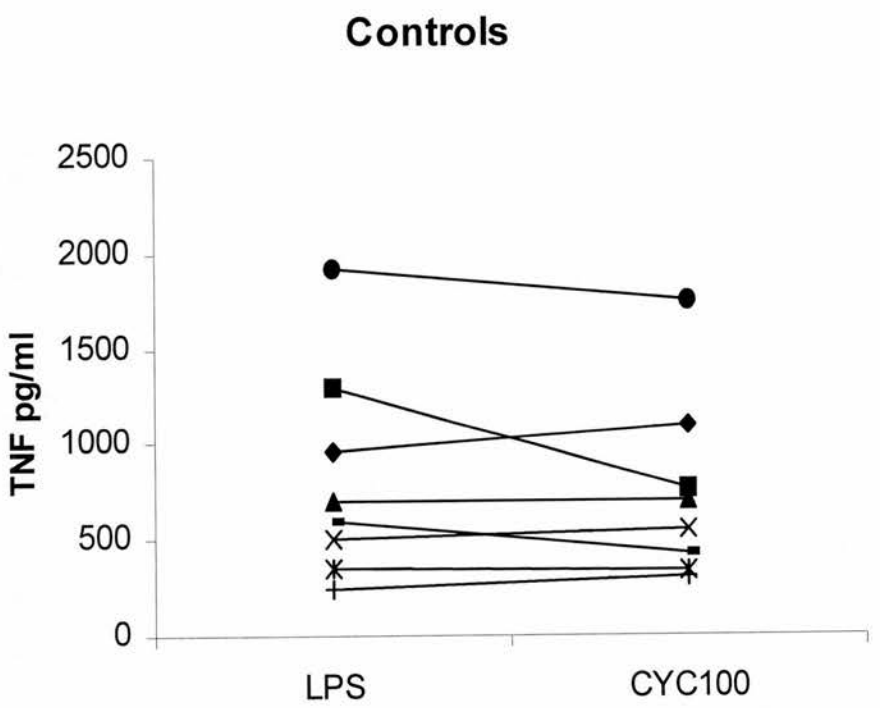
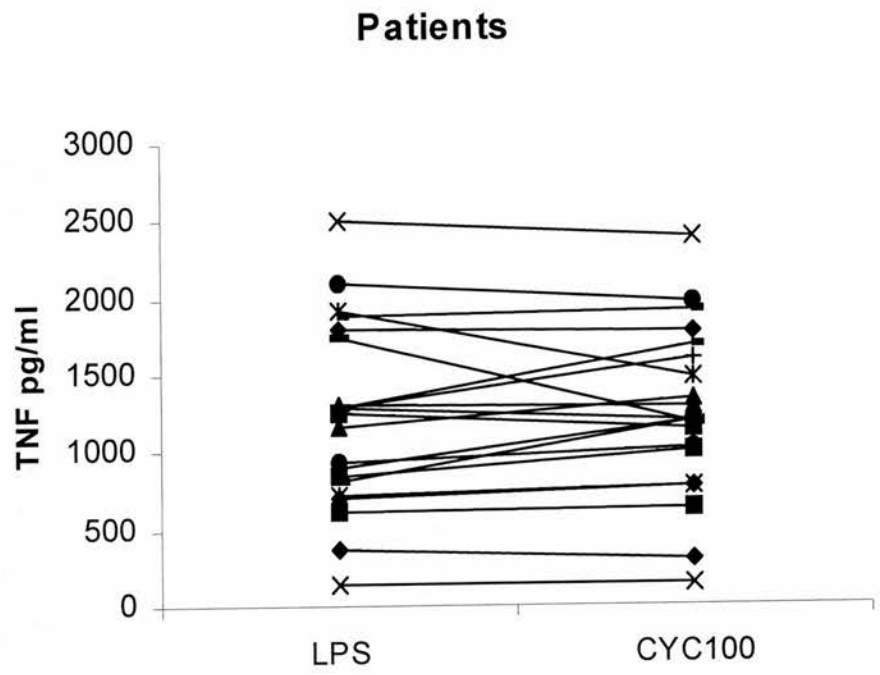
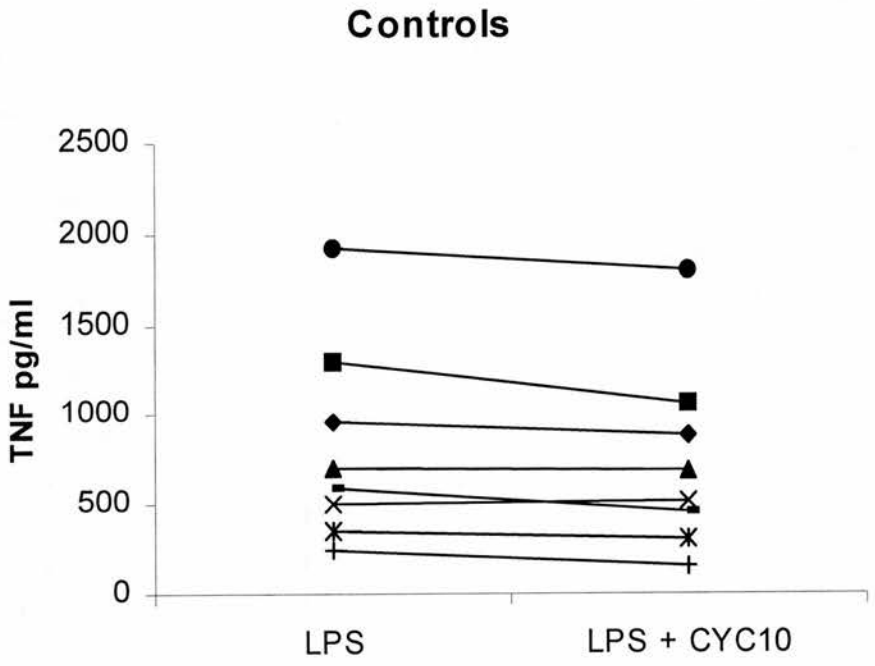

Patients
3000
2500
2000
1500
1000
500
0
TNF pg/m
LPS
LPS + TAC10

Figure 5.5. Production of TNF-α following stimulation with LPS by patients and controls with and without pre-incubation with cyclosporin 100ng and 10ng.






Controls
2500
2000
1500
1000
500
0
TNF pg/ml
LPS
LPS + CYC10

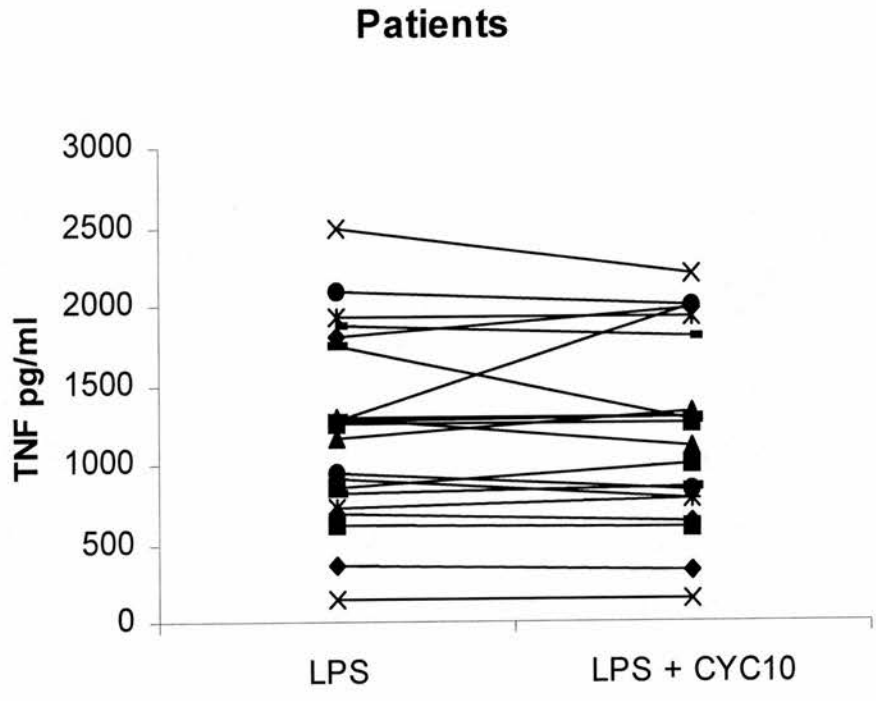
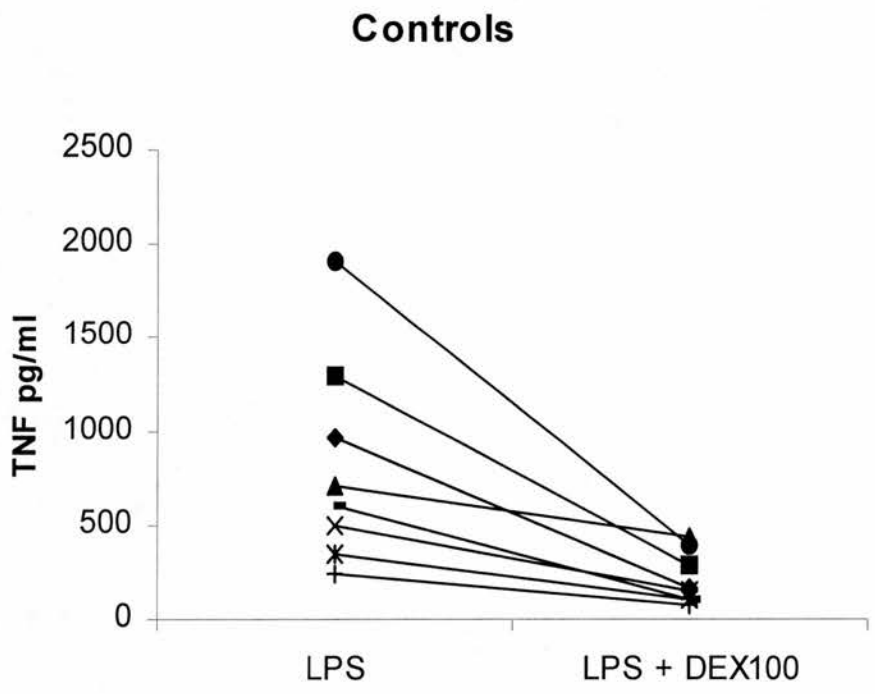
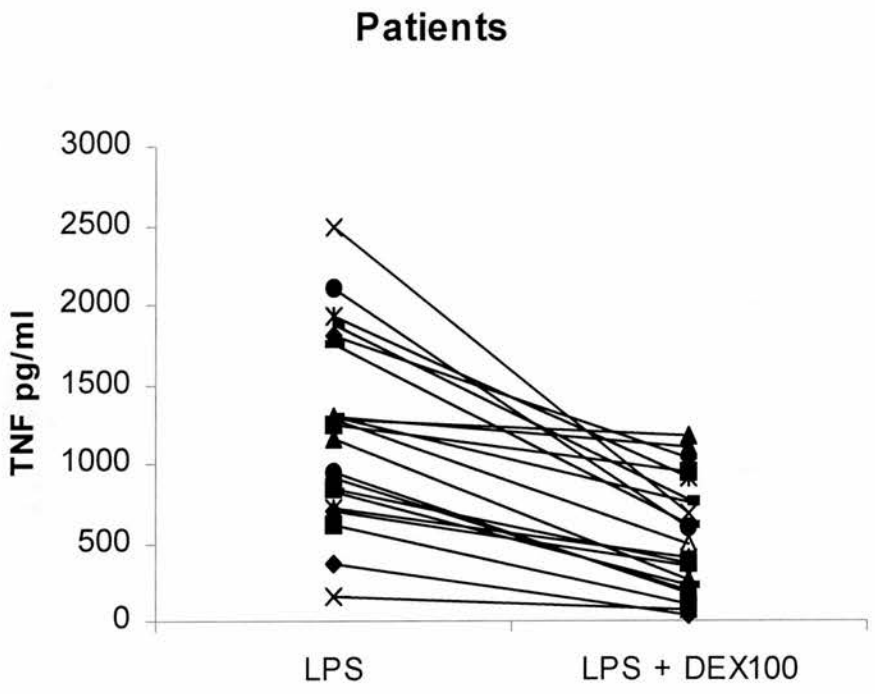
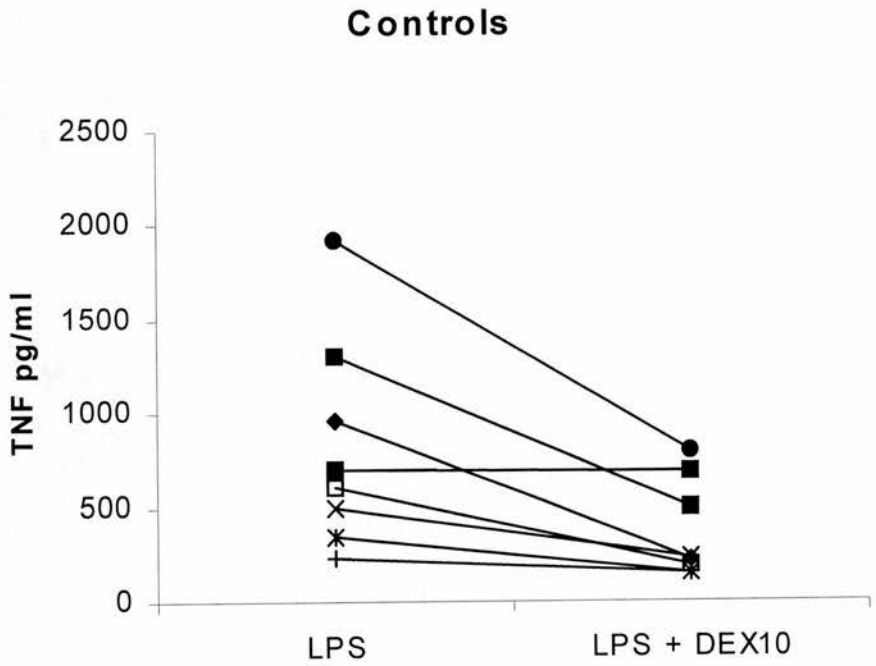

Patients
3000
2500
2000
1500
1000
500
0
TNF pg/ml
LPS
LPS + CYC10

Figure 5.6. Production of TNF-α following stimulation with LPS by patients and controls with and without pre-incubation with dexamethasone 100ng and 10ng.






Controls
2500
2000
1500
1000
500
0
TNF pg/ml
LPS
LPS + DEX10

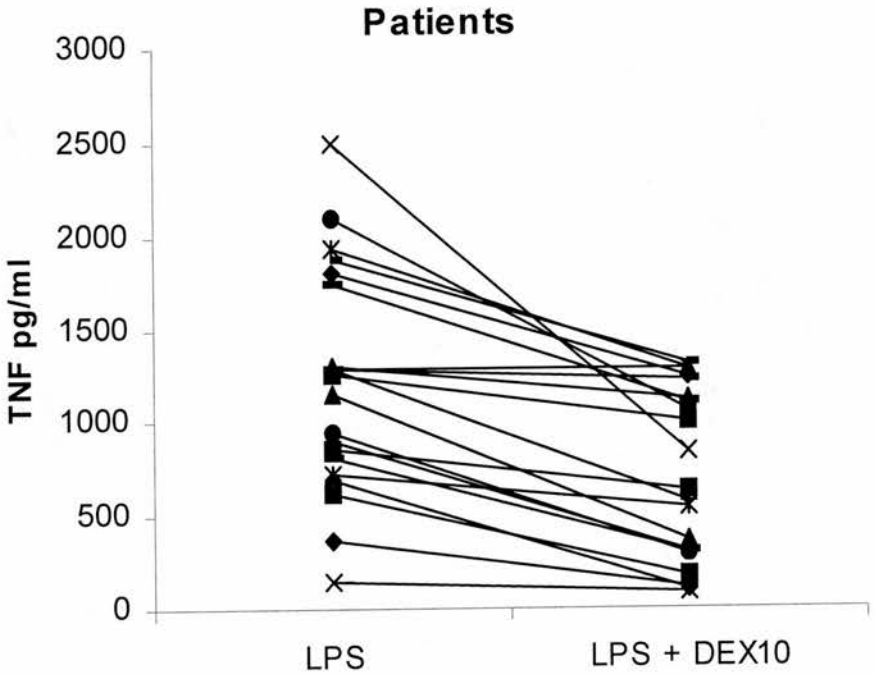
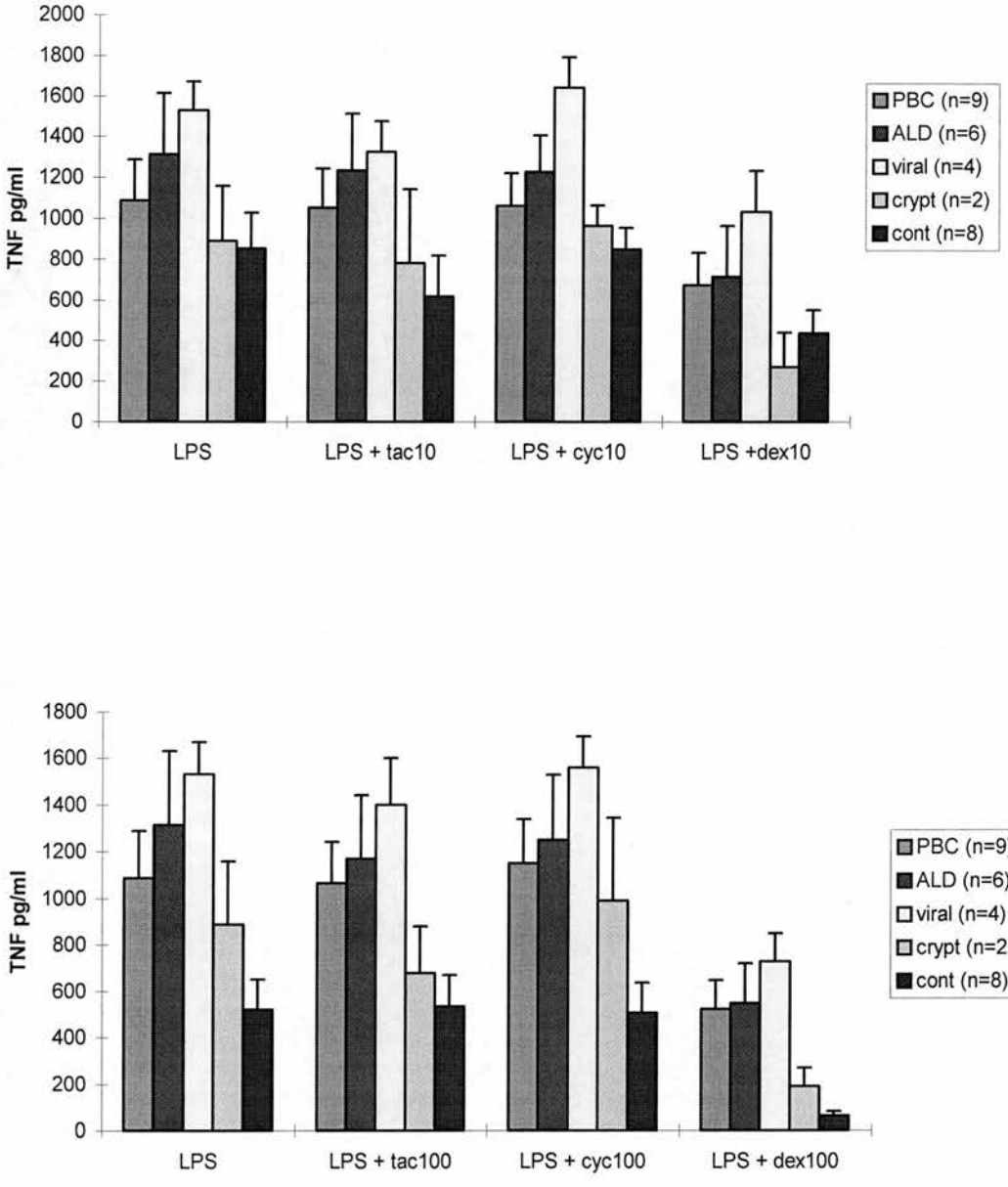

Patients
3000
2500
2000
1500
1000
500
0
TNF pg/ml
LPS
LPS + DEX10

Figure 5.7: Production of TNF-α in different aetiologies with different immunosuppressants.



3.2 INTERLEUKIN-10

The production of interleukin-10 (IL-10) in patients (770 ± 160 pg/ml) and controls (522 ± 130 pg/ml) following LPS stimulation was similar (table 5.3). Figure 5.7 shows there is no difference in the amount of IL-10 produced pre-transplant in patients who developed acute rejection (715 ± 200 pg/ml) following transplantation and those who did not develop rejection (835 ± 260 pg/ml).

The effect of pre-incubation with different immunosuppressants is shown in table 3 and more fully in figures 5.8-5.11. IL-10 production in patients was significantly inhibited by preincubation with dexamethasone 100ng ($p<0.001$) and 10 ng ($p<0.001$). Dexamethasone 100ng significantly inhibited IL-10 production more than 10ng of dexamethasone ($p=0.02$). Interleukin-10 production was augmented by pre-incubation with tacrolimus 10ng and 100ng ($p=0.04$, $p=0.03$). Cyclosporin had no significant effect on production compared with LPS. In healthy controls dexamethasone inhibited production of IL-10 ($p=0.01$ for both doses) but there was no significant difference between the two doses. Neither tacrolimus nor cyclosporin had a significant effect on the production of IL-10.There was a significant difference between the levels of IL-10 produced by patients and controls only in the presence of dexamethasone.

IL-10 production by PBMC following LPS stimulation was similar in different aetiologies. The mean values were 980 ± 380 in PBC (n=6), 910 ± 340 in alcoholic liver disease (n=6), 520 ± 90 in chronic viral disease (n=4) and 570 ± 100 in cryptogenic cirrhosis (n=2). No statistical difference was observed between

aetiologies in the effects of pre-incubation with different immnunosuppresants (Figure 5.12).

Table 5.3.Production of IL-10 (pg/ml) in patients with liver disease and normal controls. Values expressed as mean (SEM).

	Patients (n=18)	Control (n=6)
LPS alone	770 (160)	522 (130)
LPS + tac 100ng	925 (157)	535 (135)
LPS + tac 10ng	985 (170)	493 (118)
LPS + cyc 100ng	721 (159)	509 (128)
LPS + cyc 10ng	705 (166)	439 (126)
LPS + dex 100ng	305 (72)	64 (21)*
LPS + dex 10ng	415 (120)	90 (35)*
Control	49 (24)	20 (6)

* $p < 0.05$, Mann-Whitney U.

Figure 5.8: Production of IL-10 pre-transplant in patients with and without acute cellular rejection.(rej = rejection (n=9), no rej =no rejection (n=9))

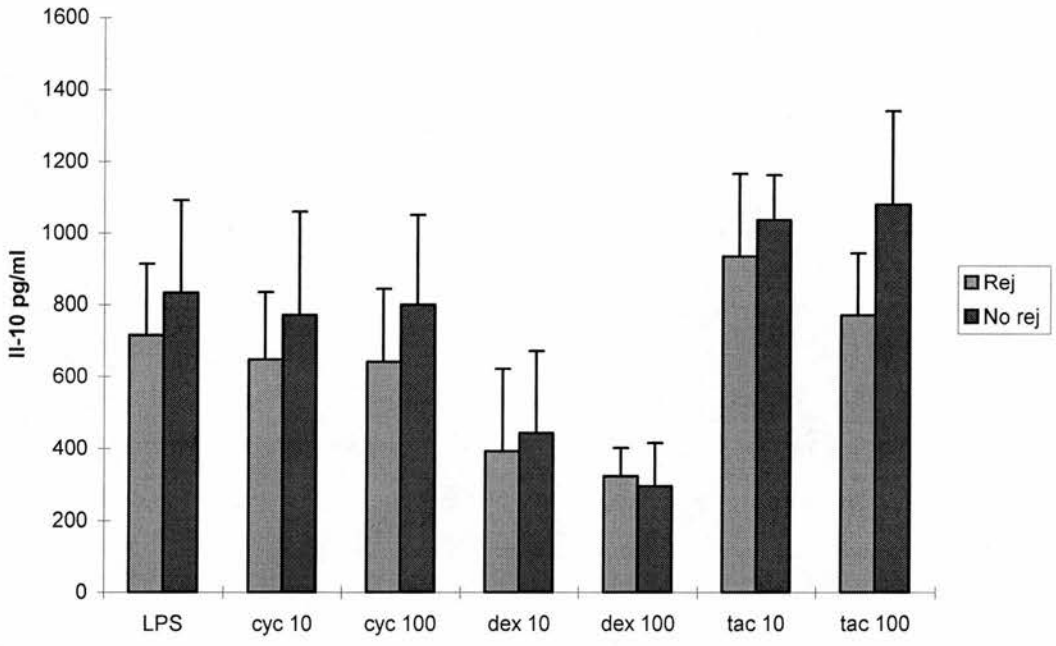
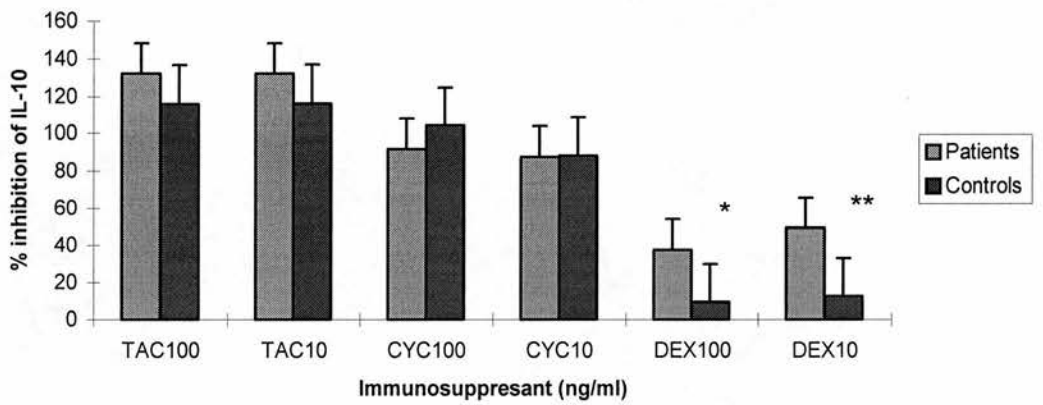
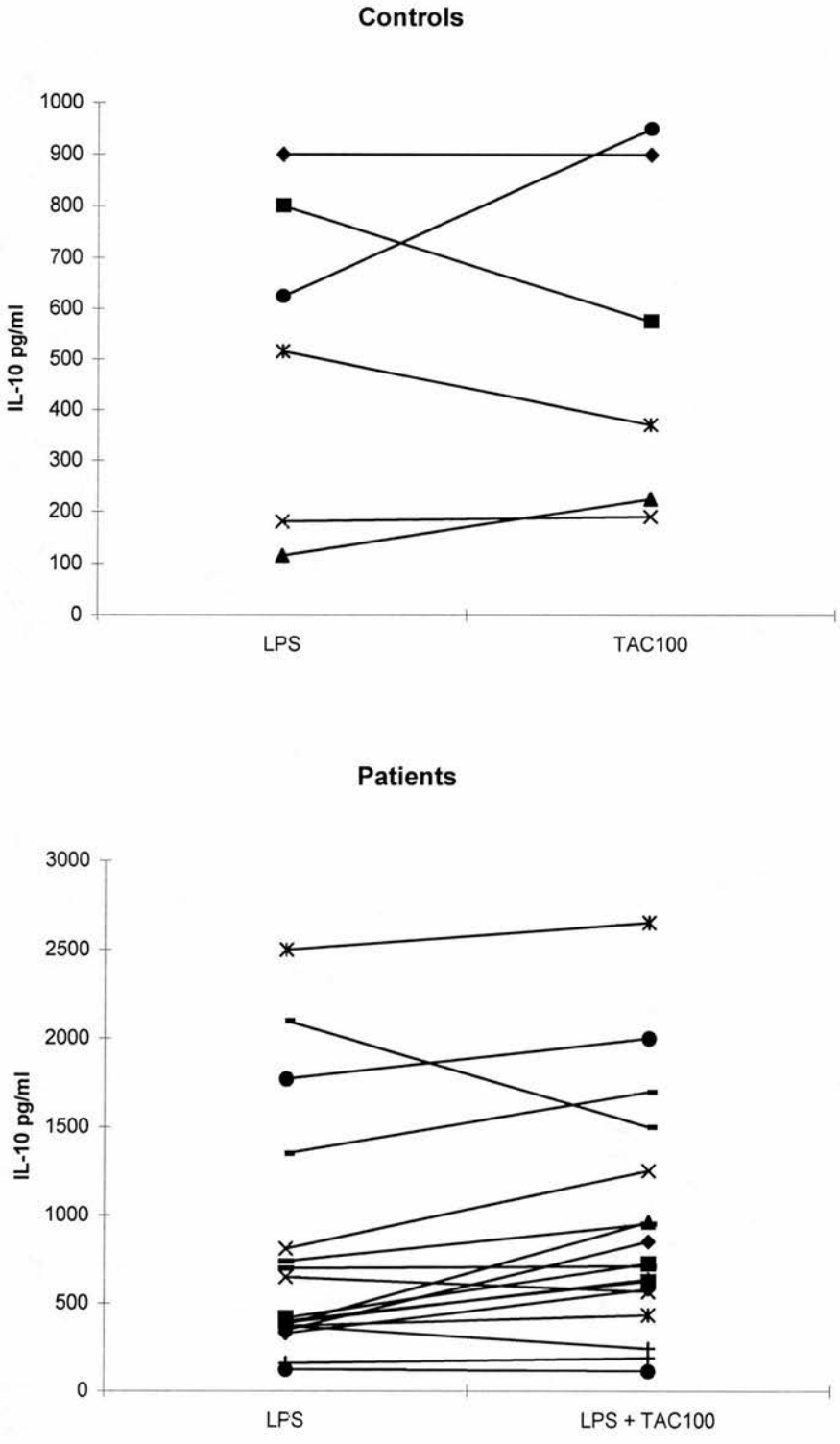


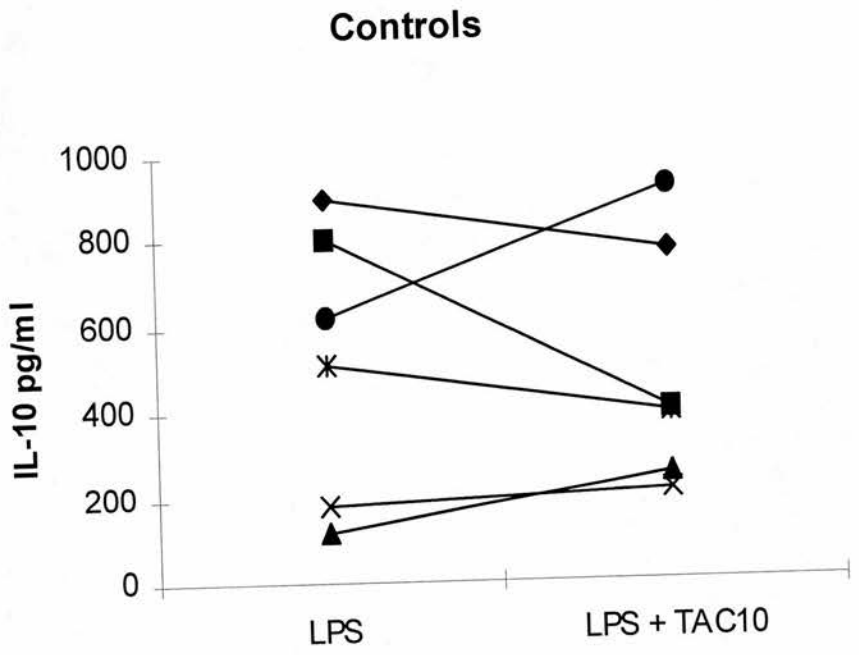
Figure 5.9. Percentage inhibition of IL-10 vs LPS alone with different immunosuppressants in patients (n=18) and controls (n=6). (mean,SEM)



* p=0.02, **p=0.01

Figure 5.10. Production of IL-10 following stimulation with LPS by patients and controls with and without pre-incubation with tacrolimus 100ng and 10ng.




Controls
1000
800
600
400
200
0
IL-10 pg/ml
LPS
LPS + TAC10

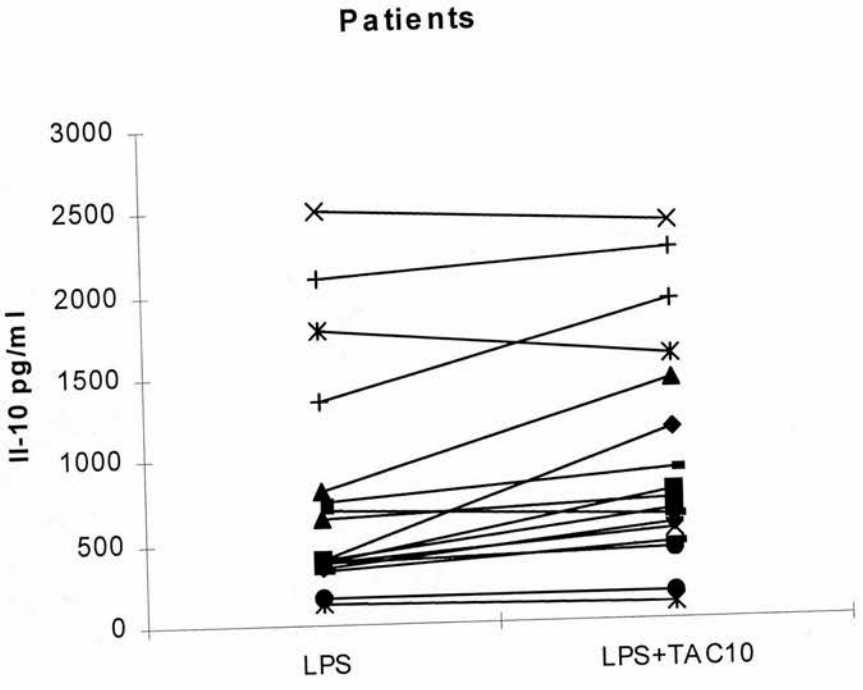
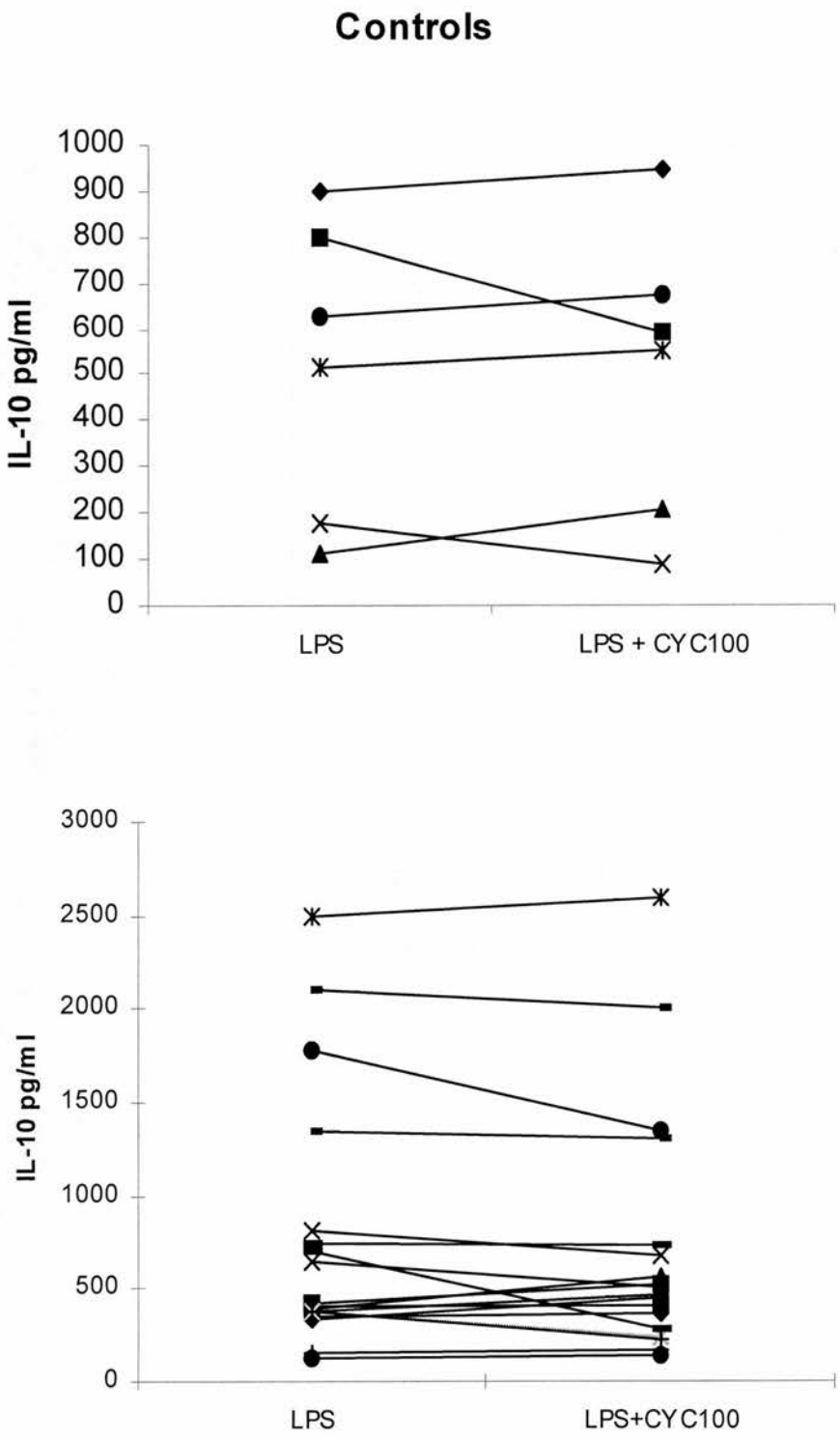
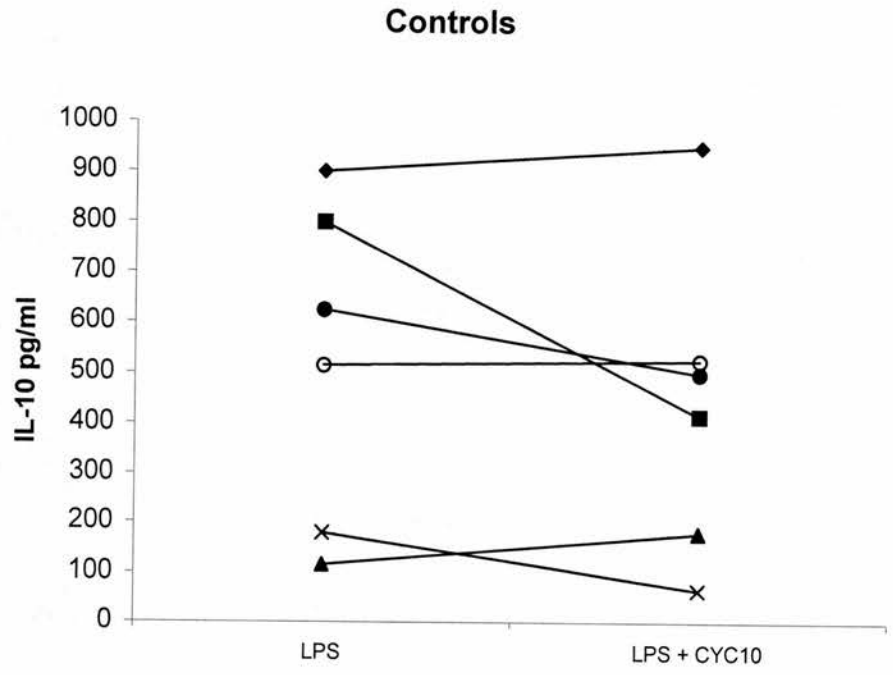

Patients
3000
2500
2000
1500
1000
500
0
Il-10 pg/ml
LPS
LPS+TAC10

Figure 5.11. Production of IL-10 following stimulation with LPS by patients and controls with and without pre-incubation with cyclosporin 100ng and 10ng.




Controls
1000
900
800
700
600
500
400
300
200
100
0
IL-10 pg/ml
LPS
LPS + CYC10

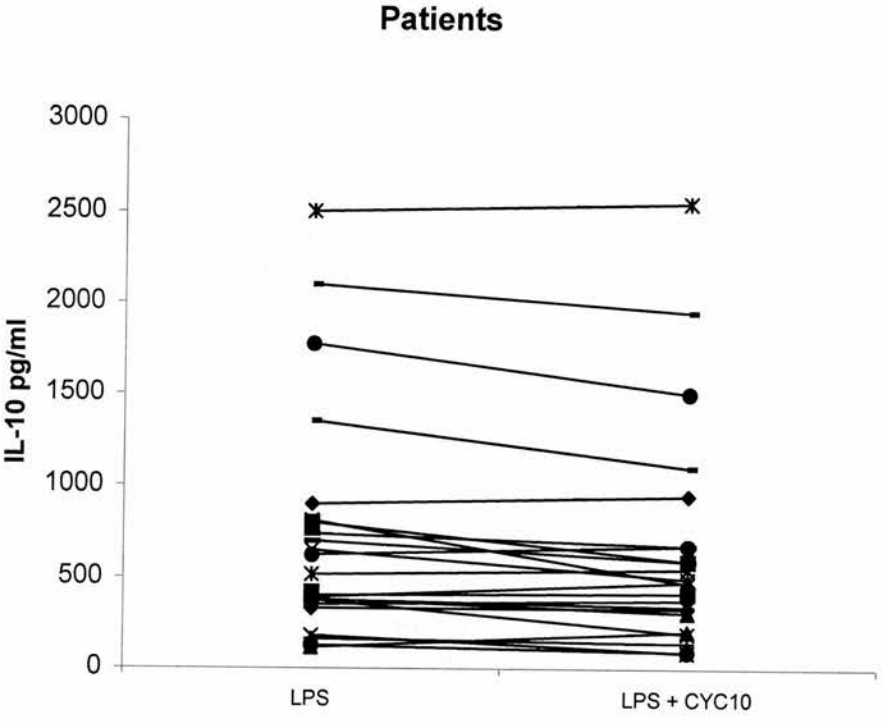
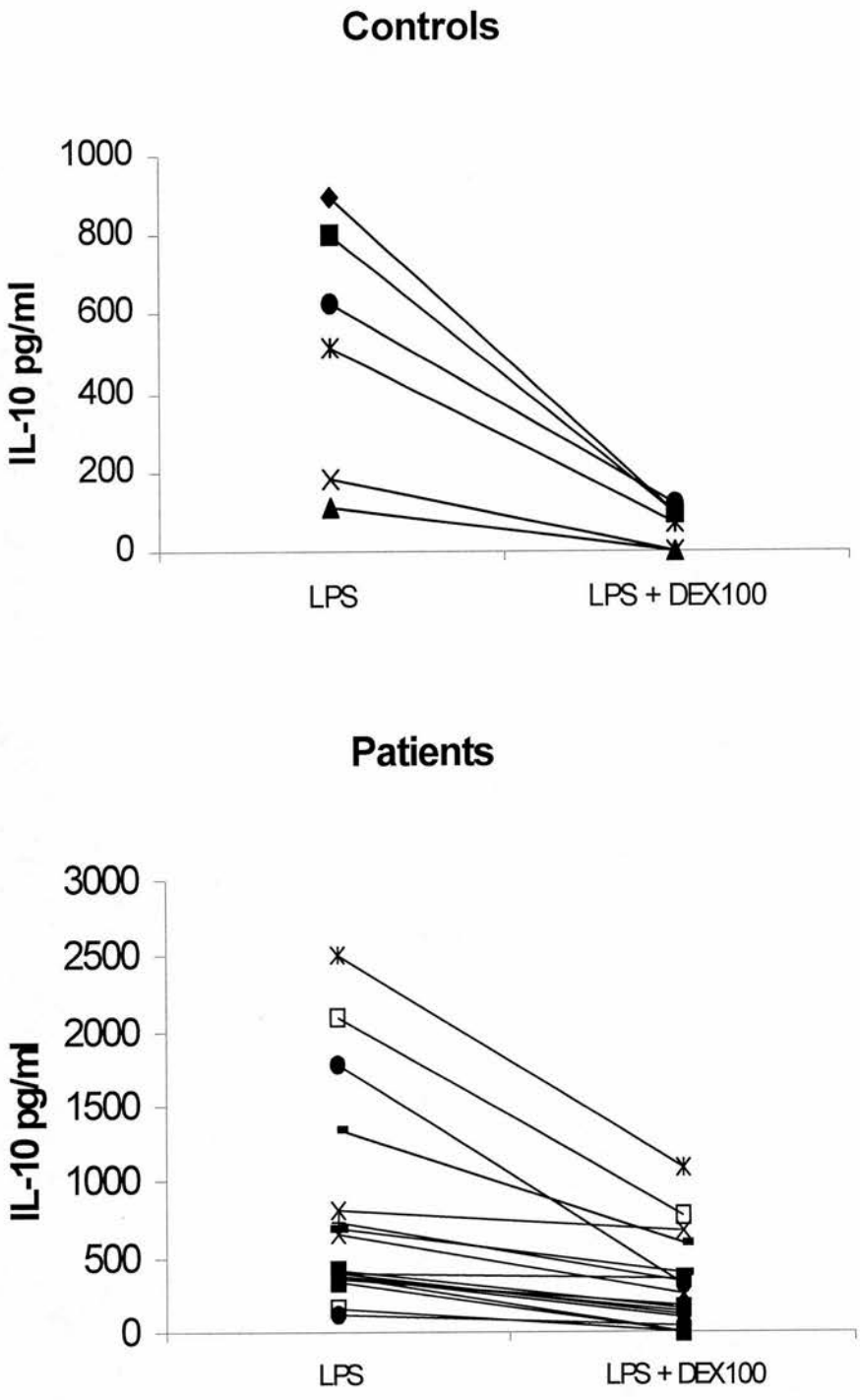
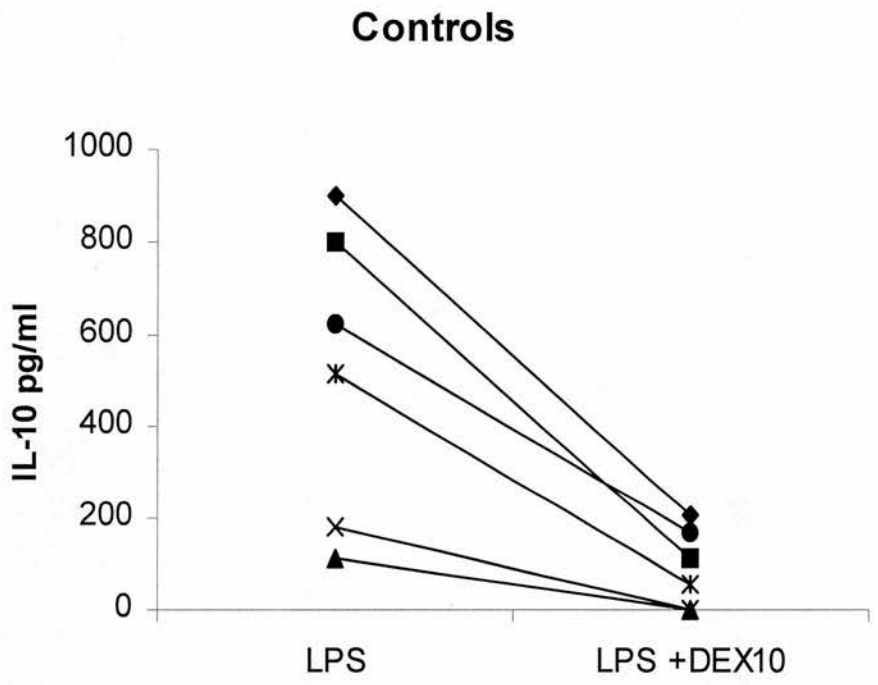

Patients
3000
2500
2000
1500
1000
500
0
IL-10 pg/ml
LPS
LPS + CYC10

Figure 5.12. Production of IL-10 following stimulation with LPS by patients and controls with and without pre-incubation with dexamethasone 100ng and 10ng.




Controls
1000
800
600
400
200
0
IL-10 pg/ml
LPS
LPS +DEX10

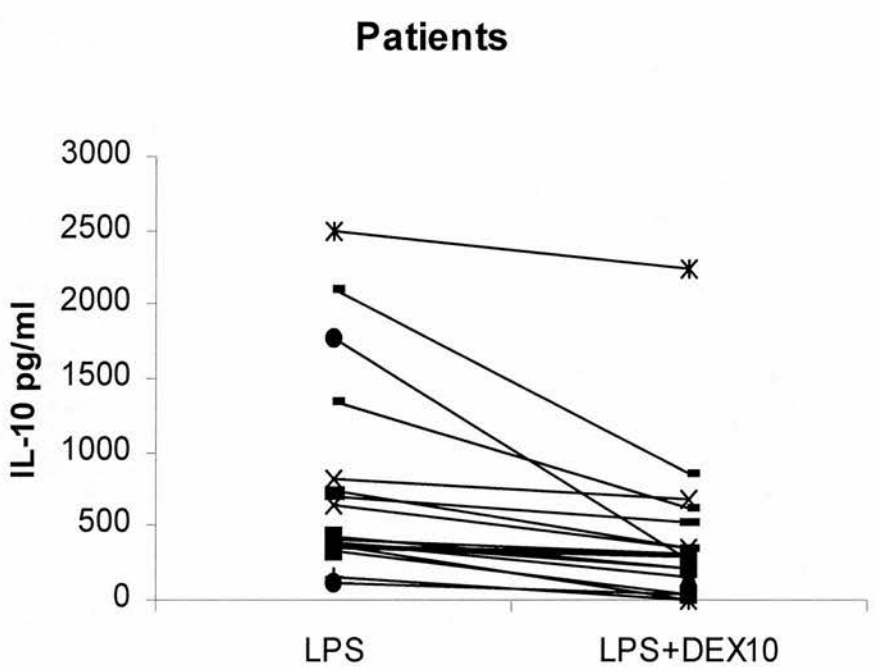
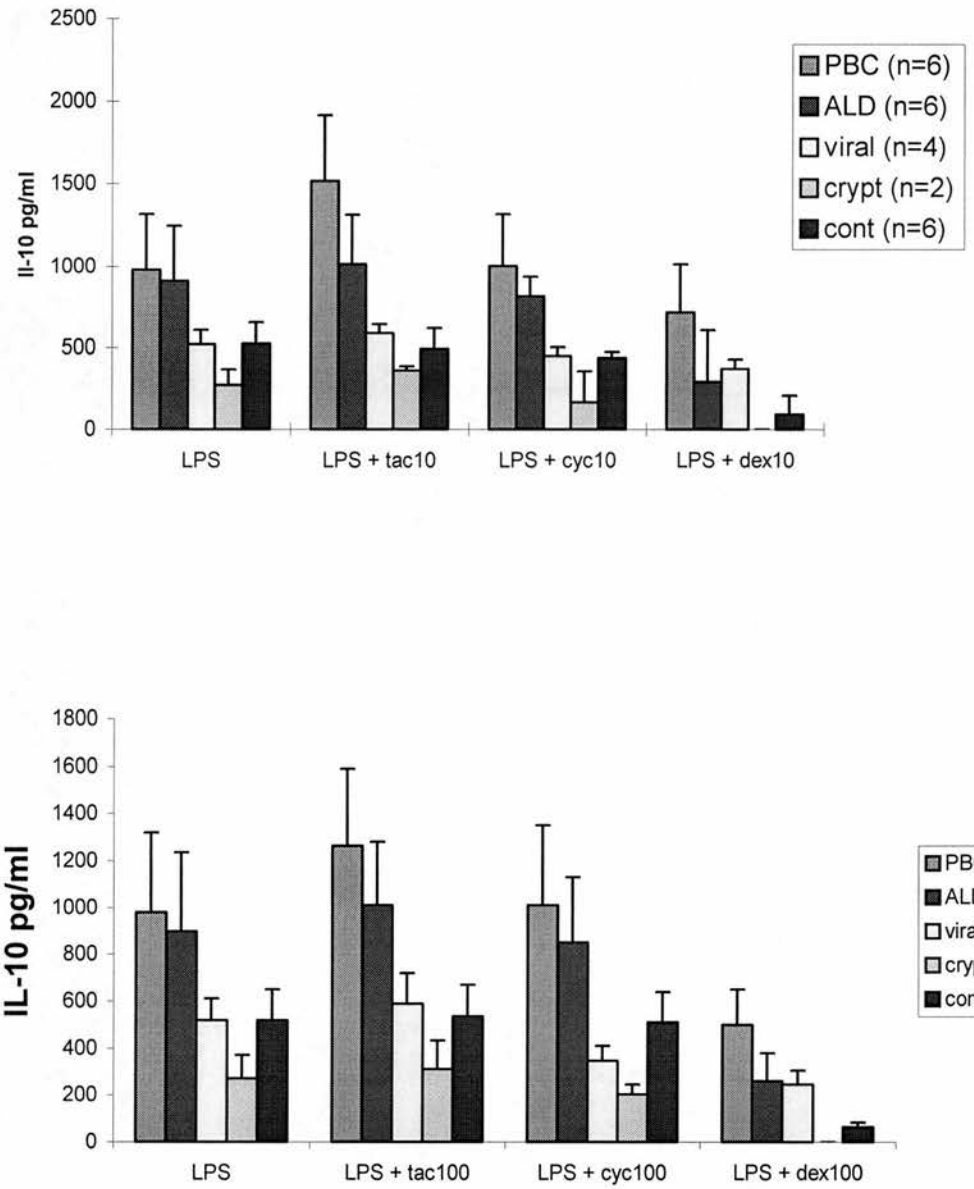

Patients
3000
2500
2000
1500
1000
500
0
IL-10 pg/ml
LPS
LPS+DEX10

Figure 5.13. Production of IL-10 in different aetiologies (PBC=primary biliary cirrhosis, ALD= alcoholic liver disease, crypt= cryptogenic, cont=controls)



3.3 CYTOKINE POLYMORPHISMS

The median values of TNF-α and IL-10 produced are shown in tables 5.4 and 5.5. There was no significant difference between the different polymorphism and production of the respective cytokine.

Table 5.4. The production of TNF–α (pg/ml) following stimulation by LPS in patients with liver disease according to polymorphism at position –308.

	Median (range) TNF-α production (pg/ml)
TNF-α -308 G/G (n=11)	1280 (150-2500)
TNF-α -308 G/A (n=6)	1220 (720-1930)
TNF-α -308 A/A (n=1)	850

Table 5.5. The production of IL-10 (pg/ml) following stimulation by LPS in patients with liver disease according to polymorphism at position –1052.

	Median (range) IL-10 production (pg/ml)
IL-10 –1082 A/A (n=7)	700 (375-2500)
IL-10 –1082 A/G (n=6)	525 (125-1775)
IL-10 -1082 G/G (n=2)	1225 (350-2100)

4.DISCUSSION

This study demonstrates that the production TNF-α pre-transplant from peripheral blood mononuclear cells stimulated by LPS is higher in patients who go on to develop acute cellular rejection. It also demonstrates a difference in response to tacrolimus and cyclosporin between patients and controls, and a difference between the immunosuppressants themselves.

The cytokine levels within hepatic allografts suffering acute rejection show differences from those grafts not undergoing rejection. TNF-α, IL-5 and IL-6 (Imagawa et al., 1990; Gorczynski et al., 1996;Martinez et al., 1992) have been shown to be increased whereas IL-10 expression was unchanged. The principal source of TNF-α is the monocyte/macrophage although many other cells produce this cytokine. In a study of human liver allograft rejection elevation of plasma TNF-α levels was seen concomitantly with large numbers of TNF-α producing monocytes within the graft (Hoffmann et al., 1993). There is also evidence to suggest that the principal source of TNF-α in acute rejection is recipient monocyte-macrophage cells (Teramoto et al., 1999). The effect of TNFα in the initiation of the rejection process presumably by activation of endothelial cells and upregulation of the expression of adhesion factors is further suggested by the improvement of survival seen in models where monoclonal antibody against TNF-α is administered post transplant (Imagawa et al., 1991) The finding that TNF-α production pre-transplant is increased in patients who then develop acute rejection is interesting. The donor liver results in an alloimmune response the magnitude of which results in either significant or insignificant acute rejection. TNF-α is involved in this immune/inflammatory reaction

and these results suggest that the pre-transplant production capabilities of the recipients may reflect the propensity for inflammation within the graft.

Interleukin-10 however has more of an immunomodulatory function and studies in liver transplantation have indicated a reduction or no change in the intragraft levels at times of acute rejection (Bishop et al., 1993;Conti et al., 1999). In other solid organ transplants IL-10 may well exacerbate rejection (Xu et al.,1995) and anti-IL-10 monoclonal antibodies have been shown to improve survival of heart transplants in animal models (Li et al.,1998).

The influence of pre-transplant cytokine levels on rejection has been investigated in renal transplantation with mean levels of γ-interferon increased in the group going on to develop acute rejection (Kaminski et al., 1995). TNF–α however was not measured in this study. This study suggests that production of TNF-α below 1260 pg/ml was good at predicting patients who were less likely to develop rejection requiring treatment.

There was no difference in TNF-α production between the aetiologies, although our numbers were small. There are reports in the literature suggesting a difference in TNF-α production between different aetiologies of liver disease (Muller at al., 1990; Broome et al., 1992). There is some evidence that as well as the underlying liver disease affecting TNF-α production there is individual variation in production determined by genotype (Louis et al., 1998). There are reports in the transplantation of various solid organs that TNF-α genotype may influence acute rejection with the polymorphism associated with increased production increased in the rejection groups

(Turner et al., 1997; Sankaran et al., 1997). No reports of TNF-α production and polymorphisms of the gene in liver disease have been published although there are data suggesting an increase in the TNF-2 polymorphism in primary sclerosing cholangitis (Bernal et al., 1999) and autoimmune hepatitis (Cookson et al.,1999).

In chapter 4 of this thesis data is presented showing an increase in acute rejection in patients who are homozygous for the TNF2 allele. This allele has been shown to lead to an increase in TNF-α production *in vitro* in healthy controls. The data regarding TNF-α production in this study did not show any correlation with genotype although only a single patient was homozygous for TNF2 and the total number of patients was small.

Long term hepatic allograft survival has been greatly enhanced by effective immunosuppressants such as cyclosporin and tacrolimus. These compounds are structurally quite different and bind to separate intracellular receptors- cyclosporin to cyclophilin and tacrolimus to FK506-binding protein 12 . Their major immunosuppressive effects appear to be through the inhibition of calcineurin, a phosphatase, by the respective drug-immunophilin complex. There does appear to be some difference in the effects of these drugs *in vitro* depending on the cell type used and the inductor. In the case of cytokine production there are reports of enhanced production of IL-6 (Murayama et al., 1994), while many other cytokines are inhibited e.g. IL-10, IL-2, IL-4 and IL-8 (Ruhlmann and Nordheim, 1997).

Tumour necrosis factor alpha production is inhibited *in-vitro* by both cyclosporin and tacrolimus in cells from healthy individuals although the mode of activation has been

shown to influence inhibition. Monocytes in one study were not inhibited by cyclosporin or tacrolimus when activated by LPS (Andersson et al., 1992), although this has not been a universal finding (Murayama et al., 1994). The inhibition of TNF-α production by tacrolimus and cyclosporin is partly through the inhibition of calcineurin (Staruch et al., 1998). Our results suggest that patients with liver disease do not reduce production of TNF-α from PBMC following pre-incubation with calcineurin inhibitors whereas control patients do. It has been shown that PBMC from patients with cirrhosis after resting *in vitro* for 24 hours produce TNF-α levels similar to healthy controls (Devière et al., 1990). It may be that this primed state in cirrhotics overcomes the proposed mechanism of inhibition by calcinuerin inhibitors. The inhibition of TNFα production by dexamethasone is likely to be a different mechanism unaffected by liver disease. Other studies have shown corticosteroids to be inhibitors of other cytokines with no effect from either cyclosporin or tacrolimus (van Asmuth et al., 1994).

The differential effect of tacrolimus on the production of IL-10 in patients compared with normal controls and with the other immunosuppresants is interesting. The studies on the effect on immunosuppressants on interleukin-10 production are conflicting. In most studies (Blancho et al., 1995; Naora et al., 1995), as in ours, the production is unchanged although one study did suggest an upregulation (Blancho et al., 1997). The literature on tacrolimus is similarly conflicting with reports of no effect (Wang et al., 1993) and an increase in production (Woo et al., 1995). The mechanisms that control and regulate IL-10 production are still unclear. There are studies reporting the upregulation of IL-10 production with substance P, IFN-α and IFN-β (Ho et al., 1996; Schandene et al., 1996; Porrini et al., 1995;) It is of interest that the mouse

IL-10 gene has shown regulatory motifs in the enhancer region similar to that in the Il-6 enhancer as tacrolimus has been shown to enhance the IL-6 production in human monocytes. Interleukin-10 has been shown to suppress inflammatory cytokine production in humans (de Waal et al., 1991). It also downregulates MHC class II molecule production in monocytes therefore inhibiting proliferative T cell and cytotoxic T cell responses (Bejerano et al., 1992). It is possible that the effect of tacrolimus on interlukin-10 production may contribute to the difference in immunosuppressive capabilities between tacrolimus and cyclosporin.

5.CONCLUSIONS

The pre-transplant level of the pro-inflammatory cytokine TNF-α is increased in the group of patients who develop acute cellular rejection post-transplant, suggesting that a patient's "inflammatory" potential may have some influence on their response to the allograft.

There is enhanced production of interleukin-10 by peripheral blood mononuclear cells from patients with liver disease in the presence of tacrolimus and a lack of inhibition of TNF-α production by cyclosporin and tacrolimus.

CHAPTER SIX

CONTACT SENSITISATION PRE-TRANSPLANT AS A PREDICTOR OF ACUTE REJECTION

1.INTRODUCTION

2.MATERIALS AND METHODS

- 2.1 Sensitisation Agent
- 2.2 Patients
- 2.3 Cytokine Polymorphisms
- 2.4 Follow up and Statistics

3. RESULTS

- 3.1 Skin tests
- 3.2 Correlation with Rejection
- 3.3 Cytokine Polymorphisms

4.DISCUSSION

5.CONCLUSIONS

1.INTRODUCTION

A recent report of pre-transplant parameters of patients which predict subsequent development of acute rejection identified recipient age, donor age, HLA-DR mismatch, serum creatinine, aetiology of liver disease and cold ischaemia time as risk factors (Wiesner at al.1998). Data presented earlier in this thesis and recently published largely agreed with these findings (Bathgate et al., 1999). The acute rejection of hepatic allografts is principally a T cell mediated response with a characteristic histological appearance of bile ductulitis, venous endotheliitis and portal tract inflammation (Snover et al., 1984). The histological severity of acute rejection influences the decision to treat as graft outcome is worse following severe rejection (Wiesner et al., 1998).

Contact hypersensitivity reactions are T cell mediated responses that are diminished in liver disease (Pirisi et al, 1997). The aim of this study was to investigate the relationship between contact sensitisation to a neo-antigen pre-transplant in patients with chronic liver disease and acute cellular rejection post-transplant.

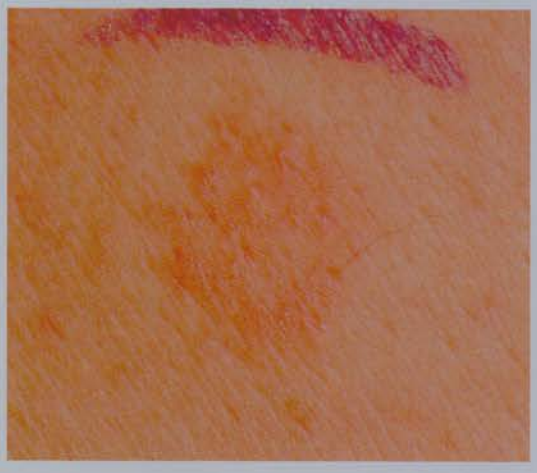
2.MATERIALS AND METHODS

2.1 SENSITISATION AGENT

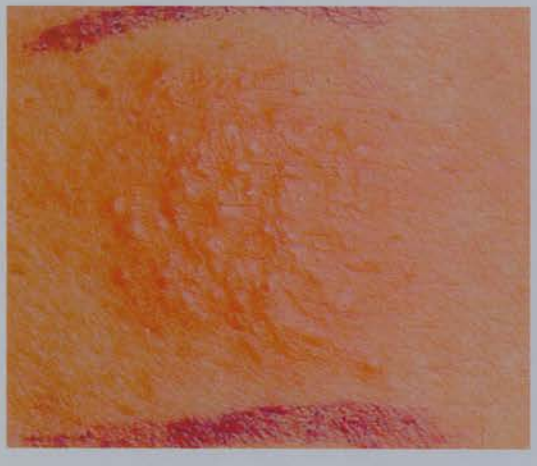
Diphenylcyclopropenone is an agent used by dermatologists for the treatment of alopecia areata. Preparation of the diphenylcyclopropenone was carried out by the hospital pharmacy using acetone as the solvent. The solutions were kept at 4° C with a shelf life of 4 weeks.

2.2 PATIENTS

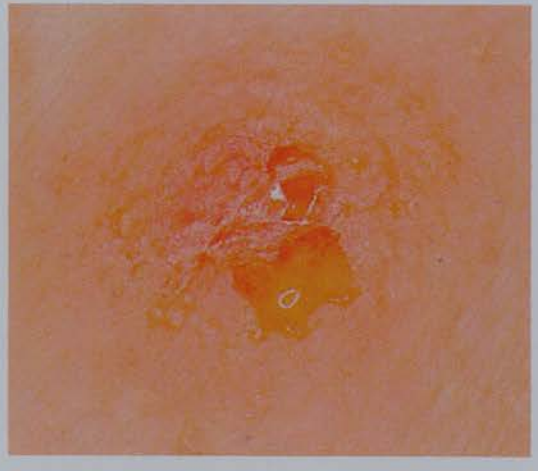
Consecutive patients with chronic disease listed for orthotopic liver transplantation between February 1997 and August 1998 were sensitised with 100µl of 0.1% diphenylcyclopropenone (University of Nijmengen, Netherlands) applied on a filter paper under a 2cm Finn chamber (Epitest Ltd Oy, Tuusula, Finland) to the upper arm for 48 hours. An elicitation test was applied to the opposite forearm 12 days later with 15µl of diphenylcyclopropenone at concentrations of 0.001%,0.0025%,0.005%, 0.01% and 0.025% placed on 8mm filter paper discs on a strip of Finn chambers (Epitest Ltd Oy, Tuusula, Finland).The elicitation reaction was recorded by a single observer to reduce any variation. Each concentration was given a score - erythema and induration = 1, vesicles = 2, bulla = 3. Figure 6.1 indicates the reactions seen. The scores for each concentration were summated giving a total score out of 15. The elicitation reaction of a patient is shown in figure 6.2 .



Positive reaction
score = 1



Positive reaction
score = 2



Positive reaction
score = 3

Figure 6.1. Elicitation reactions.

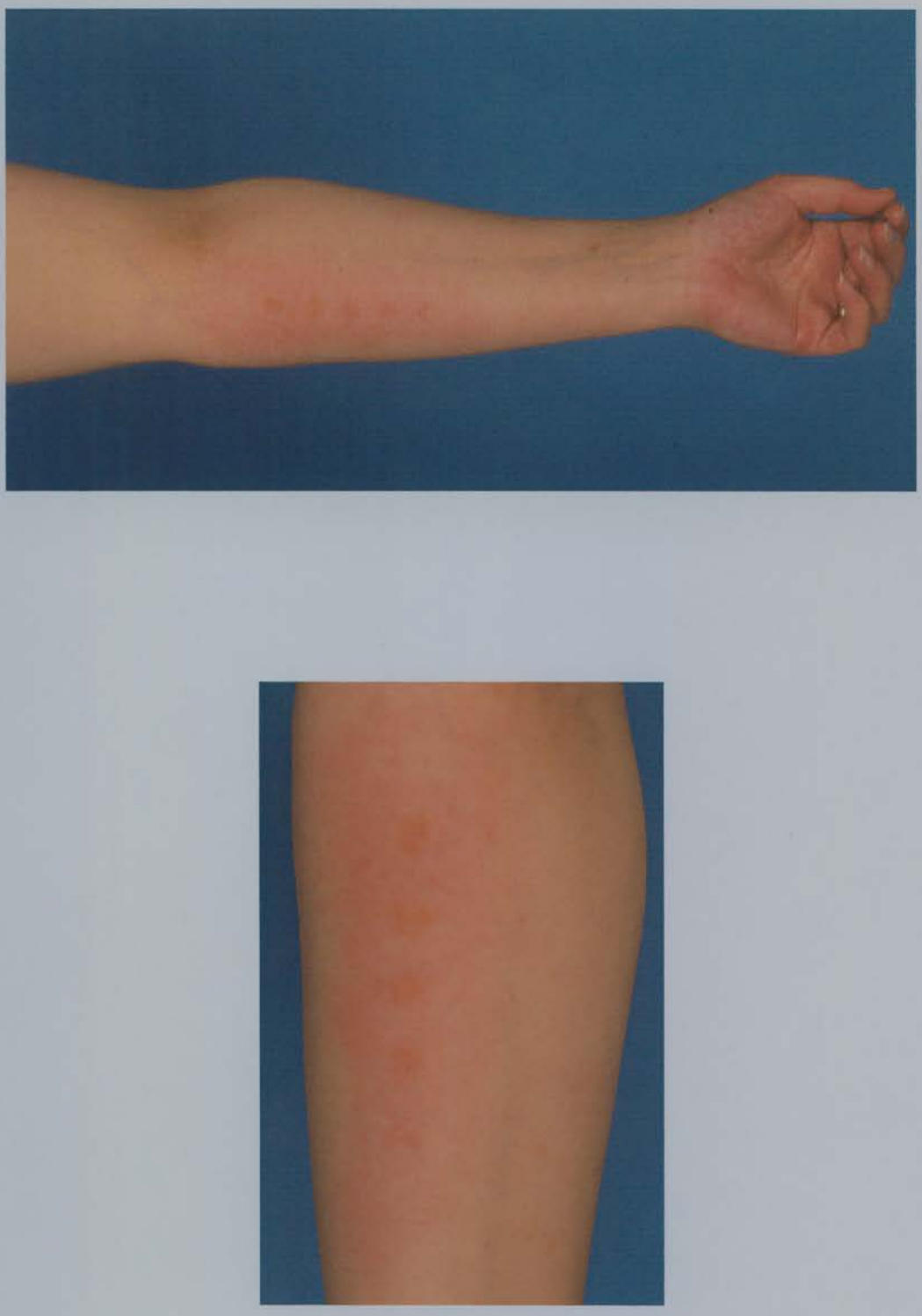


Figure 6.2. An elicitation reaction in a patient.

The doses of diphenylcyclopropenone used were established by sensitising healthy controls with varying concentrations. The final test doses as used above all elicited skin test scores between 8 and 13 in healthy controls (n=5) with a median age of 35 (range, 32-42).

All transplants were carried out at the Scottish Liver Transplant Unit between April 1997 and December 1998. The immunosuppresion regimen was triple therapy with either microemulsion cyclosporin 10mg/kg/d or tacrolimus 0.1mg/kg/d (target trough levels were 175-200ng/l for cyclosporin and 10-15 ng/l for tacrolimus,azathioprine (2mg/kg) and prednisolone 20mg/d. Tacrolimus or cyclosporin (Neoral) administration was randomly assigned to patients.

Acute rejection was defined as rejection requiring treatment with high dose corticosteroids. This decision was based on clinical and histological evidence, and was made by the clinicians on the unit at the time. These clinicians were blinded to the skin test score. Protocol biopsies were performed at seven days and further biopsies performed if liver function tests deteriorated or were slow to improve after rejection therapy. All biopsies in the first thirty days following transplantation were assessed. The histologic severity of acute rejection was graded according to the Banff criteria (Demetris et al., 1997). When more than one allograft biopsy was performed the most severe degree of rejection was used in analyses.

Details of the donor age and cold ischaemia time were collected. Recipient age, aetiology, severity of liver disease and nutritional status (anthropometry assessed by a single dietician) was noted pre-transplant. Lymphocytotoxicity testing and donor recipient HLA DR mismatching was performed using standard techniques by the tissue typing laboratory in our institution. Calcineurin inhibitor levels were collected for each patient.

Chronic rejection was diagnosed using standard histologic criteria after appropraite clinical/radiologic exclusions (International panel, 2000). Graft loss was defined as patient death or re-transplantation.

2.3 CYTOKINE POLYMORPHISMS

Cytokine polymorphisms were analysed as described in Chapter 4.

2.4 FOLLOW UP AND STATISTICS

All patients were followed up for at least one year. Univariate analysis was assessed by the Students t-test for the difference between means for continuous variables and Chi square testing with Fisher's exact test where appropriate for categorical variables. Logistic regression for univariate predictors was carried out using SPSS statistical package (version 9.0, SPSS Inc, Chicago,US)

3.RESULTS

3.1 SKIN TESTS

Forty one patients were sensitised and had elicitation tests performed. Three patients were transplanted before the elicitation phase could be completed. The age and nutritional status of the patients completing testing is shown in table 6.1. Nineteen patients were responders having a skin test score varying from 1-9. Twenty two patients had no response. The variation in skin test response in the different aetiologies is shown in table 6.2.

3.2 CORRELATION WITH REJECTION

Three patients, all non-responders, did not survive to transplantation. 38 patients were transplanted with the mean length of time from elicitation to transplantation being 58 (SEM 11) days.

Thirty eight biopsies in 31 patients were assessed. Seven patients did not have biopsies at seven days at the discretion of the clinical team at the time. None of these 7 patients required allograft biopsy in the first 30 days and were regarded as negative for acute rejection.

Acute rejection requiring treatment occurred in 19 (50%) of 38 patients. Five patients, all responders, required multiple biopsies (2-4). Three of the 5 had additional therapy for acute rejection. Table 6.3 shows the occurrence of acute rejection according to

skin test response, donor and recipient age, cold ischaemia time , HLA DR mismatch, lymphocytotoxic crossmatch, immunosuppression levels, nutritional status and severity of liver disease. No difference in rejection was seen according to aetiology of liver disease. Median values for age and donor age were used as cut-off values.

On univariate analysis skin test reponse ($p<0.001$), donor age ($p=0.05$), recipient age ($p=0.05$) and primary immunosuppression ($p=0.003$) were significantly associated with acute rejection. On multivariate analysis skin test response was the only independent factor associated with rejection ($p=0.02$).

Table 6.4 shows the severity of rejection in the different skin test scores using the original histological assessments which were performed by one of three pathologists. A fourth pathologist re-scored all the biopsies in order to eliminate inter-observer variation in scoring. These results are shown in table 6.5. There was no increase in the score of severity of rejection as assessed by the single pathologist but 6 biopsies were scored as mild compared with moderate and 6 biopsies were scored as having no rejection compared with mild rejection.

All patients who required more than a single biopsy in the first 30 days had a skin test score greater than 1(Table 6.6).

Two patients died within the first 30 days from sepsis and multi organ failure, both were non-responders. One graft was lost to chronic rejection at 8 weeks (skin test score 9) and one was lost to hepatic artery thrombosis at 6 weeks (non-responder). All other grafts were functioning well at 12 months.

3.3 CYTOKINE POLYMORPHISMS

Table 6.7 shows the cytokine genotype polymorphisms of the patients who responded and those who had no response. There was no significant difference for any of the polymorphisms investigated between responders and non-responders.

Table 6.8 shows the cytokine genotype polymorphisms in those with skin tests greater than or less than or equal to one. There were no significant differences between the two groups for any of the polymorphisms investigated.

Table 6.1. Contact sensitisation response according to age aetiology and mid-arm muscle circumference (n=41)

	Non-responders	Responder	Significance
Age (mean, SEM)	55.8 (7.6)	46.6(8.9)	P= 0.001
Childs class- A B C	1 5 16	1 11 7	P = N.S.
MAMC – normal marginal deplete	5 5 12	7 2 9	P=N.S.

MAMC = midarm muscle circumference. N.S. = non significant.

Table 6.2. Skin test score according to aetiology (n=41)

	No response	Test score 1-3	Test score 4-6	Test score 7-9
Primary biliary cirrhosis (n=17)	9	4	3	1
Alcoholic liver disease (n=10)	7	3	0	0
Primary sclerosing cholangitis (n=5)	2	0	1	2
Chronic viral hepatitis (n=4)	0	2	2	0
Autoimmune hepatitis (n=2)	2	0	0	0
Cryptogenic cirrhosis (n=3)	2	1	0	0

Table 6.3. The occurrence of acute rejection. (n=38)

	No rejection	Acute rejection	Significance
Elicitation response-			
no	18	1	p< 0.001
yes	5	14	
Age < 53	8	10	p= 0.05
≥ 53	15	5	
Donor age < 45	8	10	p= 0.05
≥ 45	15	5	
DR mismatch- 0	1	0	p= n.s
1	12	6	
2	7	6	
Crossmatch - negative	16	13	p= n.s
positive	4	2	
Midarm muscle circumference-			
normal	6	6	p= n.s.
marginal	4	2	
deplete	11	7	
Immunosuppression -			
tacrolimus	16	3	p= 0.003
cyclosporin	7	12	
Mean level first 7days			
tacrolimus (95% C.I.)	9.5(7.8-11)	13 (4-21)	p = n.s.
cyclosporin (95% C.I.)	185 (142-228)	218(190-245)	
Child-Pugh class - A	1	1	p= 0.03
Child –Pugh class-B	6	10	
Child-Pugh class - C	16	4	

Significance test = χ^2 test.

Table 6.4. Skin test and histological severity of rejection (n=31)

Skin test score	No rejection	Mild rejection	Mod. Rejection	Sev. rejection
0	3	9	0	0
1-2	0	2	5	1
3-4	0	0	4	1
5-6	0	0	3	0
7-8	0	0	1	2

Table 6.5. Skin test score and histological severity of rejection as scored by a single pathologist.(n=31)

Skin test score	No rejection	Mild rejection	Mod rejection	Sev. Rejection
0	7	5	0	0
1-2	2	3	2	1
3-4	0	2	2	1
5-6	0	0	3	0
7-8	0	0	1	2

Table 6.6. Severity of rejection in responders and non-responders ($p<0.001$).

	No rej/mild rejection	Mod/severe rejection
Non -responder	12	0
Responder	7	12

Table 6.7. Contingency table for repeat biopsy (p=0.03, Fisher's exact test).

	< 2 biopsies	2 or more biopsies
Skin test ≤1	24	0
Skin test > 1	9	5

Table 6.8. Cytokine polymorphisms in responders and non-responders.

TNF-α -308 genotype	G/G	A/G	A/A
No response	11	6	1
Response	12	2	3

IL-10 –1082 genotype	A/A	A/G	G/G
No response	7	8	3
Response	5	9	4

	$TGF\beta_1$+869 genotype			$TGF\beta_1$ +915 genotype		
	C/C	C/T	T/T	C/C	C/G	G/G
No response	3	7	8	1	2	15
Response	5	10	2	1	0	16

Table 6.9. Cytokine polymorphisms and skin test scores greater than one.

TNF-α -308 genotype	G/G	A/G	A/A
Skin test score ≤1	15	6	2
Skin test score >1	6	2	2

IL-10 –1082 genotype	A/A	A/G	G/G
Skin test score ≤1	9	10	4
Skin test score >1	3	7	3

	$TGF\beta_1$ +869 genotype			$TGF\beta_1$ +915 genotype		
	C/C	C/T	T/T	C/C	C/G	G/G
Skin test score ≤1	5	9	9	1	2	20
Skin test score >1	3	8	1	1	0	11

4.DISCUSSION

This study demonstrates that the inability to mount an immune response to a contact neo-antigen predicts those patients who are less likely to develop acute cellular rejection. It also demonstrates a relationship between magnitude of reaction to the contact antigen and severity of acute rejection.

The occurrence of acute cellular rejection, unless it is severe, does not detrimentally affect graft outcome in patients without hepatitis C infection. There is also evidence to suggest that patients with a single episode of acute rejection have in fact an improved outcome (Wiesner et al.,1998; Avolio et al, 1998). It may therefore be possible to reduce immunosuppression in patients with a decreased risk of acute rejection in the knowledge that should they develop acute rejection as a consequence there is no evidence to suggest that they will detrimentally affect graft outcome.

Contact sensitisation to a neo-antigen has been investigated in the past in renal transplantation, with responders being at an increased risk of losing their graft in the first year (Watson et al., 1980). As already indicated, acute rejection does not have the same detrimental effect on graft outcome in liver transplantation but the findings of this study could allow a reduction in immunosuppression in the non-responders.

Skin testing in liver disease has been investigated in the past with an impairment of contact sensitisation found in primary biliary cirrhosis (Sherlock et al.,1969). Perhaps surprisingly, this did not appear to be related to bilirubin or histological stage.

Alcoholic liver disease (Snyder et al., 1978) has also been shown to impair contact sensitisation, as has fulminant liver failure (O'Keefe et al.,1980).

The effect of nutrition on contact sensitisation is not clearly established. Studies investigating sensitisation with dinitrochlorobenzene (DNCB) have shown no attenuation of responses with lesser degrees of malnutrition (Harrison et al, 1975). In our study there was no significant difference between patients with protein malnutrition as measured by mid-arm muscle circumference and those with normal nutrition in their sensitisation. Initially investigators felt that skin testing in hospitalised patients would help identify patients who may well respond to intervention, but no study has shown any improvement in outcome following dietary intervention (Twomey et al., 1982). Skin testing before major surgery has been shown to predict mortality and sepsis (Meakins et al., 1977; Johnson et al., 1979), although this has not been every centre's experience (Brown et al., 1982).

The contact sensitisation reaction at a cellular level has not been fully established in humans. However murine experiments have shown this to be complex, involving three different subsets of T cells, of which CD4 cells are the effectors (Salerno et al.,1998). The mechanism of acute rejection is principally the direct recognition of donor MHC on donor antigen presenting cells by host T cells (Orosz et al.). Studies investigating the T cells present within the portal tracts in acute cellular rejection in humans have shown variable results with some investigators finding a predominance of $CD8^+$ T cells (Ibrahim et al., 1993; Wong et al., 1998) while others have found a predominance of $CD4^+$ T cells (Dollinger et al., 1998).

The ability of a contact sensitisation reaction to predict those patients less likely to develop acute early rejection post-transplantation is interesting. The pre-transplant parameters investigated that affect post transplant rejection have been age, aetiology of liver disease, preoperative creatinine, severity of liver disease and nutritional status (Adams et al., 1993;Farges et al., 1996; Wiesner et al., 1997; Wiesner et al., 1998; Bathgate et al., 1999). There have been some reports suggesting that cold-ischaemia time and donor age may also influence acute rejection, although this is not every centre's experience (Shackleton et al.,1995). All these parameters were investigated in our population, and none was found to be as useful as skin testing in predicting those less likely to suffer acute rejection. The threshold for treatment varies from unit to unit, with mild acute rejection often not being treated (Dousset et al., 1993). In patients without a response to diphenylcyclopropenone, the most severe rejection seen was mild rejection suggesting there is scope to diminish immunosuppression in these patients.

The finding of increased severity of acute rejection in patients with high skin test scores is also interesting. There are reports suggesting that severe acute rejection does lead to a decrease in graft survival. The number of patients in this study is small but the one patient who developed chronic rejection had the highest skin test score. The only other patients who required additional therapy to a single course of methylprednisolone had skin test scores greater than one. The immunosuppressive agents used also affected acute rejection in our study. There is evidence to suggest that tacrolimus is more effective in preventing acute rejection from the two multi-centre trials (U.S multicenter FK506 liver study group, 1994; European multicentre

FK506 liver study group, 1994), although our study involved the new microemulsion preparation of cyclosporin of which there is less comparative data.

There are studies suggesting an increased suscepitibility to infection in patients with impaired responses to skin testing. Two of the three patients who died while on the waiting list died from sepsis and the two patients who died in the first two months also had infection and multi-organ failure. The potential benefit of reducing immunosuppression in patients with no response on skin testing may be to reduce the early problems associated with infection in the post-operative period. There is also evidence to suggest that the long term renal problems seen with cyclosporin are influenced by levels in the first 30 days, although this study investigated patients given the older preparation of cyclosporin.

The finding that there was no relationship between skin test response and cytokine genotyping is not too surprising. The cell mediated response required for contact sensitisation as indicated above is complex and will involve many different cytokines and mediators. It may be that other cytokines such as interferon gamma are important in this reaction although the literature on diphenylcyclopropenone in humans suggests that there is an increase in TNF-α (Hoffmann et al., 1995).

5.CONCLUSION

This study demonstrates that the inability to mount a response to a contact neo-antigen pre-transplant predicts a reduced likelihood of developing acute cellular rejection following transplantation. A relationship between skin test scores and severity of acute rejection was also found. These findings may provide a basis for individualising immunosuppressive regimens.

CHAPTER SEVEN

DISCUSSION

Orthotopic liver transplantation is now the established treatment of choice for end-stage chronic liver disease. The long-term outcome of patient and graft is excellent for solid organ transplantation and the loss of grafts to either acute or chronic rejection is now relatively rare. The present immunosuppressive regimens are very effective and one of the major advances for the future will be to minimise adverse effects of the present immunosuppressive agents and to consider new agents with less adverse effects.

Acute cellular rejection in orthotopic liver transplantation, in most cases, does not affect graft outcome in the long term and may in fact improve patient survival. The prevention of acute rejection is therefore not a major priority. However the avoidance of severe acute rejection and acute rejection in patients with hepatitis C infection is probably desirable given that these two groups appear to have a worse outcome (Wiesner et al.,1998). It is unknown if there is some long term immunological benefit from having an episode of acute rejection or whether allowing patients to develop acute rejection by reducing immunosuppression improves outcome.

The immunosuppression required to prevent acute rejection does have a downside both in the short and long term. Infection and renal impairment are frequent early postoperative complications exacerbated by immunosuppressive agents. There is evidence that the early postoperative levels of cyclosporin has consequences on late renal impairment (Fisher et al., 1997). In the longer term, the risk of the development of malignancy both lymphoproliferative and solid organ is also increased by the immunosuppressive regimens used. Other adverse effects that can seriously affect

quality of life and are relatively commonly experienced include diabetes mellitus, gingival hypertrophy, headache and hirsutism.

These factors have led to an interest in attempting to assess pre-transplant an individual's propensity for developing acute rejection. The largest study in the literature is by Wiesner et al. (1998) who found recipient age, preoperative creatinine, donor age, cold ischaemia time and HLA-DR mismatch to be associated with acute rejection. Other earlier studies had shown aetiology to have some relevance with patients with alcoholic liver disease and Hepatitis B experiencing less episodes of rejection (Farges et al., 1996; Berlakovich et al., 1996; Adams et al., 1991).

As the majority of chapters included in this thesis involve the prediction of acute rejection it must be emphasised that acute rejection in a clinical setting is difficult to define. For the sake of simplicity throughout the thesis acute rejection requiring treatment with high dose steroids has been used. This is not the same as histological acute rejection, as the vast majority of protocol biopsies would show evidence of some degree of acute rejection. The decision to treat was therefore taken by the clinical team looking after the transplant unit, which changes weekly and all clinicians do not have the same threshold for treating acute rejection. The definition of acute rejection therefore represented everyday practice but it may not reflect the severity of acute rejection precisely. These issues were addressed in chapters 2 and 7 where histologic severity was included and revealed that no patients with mild acute rejection received therapy but not all patients with moderate severity were treated.

The results of a retrospective analysis of the first 123 patients transplanted at the Scottish Liver Transplant Unit confirmed that patients who are younger are more likely to develop acute rejection. Severity of liver disease, mid-arm muscle circumference and aetiology of liver disease was also found to influence rejection whereas no affect of HLA-DR mismatch or cold-ischaemia was found. The geography of the United Kingdom compared with the United States means that the cold ischaemia times are different. The patients who appear less likely to develop acute rejection from this study were older patients, patients with Child's C liver disease, depleted muscle mass and those transplanted for alcoholic liver disease. This chapter involved a retrospective analysis, which is not as stringent as collecting data prospectively. For example, not all of the nutritional data were available reducing the number of patients that could be assessed for all parameters. The purpose of this initial chapter was to assess parameters that could be compared with skin testing where data was collected prospectively.

Chapter 3 addressed the issue of hyperacute rejection and the effect of a positive lymphocytotoxic crossmatch on acute rejection. Hyperacute rejection is well described in renal transplantation although reports in orthotopic liver transplantation are limited to a few cases. The finding in this thesis that patients with a positive lymphocytotoxic crossmatch have an increase in early graft loss may be related to damage sustained from preformed antibodies and complement fixation. The numbers involved are relatively small and again this involved retrospective analysis. The published literature remains split on the role of a positive lymphocytotoxic crossmatch and it does seem unlikely that transplants will be stopped or therapy altered on the basis of a positive crossmatch.

The influence of a positive lymphocytotoxic crossmatch on acute cellular rejection is also unclear. As reported in this thesis there appears to be no increased incidence of acute rejection but an increase in recurrent acute rejection. The lack of crossmatch results in all patients with recurrent rejection in chapter 2 meant that no relationship was seen in this larger group and it may well be that the small numbers introduced a type 2 error. There has however been a further report in abstract form of an increase in recurrent rejection in patients with a positive flow cytometry crossmatch (McCarthy et al.,1999) but a further negative study has also been reported(Lang et al., 1999). The influence of a positive crossmatch on acute cellular rejection is therefore still unclear despite many studies, suggesting that at most there is only a small effect.

Chapters 4 and 5 explore the role of cytokines in predicting acute rejection. It is difficult to accept that any single cytokine would be responsible for acute rejection given the redundancy seen in the cytokine networks. However modulation of the effect of tumour necrosis factor alpha by inhibiting monoclonal antibody has been shown to be of benefit in the inflammatory conditions rheumatoid arthritis and Crohn's disease (Maini et al., 1999; Present et al., 1999). The role of cytokine polymorphisms in acute rejection at most appears minimal. There does appear to be an association with the polymorphism commonly called TNF2 when this is homozygous. The homozygous state is relatively low in the population at large varying from 0-12% in reported series. The lack of association with severity of rejection and recurrent rejection suggest that any predilection for acute rejection will not influence graft outcome. Although the identification of recipient polymorphisms could be carried out in the pre-transplant period it does appear that this relatively time

consuming investigation would be of little benefit in allowing tailoring of immunosuppression.

The role of cytokine polymorphisms in disease susceptibility and progression does look more promising. The numbers of patients with each aetiology was small but the findings of the TNF2 allele increase in primary sclerosing cholangitis and autoimmune hepatitis has been reported with larger series, since this work was done, by other authors (Cookson et al., 1999). There does appear to be an influence of these alleles on progression of disease and response to therapy (Czaja et al., 1999) in keeping with the finding of this allele in a population with end-stage disease.

The measurement of cytokines in peripheral blood may not reflect what is going on in the liver and we therefore extracted peripheral blood mononuclear cells and stimulated them lipopolysaccharide, which is a potent stimulator of monocytes. In the acute rejection process inflammatory cells will be stimulated within the liver by cytokines and other mediators produced in the inflammatory reaction. PBMC production of cytokines may not reflect exactly what is going on in the liver at the time of acute rejection. This study was not designed to determine mechanisms of acute rejection but to identify an individuals "inflammatory potential" when cells involved in the inflammatory process are stimulated. The finding that TNF-α is increased pre-transplant in patients who go on to develop acute rejection is in-keeping with the hypothesis that an individual's propensity to produce this inflammatory cytokine following a stimulus, in this case with allostimulation, has a bearing on the severity of the inflammatory reaction seen in acute rejection. However the sensitivity and specificity in predicting acute rejection do not suggest that this test would be of benefit in the clinical situation regarding tailoring of immunosuppression.

Contact sensitisation, however, does appear to accurately predict patients who will not develop significant acute rejection. The number of patients studied was relatively small but the results do suggest further study would be appropriate. The test itself is easy to carry out although slightly inconvenient as it involves either the patient coming to you or you going to the patient on two occasions to carry out the elicitation reaction and scoring. It would be possible to let the patient or their spouse read the test result by giving them photographs of the possible reactions. Given that a test result of greater than 1 indicated the possibility of troublesome rejection the elicitation phase could be modified to only include the two strongest concentrations which would reduce the irritation of a positive reaction in the lower concentrations.

The next step in applying this to clinical practice would be to study a reduction in immunosuppression in the patients with no elicitation response in the early transplant period to determine any reduction in adverse effects and also any effect on graft outcome. The possibilities include using no steroids in patients with no skin test response given that steroids have a broad immunosuppressive effect and the major problem initially is with infective complications. Alternatively the dose of calcineurin inhibitors could be reduced to achieve target trough levels half of the presently accepted levels. This could potentially improve long-term renal outcome and possibly reduce early infection.

There is also potential for investigating patients in whom a reduction in immunosuppresion is desired because of established adverse effects such as renal impairment or malignancy following transplantation to determine if there is any

relationship to skin test scores while on immunosuppresion and propensity to develop acute rejection following a reduction in immunosuppression. The role of immuosuppression in patients with hepatitis C infection is still unclear but it may be that skin testing would prove useful in tailoring immunosuppression if recurrence of hepatitis C merited changes in immunosuppression.

The literature does support a contribution to the acute rejection process by donor factors. It does make sense that a foreign antigen the size of a liver would have variable potency in allostimulation. Our study suggests, however, that the recipient's own immune status is the principal determinant in the early acute rejection process.

The recipient's well being in the post-operative period also has some bearing on the rejection process. In the final study some of our patients with positive skin tests had prolonged ITU stays and repeat laparotomies which may be the reason they did not require treatment for acute rejection. Overall "fitness" may be part of the reason that patients with acute rejection appear to have a better outcome. The other proposed explanation for this finding is that a mild to moderate rejection episode leads to a beneficial immunological effect affecting long term graft outcome.

The aim of this thesis was to investigate the possibility of predicting acute rejection following transplantation in the pre-transplant period by various measurements of the recipient's immune status. In the studies performed the best predictor appears to be a contact sensitisation reaction with a neo-antigen. Further studies will clarify if this finding has a clinical application.

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APPENDIX

The Effect of a Positive T-Lymphocytotoxic Crossmatch on Hepatic Allograft Survival and Rejection

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The influence of crossmatching in liver transplantation is still controversial, and at present, our unit does not alter management according to the result of standard lymphocytotoxicity testing. This study retrospectively assessed outcome of grafts transplanted in the presence of preformed antidonor cytotoxic antibody. One hundred twelve patients undergoing their first orthotopic liver transplantation had results available (mean follow-up: 18 months). Twelve patients had a positive crossmatch and 100 negative. The 1-year graft survival was 58% in the positive crossmatch group, compared with 81% in the negative crossmatch group (P = .02). The 1-year patient survival was 83% in the positive crossmatch group compared with 90% in the negative group (P = .41). Acute cellular rejection occurred in 6 of 7 (86%) grafts surviving more than 7 days in the positive crossmatch group compared with 46 of 88 (52%) grafts in the negative group (P = .09). However, episodes of further acute cellular rejection requiring treatment occurred in 4 of the 6 grafts in the positive crossmatch group but in only 4 of the 46 grafts with a negative crossmatch (P = .0006). The authors conclude that evidence exists in our population that preformed antidonor antibodies adversely affect the outcome of hepatic allografts but not patient survival.

In the early days of solid organ transplantation, it became apparent that a positive lymphocytotoxic crossmatch was associated with hyperacute rejection of renal allografts.[1,2] Hepatic allografts appeared to be resistant to these preformed antibodies, and early reports suggested no adverse effects on graft rejection or outcome.[3-6]

Reports of cases of hyperacute hepatic allograft rejection began to emerge,[7-9] and a subsequent series of reports indicated poorer graft outcome in those grafts transplanted against a positive lymphocytotoxic crossmatch.[10-14] However, this is not every center's experience.[15-18]

The liver transplant unit in Scotland was initiated in 1992 and, like many units, performed liver transplants without reference to cytotoxic donor-specific antibody status. We have examined the role of these antibodies in allograft rejection and graft survival.

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Patients and Methods

Patients

During the period from December 1992 to June 1997, 145 adult patients underwent orthotopic liver transplantation (OLT) for the first time. Thirty-three patients were not tested for lymphocytotoxic antibody against a specific donor for technical reasons. No blood group–incompatible transplantation was performed. Immunosuppression was begun within the first 6 hours with intravenous hydrocortisone (100 mg twice daily) and azathioprine (2 mg/kg/d). Treatment with cyclosporine (5 mg/kg every 12 hours) or tacrolimus (0.05 mg/kg every 12 hours) was begun within the first 24 hours.

Crossmatch Test

The recipients' sera obtained immediately before OLT were tested for cytotoxic antibody against donor lymphocytes by use of the standard National Institutes of Health (NIH) technique. Donor T-lymphocytes were obtained from lymph nodes, and 1 μL of the patient's serum was added for 30 minutes at room temperature. Five microliters of rabbit complement was added for an additional 1 hour at room temperature, and ethidium bromide and acridine orange were added to stain cells. The crossmatch test was interpreted as positive when more than 20% of donor T-lymphocytes were killed by recipient serum and negative when fewer than 20% of cells were killed.

Organ Preservation

All liver allografts were preserved with University of Wisconsin solution.

Follow-Up and Statistics

Acute cellular rejection was defined as clinical, biochemical, and histologic evidence of rejection requiring treatment with high doses of steroids. Hepatic allografts were considered lost if the recipient died or the graft was replaced because of poor or no function.

Mean follow-up was 18 months (range, 2 to 51 months).

Survival rates were calculated by the Kaplan-Meier method with Breslow log-rank tests for significance. Statistical comparisons were made by Student's *t* test for the difference between means, chi-squared analysis, and Fisher's exact test.

Results

Twelve (10.7%) of the 112 recipients who received first hepatic allografts had positive antidonor lymphocytotoxic antibody test results. One hundred (89.3%) had negative crossmatches. The characteristics of these patients are detailed in Table 1 and show no statistically significant differences between the two groups. However, there was a preponderance of women in the positive-crossmatch group (82% *v* 59%). In addition, 67% of the positive-crossmatch group had autoimmune liver disease, compared with 38% of the negative-crossmatch group. The donor factors that may be important in graft function did not differ between the positive- and negative-crossmatch groups, i.e., mean donor age (42.1 *v* 43.1 years), use of pressors/inotropes (67% *v* 63%), peak aminotransferase level (36.0 *v* 40.7 U/L), and hypernatremia (150 *v* 147 mmol/L).

Two patients in the positive-crossmatch group had not been pregnant or received a blood transfusion. Six patients in this group had been pregnant and received a transfusion.

Figure 1 shows the graft survival for those with positive and a negative crossmatches. The 12-month graft survival was 58% in the positive-crossmatch group and 81% in the negative-crossmatch group ($P = .03$). Twelve-month patient survival was 83% and 90% ($P = .41$) for those with positive and negative crossmatches.

Three of the 5 patients who lost grafts in the positive-crossmatch group underwent retransplantation in 1 to 4 days. The other 2 patients died within 5 days. The first had fibrin thrombi in the lungs and dilatation of the right side of the heart, and the second died of sepsis. In the negative-crossmatch group, 4 patients underwent retransplantion within 5 days—3 for primary nonfunction and the other for anastomotic hepatic artery thrombosis. The other eight early losses were from sepsis (n = 4), cerebral complications (n = 2), or cardiac arrest during surgery (n = 2). The reason for the other seven graft losses were death from sepsis (n = 1), retransplantation for chronic rejection (n = 4), hepatic artery thrombosis (n = 1), and disease recurrence (n = 1).

Table 2 shows the episodes of acute cellular rejection in both groups in the grafts that survived longer than 7 days, demonstrating a significant increase in rejection that required treatment with more than one dose of high-dose steroids in the positive-crossmatch group.

Table 3 shows the outcome of grafts transplanted against a positive crossmatch. This shows that 3 of the 7 patients with a strongly positive

Table 1. Patients and Immunosuppression

Crossmatch	Positive	Negative	*P*
No. of grafts	12	100	
Age (y) (SEM)	54.1 (10.2)	47.4 (12.9)	.08
Male/female	2/10	41/59	.07
Cold ischemia time (h) (SEM)	10.5 (3.2)	10.4 (2.5)	NS
Immunosuppression*	9/3	91/9	NS
Indication for transplantation			
Primary biliary cirrhosis	7	33	NS
Alcoholic liver disease	1	20	NS
Primary sclerosing cholangitis	1	9	NS
Viral disease	0	4	NS
Fulminant hepatic failure	0	18	NS
Tumor	1	0	NS
Autoimmune hepatitis	1	5	NS
Cryptogenic cirrhosis	1	7	NS
Other	0	4	NS

*Cyclosporine, prednisolone, azathioprine/tacrolimus, prednisolone, azathioprine.

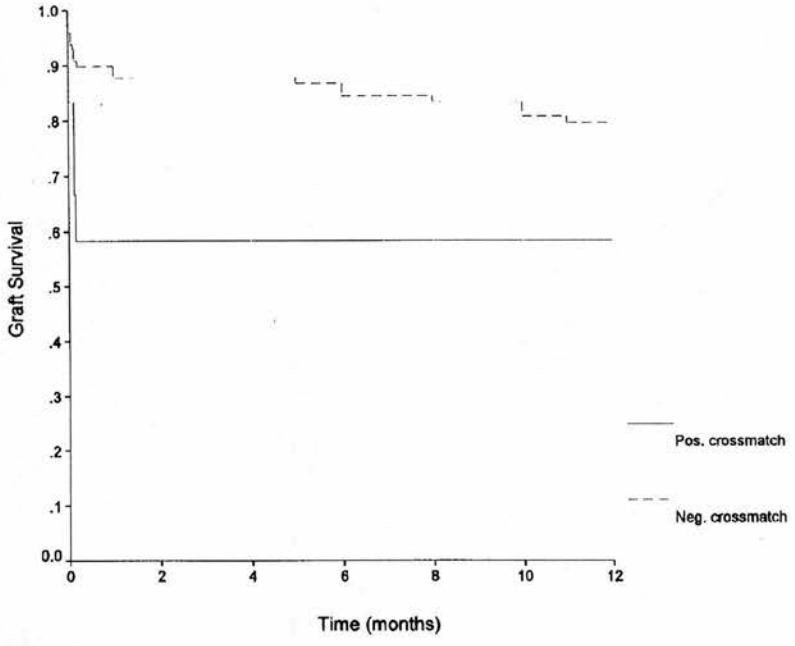

Figure 1. One-year graft survival in patients with positive (n = 12) and negative (n = 100) crossmatches (*P* = .02).

crossmatch lost their grafts; the remaining patients had a mean of 2.25 rejection episodes compared with two graft losses and a mean of one rejection episode in the 5 patients with either weakly positive or positive crossmatches.

Discussion

The results from our single-center study of 112 patients who received a first hepatic allograft for either acute or chronic disease showed overall graft and patient survival of 78% and 89%. Initial graft loss was significantly worse where preformed cytotoxic antidonor antibodies existed.

In recent years, the standard NIH crossmatch has been joined by more sensitive methodologies for detection of immune reactivity, i.e., long-incubation NIH assay, anti-human globulin (AHG) procedure, and indirect immunofluorescent flow cytometry.[19]

Table 2. Acute Cellular Rejection in Positive and Negative Crossmatches

Crossmatch	Positive (n = 7)	Negative (n = 88)	*P*
One episode of acute cellular rejection	6 (86%)	46 (52%)	.09
More than one episode of acute rejection within 30 days	4 (57%)	4 (5%)	.0006

The clinical relevance of these procedures in liver transplantation is questionable because they may be oversensitive, and recent studies with flow cytometry show no impact on graft survival.[17,20] It is possible that the liver is able to neutralize antibody in low titer, but in some grafts, the protective mechanisms are overwhelmed and the antibody persists. The extremely poor outcome of these patients was shown by Manez et al.[21]

The data in our study do not distinguish between immunoglobulin (Ig) M and G antidonor antibody. There is evidence in primary recipients of renal allografts that IgM reactivity does not confer an adverse effect on allograft outcome.[22,23] However, in liver transplantation, IgM reactivity has been shown to affect graft outcome adversely, albeit less so than IgG reactivity.[12]

Our rate of positive crossmatches is similar to those of other centers,[11-18] although the studies reported to date have used different criteria for a positive crossmatch, varying from greater than 10% cell death to 50% cell death. The preponderance of women did not quite reach significance, unlike the results in the study of Charco et al,[14] who also used 20% cell death as a positive crossmatch.

Table 3. Outcome of Grafts With Positive Crossmatches

Patient	Cell Death (%)	Immunosuppression	Graft Status	Episodes of ACR Requiring Therapy
1	41-80	cyc, aza, pre	Patient died at 48 mo	0
2	41-80	cyc, aza, hyd	Patient died on day 5	
3	81-100	None	Patient died on day 1	
4	21-40	cyc, aza, hyd	Failed on day 1	
5	81-100	cyc, aza, pre	Functioning at 27 mo	2
6	81-100	cyc, aza, pre	Functioning at 22 mo	4
7	81-100	cyc, aza, hyd	Failed on day 1	
8	81-100	cyc, aza, hyd	Functioning at 11 mo	2
9	21-40	tac, aza, pre	Functioning at 9 mo	2
10	81-100	tac, aza, pre	Functioning at 4 mo	1
11	81-100	tac, aza, hyd	Failed on day 2	
12	21-40	tac, pre	Functioning at 2 mo	1

Abbreviations: ACR, acute cellular rejection; cyc, cyclosporine; aza, azathioprine; pre, prednisolone ; hyd, hydrocortisone; tac, tacrolimus.

The poor early outcome of grafts transplanted across a positive crossmatch found in other centers was confirmed in our population. Of particular interest was the loss of grafts with so-called primary nonfunction. The preperfusion biopsy specimens of these grafts did not show any architectural abnormalities or steatosis, and the donor risk factors for graft loss were not different between the two groups. The histologic findings of these failed grafts were not dissimilar from those described by Demetris et al.[24] The strict criteria that have been suggested by the same group for hyperacute rejection were not met, but it is possible that immunoligic damage contributed to graft loss.

The findings of acute cellular rejection requiring treatment in addition to a single course of methylprednisolone is also interesting. This has been reported by other groups,[10,17] and the reason for this finding is not entirely clear. There were more episodes of rejection in the group with strongly positive crossmatches than in the group with less positive crossmatches, and it is possible that the liver cell injury resulting from antidonor antibodies results in a clinicopathologic syndrome similar to acute cellular rejection. The finding in this study of no increase in the incidence of chronic rejection in our positive-crossmatch group supports this conclusion because steroid-resistant and relapsing acute cellular rejection have been shown to be more likely to progress to chronic rejection.[25] An early study from the Mayo Clinic that used AHG lymphocytotoxicity testing suggested an increase in the vanishing bile duct syndrome[26] in patients with a positive crossmatch; this was supported by findings in other centers.[11,12] However, recent studies from other centers did not find this association.[13-17]

Our experience supports the findings of other groups in the adverse initial outcome of grafts transplanted against a positive crossmatch. More recent reports that do not show a detrimental effect on graft survival have used different immunosuppressive regimens from ours and include high-dose methylprednisolone and in some cases prostaglandin E_1, which may be beneficial in a positive-crossmatch situation.[27] In view of our findings, it is possible that we need to consider altering our treatment of patients who have preformed antidonor antibodies.

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The Prediction of Acute Cellular Rejection in Orthotopic Liver Transplantation

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The occurrence of acute cellular rejection after orthotopic liver transplantation is common. At present, no allowance is made in immunosuppressive regimens for parameters other than weight. We investigated parameters in 121 consecutive patients receiving their primary allograft to determine if there are pretransplantation factors predicting the occurrence of acute cellular rejection after transplantation. The case notes and dietetic notes of these patients were reviewed for age at transplantation, cause of liver disease, preoperative albumin and creatinine levels, lymphocyte count, anthropometric measurements, donor age, HLA DR mismatch, and cold ischemia time. Acute cellular rejection was more likely to occur in younger patients, patients with Child's class A disease, and those with normal midarm muscle circumference. Acute rejection was increased in transplant recipients from donors aged younger than 30 and older than 50 years. Acute cellular rejection was less likely to occur in patients who underwent transplantation for alcoholic liver disease. Chronic rejection was significantly increased in women and those patients who experienced recurrent acute rejection. On multivariate analysis, the only significant predictor was the decreased likelihood of acute rejection in patients with depleted midarm muscle circumference. In conclusion, it may be possible to individualize immunosuppressive regimens on the basis of pretransplantation characteristics.

Acute cellular rejection is common after orthotopic liver transplantation, usually occurring in the first 3 weeks after transplantation. There is an increase in morbidity associated with acute cellular rejection,[1] although more recent reports suggest that a single episode of acute rejection may improve graft outcome.[2,3] Most, if not all, centers administer a protocol immunosuppression regimen to all transplant recipients, with adjustments made only for weight and with the same target levels for calcineurin inhibitors for all recipients. The adverse effects of immunosuppression are multiple, both in the short and longer term. It may be appropriate to tailor immunosuppression on an individual basis because all patients do not have the same risk for developing acute rejection.

Retrospective studies have shown original disease to be important in the occurrence of acute rejection,[4-6] and more recent reports have suggested other risk factors, such as age of the recipient.[3] We investigated markers of immune status pretransplantation in our patients with chronic liver disease to identify their value in predicting the risk for developing acute cellular rejection.

Methods

Patients

The case notes and dietetic records of 121 consecutive patients who underwent transplantation between November 1992 and April 1997, receiving their first hepatic allograft, were examined for the following information: sex, cause of liver disease, age at liver transplantation, Child's class, triceps skinfold thickness and midarm muscle circumference, preoperative albumin and creatinine levels, and lymphocyte counts. Nutritional parameters were compared with normal values for age and sex and classified as depleted if they were less than the 5th percentile and marginal if between the 5th and 15th percentile.[7] Other factors investigated were donor age, cold ischemia time, and number of HLA DR mismatches between donor and recipient. HLA typing was performed at the Scottish Blood Transfusion Service Tissue Typing Laboratory using standard techniques (only 81 recipient/donor types were available). Mean follow-up was 36 months (range, 1 to 60 months).

Acute cellular rejection was defined as rejection requiring treatment with high-dose steroids. Most patients (83%) had a protocol biopsy performed at approximately 7 days, although this was removed from the protocol for 1 year. Indications for liver biopsy at other times were abnormal liver enzyme levels or slowly resolving liver function test results after rejection therapy.

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Table 1. Severity of Acute Rejection on Day 7 Biopsy in Patients With No Treatment and Early and Recurrent Acute Rejection

	No Rejection	Mild	Moderate	Severe
No treatment	5	31	6	0
Single episode of acute rejection	—	2	22	8
Recurrent episodes of acute rejection	—	0	13	6

All patients with acute rejection except 1 underwent a biopsy before treatment was commenced. Acute rejection was graded as mild, moderate, or severe according to the rejection activity index.[8] Late acute rejection was defined as occurring after the first 30 days.

Immunosuppression used triple therapy consisting of cyclosporine (10 mg/kg/d) or tacrolimus (0.1 mg/kg/d), azathioprine (2 mg/kg/d), and prednisolone (20 mg/d). The target trough levels were 175 to 200 ng/mL for the first 6 months, 125 to 150 ng/mL for 6 to 12 months, and then 100 to 125 ng/mL. Cyclosporine was initially administered intravenously, beginning within the first 6 hours posttransplantation for the first 48 hours, until the microemulsion preparation became available (in 1995), after which it was then administered through a nasogastric tube. Tacrolimus was used in 5 patients (randomly assigned), with target levels of 10 to 15 ng/mL for the first 3 months and then 5 to 10 ng/mL.

Statistical Analysis

The numerical variables were assessed using Student's *t*-test for the difference between two means. The existence of an association between acute rejection and specific categorical variables was verified by use of Pearson's Chi-squared tests. Those categorical or numerical variables significantly associated with acute rejection were tested on multivariate analysis using a stepwise approach. All statistical tests were performed by means of the SPSS statistical software package, release 6.1.3 (1995, SPSS Inc., Chicago).

Results

The overall incidence of acute cellular rejection in 121 patients was 53%. The severity of these episodes is shown in Table 1. Nineteen patients (15%) had more than one episode of acute rejection treated. Nine of these patients required a second course of methylprednisolone within 2 weeks of the initial early rejection episode. Two of these patients went on to receive OKT3. One patient with two episodes of acute rejection developed chronic rejection. Five patients had three or more episodes of acute rejection requiring treatment, 4 of whom went on to lose their graft from chronic rejection. Eighteen of these patients (95%) were women ($P = .007$). No other variable was significantly different in the group with recurrent rejection.

Eleven patients had an episode of late acute rejection, five of which were associated with immunosuppression levels less than target levels, three episodes at the time of cytomegalovirus (CMV) infection, and three episodes with no known attributable factor. The histological severity of these lesions was mild in 4 patients and moderate in 7 patients. One of these patients developed chronic rejection resulting from poor compliance.

Eight patients (7%) developed chronic rejection. All patients were women ($P = .04$) aged 47.5 ± 5.2 years (mean ± SE) compared with 47.6 ± 1.2 years ($P = .34$) for patients without chronic rejection. There was no difference between the two groups in the variables measured, including cause of liver disease, CMV infection, HLA DR mismatch, or initial severity of acute rejection. Patients who had recurrent (5 of 19 patients) or late rejection (2 of 11 patients) were more likely ($P = .03$) to develop chronic rejection than those who had a single episode of early acute rejection (1 of 34 patients).

The occurrence of acute rejection related to original disease is listed in Table 2. Patients with

Table 2. Acute Cellular Rejection in Different Causes of Liver Disease

Cause	No Rejection	Acute Rejection
Primary biliary cirrhosis	24 (20)	25 (21)
Alcoholic liver disease	14 (12)	4 (3)
Primary sclerosing cholangitis	6 (5)	8 (7)
Chronic viral disease	2 (2)	2 (2)
Autoimmune hepatitis	1 (1)	5 (4)
Cryptogenic cirrhosis	2 (2)	3 (2)
Paracetamol-induced acute liver failure	5 (4)	7 (6)
Non A-E acute liver failure	3 (2)	4 (3)
Others	0	6 (4)

NOTE. Values expressed as number of patients (%). Abbreviation: Non A-E, Non A Non B Non C Non E.

alcoholic liver disease had less acute rejection than patients with nonalcoholic liver disease ($P = .01$).

Table 3 lists recipient age, preoperative albumin levels, lymphocyte counts, and the cold ischemia times. The patients with acute cellular rejection were younger than those without ($P = .007$). The occurrence of acute rejection with respect to recipient sex, donor age, serum creatinine level pretransplantation, and the number of HLA DR mismatches is listed in Table 4. Patients with Child's class A disease had more acute rejection than those with classes B and C ($P = .04$).

Nutritional parameters of patients with chronic liver disease, measured by anthropometry (n = 75), listed in Table 5, predicted less acute rejection in those patients who had depleted midarm muscle circumference ($P = .01$).

Stepwise logistic regression analysis of those variables found to be significant on univariate analysis indicated that midarm muscle circumference was the only factor independently associated with acute rejection ($P = .01$).

Discussion

The occurrence of acute cellular rejection in 53% of the patients is in keeping with other reported series. Recent evidence suggests that a single episode of acute cellular rejection does not adversely affect graft outcome.[2,3] However, the use of immunosuppressive agents is not without its problems, with infection as the main early complication and recent evidence that suggests early levels of cyclosporine are the principal predictors of late renal failure.[9] The value of predicting acute cellular rejection may be twofold. Those more likely to experience acute rejection could have their levels of immunosuppressives maintained at the present accepted levels, but those predicted to be less likely to experience acute rejection could reduce immunosuppression.

Acute rejection is principally a T-lymphocyte–mediated response; therefore, the parameters investigated were principally those known to affect cell-mediated immunity, as well as a few others that

Table 3. The Occurrence of Acute Rejection Related to Age at Transplantation and Preoperative Levels of Albumin, Creatinine, and Lymphocytes

Variable	No Rejection	Acute Rejection	*P*
Age (yr)	51.3 ± 1.44	44.3 ± 1.66	.007
Albumin (g/L)	30.2 ± 0.77	31.8 ± 0.76	.59
Lymphocyte count ($\times 10^9$)	1.26 ± 0.10	1.41 ± 0.12	.22
Cold ischemia time (h)	10.6 ± 0.4	10.6 ± 0.4	.30

NOTE. Values expressed as mean ± SE.

Table 4. Sex, Donor Age, HLA DR Mismatches, Child's Class, and Pretransplantation Creatinine Levels in Acute Rejection

	No Rejection	Rejection	*P*
Sex			
Women	37	45	.53
Men	20	19	
Donor age (yr)			
1-29	7	19	.03
30-49	32	26	
>50	10	18	
HLA DR mismatches			
0	2	5	.48
1	20	20	
2	19	15	
Severity of liver disease			
ALF	8	12	.04
Child's class A	1	9	
Child's class B	17	15	
Child's class C	31	28	
Pretransplant creatinine (mg/dL)			
<2.0	41	58	.13
>2.0	13	9	

Abbreviation: ALF, acute liver failure.

Table 5. Acute Rejection in Different Nutritional Groups

	No Rejection	Acute Rejection	*P*
Triceps skinfold thickness			
Normal	15	14	
Marginal depletion	5	5	.97
Depleted	21	21	
Midarm muscle circumference			
Normal	17	25	
Marginal depletion	5	8	.01
Depleted	18	6	

have been suggested to influence the incidence of acute rejection. The finding that the cold ischemia time was not different between the rejection and nonrejection groups is contrary to the results of Wiesner et al,[3] although the longest cold ischemia time in our group was 15 hours, which was the time beyond which the incidence of acute rejection was increased in the study of Wiesner et al.[3] The donor age findings were also different because the increases in rejection were in the younger and older donor age groups compared with the donor age groups of 30 to 39 years in the previous study.

The role of HLA mismatching in acute cellular rejection is not clear, with many centers such as ours finding no association with DR mismatches,[10,11] although this is not every center's experience.[3]

The finding that acute cellular rejection is more likely to occur in younger patients is not surprising because cell-mediated immunity is known to decrease with age.[12,13] Similarly, the finding that patients with less severe liver disease are more susceptible to rejection is not unexpected because cell-mediated reactivity has been related to severity of liver disease.[14,15]

The influence of original disease has been known for some time, and other investigators have noted a reduction in acute cellular rejection in alcoholic liver disease compared with primary biliary cirrhosis or nonalcoholic liver disease.[4-6] The reason for this may be that patients with alcoholic cirrhosis are often severely compromised nutritionally, although recent evidence suggests that the degree of nutritional disturbance is related to severity of disease, rather than cause.[16]

The effect of renal impairment on cell-mediated immunity in chronic liver disease has not been investigated. Renal impairment per se affects cell-mediated immunity as measured by skin testing[17] and may have clinical implications.[18] One previous report[3] suggested that liver transplant patients with greater creatinine levels pretransplantation are less susceptible to acute rejection after transplantation. Our data, although showing the same trend, do not show statistical significance. Serum creatinine level is not a reliable indicator of creatinine clearance, which may be a more potent predictor of impaired cell-mediated immunity. Further studies may confirm this.

An involvement of nutritional status in cell-mediated immunity has been proposed for some time.[19,20] Midarm muscle circumference pretransplantation in our experience was the only independent predictor of the absence of acute cellular rejection. This may reflect a relative inability to mount a cell-mediated response to foreign antigen in patients with protein energy malnutrition. Infection after transplantation has been found by some to cause a significant difference in survival in those with a poor nutritional state.[21] Fat stores, measured by triceps skinfold thickness, do not appear to reflect immune capabilities to the same extent.

Chronic rejection is now uncommon, and in our small experience, the only risk factors identified were female sex and recurrent acute cellular rejection. The latter risk factor has been found in other centers.[22] CMV infection has been found to be a risk factor in some centers.[22,23] It was the policy of our unit to transplant CMV-negative patients with CMV-negative donor livers until 1997, which may account for this not being a risk factor in our group, as infection was rare.

The significance of these findings in clinical practice are unknown, but it may be appropriate to administer immunosuppression at a lower level in patients who are muscle depleted, especially if they are undergoing transplantation for alcoholic liver disease. It may be that more dynamic tests of cell-mediated immunity, such as skin testing,[24] may be more helpful in predicting pretransplantation the patients more or less likely to experience acute cellular rejection.

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Pretransplantation Tumor Necrosis Factor-α Production Predicts Acute Rejection After Liver Transplantation

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Immunosuppressive therapy has many adverse effects in both the short and longer term. Tailoring immunosuppression might be possible if pretransplantation parameters predicted rejection. We investigated production of the proinflammatory cytokine, tumor necrosis factor-α (TNF-α), and the anti-inflammatory cytokine, interleukin-10 (IL-10), pretransplantation to determine whether there is a relation with acute rejection. Peripheral-blood mononuclear cells were obtained from patients with chronic liver disease on the waiting list for orthotopic liver transplantation and healthy controls. Cells (0.5×10^6) were stimulated with 200 ng of lipopolysaccharide. Preincubation for 30 minutes with tacrolimus, cyclosporine, and dexamethasone at concentrations of 10 and 100 ng was also performed. TNF-α and IL-10 levels were measured by enzyme-linked immunosorbent assay. Acute rejection was defined on clinical and histological grounds. Pretransplantation in vitro production of TNF-α significantly ($P < .05$) increased in the group of patients with acute rejection (n = 9) compared with those who did not develop rejection (n = 12). Preincubation with dexamethasone significantly ($P < .001$) reduced TNF-α and IL-10 production in both patients and controls (n = 8). IL-10 production pretransplantation was not different in those who developed acute rejection (n = 9) compared with those who did not (n = 9). Preincubation with tacrolimus augmented ($P < .05$) the production of IL-10 in patients (n = 18), but not controls (n = 6). Pretransplantation TNF-α production is increased in patients who go on to develop acute rejection posttransplantion. (*Liver Transpl 2000;6:721-727.*)

The treatment of choice for end-stage liver disease is orthotopic liver transplantation. The prevention of acute cellular rejection requires immunosuppression, with the mainstay of treatment provided by the calcineurin inhibitors, cyclosporine and tacrolimus. The principal mechanism of their immunosuppressive action is inhibition of interleukin-2 (IL-2) transcription by preventing the activation of nuclear factor of activated T cells.[1] The effect of these drugs on the production of other cytokines is largely inhibitory,[2] although some, such as IL-6, have been shown to be enhanced.[3] Moreover, tacrolimus and cyclosporine have differential effects on the production of some cytokines.[4,5]

Acute rejection is not an entirely predictable event, and the powerful immunosuppressive agents have multiple adverse effects, including acute renal impairment, increased susceptibility to infection, and, in the longer term, chronic renal damage and malignancy. Liver allograft loss is very infrequently a consequence of rejection, and some patients may be overimmunosuppressed. The effects of pretransplantation parameters on acute rejection have recently been reported.[6] Age, severity of liver disease, renal impairment, and original disease have been shown to influence the occurrence of acute rejection. If pretransplantation parameters can reliably identify a propensity for rejection, it may allow tailoring of immunosuppression for individual patients.

The role of cytokines in acute cellular rejection after orthotopic liver transplantation is unclear. There is an increase in such proinflammatory cytokines as tumor necrosis factor-α (TNF-α) and IL-6 in both graft and serum at the time of acute cellular rejection.[7-9] Intragraft IL-5 levels are also increased during acute rejection.[10] Conversely, IL-10 levels in serum and graft appear to be unchanged at the time of acute rejection.[11,12]

The proinflammatory cytokines, TNF-α and IL-6, can activate endothelial cell and up-regulate cell adhesion molecules and major histocompatibility complex (MHC) expression, as well as recruit other immune cells to the site of inflammation. Other cytokines, such as IL-4 and IL-10, may have a modulatory function.

IL-10 inhibits the proliferation of T cells by inhibiting IL-2 production and reducing the synthesis of such proinflammatory cytokines as TNF-α, IL-6, and IL-1β. It also down-regulates surface expression of MHC class II molecules on antigen-presenting cells.

Cytokine production varies with the cause of end-stage liver disease,[13,14] and pretransplantation levels have not been studied with respect to acute rejection posttransplantation. The aim of this study was to inves-

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tigate TNF-α and IL-10 production by patients with end-stage liver disease awaiting transplantation and explore their relation with acute cellular rejection. The effect of different immunosuppressants was also investigated. We found increased in vitro production of TNF-α by patients who developed acute rejection, but no difference in IL-10 production.

Patients and Methods

Patients

All patients studied were free from sepsis and in a stable condition while on the waiting list for orthotopic transplantation (9 women, primary biliary cirrhosis [6 for IL-10 production]; 6 men, alcoholic cirrhosis; 4 men, chronic viral disease; and 2 women, cryptogenic cirrhosis).

Ethical approval and informed consent were obtained. Thirty milliliters of heparinized blood was obtained from patients and healthy controls. Blood was separated on a density gradient (Histopaque; Sigma Chemical Co, St Louis, MO), and 0.5×10^6/mL of peripheral-blood mononuclear cells (PBMCs) suspended in RPMI 1640 with L-glutamine (Sigma Chemical Co) supplemented with 10% fetal calf serum (Gibco, Paisley, UK) and streptomycin was added to 12-well plates (Iwaki microplate, supplied by Bibby Sterilin, Stone, UK). One milliliter (0.5×10^6 cells) of suspension was added to each well and placed in a 5% carbon dioxide incubator at 37°C.

Tacrolimus (a gift from Fujisawa Co, Munich, Germany), cyclosporine (Sigma Chemical Co), and dexamethasone (Sigma Chemical Co) were added to 10- and 100-ng/mL final concentrations in duplicate and preincubated for 30 minutes before stimulating with 200 ng of lipopolysaccharide (LPS) *Escherichia coli* O111:B4 (Sigma Chemical Co). Supernatants were removed after 20 hours and stored at –20°C until cytokine level measurement.

TNF-α and IL-10 concentrations were determined by enzyme-linked immunosorbent assay (Quantikine; R & D Systems Europe, Abingdon, UK) according to the manufacturer's instructions. Briefly, supernatant was added to 96-well precoated monoclonal antibody microtiter plates in duplicate. After incubation for 2 hours, the wells were washed 4 times, and conjugate was added to each well. After incubation for 1 hour and further washing, substrate was added and incubated for 30 minutes. Optical density was measured at 450 nm and compared with the standard curve.

Samples obtained at different times pretransplantation in the same patients showed a variation in measured levels of 1% to 14% (mean, 6%) for TNF-α and 3% to 12% (mean, 8%) for IL-10.

Acute rejection was defined as rejection requiring treatment with high-dose corticosteroids. The diagnosis was made on clinical and histological grounds.[15]

Table 1. Occurrence of Acute Rejection According to Cause of Liver Disease, Age, and Pretransplantation Creatinine Level

	Acute Rejection	No Rejection
Primary biliary cirrhosis	3	6
Alcoholic liver disease	2	4
Others	4	2
Age (yr)		
<50	7	4
>50	3	7
Serum creatinine (μmol/L)		
>70	5	5
<70	7	3

NOTE. Pretransplantation creatinine level not significant.

Statistical Analysis

The sign test was used to compare paired samples of cytokine concentrations. Mann-Whitney *U* test was used to compare differences between patients and controls; patients with and without acute rejection. Analysis of variance (ANOVA) was used to investigate a difference between causes of liver disease.

Results

Acute Rejection

Acute rejection requiring treatment occurred in 9 of 21 patients (43%). The occurrence of acute rejection with respect to cause of liver disease, recipient age, and pretransplantation creatinine level is listed in Table 1. There was a trend for acute rejection to occur in younger recipients ($P = .1$).

TNF-α

Production of TNF-α in patients (1,220 ± 130 pg/mL; mean ± SEM; n = 21) and controls (820 ± 190 pg/mL; n = 8) after LPS stimulation of PBMCs is listed in Table 2. There was no significant difference. Production of TNF-α pretransplantation significantly increased in patients (1,575 ± 190 pg/mL) who went on to develop acute cellular rejection requiring treatment after transplantation compared with patients who did not have rejection (950 ± 130 pg/mL; $P = .02$; Fig. 1). TNF-α production remained significantly greater in patients who developed acute cellular rejection after preincubation with cyclosporine or tacrolimus, but was similar with 100 ng of dexamethasone.

The best threshold for TNF-α production was determined using a receiver operator curve. A contingency

Table 2. Production of TNF-α in Patients With Liver Disease and Healthy Controls

	Patients (n = 21)	Healthy Controls (n = 8)
LPS alone	1,220 ± 130	820 ± 190
LPS + tac 100 ng	1,125 ± 117	605 ± 100*†
LPS + tac 10 ng	1,130 ± 106	590 ± 125‡§
LPS + cyc 100 ng	1,240 ± 120	740 ± 171‡
LPS + cyc 10 ng	1,210 ± 130	733 ± 184*
LPS + dex 100 ng	540 ± 78§	216 ± 50*§
LPS + dex 10 ng	715 ± 100§	370 ± 90§
Control	6 ± 3	20 ± 6

NOTE. Production of TNF-α expressed in picograms per milliliter. Values expressed as mean ± SEM.
Abbreviations: tac, tacrolimus; cyc, cyclosporine; dex, dexamethasone.
*$P < .05$ in comparisons between patients and controls.
†$P < .05$ in comparisons between LPS stimulation and preincubation with different immunosuppressants.
‡$P < .01$ in comparisons between patients and controls.
§$P < .01$ in comparisons between LPS stimulation and preincubation with different immunosuppressants.

table using a cutoff value of TNF-α production of 1,260 pg/mL is listed in Table 3. Sensitivity was 78%, specificity was 75%, positive predictive value was 0.70, and negative predictive value was 0.81.

The effects of preincubation with the different immunosuppressants in the inhibition or augmentation of TNF-α production are also listed in Table 1. TNF-α production by patients was significantly inhibited by dexamethasone, but not cyclosporine or tacrolimus (all $P = .000$ with respect to either dose of dexamethasone compared with LPS, both doses of tacrolimus, and both doses of cyclosporine). Dexamethasone, 100 ng, significantly inhibited TNF-α production more than did 10 ng of dexamethasone ($P = .000$). In healthy controls, TNF-α production was also significantly inhibited by dexamethasone in a similar dose-dependent manner. However TNF-α production was also inhibited by tacrolimus, 10 ng ($P = .008$) and 100 ng ($P = .04$), but not cyclosporine, 10 ng ($P = .18$) or 100 ng ($P = 1.00$). The differential effect of immunosuppressants led to significant differences in TNF-α production between patients and controls that were not seen with LPS alone.

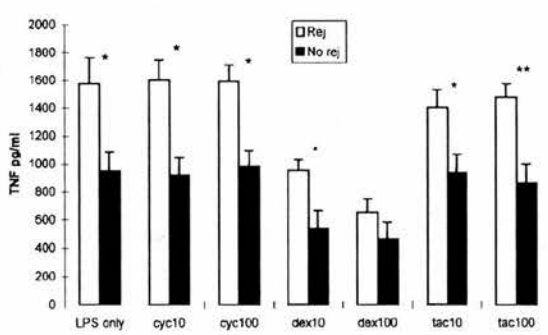

Figure 1. Production of TNF-α pretransplantation by patients with (n = 9) and without acute cellular rejection (n = 12). Values shown as mean + SEM. *$P < .05$. **$P < 0.01$. (tac, tacrolimus; cyc, cyclosporine; dex, dexamethasone.)

There was no statistically significant difference in the production of TNF-α after LPS stimulation in patients with different causes of liver disease. Mean values were 1,089 ± 200 pg/mL in patients with primary biliary cirrhosis (n = 9), 1,315 ± 300 pg/mL in patients with alcoholic liver disease (n = 6), 1,530 ± 140 pg/mL in patients with chronic viral disease (n = 4), and 890 ± 270 pg/mL in patients with cryptogenic cirrhosis (n = 2). No statistically significant difference was observed between causes of liver disease in the effects of preincubation with different immunosuppressants.

There was no correlation of TNF-α production with age or serum creatinine level.

In the 8 patients with less severe liver disease (1 patient, Child's class A; 7 patients, Child's class B), mean production of TNF-α was 1,300 ± 225 pg/mL, which was not significantly different from that of the 13 patients with Child's class C cirrhosis (1,170 ± 165 pg/mL; $P = .6$, Mann-Whitney U test). No statistically significant difference was seen in the effects of preincubation with different immunosuppressants in the different Child's class groups.

IL-10

Production of IL-10 in patients (770 ± 160 pg/mL; n = 18) and controls (522 ±130 pg/mL; n = 6) after LPS stimulation was similar. Figure 2 shows there was no difference in the amount of IL-10 produced pretransplantation in patients who developed acute rejec-

Table 3. Contingency Table of TNF-α Production Pretransplantation and Acute Rejection

TNF-α (pg/mL)	No Rejection	Acute Rejection
<1,260	9	2
>1,260	3	7

NOTE. $P = .03$, Fisher's exact test.

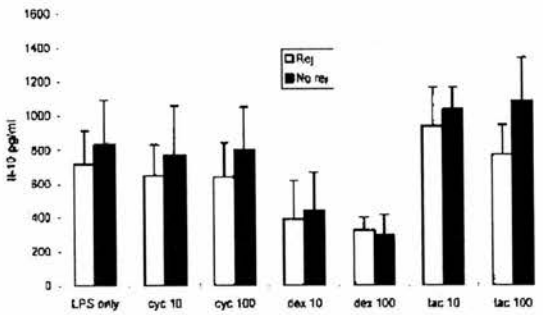

Figure 2. IL-10 production by patients with acute rejection (n = 9) and no rejection (n = 9). Values shown as mean + SEM. (tac, tacrolimus; cyc, cyclosporine; dex, dexamethasone.)

tion (715 ± 200 pg/mL) after transplantation and those who did not develop rejection (835 ± 260 pg/mL).

The effect of preincubation with different immunosuppressants is listed in Table 4. IL-10 production in patients was significantly inhibited by preincubation with dexamethasone, 100 ng ($P = .00$) and 10 ng ($P = .00$). Dexamethasone, 100 ng, significantly inhibited IL-10 production more than did 10 ng of dexamethasone ($P = .02$). IL-10 production was augmented by preincubation with tacrolimus, 10 ng ($P = .04$) and 100 ng ($P = .03$). Cyclosporine had no significant effect on production compared with LPS. In healthy controls, dexamethasone inhibited IL-10 production, but there was no significant difference between the 2 doses ($P = .01$ for both doses). Neither tacrolimus nor cyclosporine had a significant effect on IL-10 production. There was a significant difference between levels of IL-10 in patients and controls only in the presence of dexamethasone.

IL-10 production by PBMCs after LPS stimulation was similar for patients with different causes of liver disease. Mean values were 980 ± 380 pg/mL in patients with primary biliary cirrhosis (n = 6), 910 ± 340 pg/mL in patients with alcoholic liver disease (n = 6), 520 ± 90 pg/mL in patients with chronic viral disease (n = 4), and 570 ± 100 pg/mL in patients with cryptogenic cirrhosis (n = 2). No statistically significant difference was observed between causes of liver disease in the effects of preincubation with different immunosuppresants.

In the 8 patients with less severe liver disease (1 patient, Child's class A; 7 patients, Child's class B), mean production of IL-10 was 1,080 ± 290 pg/mL, which was not significantly different from the 10 patients with Child's class C cirrhosis (530 ± 150 pg/mL; $P = .1$).

Discussion

This study shows that pretransplantation TNF-α production from PBMCs stimulated by LPS is greater in patients who go on to develop acute cellular rejection. We also report a difference in response to tacrolimus in patients and controls, with inhibition of TNF-α in healthy controls but not patients, and augmentation of IL-10 in patients but not controls. Dexamethasone inhibited production of both cytokines by patients and controls.

Cytokine levels in hepatic allografts with acute rejection show differences from those grafts without rejection. TNF-α, IL-5, and IL-6[7,8,10] levels have been shown to be increased. The principal source of TNF-α is the monocyte/macrophage, although many other cells produce this cytokine. In a study investigating human liver allograft rejection, elevated plasma TNF-α levels occurred concomitantly with large numbers of TNF-α–producing monocytes within the graft.[16] Evidence also suggests that the principal source of TNF-α in acute rejection is recipient monocyte/macrophage cells.[17] The effect of TNF-α in the initiation of the rejection process, presumably by activation of endothelial cells and up-regulation of the expression of adhesion factors, is further suggested by the improved survival seen in models in which monoclonal antibody against TNF-α is administered posttransplantation.[18] The finding that TNF-α production pretransplantation is increased in patients who then develop acute rejection is

Table 4. Production of IL-10 in Patients With Liver Disease and Healthy Controls

	Patients (n = 18)	Controls (n = 6)
LPS alone	770 ± 160	522 ± 130
LPS + tac 100 ng	925 ± 157*	535 ± 135
LPS + tac 10 ng	985 ± 170*	493 ± 118
LPS + cyc 100 ng	721 ± 159	509 ± 128
LPS + cyc 10 ng	705 ± 166	439 ± 126
LPS + dex 100 ng	305 ± 72†	64 ± 21†‡
LPS + dex 10 ng	415 ± 120†	90 ± 35†‡
Control	49 ± 24	20 ± 6

NOTE. IL-10 expressed in picograms per milliliter. Values expressed as mean ± SEM.
Abbreviations: tac, tacrolimus; cyc, cyclosporine; dex, dexamethasone.
*$P < .05$ for comparisons between LPS stimulation and preincubation with immunosuppressants.
†$P < .01$ for comparisons between LPS stimulation and preincubation with immunosuppressants.
‡$P < .05$ for comparisons between patients and controls.

interesting. The donor liver results in an alloimmune response, the magnitude of which results in either significant or insignificant acute rejection. As previously indicated, TNF-α has a major role in this immune/inflammatory reaction, and our results suggest that the pretransplantation production capabilities of the transplant recipients may reflect the propensity for inflammation within the graft. However, IL-10 has more of an immunomodulatory function, and studies of liver transplantation have indicated a reduction or no change in the intragraft levels at times of acute rejection.[11,12] In other solid-organ transplants, IL-10 may exacerbate rejection,[19] and anti–IL-10 monoclonal antibodies have been shown to improve survival of heart transplants in animal models.[20]

The influence of pretransplantation cytokine levels on rejection has been investigated in renal transplantation, with increased mean levels of interferon-γ (IFN-γ) in the group going on to develop acute rejection.[21] However, TNF-α was not measured in this study. Our study suggested that TNF-α production less than 1,260 pg/mL predicted patients less likely to experience acute rejection. The benefit of being able to predict patients less likely to experience acute rejection is that from day 1, immunosuppression could be reduced, hopefully reducing such adverse effects as renal impairment or infection. Evidence suggests that a single episode of acute rejection improves graft survival.[6] It is therefore unlikely that reducing immunosuppression in patients less likely to develop acute rejection will adversely affect their graft if they go on to have acute rejection as a result.

The effect of other pretransplantation parameters on acute rejection was reported recently.[6] In a large study, pretransplantation creatinine level and recipient age were found to be predictors of rejection, whereas other studies have shown cause of liver disease to influence rejection.[22,23] Recipient age in this small study did not quite reach statistical significance. There are no reports of TNF-α levels declining with age, and we found no correlation of production with age or severity of liver disease.

Long-term hepatic allograft survival has been greatly enhanced by effective immunosuppressants, such as cyclosporine and tacrolimus. These compounds are structurally different and bind to separate intracellular receptors: cyclosporine to cyclophilin and tacrolimus to FK506-binding protein 12. Their major immunosuppressive effects appear to be through the inhibition of calcineurin, a phosphatase, by the respective drug-immunophilin complex. There appears to be some difference in the effects of these drugs in vitro, depending on the cell type used and the inductor. In the case of cytokine production, there are reports of enhanced production of IL-6,[3] whereas many other cytokines are inhibited, e.g., IL-1β, IL-2, IL-4, and IL-8.[2]

TNF-α production is inhibited in vitro by both cyclosporine and tacrolimus in cells from healthy individuals, although the mode of activation has been shown to influence inhibition. Monocytes in one study were not inhibited by cyclosporine or tacrolimus when activated by LPS,[24] although this has not been a universal finding.[3] The inhibition of TNF-α production by tacrolimus and cyclosporine is partly through the inhibition of calcineurin.[25] Our results suggest that patients with liver disease do not reduce production of TNF-α from PBMCs after preincubation with calcineurin inhibitors, whereas control patients do. It has been shown that PBMCs from patients with cirrhosis after resting in vitro for 24 hours produce TNF-α levels similar to those of healthy controls.[26] It may be that this primed state in patients with cirrhosis may overcome the proposed mechanism of inhibition by calcineurin inhibitors. The inhibition of TNF-α production by dexamethasone is likely to be a different mechanism unaffected by liver disease. Other studies have shown corticosteroids to be inhibitors of other cytokines, with no effect from either cyclosporine or tacrolimus.[27]

The differential effect of tacrolimus on the production of IL-10 in patients compared with healthy controls and with the other immunosuppressants is interesting. Studies of the effect of cyclosporine on IL-10 production are conflicting. In most studies,[28,29] as in ours, production is unchanged, although one study suggested an up-regulation.[30] The literature on tacrolimus is similarly conflicting, with reports of no effect[31] and an increase in production.[32] The mechanisms that control and regulate IL-10 production are still unclear. Some studies report the up-regulation of IL-10 production by substance P,[33] IFN-α,[34] and IFN-β.[35] It is of interest that the mouse IL-10 gene has shown regulatory motifs in the enhancer region similar to that in the IL-6 enhancer region because tacrolimus has been shown to enhance IL-6 production in human monocytes. IL-10 has been shown to suppress inflammatory cytokine production in humans.[36] It also down-regulates MHC class II molecule production in monocytes, therefore inhibiting proliferative T-cell and cytotoxic T-cell responses.[37] It is possible that the effect of tacrolimus on IL-10 production may contribute to the difference in immunosuppressive capabilities between tacrolimus and cyclosporine.

In conclusion, we report increased pretransplantation levels of the proinflammatory cytokine, TNF-α, in

the group of patients who develop acute cellular rejection posttransplantation, suggesting that a patient's inflammatory potential may have some influence on response to the allograft. Furthermore, we report enhanced production of IL-10 by patients with liver disease in the presence of tacrolimus and a lack of inhibition of TNF-α production by cyclosporine and tacrolimus. This indicates that studies investigating mechanisms in healthy volunteers may not apply to patients.

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duration of vancomycin administration (18.4±7.9 vs. 17.3±8 days, $P=0.566$) and can only be explained by the timing of the first doses of vancomycin, almost a week earlier in the prophylaxis group. We speculate that renal function may be particularly vulnerable during the first days after the end of the conditioning regimen so that the nephrotoxicity of vancomycin may be increased.

We conclude that GPB significantly contributes to overall morbidity during the early post-BMT episode but has no major impact on early mortality. The prophylactic use of vancomycin reduces the risk of GPB, especially of potentially fatal streptococcus sepsis. However, this advantage may be outweighed by a negative impact on renal function, which seems to be particularly vulnerable to vancomycin toxicity within the first week after BMT. The results of our study support a restrictive use of vancomycin in the management of neutropenia after BMT.

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THE EFFECT OF POLYMORPHISMS IN TUMOR NECROSIS FACTOR-α, INTERLEUKIN-10, AND TRANSFORMING GROWTH FACTOR-β1 GENES IN ACUTE HEPATIC ALLOGRAFT REJECTION

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Background. **The occurrence of acute rejection in orthotopic liver transplantation is unpredictable. The role of cytokines in the process of rejection is not entirely clear. We investigated polymorphisms in the genes encoding tumor necrosis factor (TNF)-α, interleukin (IL)-10, and transforming growth factor (TGF)-β1, which affect the amount of cytokine produced in vitro, in a liver transplant population to determine any association with acute rejection.**

Method. **DNA was extracted from whole blood of liver transplant patients. After amplification with polymerase chain reactions, the polymorphisms at TNF-α −308, IL-10 −1082, and TGF-β1 +869 and +915 were determined using sequence-specific oligonucleotide probes. Acute cellular rejection was a clinical and histological diagnosis.**

Results. **Acute cellular rejection requiring treatment occurred in 68 (48%) of 144 patients. Acute cellular rejection was significantly associated with the TNF-α −308 A/A genotype ($P<0.02$). There was no significant association with either IL-10 or TGF-β1 polymorphisms in acute rejection.**

Conclusion. **Patients with a homozygous TNF-α −308 genotype A/A are more likely to suffer from acute cellular rejection after liver transplantation.**

Acute cellular rejection after orthotopic liver transplantation occurs in about 50% of recipients (*1*). Cytokines play a major role in the inflammation and immune responses that take place in allorecognition and rejection. There are a number of cytokines that have been implicated in these responses. The proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-6 can cause endothelial cell activation, up-regulate cell adhesion molecules and MHC expression, as well as recruit other immune cells to the site of

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flammation. Other cytokines, such as IL-4 and IL-10, may ave a modulatory function.

IL-10 inhibits the proliferation of T cells by inhibiting IL-2 oduction and reducing the synthesis of proinflammatory tokines such as TNF-α, IL-6, and IL-1β. It also down-gulates surface expression of MHC class II molecules on ntigen-presenting cells.

There is evidence for increased production of TNF-α in atients undergoing acute cellular rejection after orthotopic ver transplantation in both measurement of serum levels nd intragraft messenger RNA (*2*, *3*). Other studies have vealed increased intragraft expression of other proinflam-atory cytokines such as IL-6 (*4*). The measurement of IL-10 serum and graft have shown no changes during liver lograft rejection (*5*, *6*), although one study suggested an crease before episodes of acute rejection (*7*).

An individual's propensity to develop acute rejection after ver transplantation is difficult to predict. One possible rea-n for individual variation may be differences in cytokine oduction related to polymorphisms in cytokine genes. We vestigated polymorphisms known to affect the production cytokines in vitro for the cytokines TNF-α (*8*), IL-10 (*9*), nd transforming growth factor (TGF)-β1 (*10*) in a liver ansplant population with respect to acute rejection.

Patients underwent transplantation at the Scottish Liver ransplant Unit between November 1992 and November 998. All patients were adults receiving primary transplants. here were no ABO-incompatible transplants in this group. mmunosuppression was with triple-drug therapy consisting cyclosporine or tacrolimus, azathioprine (1–2 mg/kg), and rednisolone (20 mg tapering to 5 mg at 3 months after ansplantation).

Acute rejection was defined as rejection treated with high-se corticosteroids based upon clinical, biochemical, and stological evidence—using the accepted Banff criteria. hronic rejection was diagnosed histologically—foamy cell teriopathy and/or 50% of portal tracts without a bile duct. he technical aspects of determining cytokine genotypes are etailed elsewhere (*11*).

Briefly, genomic DNA was obtained from EDTA anticoag-ated whole blood by phenol extraction and ethanol precip-ation after proteinase K (Boehringer Mannheim, Mann-eim, Germany) digestion. DNA was amplified by a olymerase chain reaction carried out using standard tech-ques.

After specific polymerase chain reaction reactions were erformed, two 5′ biotinylated oligonucleotide probes (Geno-s Biotechnologies Ltd, Pampisford, UK) were used to pos-ively identify each polymorphism in cytokine gene promot-s by sequence-specific oligonucleotide probing. The olymorphisms we investigated in the TNF-α and IL-10 enes were those located in the promoter regions at position 308 (G to A) and −1082 (G to A), respectively, relative to e transcription start site. The homozygous genotype TNF-α 308 A/A is associated with high production of TNF-α, and e homozygous genotype IL-10 −1082 G/G is associated ith high production of IL-10. The polymorphisms for GF-β_1 were those located in the leader sequence at position 869 (C to T) and position +915 (G to C). The polymorphisms 869 result in leucine (T) or proline (C) residues at codon in the protein signal sequence, with the former associated ith high production of TGF-β1. Polymorphisms at position +915 result in either an arginine (G) or leucine (C), with arginine at codon 25 associated with high production. Heterozygotes produce intermediate amounts of the respective cytokines.

Statistical analysis was carried out using chi-square tests, with a value of $P<0.05$ deemed as significant. The Bonferroni correction was applied in multiple comparisons. Analysis of genotypes was carried out in 144 patients who received a transplant.

The overall incidence of acute cellular rejection in this group was 48%. The incidence of acute rejection in the different etiologies is shown in Table 1. No significant association between acute rejection and HLA DR mismatches was found.

The occurrence of acute rejection in patients with the different TNF-α genotypes is shown in Table 2. The results for the IL-10 and TGF-β1 genotypes are shown in Tables 3 and 4. There was a significant difference between the rejection and nonrejection groups with respect to the TNF-α −308 genotype. The increased incidence of acute rejection was in those patients who were homozygous A/A ($P<0.02$, Bonferroni correction). When acute rejection was further classified as a single episode or recurrent, there was no significant difference between genotypes, with recurrent acute rejection occurring in six, eight, and two patients for the genotypes G/G, G/A, and A/A, respectively. Combining the TNF-α genotype with the IL-10 genotype did not show significant differences between groups (data not shown).

Chronic rejection occurred in only six patients. This group was not thought large enough to subject to any meaningful analysis.

Acute rejection after liver transplantation is an unpredictable event that often requires treatment with an increase in immunosuppression usually in the form of high-dose corticosteroids. The ability to predict which patients are more likely to suffer from acute rejection may allow individualization of immunosuppression, therefore preventing over immunosuppression in patients less likely to develop rejection. A single episode of acute rejection does not seem to adversely affect long-term graft outcome (*12*, *13*).

The role of polymorphisms in individual cytokine genes that influence production of that cytokine is largely unknown with respect to acute cellular rejection in liver transplantation. This may have some bearing on future immunosuppressive strategies if there is a relation between genotype and rejection.

The proinflammatory cytokine TNF-α has many effects in the inflammatory process, and there is evidence to suggest that the polymorphism at position −308 in the promoter

TABLE 1. The occurrence of acute rejection according to etiology of liver disease

etiology	No rejection	Acute rejection
Primary biliary cirrhosis (n = 61)	30	31
Alcoholic liver disease (n = 25)	17	6
Primary sclerosing cholangitis (n = 16)	6	10
Autoimmune hepatitis (n = 8)	4	4
Chronic viral hepatitis (n = 7)	3	4
Acute liver failure (n = 20)	12	8
Others (n = 9)	4	5

TABLE 2. The incidence of acute rejection with respect to the TNF-α − 308 genotype

TNF-α − 308 genotype	G/G	A/G	A/A
No acute rejection	42	30	4
Acute rejection	28	25	15[a]

[a] $P < 0.02$ (Bonferroni correction).

TABLE 3. Acute rejection with respect to the IL-10 − 1082 genotype (not significant)

IL-10 − 1082 genotype	A/A	A/G	G/G
No acute rejection	22	38	16
Acute rejection	16	30	22

TABLE 4. Acute rejection with respect to the TGF-β1 +869 and +915 genotype (not significant)

	TGF-β1 +869 genotype			TGF-β1 +915 genotype		
	C/C	C/T	T/T	C/C	C/G	G/G
No rejection	8	36	32	1	11	64
Acute rejection	10	35	23	3	9	56

region of the gene influences disease. Individuals possessing the so-called TNF2 genotype (−308 A) have been shown to have worse outcome in cerebral malaria and non-Hodgkin's lymphoma (*14*, *15*). The influence of this polymorphism in rejection of solid organ allografts has been reported in both kidney and heart transplantation (*16*, *17*), the latter only showing a statistical difference when combined with the IL-10 genotype. This is the first report of an effect in liver transplantation, although a recent study reported no effect of another polymorphism, the so-called *Nco*I polymorphism in the TNF-β gene, on the occurrence of acute rejection (*18*). The large increase in relative risk associated with cerebral malaria (*14*) outcome was associated with the homozygous TNF2 genotype, and it may be that this genotype has more of an effect on TNF-α production than the *Nco*I polymorphism.

The finding that 79% of patients who were homozygous for TNF2 required treatment for acute rejection compared with 40% of patients who were homozygous for TNF1 suggests that the TNF2 genotype may predispose to the occurrence of acute cellular rejection in liver transplantation. The finding that there was no difference in genotypes in patients with recurrent acute rejection may indicate that the initial recipient recognition of donor antigen is the only response influenced to a sufficient level by the TNF-α genotype.

The role of IL-10 in acute cellular rejection is not straightforward. It has many anti-inflammatory properties, and one may therefore expect that a polymorphism leading to high production both in vitro (*10*) and in vivo (*19*) may protect against acute rejection. However the studies investigating IL-10 in acute rejection in liver transplantation both in the graft and serum have failed to show differences between grafts with or without rejection. Studies investigating acute renal allograft rejection have shown increased IL-10 intragraft expression (*20*, *21*), and the recent study reporting cytokine genotypes in renal allografts showed an increase in rejection in the polymorphism associated with high IL-10 production (*16*). Cardiac allografts in animal studies have shown an exacerbation of rejection with the addition of IL-10, and the measurement of intragraft levels of IL-10 in human grafts does not predict acute rejection (*22*). As already indicated, the single study of cytokine genotyping in heart transplantation has shown that a combination of a genotype corresponding to high TNF-α production and low IL-10 production was associated with high levels of rejection in the early posttransplantation period (*17*). There was, however, no reduced rejection in those with the high IL-10 producer genotype. The effect that IL-10 has on B cells, causing them to proliferate and increase the humoral response, may be important in renal transplantation; this may be one of the reasons for the apparent difference in the effect of IL-10 in the rejection of different organs.

In our study there is no evidence that IL-10 polymorphisms either alone or in combination with specific TNF-α polymorphisms influence the occurrence of acute cellular rejection. There is a widespread belief that acute cellular rejection in hepatic allografts is different from that in other solid organ allografts as evidenced by the lack of requirement for HLA matching and the improved outcome of grafts suffering a single episode of acute rejection (*12*, *13*). Therefore it may not be too surprising that the influence of polymorphisms differs between organs.

TGF-β is a cytokine with immunosuppressive and profibrotic actions. The data presented suggest no influence of the TGF-β1 genotype on acute rejection. There is evidence to suggest that in the case of lung allografts, there is a significant effect on graft outcome with respect to lung fibrosis, which is influenced by the TGF-β genotype (*10*), and recent evidence also suggests an effect on cardiac transplant vasculopathy (*23*). Chronic rejection of hepatic allografts is not common and it occurred in less than 5% of this study population. Chronic rejection of hepatic allografts is not common and it occurred in less than 5% of this study population. Larger numbers of patients will be required to determine whether the TGF-β1 genotype influences hepatic allografts in a similar fashion.

In conclusion we report the first study of cytokine genotypes in a liver transplant population, showing that TNF-α polymorphism associated with high production is significantly associated with acute cellular rejection. The influence of these findings on clinical practice is not clear at present but may help with tailoring immunosuppressive strategies.

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TRANSFORMING GROWTH FACTOR-β AND INTERLEUKIN-10 SUBVERT ALLOREACTIVE DELAYED TYPE HYPERSENSITIVITY IN CARDIAC ALLOGRAFT ACCEPTOR MICE[1]

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We have previously reported that temporary treatment of cardiac allograft recipients with gallium nitrate (GN) results in indefinite graft survival, and the inability to mount donor-reactive delayed type hypersensitivity (DTH) responses. We report that antibodies to either transforming growth factor-β (TGFβ) or interleukin-10 (IL10) can uncover DTH responses to donor alloantigens in cardiac allograft acceptor mice. The DTH responses uncovered with TGFβ-reactive antibodies can be blocked by exogenous IL10, and those uncovered with IL10-reactive antibodies can be blocked by exogenous TGFβ. These data demonstrate that allograft acceptor mice are fully allosensitized, and poised to make donor-reactive cell-mediated immune responses. However, such responses are subverted by a donor alloantigen-dependent mechanism that involves TGFβ and IL10, which in turn interfere with local cell-mediated immune responses.

The acute rejection of murine cardiac allografts can be blocked by short-term treatment of the allograft recipients with selected immunosuppressive agents, including anti-CD4 monoclonal antibody (*1*, *2*) and gallium nitrate (GN) (*3*, *4*). Despite numerous studies on this phenomenon, little is known about the immunological mechanisms that operate to prevent acute allograft rejection under these conditions. We and others have demonstrated that the mechanism of allograft acceptance is: (1) mediated by an active, antigen-dependent immunologic process that overtly protects the allograft, (2) transferable via splenocytes to severe combined immuno-

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